Practical Pharmaceutical Chemistry

Fourth Edition – Part Two

Edited by

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Preface to the Fourth Edition

A spate of official publications, including the *British Pharmacopoeia* 1980, and its Addenda (1981, 1982, 1983 and 1986), the *British Pharmacopoeia (Veterinary)* 1985, the combined *United States Pharmacopoeia* XXI and *National Formulary* XVI and the second edition of the *European Pharmacopoeia*, call for yet another revision of *Practical Pharmaceutical Chemistry*. In this, the fourth edition, we have endeavoured to reflect the growing international convergence of policy and practice both in the change of subtitle of Part 1 to Pharmaceutical Analysis and Quality Control, and in the breadth of its content.

The objectives of this revision have been achieved in part by reducing the heavy dependence of earlier editions on the methods of the *British Pharmacopoeia*. Wherever possible, examples are based on drugs and dosage forms that are in widespread and common use in Britain, continental Europe and North America. Additionally, some reference to veterinary pharmaceuticals is made where they provide appropriate examples. As in previous editions, substances that are the subject of monographs in the British Pharmacopoeia are denoted by their British Approved Names (BAN), and are distinguished from United Adopted Names (Usan) where these are given by setting BANs in italics.

The discussion of drug registration has also been broadened to include reference to FDA and EEC procedures, the control of veterinary as well as human medicines, and the need in the United Kingdom for biologically based products to be manufactured in conformity with the requirements of the *Biological Compendium* (1977).

The detailed chapter-by-chapter revision in Part 1 encompasses the changeover in European analytical practice from NORMALITY to MOLARITY, which despite the reservations of some analysts, particularly in relation to oxidation-reduction titrations, is now virtually complete. The expansion of rapid complexometric titration methods, with the consequent almost complete demise of the older much slower gravimetric methods, is reflected in the regrouping of complexometric, argentometric and gravimetric methods into a single chapter. A brief treatment of variables in quantitative analysis appropriate to the application of chemical methods has been included for the first time, and the chapter on the analysis of dosage forms has been updated to better reflect both the range of products and the methods used in their control. In this respect the need for control analysts to embrace an

appreciation of biological methods is reflected in the inclusion of short sections on sterility testing, on microbiological contamination and challenge tests for antimicrobial preservatives, on microbiological assays and on enzymes in pharmaceutical analysis.

There have been few, if any, major innovations in physical methods applicable to pharmaceutical analysis since the publication of the third edition in 1976. However, there have been a number of improvements and changes of emphasis. These are reflected in Part 2 of the present edition, which has been extensively revised in an endeavour to give a broader coverage of the most widely used techniques and a better balance of material that fairly reflects modern practice. In particular, the steady growth in importance of quantitative chromatographic techniques is recognised in the broader coverage and depth accorded to gas chromatography and high performance liquid chromatography, the inclusion of a short section on capillary column gas chromatography, and the transfer from Part 1 of important sections on ion exchange and size exclusion chromatography. The treatment of NMR spectroscopy has also been extended to include a brief introduction to ^{13}C NMR, and the coverage of radiopharmaceuticals increased to include radionuclide generators and quality control of radiopharmaceuticals – a subject of special interest to those engaged in hospital pharmacy. To balance these expansions, coverage of electrochemistry and polarography has been compressed into a single chapter – a degree of emphasis that is more in keeping with the rather modest role that these techniques continue to play in the practice of pharmaceutical analysis.

Two new chapters have been added to improve cohesion in Part 2. The first, by way of introduction, sets out the contribution and role of physical methods of analysis in the various phases of drug development, in quality control within the factory and in independent control laboratories, and in the clinic. The second, by way of conclusion, consists of a series of 'workshop style' exercises, with separate solutions, to illustrate and give practice in the application of spectroscopic techniques in structural elucidation and verification of identity.

Many analytical methods serve a common purpose in their suitability both for the quality control of pharmaceutical products and the study of their absorption, distribution, metabolism and excretion whether in laboratory animals or human subjects. The importance of developing sensitive, cold methods, as opposed to those based on radiolabelled compounds, is widely recognised in clinical pharmacology. Hospital pharmaceutical departments well equipped for essential quality control work are in a unique position with staff, equipment and laboratory facilities to undertake pharmacokinetic and metabolic studies, and even to provide a routine pharmacokinetic monitoring service if this is required. With such developments in mind, we have continued to feature applications of the various separation and spectroscopic methods to drug metabolism and pharmacokinetics together with relevant practical exercises.

J.B.S.

Part 2

Physical Methods of Analysis

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attaches to such studies is related to both dosage and the complexity of the metabolic pathways, but highly sensitive methods are essential. For this reason, radiolabels, primarily ^{14}C and ^{3}H, are widely used in primary animal studies (Chapter 13) of tissue distribution, blood levels, and excretion via the lungs, liver and kidney. Recovery studies are necessarily dependent on the use of satisfactory extraction methods (Part 1, Chapter 9) and the powerful separative techniques of thin-layer, gas and high performance liquid chromatography (Chapter 4) to separate labelled metabolites from unchanged drug for identification and quantitative determination.

Human metabolic studies, on the other hand, are constrained by the unacceptability of exposure to radioactivity. Pharmacokinetic studies, too, often require measurements in the microgram, nanogram or picogram/ml range. Much depends on the capability of the separative and ultimate detection system. Gas chromatography, capillary gas chromatography and high performance liquid chromatography all have their place, but much use is now made of such powerful coupled separative–detector systems as gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS) (Chapters 4 and 11). These systems are extremely versatile but very expensive, and much valuable work is still achieved with the less sophisticated and less versatile though sensitive detection capabilities of ultraviolet spectrophotometry, spectrofluorimetry (Chapter 9) and polarography (Chapter 5). In a few special cases where there is sufficient clinical demand for the development of a sensistive routine method for pharmacokinetic monitoring of the patient's clinical status, radioimmunoassay (Chapter 13) is a valuable tool as in the case of *Digoxin*.

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General physical methods

G. C. WOOD

Density

Density

Density, ρ, is the mass of a unit volume of a material. The millilitre is usually chosen to express volume, this being the volume of 1 g water at 3.98°C, the temperature of maximum density of water. Thus the density of water at 3.98°C is 1.0000 g ml^{-1} (for most purposes the difference between densities expressed in g ml^{-1} and in g cm^{-3} can be neglected). Density depends on temperature which is therefore specified by a subscript (ρ_t), t being in degrees centigrade. The density of water at various temperatures is given in Table 2.1.

Table 2.1 Densities of water (g ml^{-1}) at various temperatures

t°C	0	3.98	10	15	20	25
ρ_t	0.99987	1.0000	0.99973	0.99913	0.99823	0.99707
t°C	30	40	50	60	70	80
ρ_t	0.99569	0.99224	0.98807	0.98324	0.97781	0.97183

Specific gravity

Specific gravity or relative density, $d_{t_1}^{t_2}$, is the ratio of the mass of a certain volume of the material at a particular temperature (t_2) to the mass of an equal volume of water at the same or some other specified temperature (t_1).

Thus, $d_{t_1}^{t_2} = \rho_{t_2}/\rho_{t_1 H_2O}$. It follows that d_4^t is numerically equal to ρ_t (the difference between the densities of water at 4°C and 3.98°C being neglected) though, unlike density, it is dimensionless.

Molar volume

The molar volume of a compound, a quantity often used in calculations, is expressed as:

$$\text{molar volume} = \text{molecular weight/density}$$

Physically the molar volume is a measure of molecular volume plus any free space between the molecules. Attempts have been made to use molar volumes as an additive property of the number and types of

atoms and groupings in a molecule, but the additivity is only approximate.

Partial molar volume

When solutions are formed from pure components, unless the solution is ideal (obeying Raoult's Law, and giving no heat or volume changes on mixing the components), the final volume is not simply the sum of the constituent volumes. Volume changes nearly always occur on mixing. It is rare to find that:

$$V = n_1V_1 + n_2V_2 \tag{1}$$

where V is the total volume of solution, and V_1 and V_2 are the molar volumes of components 1 and 2 respectively. For non-ideal solutions, which are those normally encountered, eq. (1) becomes:

$$V = n_1\overline{V}_1 + n_2\overline{V}_2 \tag{2}$$

where $\overline{V}_1$ and $\overline{V}_2$ are the partial molar volumes of components 1 and 2 and n_1 and n_2 are the numbers of moles of components 1 and 2. A partial molar volume is the change in volume when one mole of a particular component is added to an infinitely large volume of solution at constant temperature and pressure.

For component 1:

$$\overline{V}_1 = \left(\frac{\delta V}{\delta n_1}\right)_{T,P,n_2} \tag{3}$$

A simple means of evaluating the partial molar volume is to plot V, the volume of solution containing containing 1000 g solvent, against the molality, m, of the solute. The slope of (or tangent to) the line at a particular value of m, gives $\overline{V}_2$ at this concentration.

Interconversion for concentrations

Solution concentrations are expressed in a number of different ways, and it is often useful to be able to convert from one system to another. Consider a solution of total volume, V, and density, ρ, in which:

W_1 = weight of solvent of molecular weight M_1
W_2 = weight of solute of molecular weight M_2
Total weight of solution, $W = W_1 + W_2$
number of moles of solvent, $n_1 = W_1/M_1$
number of moles of solute, $n_2 = W_2/M_2$

Molarity (C):

C = number of moles of solute per litre of solution

$$C = 1000n_2/V = \frac{1000n_2\rho}{W_1 + W_2} = \frac{1000\rho W_2}{M_2(W_1 + W_2)} \tag{4}$$

Molality (m):

m = number of moles solute per 1000 g solvent

$$m = \frac{1000n_2}{W_1} = \frac{1000W_2}{M_2W_1} \tag{5}$$

Interconversion of molarity and molality:

$$C = \frac{1000m\rho}{1000 + M_2m} \tag{6}$$

Interconversion of percentages:

$$(\%\text{w/w}) = (\%\text{w/v})/\rho \tag{7}$$

Mole fraction:

$$X = \frac{\text{number of moles of component}}{\text{total number of moles present}}$$

$$\text{Mole fraction of solvent} = X_1 = n_1/(n_1 + n_2) \tag{8a}$$

$$\text{Mole fraction of solute} = X_2 = n_2/(n_2 + n_2) \tag{8b}$$

Interconversion of molarity and mole fraction:

$$C = \frac{1000X_2\rho}{(X_1M_1 + X_2M_2)} \tag{9}$$

Practical experiments

Experiment 1 *Determination of the specific gravity and the density of a liquid*

A pycnometer is used for the accurate determination of liquid densities. A convenient form is shown in Fig. 2.1, although many different types are available. For example, the vessels for volatile liquids are

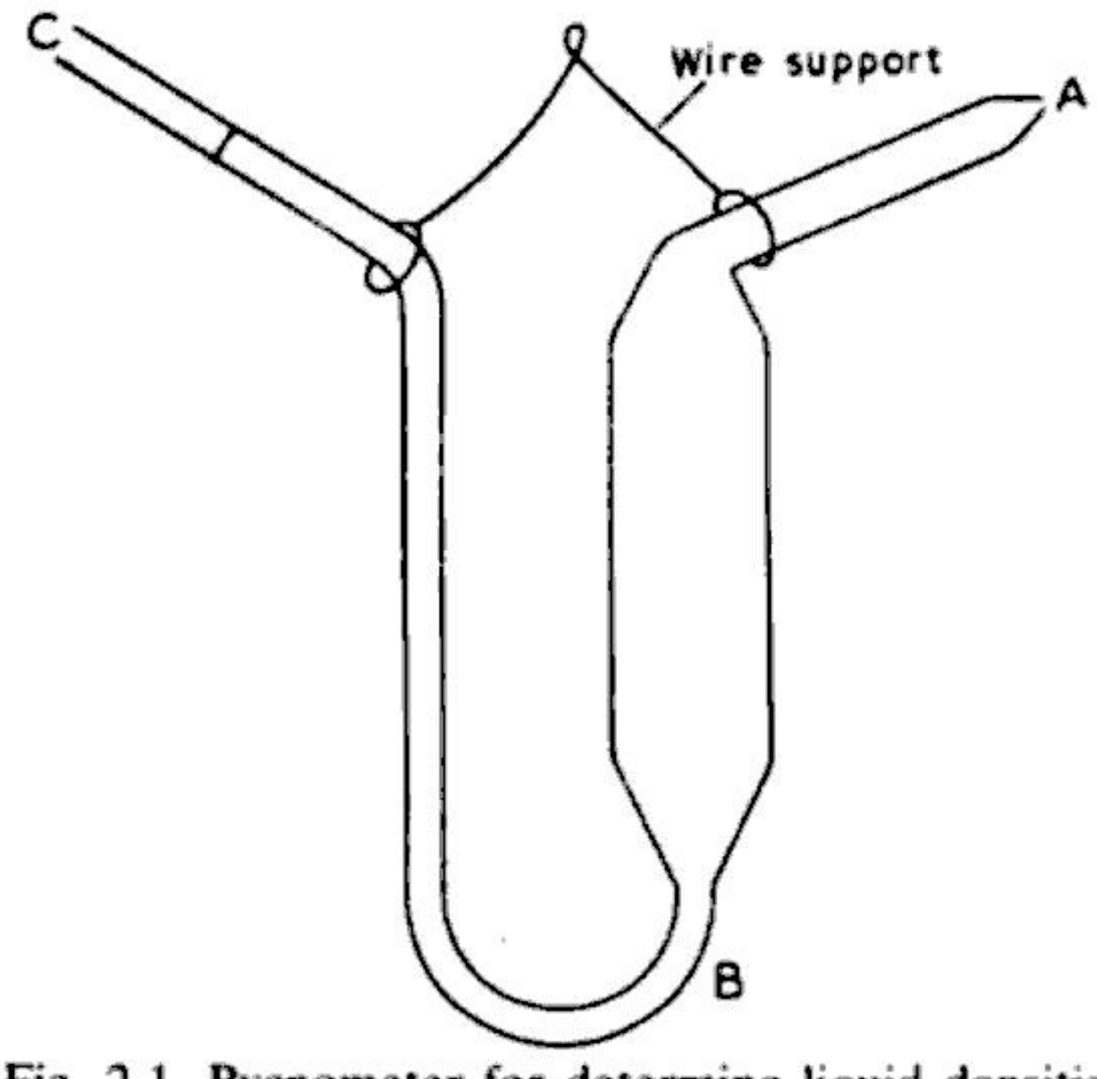

Fig. 2.1. Pycnometer for determing liquid densities

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Practical experiments

Experiment 4 *Determination of the mutual solubility curve of phenol and water*

Method Use test tubes of 20 ml capacity, fitted with ground glass stoppers. Weigh accurately about 1, 2, 3, 4, 5, 6, 7, 8, and 9 g phenol into the tubes. Add sufficient *water* to each tube to make the total contents weigh about 10 g, and weigh accurately. Fit up a 2 l beaker with a hand stirrer, and a thermometer (0–100°, graduated to 0.1°). Attach a piece of copper wire to the tube containing the most dilute solution of phenol in water, and hang it on the rim of the beaker. Place sufficient water in the beaker to bring the level to the bottom of the ground glass stopper of the tube. Heat the water in the beaker, shaking the tube frequently, and raising the temperature slowly when the turbidity of the phenol–water mixture shows signs of disappearing. Determine the temperature at which the turbidity disappears on shaking. Return the tube to the beaker, allow the temperature to fall, and note the temperature at which the turbidity just reappears. Repeat the heating and cooling, and take the mean of all four temperature readings. Detertmine the temperature at which miscibility occurs for the other solutions (cooling may be required for the most concentrated solutions).

Treatment of results Plot the temperatures at which a single phase occurs as ordinates, against percentage (w/w) phenol in water as abscissae. Determine the critical solution temperature from this graph.

Experiment 5 *Determination of solubility of adipic acid in water*

A simple quick method for determining the solubility of a solid in a liquid is to prepare a set of test tubes each containing a weighed amount of solid, add varying quantities of solvent, shake thoroughly, and note the concentration at which all the solid dissolves.

For accurate solubility determinations it is necessary to ensure that the solvent is completely saturated with solute at a particular temperature, withdraw a sample of saturated solution without disturbing the equilibrium, and analyse it to determine concentration. The solid and solvent are mixed by stirring or shaking in a thermostat. Samples are withdrawn at intervals and assayed. Equilibrium is reached when there is no further uptake of solute by solvent. This method can also be applied to the determination of liquid–liquid solubilities.

Method Place adipic acid (about 4 g, roughly weighed) in each of two flasks (100 ml) fitted with B24 size ground glass stoppers, and add *water* (about 60 ml) to each flask. Immerse one flask at a level just above the bottom of its stopper in a thermostat bath set at 20°, warm the second flask at 50° for 10 min, shaking it occasionally, and then place it similarly in the thermostat bath. This procedure is adopted so that equilibrium is approached from both over- and under-saturation. Shake the flasks every 20 min for 2 h. Allow the flasks to remain undisturbed for 10 min. Fit a one inch length of polythene tubing to the tip of a 10 ml pipette, and pack the tubing with cotton wool to act as a filter. Cautiously withdraw a sample of solution from the flask, examining the contents of the pipette against a bright light to make sure the filtration was efficient. Remove the filter, adjust the volume of the solution in the pipette to 10 ml, and run the solution out into a tared flask. Weigh the sample.

Titrate the sample with standard 0.2M sodium hydroxide using phenolphthalein as indicator. Continue to shake the flasks during a further hour, withdraw a sample from each, and re-assay. Equilibrium should have been reached after 2 h, as shown by agreement between the four sets of results.

If the heat of solution is to be determined, repeat the experiment at 30°. The pipette should be preheated to the temperature of the experiment.

Treatment of results

1. Calculate the solubility of adipic acid in g solute/100 g water, and mol l^{-1} solution (molecular weight of adipic acid = 146). Present the results to show the equilibrium has been reached.

2. Calculate ΔH from eq. (12b) using the solubility in mol l^{-1}.

Molecular weight

Excluding very large molecules, the determination of the molecular weight of compounds is based on the use of colligative properties, generally freezing point depression or boiling point elevation. Osmotic pressure can be used in the 5000–300 000 molecular weight range.

The presence of a non-volatile solute in a solution lowers the vapour pressure from p_1^0 (that of the pure solvent) to p_1 (that of the solution). For ideal solutions, Raoult's law gives

$$p_1 = X_1 p_1^0 = (1 - X_2)p_1^0 \tag{13}$$

where X_1 is the mole fraction of solvent, and X_2 that of the solute. In dilute solution the term W_2/M_2 in the denominator of X_2 can be neglected:

$$X_2 = \frac{W_2/M_2}{W_1/M_1 + W_2/M_2} \simeq \frac{W_2 M_1}{M_2 W_1} \tag{14}$$

giving for the relative lowering of the vapour pressure

$$\frac{p_1^0 - p_1}{p_1^0} = \frac{W_2 M_1}{M_2 W_1} \tag{15}$$

Equation (15) can be used to determine M_2 from measurements of vapour pressure, assuming that no dissociation or association of solute occurs.

Using Fig. 2.2 consider the boiling point elevation and freezing point depression. AB represents the sublimation curve of pure solid solvent, and BC the vapour pressure curve of liquid solvent, reaching the boiling point at C. When solute is present, the vapour pressure of the solution is lower than that of the solvent (DE), giving a freezing point at T_f, a depression of the freezing point $\Delta T_f = T_0 - T_f$; the boiling point of the solution is raised to T_b, giving elevation of boiling point $\Delta T_b = T_b - T_{b_0}$.

At the freezing point of a solution, there is an equilibrium between solid solvent and solution, such that the chemical potential of the separated (frozen) solvent and of solvent in the solution are equal. By

considering the variation of chemical potential with temperature the following relationship is obtained

$$\Delta T_f = \frac{RT_0^2 X_2}{\Delta H_{fus}} \tag{16}$$

where ΔH_{fus} is the latent heat of fusion per mole of solvent. In dilute solution eq. (14) is used for the mole fraction of solute, and taking molality from eq. (5):

$$X_2 = M_1 / m1000$$

hence:

$$\Delta T_f = \frac{RT_0^2 M_1 m}{\Delta H_{fus} 1000}$$

and

$$\Delta T_f = K_f m \tag{17}$$

in which all the constants for a particular solvent are combined in a new constant K_f.

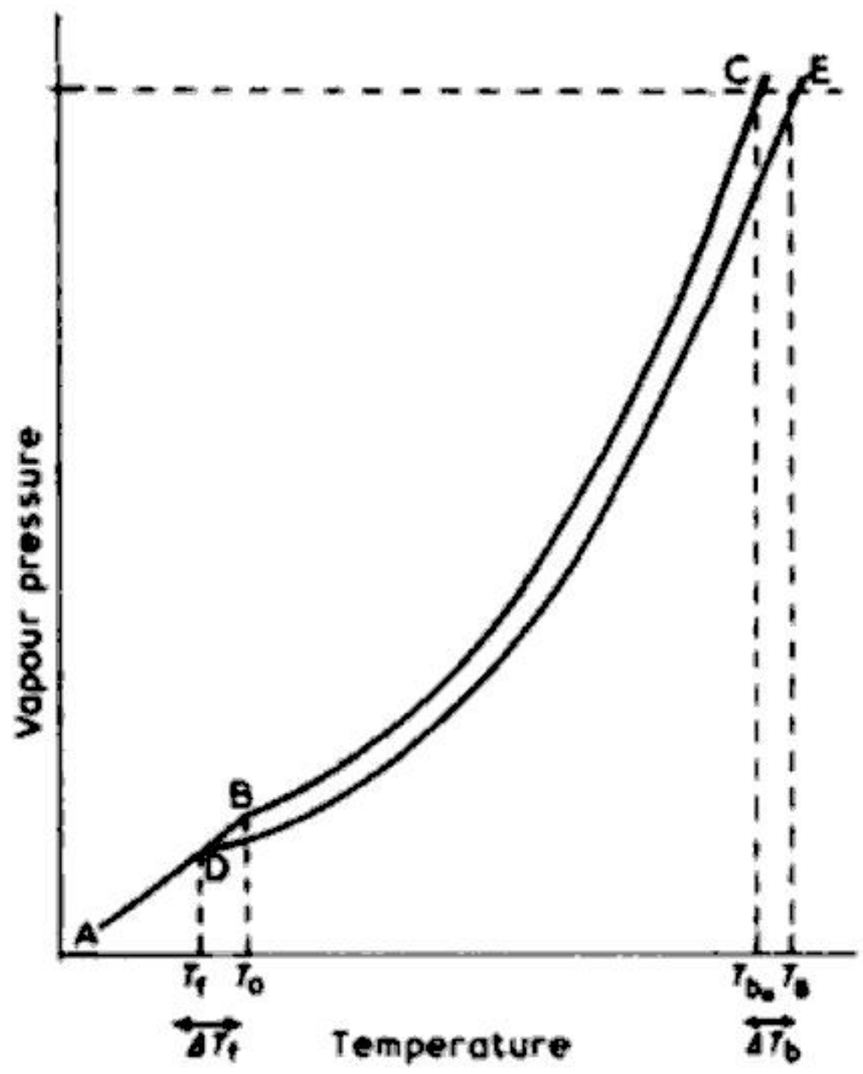

Fig. 2.2. Vapour pressure-temperature curves for pure solvent and solution

By an analogous treatment, considering the equilibrium between solvent vapour and liquid solvent in solution at the boiling point, the chemical potential of solvent in the vapour phase must be the same as that in the liquid phase, and

$$\Delta T_b = \frac{RT_b^2 X_2}{\Delta H_{vap}} \tag{18}$$

where ΔH_{vap} is the molar latent heat of vaporisation. Simplifying as before for dilute solution:

$$\Delta T_b = \frac{RT_b^2 M_1 m}{\Delta H_{vap} 1000}$$

and

$$\Delta T_b = K_b m \tag{19}$$

Examples of molal cryoscopic and ebullioscopic constants are given for commonly used solvents in Table 2.2. The table shows that for a certain solute concentration, the depression of the freezing point will be greater than the elevation of the boiling point; freezing points are also easier to determine than boiling points.

Table 2.2 Cryoscopic and ebullioscopic constants

Solvent	T_f	T_b	K_f	K_b
Acetic acid	16.7	118	3.9	2.93
Benzene	5.4	80	5.12	2.53
Chloroform		61		3.63
Ethanol		79		1.22
Water	0	100	1.86	0.51

The osmotic pressure can be considered in relation to the other colligative properties using Fig. 2.3. Solvent and solution are placed in the two arms of the vessel shown. As $p_1^0 > p_1$ solvent distils through the vapour space, which in this case is acting like a semi-permeable membrane. To prevent distillation, the pressure on the solution must be increased; this raises the chemical potential of the solvent in solution, so that at equilibrium there is no further distillation.

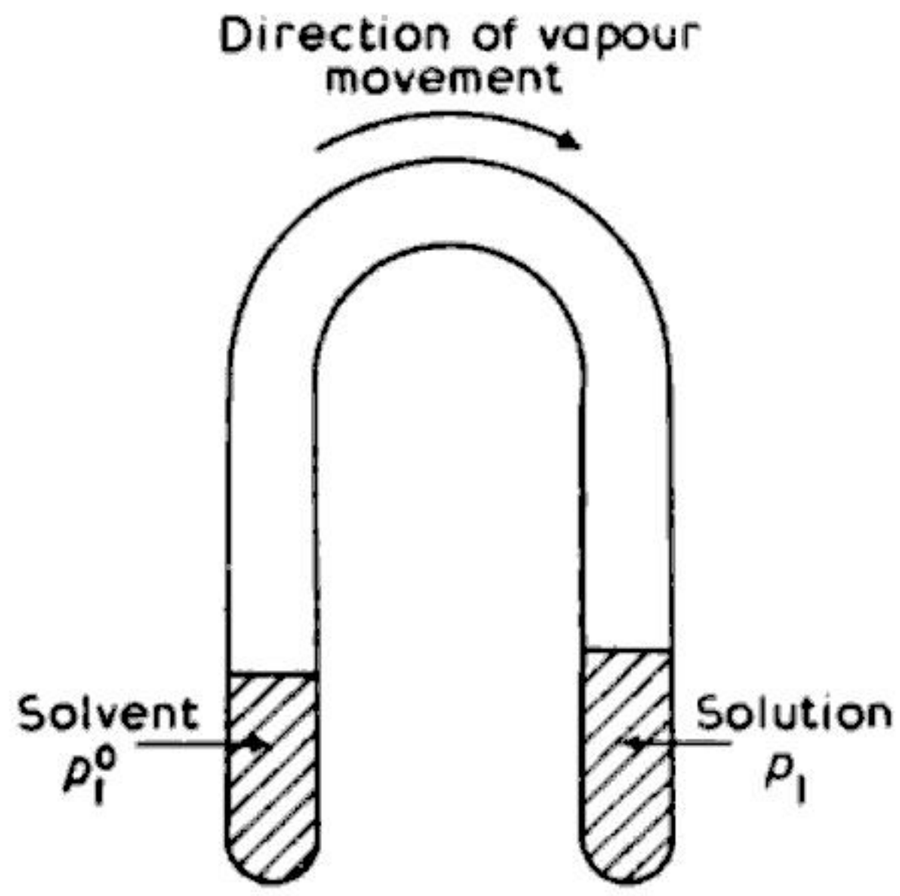

Fig. 2.3. Apparatus for equilibrium of solvent and solution

From a thermodynamic consideration of the effect of pressure

$$\Pi \bar{V}_1 = RT \ln (p_1^0/p_1) \tag{20}$$

where Π = osmotic pressure and $\bar{V}_1$ = partial specific volume of

solvent. If it is assumed that Raoult's law holds, and that solutions are dilute, this equation can be simplified:

$$\Pi = W_2RT/M_2 \tag{21}$$

This enables the molecular weight of the solute to be calculated from measurements of osmotic pressure, W_2 being the concentration of solute in $g\,l^{-1}$.

Solute mixtures

If the solute under test is impure an average molecular weight will be obtained. The average is due to materials of different molecular weight depressing the freezing point (or elevating the boiling point) by different amounts. Colligative methods depend on the number of molecules present in the solution, and the methods give a number average molecular weight, M_N,

$$M_N = \frac{\Sigma n_i M_i}{\Sigma n_i}$$

where n_i = number of molecules of molecular weight M_i, and so on. Hence contamination of the material under test with low molecular weight impurities can give erroneous values for the molecular weight.

Practical experiments

Experiment 6 *Determination of molecular weight by freezing point depression*

The conventional apparatus of Beckmann (Fig. 2.4) consists of an outer bath, A, holding the freezing mixture, which cools the solution in the tube, B, which is surrounded by an air jacket, C. A Beckmann thermometer, D, and stirrer, E, dip into the solution. Samples may be introduced through the side arm, F. The freezing bath is covered by a lid, and kept mixed by a stirrer, G.

When using this apparatus, some supercooling of the sample occurs, as separation of solid solvent does not take place until the temperature is a little below the freezing point. The latent heat of crystallisation then raises the temperature. This effect may be minimised if the temperature of the freezing mixture is too low, and a temperature lower than the true freezing point may be recorded. The freezing mixture should be kept 3–4° below the freezing point of the solution. The degree of supercooling should not be allowed to exceed 0.3–0.5°, otherwise a great deal of solvent may crystallise out, and seriously affect the concentration of the solution.

The Beckmann thermometer This is a sensitive thermometer, graduated in 0.01°, and capable of being read to 0.002°. The scale length of the thermometer is 5–6°, and provision is made for adjusting the amount of mercury in the bulb, so that the thermometer can be used over

different temperature ranges. Place the bulb in a beaker of water whose temperature has been adjusted to the freezing point of the solvent to be used. The mercury should stand near the top of the scale. If it stands above, place the thermometer in water warm enough to cause the mercury to rise up and form a drop at the end of the capillary (A) (Fig. 2.5). Shake off the drop and test again in the cool water. If necessary repeat this procedure until the mercury stands at the correct place on the scale. The initial test may show that there is too little mercury in the thermometer bulb (mercury below scale); in this case warm up the thermometer until drops form at the capillary, invert it, and tap gently to make mercury from the reservoir join with mercury in the capillary. Re-invert, and place the thermometer in a water bath held 2° above the freezing point of the solvent; the cooling draws mercury from the reservoir into the bulb. When this process is complete, tap the upper part of the thermometer gently to make excess mercury separate from the end of the capillary. Test the thermometer in water at the freezing point of the solvent to make sure that the setting is correct, i.e. the mercury is on the scale. If it is a little above, remove the excess dropwise to the reservoir as described above. Beckmann thermometers require careful handling as they are expensive, and easily broken.

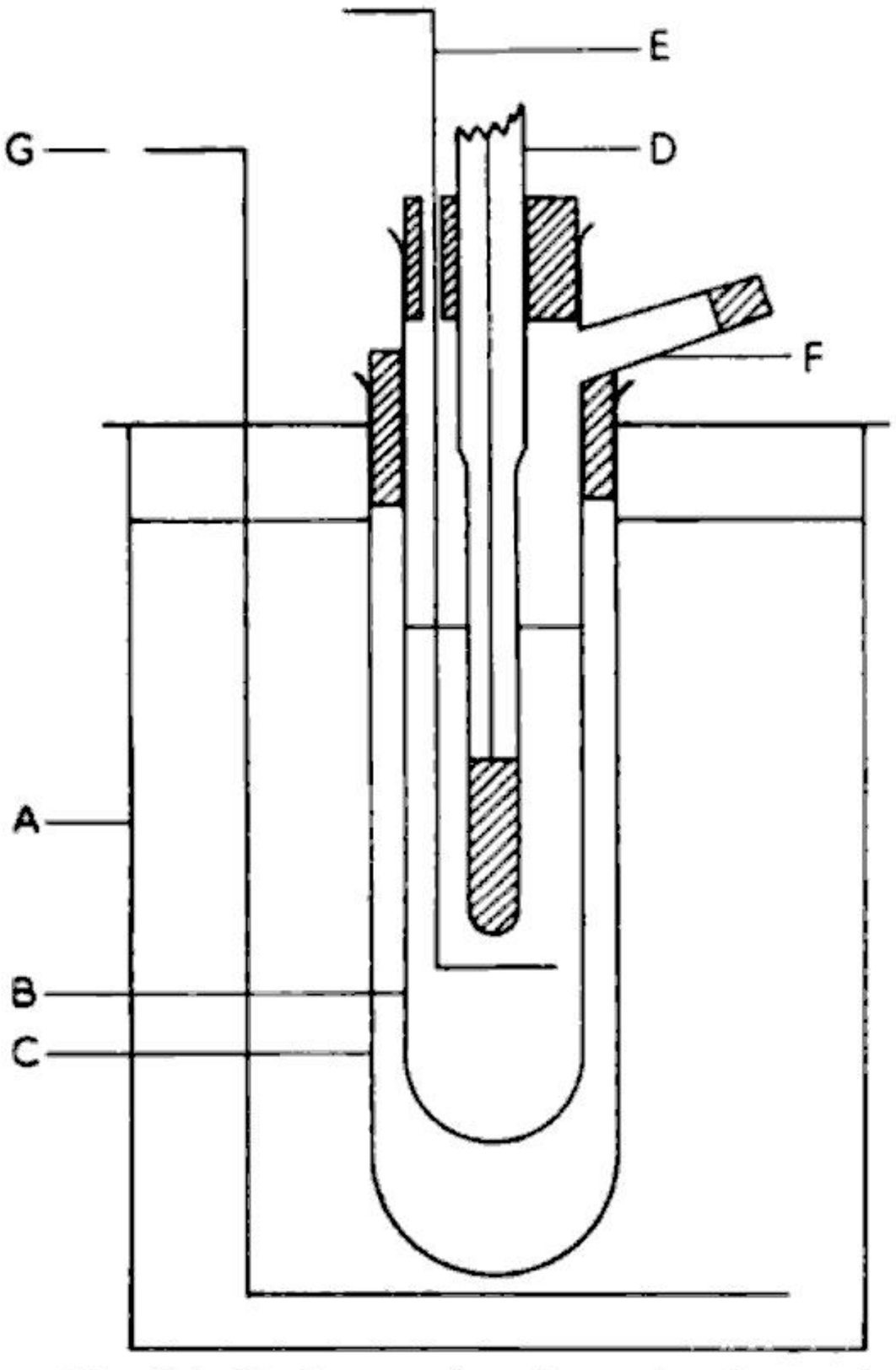

Fig. 2.4. Beckmann freezing point depression apparatus

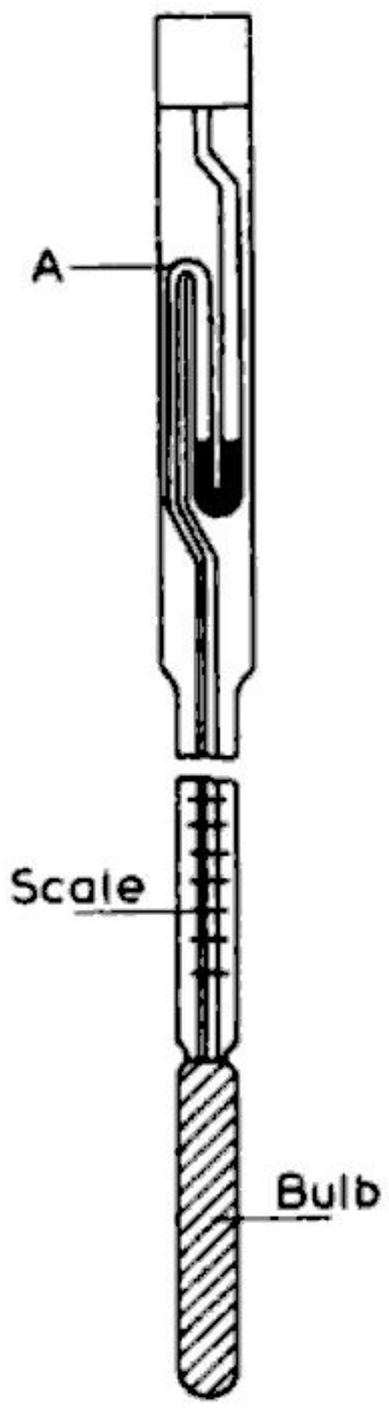

Fig. 2.5. Beckmann thermometer

Determination of freezing point Remove the tube B from the apparatus. Clean, dry, and weigh the tube. Introduce dry, crystallisable benzene (15–20 g), re-weigh and insert the Beckmann thermometer and the stirrer. Pack the bath with a freezing mixture of ice and water controlled at about 2°. Place the tube in the freezing mixture, and when solid separates, remove it, dry the outside and place it in the air jacket. Stir the solution about once every second (faster stirring can generate undue heat), and note the constant (highest) temperature on the Beckmann thermometer. The first reading is usually approximate. Remove the tube from the apparatus, and warm gently with stirring, until the temperature is about 1° above the freezing point. Replace it in the apparatus, and stir; the supercooling should not be more than 0.5°, crystallisation of solvent being induced by a bout of vigorous stirring, or by adding a crystal of benzene. Follow the temperature rise accompanying crystallisation, noting the highest temperature, and tapping the thermometer with the fingernail to prevent the mercury from sticking in the stem.

Repeat the procedure until three consistent readings for the freezing point of pure solvent have been obtained. Introduce a weighed tablet of naphthalene (about 0.12 g) through the side arm, dissolve it in the benzene with gentle warming, and determine the freezing point of the solution. When consistent results have been obtained, a second weighed tablet should be introduced to obtain a second reading.

Treatment of results Calculate the molality of the solution in both parts of the experiment using eq. (17) and the observed ΔT_f values. Calculate M_2 from eq. (5).

Experiment 7 *Detection of association on solution*

Many carboxylic acids associate in non-polar solvents, generally to form dimers. Acetic, benzoic and phenylacetic acids are among those showing this phenomenon.

Method Weigh 1, 2 and 3 g of dry phenylacetic acid into each of three graduated flasks (50 ml), make up approximately to volume with dry crystallisable benzene, stopper, and re-weigh (weight concentrations are required; the graduated flasks are merely convenient containers). Determine the freezing point of each solution, and of a sample of the solvent, by the technique of the preceding experiment.

Treatment of results

1. Calculate the apparent molecular weight, M, for phenylacetic acid in each solution. Compare this with the monomer molecular weight of 136.

2. Calculate the degree of association, α, of the phenylacetic acid in each solution, assuming that dimers are formed, e.g. $2C_6H_5CH_2COOH \rightleftharpoons (C_6H_5CH_2COOH)_2$.

For one mole of solute $(1 - \alpha)$ moles are unassociated, and $\frac{1}{2}\alpha$ are associated. The total number of moles is therefore $\frac{1}{2}\alpha + (1 - \alpha)$. Using the usual freezing point equation, calculate the freezing point depression $(\Delta T_f)_0$ assuming that no association took place, i.e., for the monomer of molecular weight, M_0.

Now,

$$(\Delta T_f)_{exp} = \left[(1 - \alpha) + \frac{\alpha}{2}\right](\Delta T_f)_0$$

$$\therefore \quad (1 - \alpha/2) = \frac{(\Delta T_f)_{exp}}{(\Delta T_f)_0} = \frac{M_0}{M}$$

Hence,

$$\alpha = \frac{2(M - M_0)}{M} \qquad (22)$$

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an extent which is indicated by the refractive index of the medium. When the latter is optically inactive both circularly polarised components are retarded to the same extent and the beam emerges from the medium polarised in the same plane as the incident beam. If the medium is optically active the components are retarded to different extents because the refractive indices of the medium for left circularly polarised light (n_L) and right circularly polarised light (n_R) differ. As a result of this circular birefringence ($\Delta n = n_L - n_R$) the beam emerges from the medium still plane-polarised but with the plane of polarisation inclined at an angle α degrees to the plane of polarisation of the incident beam, given by:

$$\alpha = \frac{1800}{\lambda} l \, \Delta n \tag{27}$$

where l is the light-path in dm and λ the wavelength in cm. α is the optical rotation of the medium and is positive when the plane of polarisation is rotated clockwise relative to that of the incident beam when viewed looking towards the light source (dextrorotation) and negative when rotation is anticlockwise (laevorotation).

If the wavelength of the light is such that the medium absorbs a fraction of the radiation, an optically active medium may show a second physical effect arising from unequal absorption of left and right circularly polarised light. As a result of this circular dichroism the beam emerges from the medium elliptically polarised. The ellipticity, ψ degrees, of the emergent beam is given by:

$$\psi = \frac{1800}{\lambda} l \, \Delta\kappa \tag{28}$$

where l and λ have the same meaning as in eq. 27, for optical rotation, and $\Delta\kappa = \kappa_L - \kappa_R$, the difference between the absorption indices* for left and right circularly polarised light. Using the more familiar absorbance, A (Chapter 7):

$$\psi = \frac{2.303 \times 1800}{4\pi} \Delta A \tag{29}$$

Any medium that shows circular dichroism must at the same time show circular birefringence and hence optical rotation. Both result from unequal interaction of the medium with left and right circularly polarised light and hence are closely related phenomena.

A more detailed account of the relationship between optical rotation and circular dichroism is given by Foss (1963).

*κ is defined by the equation:

$$I = I_0 e^{-4\pi\kappa/\lambda}$$

where I and I_0 are the intensities of the transmitted and incident light

$$\therefore \quad \kappa = \frac{2.303\lambda}{4\pi l} A$$

Effect of concentration, solvent and temperature on optical activity

The dependence of optical rotation and circular dichroism of a solution of an optically active substance on concentration may be taken into account by calculating the specific optical rotation and ellipticity or the molecular rotation and ellipticity as shown in Table 2.6.

Table 2.6 Dependence of optical rotation and circular dichroism on concentration

Specific rotation	Specific ellipticity
$[\alpha]_\lambda^t = \frac{\alpha_\lambda^t}{lc}$	$[\psi]_\lambda^t = \frac{\psi_\lambda^t}{lc}$
units – $\mathrm{deg\,cm^2\,decagram^{-1}}$	
Molecular rotation	**Molecular ellipticity**
$[\Phi]_\lambda^t = \frac{M[\alpha]_\lambda^t}{100}$	$[\Theta]_\lambda^t = \frac{M[\psi]_\lambda^t}{100}$
units – $\mathrm{deg\,cm^2\,dmol^{-1}}$	

α_λ^t = optical rotation
ψ_λ^t = ellipticity
} in degrees at temperature t°C and wavelength λ nm

c = concentration, g $\mathrm{cm^{-3}}$, of solute
l = light-path, dm
M = molecular weight of solute

It may also be shown that

$$[\Theta] = 3300\ \Delta\epsilon$$

where $\Delta\epsilon = \epsilon_L - \epsilon_R$, the difference between the molar-absorptivities of left and right circularly polarised light.

Measurements of optical rotation are frequently made with sodium D light and usually, though not necessarily, at 20°, and specific rotations based on such measurements are reported as $[\alpha]_D^{20}$. Specific rotation is generally concentration-dependent and in dilute solution an equation of the type

$$[\alpha] = A + Bc + Cc^2 \qquad (30)$$

can be used to describe results, A, B and C being constants. It is necessary always to state the concentration at which specific rotation was measured unless a procedure for extrapolating $[\alpha]$ to zero concentration and quoting $[\alpha]_{c=0}$ is followed.

Temperature may have a pronounced effect on specific rotation, and suitable temperature control is required for precise work. Effects may arise due to changes of intermolecular interactions with temperature, or to changes in equilibria between configurations. Tartaric acid provides an example of the latter effect, the variation in $[\alpha]$ being about 10%/°C, owing to the equilibrium between two forms with different optical rotatory power varying with temperature.

The particular solvent used can have a large effect on the results, e.g.

a 20% w/w solution of nicotine in chloroform has $[\alpha]_D^{20} \simeq +4°$, while the same concentration in water gives $[\alpha]_D^{20} \simeq +10°$. Chloramphenicol gives a change in sign, for example $[\alpha]_{25}^{D} = +19°$ in ethanol changes to $-25°$ in ethyl acetate. These effects, which can be very large, indicate that a statement of the solvent used must always accompany any report of $[\alpha]$.

For pure liquids $[\alpha]_D^{20} = \alpha/ld^{20}$ where d^{20} = relative density of the substance.

Optical Rotary Dispersion (ORD) and Circular Dichroism (CD) spectra

Information about the structure of organic compounds and their optical purity can be gained from measurements of optical rotation at a single wavelength. More detailed and precise information can be gained by recording the variation of α with wavelength (ORD spectra) or of ψ with wavelength (CD spectra).

The ORD, CD and absorption spectra of D-camphor-10-sulphonic acid (Fig. 2.12) illustrate many of the general features of optical activity. At wavelengths well clear of the absorption bands of the carbonyl group ($\lambda > 450\,nm$) $[\Phi]$ increases in magnitude as λ decreases and, as in many other examples, the spectrum can be fitted by an equation of the form

$$[\Phi] = \frac{K}{\lambda^2 - \lambda_0^2} \quad \text{(one-term Drude equation)} \qquad (31)$$

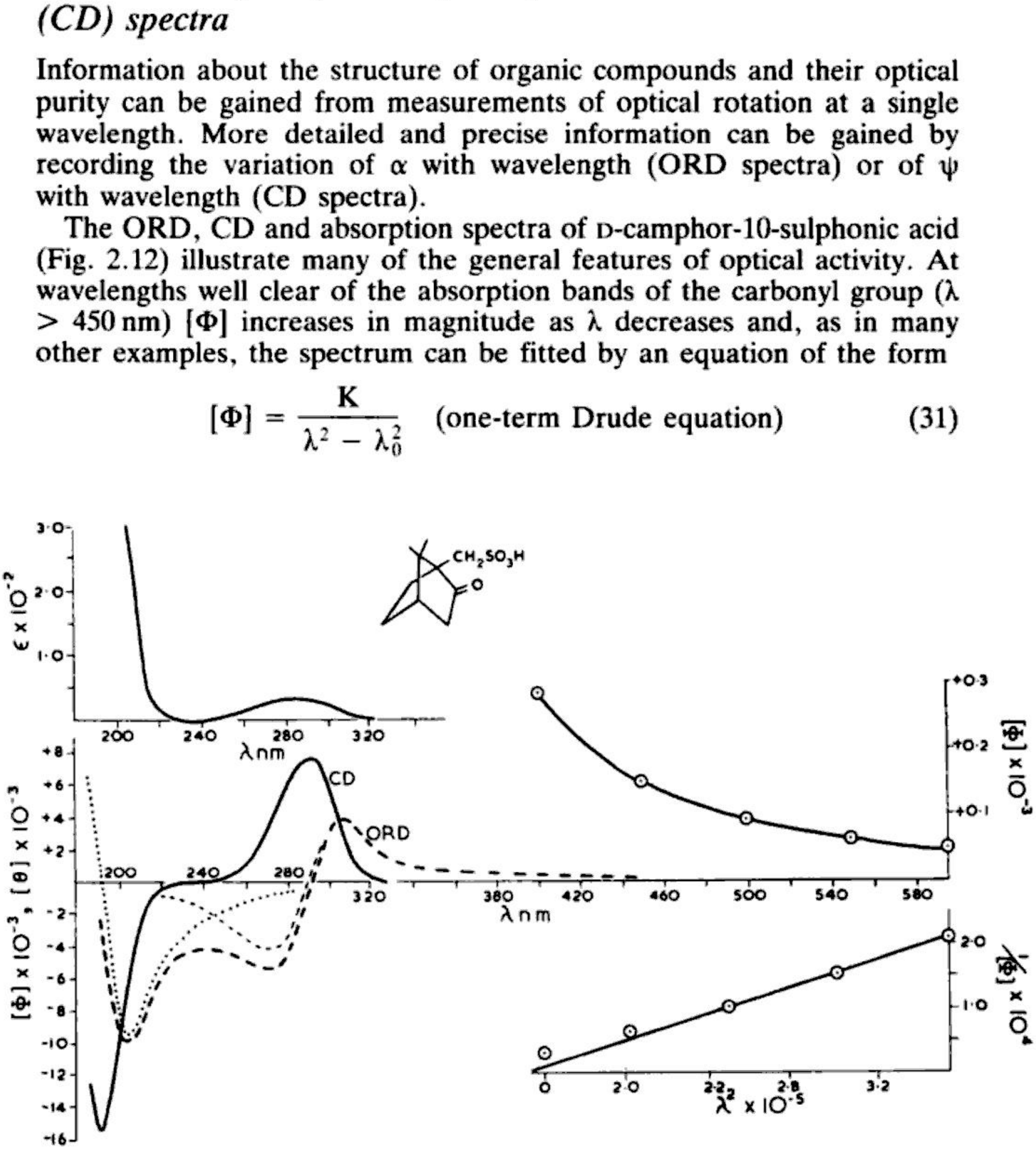

Fig. 2.12. Absorption, ORD and CD spectra of D-camphor-10-sulphonic acid in water at 27°

where K and λ_0 are constants, as shown by the linearity of the $1/[\Phi]$ *vs* λ^2 plot. For some substances more complicated Drude equations are required. The ORD spectra of many optically active compounds are of this 'plain' type which may be positive, as in this case, or negative. One obvious advantage in analytical work of measuring α at wavelengths lower than the sodium D line (589.6 nm) is the gain in sensitivity; the molecular rotation of D-camphor-10-sulphonic acid at 370 nm is about ten times its value at 589.6 nm.

In the region of the absorption band (λ_{max} = 287 nm) D-camphor-10-sulphonic acid shows circular dichroism, the shape of the CD spectrum between 240 nm and 320 nm being similar to that of the absorption spectrum. The ORD spectrum in this region takes a sigmoid course (*cf* the variation of refractive index with wavelength, Fig. 2.8) with a distinct peak and trough (extrema) at 306 nm and 270 nm and change of sign at 290 nm. This is known as a Cotton effect and by convention, when the sign of the CD is positive and the higher wavelength extremum of the ORD spectrum is positive, the Cotton effect itself is said to be positive.

Below 240 nm D-camphor-10-sulphonic acid has a second optically active absorption band ($\lambda_{max} \simeq 190$ nm) but this time the Cotton effect is negative. The CD and the higher wavelength extremum of this part of the ORD spectrum (204 nm) are both negative (the lower wavelength extremum is inaccessible).

In this relatively simple example the two absorption bands associated with the single carbonyl chromophore are well resolved, as are the corresponding CD peaks. Circular dichroism and optical rotation both result from the unequal interaction of left and right circularly polarised light with chromophores in chiral molecules and are closely related phenomena. The ORD spectrum corresponding to a particular CD band can be calculated from the CD spectrum by means of a Kronig–Kramers transform:

$$[\Phi]_\lambda = \frac{2}{\pi}\int_0^\infty [\Theta]_{\lambda'}\frac{\lambda'}{\lambda^2 - \lambda'^2}\,d\lambda' \qquad (32)$$

where $[\Phi]_\lambda$ is the molecular rotation at wavelength λ, $[\Theta]_{\lambda'}$ is the molecular ellipticity at wavelength λ', and λ and λ' are the main variable and parameter of integration respectively. The dotted lines in Fig. 2.12 are the approximate contributions to the ORD spectrum calculated from the two CD bands of D-camphor-10-sulphonic acid. The ORD Cotton effects are incompletely resolved since they spread on either side of the corresponding CD bands. In more complex molecules, possibly with several optically active chromophores, the higher resolving power of CD is advantageous in determining the contribution of each chromophore to optical activity. On the other hand, many optically active compounds have absorption bands at such low wavelengths that their solutions show no CD in the accessible spectral region; the ORD Cotton effects associated with these absorption bands extend into the accessible spectral region so that the optical activity of such substances can be studied

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Concepts of particle size: the equivalent sphere

The notion of 'particle size' is not as straightforward as might at first appear. Consider the geometrically regular parallelepiped in Fig. 3.1a:

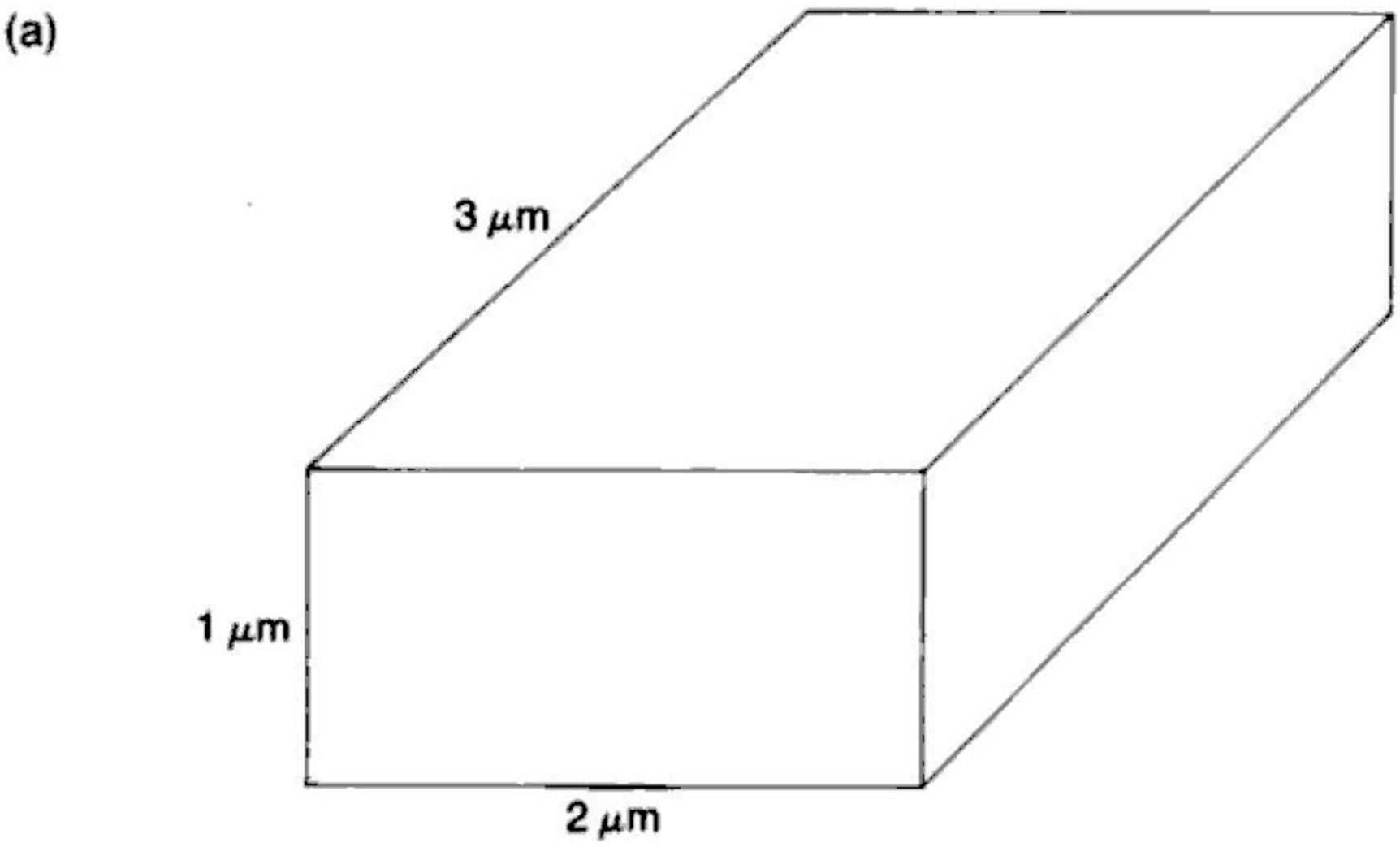

Fig. 3.1. (a) An idealised particle. (b) Different *habits* of crystalline particles: (1) tabular, (2) platy, (3) prismatic, (4) acicular, (5) bladed

clearly no one linear dimension characterises its size. Real particles are even more difficult to define because they adopt a variety of shapes (Fig. 3.1b), which are rarely geometrically regular or bound by smooth surfaces. To circumvent this difficulty the concept of an **equivalent**

sphere is utilised because, uniquely, size parameters such as surface area and volume are defined by the diameter; essentially the 'size' allocated to a particle is the diameter of a hypothetical sphere which exhibits the same measurable property as the particle. Since different techniques of particle size analysis measure different parameters, a number of **equivalent sphere diameters** are possible:

Sieve diameter (d_s):	the nominal sieve aperture width through which the particle just passes.
Projected area diameter (d_p):	diameter of a sphere of projected area equal to the projected area of the particle resting in its most stable position.
Stokes' diameter (d_{St}):	diameter of a sphere of equal density having the same settling velocity as the particle in a fluid medium within the range of Stokes' Law.
Volume diameter (d_v):	the diameter of a sphere of equivalent volume.
Volume-surface diameter (d_{vs}):	the diameter of a sphere with the same ratio of volume to surface area.

These of course differ numerically from each other, the discrepancy increasing the more anisodiametric the particle; e.g. for the particle in Fig. 3.1a:

$$d_s = 2\,\mu\text{m}$$
$$d_p = 2.76\,\mu\text{m}$$
$$d_{St} = 2.08\,\mu\text{m}\ (\rho = 1\,\text{g cm}^{-3}),$$
$$d_v = 2.25\,\mu\text{m}$$

It is important to remember therefore that the validity of particle size data is linked to the particular technique used, which if possible should be chosen in view of the end-use of the data; e.g. d_{St} may be more appropriate for a powder destined for formulation as a suspension. Semi-empirical mathematical procedures are available for the interconversion of equivalent sphere diameters.

Size distribution

Consider a sample of powder examined under a microscope from which 500 particles are sized by comparing them with some suitable scale so that the particles can be grouped into classes of different sizes. The number of particles lying in each interval of 2 μm is shown in Table 3.2. The particles in this particular example all lie between 2 and 22 μm. The data can be expressed in the form of a histogram (Fig. 3.2) where the abscissa represents the particle size interval and the ordinate the frequency per interval. However, the histogram does not provide a unique pattern for a given particle size distribution, since its shape varies if the scale of particle size intervals is changed.

Table 3.2 Size distribution in a sample of 500 particles

Size interval in μm	2–4	4–6	6–8	8–10	10–12	12–14	14–16	16–18	18–20	20–22
Number of particles	25	88	107	110	55	45	30	18	12	10
% Frequency in each interval	5	17.6	21.4	22	11	9	6	3.6	2.4	2
% Cumulative undersize	5	22.6	44	66	77	86	92	95.6	98	100

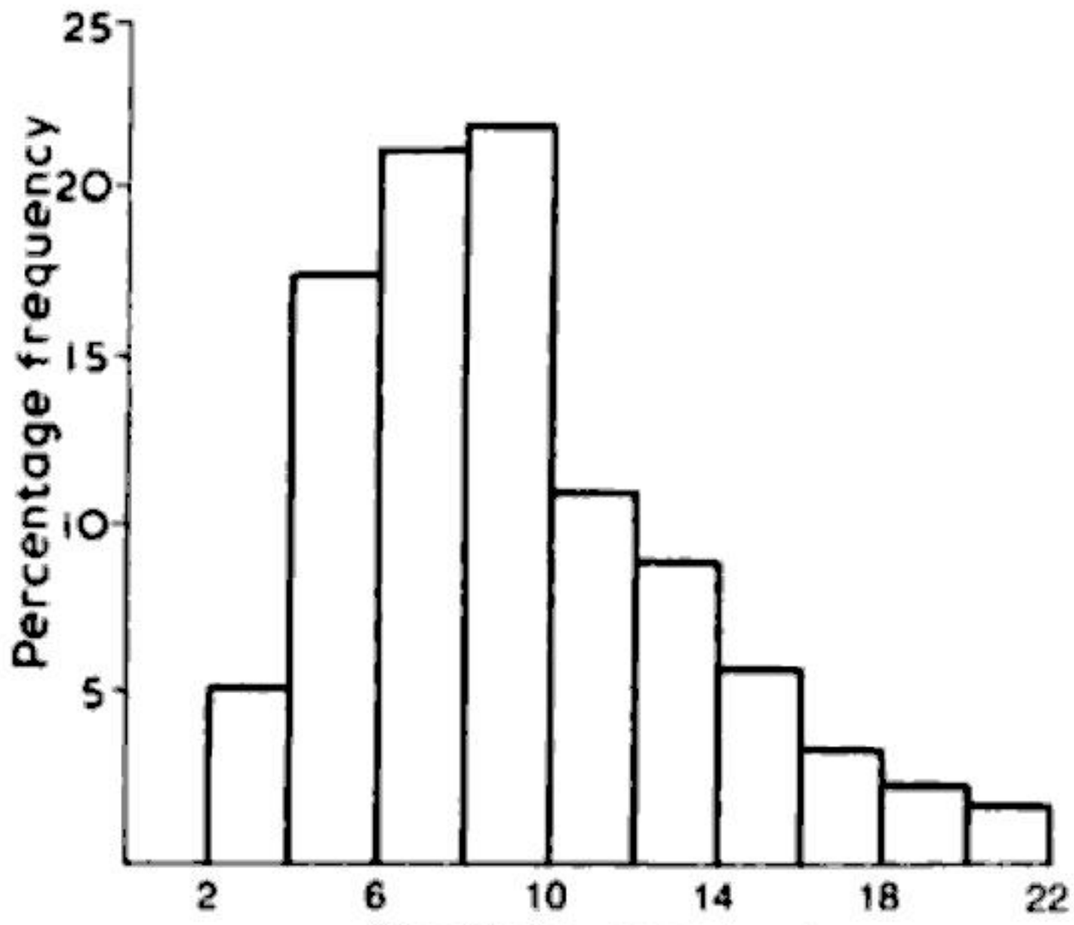

Fig. 3.2. Number-frequency histogram (from data in Table 3.2)

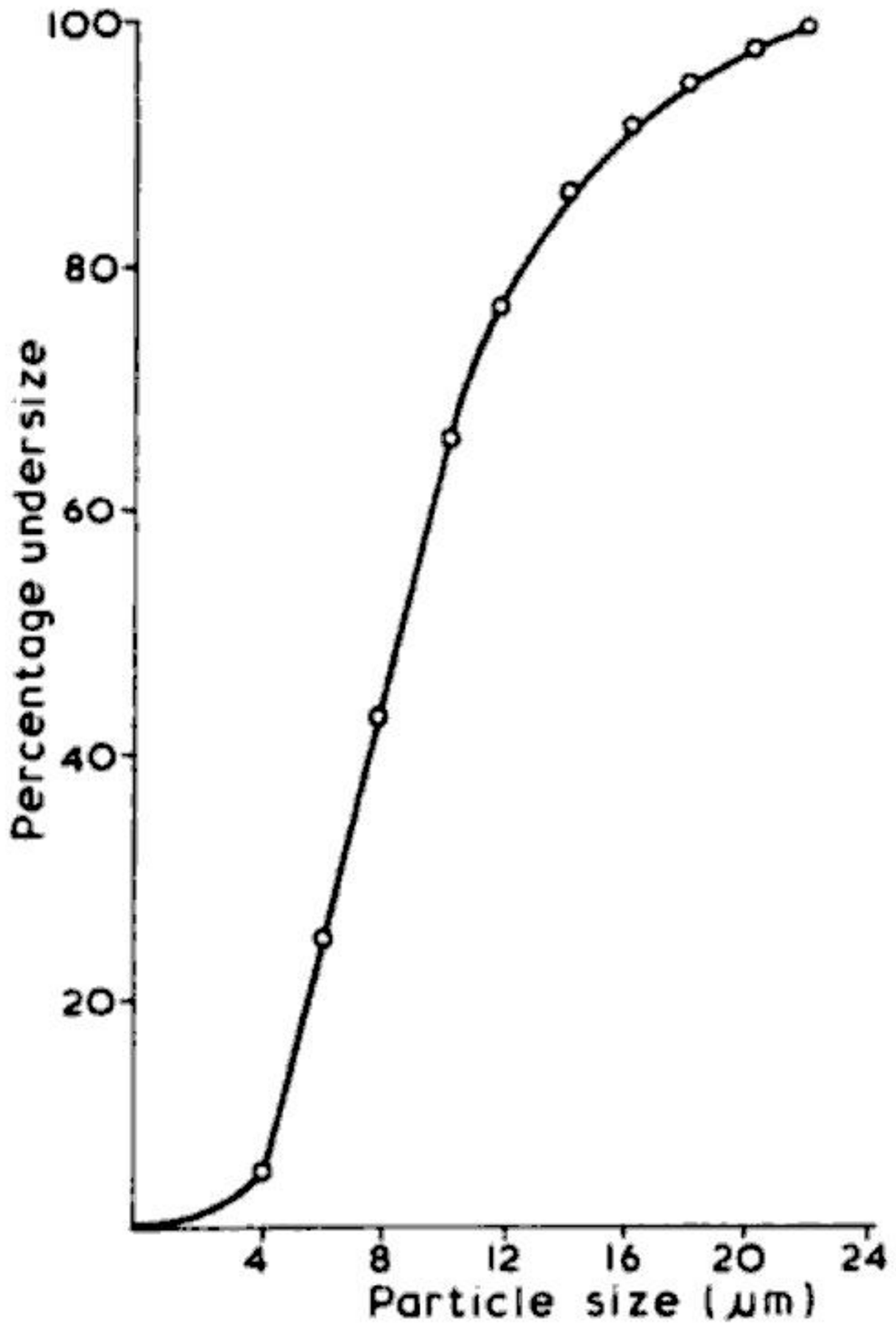

Fig. 3.3. Cumulative number-frequency undersize curve (from data in Table 3.2)

An alternative method is to express the data in the form of a cumulative distribution curve where the cumulative percentage larger (oversize) or smaller (undersize) is plotted against the particle size as shown in Fig. 3.3.

The example just discussed was a size distribution based on numbers of particles. In sieving and sedimentation methods the distribution is calculated on a weight basis. If it is necessary to convert from a size distribution by number to one by weight, the conversion is usually made by assuming that all the particles have the same shape and density.

Mean size of a particulate system

The concept of *mean size* requires some explanation since it may be calculated on the basis of numbers of particles, weight of particles or surface area of particles. This acquires importance in correlation problems.

The simplest average diameter is the arithmetic mean diameter $\bar{d}_n$ which we define for two diameters d_1 and d_2 as

$$\bar{d}_n = (d_1 + d_2)/2$$

In general terms we define the **length-number mean diameter** by

$$\bar{d}_{ln} = \frac{\Sigma\, n \cdot d}{\Sigma\, n} \tag{1}$$

where n is the number of particles with diameter d. In many pharmaceutical applications the surface area of the particles is important and the **volume-surface mean diameter** can be employed. This is defined as

$$\bar{d}_{vs} = \frac{\Sigma\, nd^3}{\Sigma\, nd^2} \tag{2}$$

because the surface area is proportional to nd^2. The significance of volume-surface mean diameter $\bar{d}_{vs}$ is that the **specific surface area** (surface area per unit weight) can be calculated from it by the equation

$$S = \frac{\Sigma\, n\, \pi d^2}{\Sigma\, n\, (\pi/6)d^3\sigma} = \frac{6}{\bar{d}_{vs}\sigma} \tag{3}$$

where σ is the density of the solid. This equation is strictly correct only for spherical or cubical particles but may be used without correction for shape if the particles are not too asymmetrical.

If the weight of each fraction, rather than number or surface area, is noted then the **weight-moment mean diameter** is obtained from

$$\bar{d}_{wm} = \frac{\Sigma\, nd^4}{\Sigma\, nd^3} \tag{4}$$

This quantity emphasises the larger particles in a sample.

It is important that the terminology of mean diameters used above is strictly adhered to. For example, the weight-moment mean diameter is not the same as the diameter of a particle of mean weight. The latter

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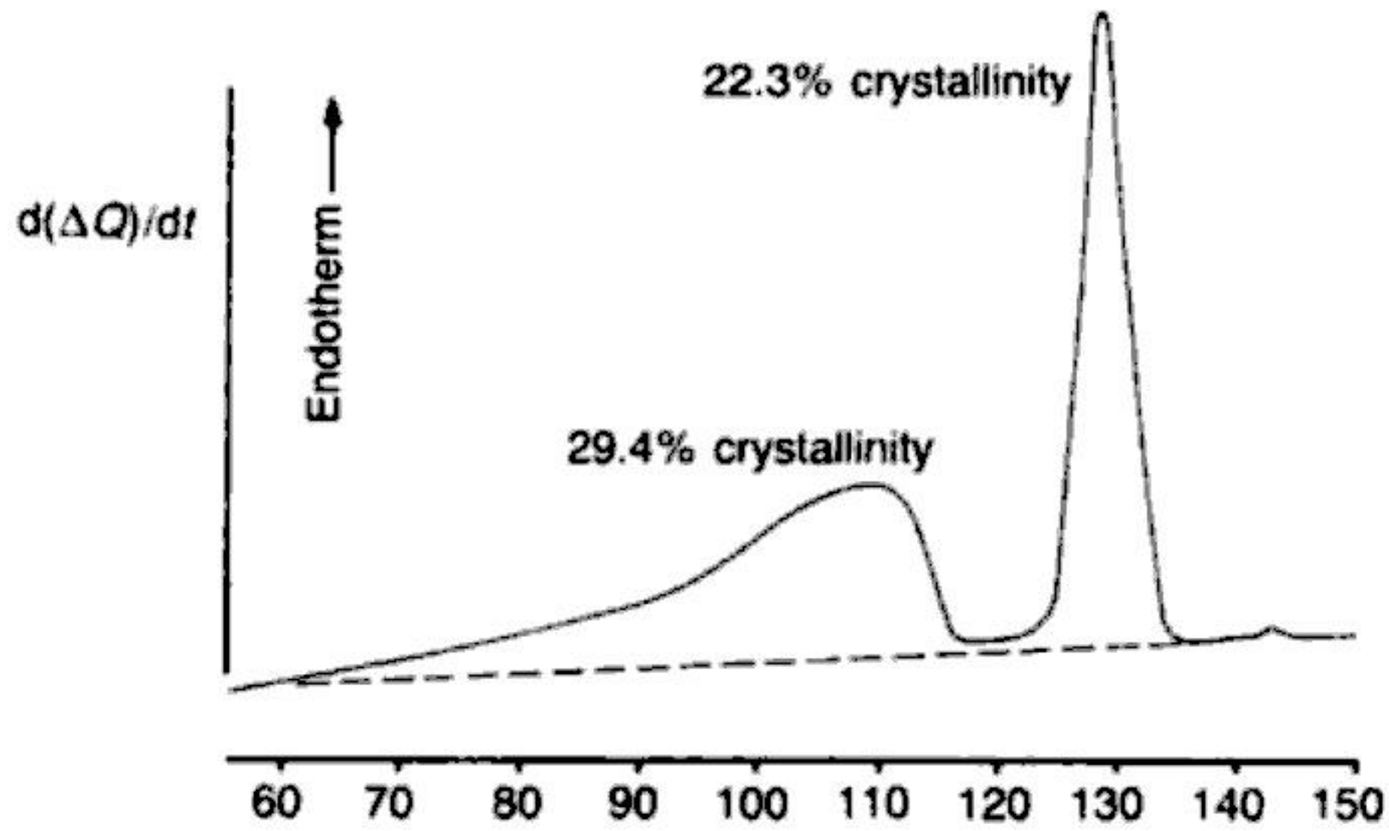

Fig. 3.15. Illustration of the use of DSC in analysis of polymer blends, which are often difficult to distinguish from copolymers by usual analytical techniques. The figure shows the melting region of a blend of 25% linear and 75% branched polyethylene. The high melting crystallites produce a peak well-resolved from that of the crystallites consisting of shorter linear and branched molecules. Total crystallinity 51.7%, from which pharmaceutically relevant parameters such as relative water vapour permeability may be calculated (redrawn, with permission, from Perkin-Elmer Corp. literature.)

where $\Delta H_{f,1}$ is the heat of fusion of the sample in J mol^{-1}, R the gas constant, T_1 (K) is the melting point of a pure sample, T_s is the temperature of the sample, F is the fraction of sample melted at T_s, and $\Delta T = T_1 - T_s$. On rearrangement and substitution we obtain

$$T_s = T_1 - \frac{RT_1^2 x_2}{\Delta H_{f,1}} \cdot \frac{1}{F}$$

For compounds which are 99.5 mol % pure ($x_2 = 0.005$) or more, the melting point depression is very small but even at this level of purity the melting range will have increased considerably. It is thus the range combined with the melting depression that is used in the assessment of thermally stable compounds of high purity by DSC. In the *dynamic method* the small (1–3 mg) sample is heated slowly (~1°C min^{-1}) through the melting range in order to minimise thermal lag and allow equilibrium. The fraction melted (F) at any temperature T_s is obtained from the ratio of the area under the curve (AUC) at T_s to the total AUC of the endotherm. A plot of sample temperature versus $1/F$ should be a straight line with a slope equal to $-RT_1^2 x_2/\Delta H_{f,1}$ and an intercept of T_1. The procedure is illustrated in Fig. 3.16; in this example the slope is 0.227, and from the DSC curve AUC $\Delta H_{f,1} = 27.6$ kJ mol^{-1}. Substituting the values in eq. (15)

$$0.227 = \frac{8.314 \times (428.28)^2}{27.6 \times 1000} x_2$$

$$\therefore\ x_2 = 0.004$$

$$\therefore\quad \text{sample purity} = 99.6\ \text{mol\%}$$

The method has been critically reviewed by van Dooren and Muller (1984).

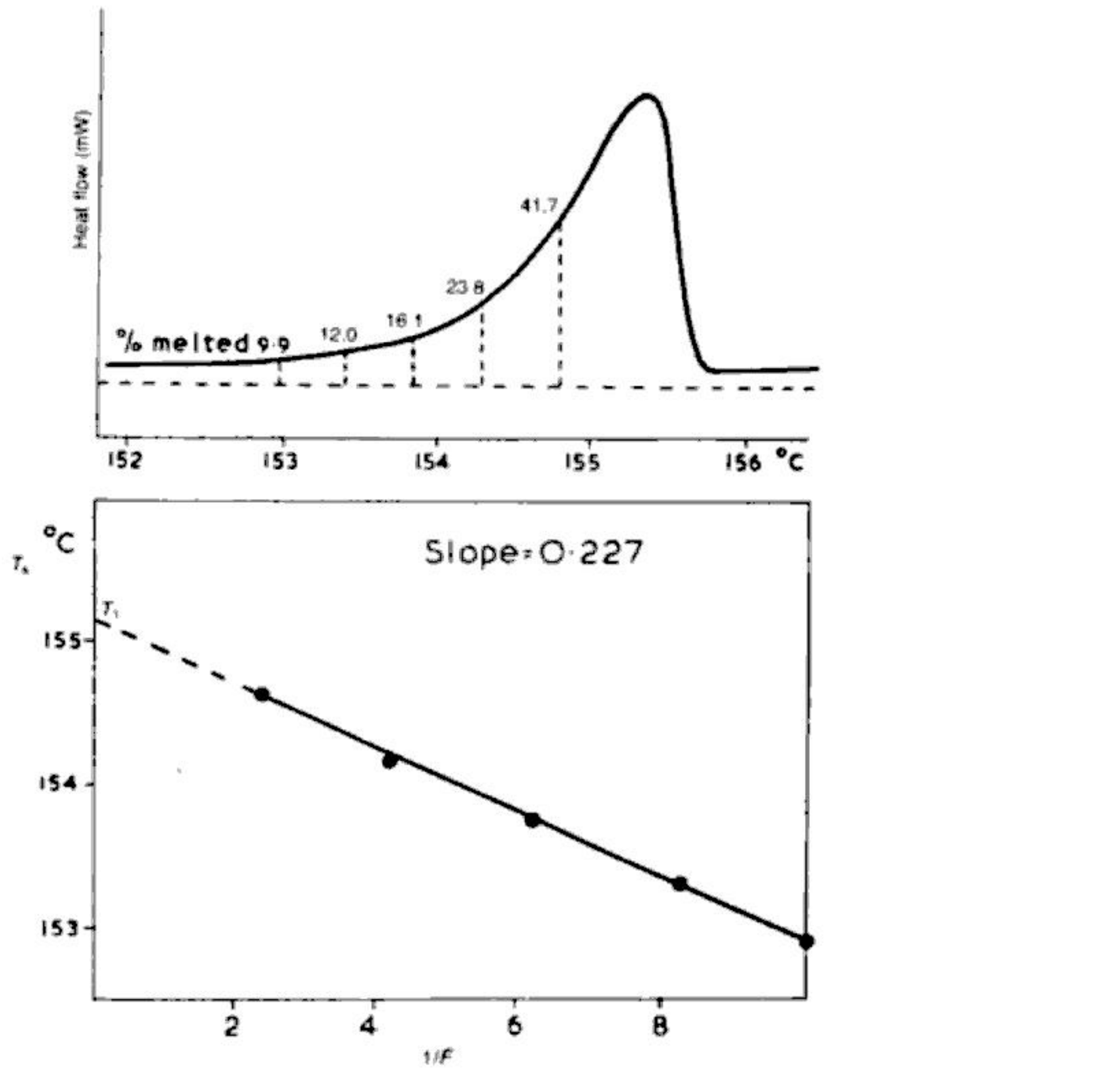

Fig. 3.16. Purity determination of testosterone by DSC. The lower diagram is a plot of the reciprocal of the fraction melted ($1/F$) as a function of the temperature of a sample of testosterone heated at $1.25° \text{ min}^{-1}$

Thermogravimetry

In this technique the mass of a sample is monitored while it is being subjected to a controlled temperature programme. Although only events associated with changes in mass under *dynamic* or *isothermal* conditions are recorded, TG may be considered the modern equivalent of gravimetry and is one of the most widely used thermoanalytical methods.

The essential parts of a thermobalance are the programmable furnace, microbalance and recorder (Fig. 3.17). Commercially available instruments can heat samples to 1500° at rates of $2.5° \text{ min}^{-1}$, under special atmospheric conditions (e.g. inert gas, vacuum), and detect mass changes as small as $0.1\ \mu g$. The rate of mass loss can also be monitored, and the exhaust gas from the furnace purged into a gas chromatograph or mass spectrometer for analysis.

The usefulness of thermogravimetry is illustrated by the TG curve obtained on heating hydrated calcium oxalate (Fig. 3.18a): from ambient temperature to 100° the monohydrate is stable, losing its water of crystallisation between 100 and 226° to give the anhydrous form, which in turn remains stable to 420°, when it decomposes to calcium carbonate and carbon monoxide, the residual solid finally decomposing to calcium oxide with the evolution of carbon dioxide between 660 and 840°. The

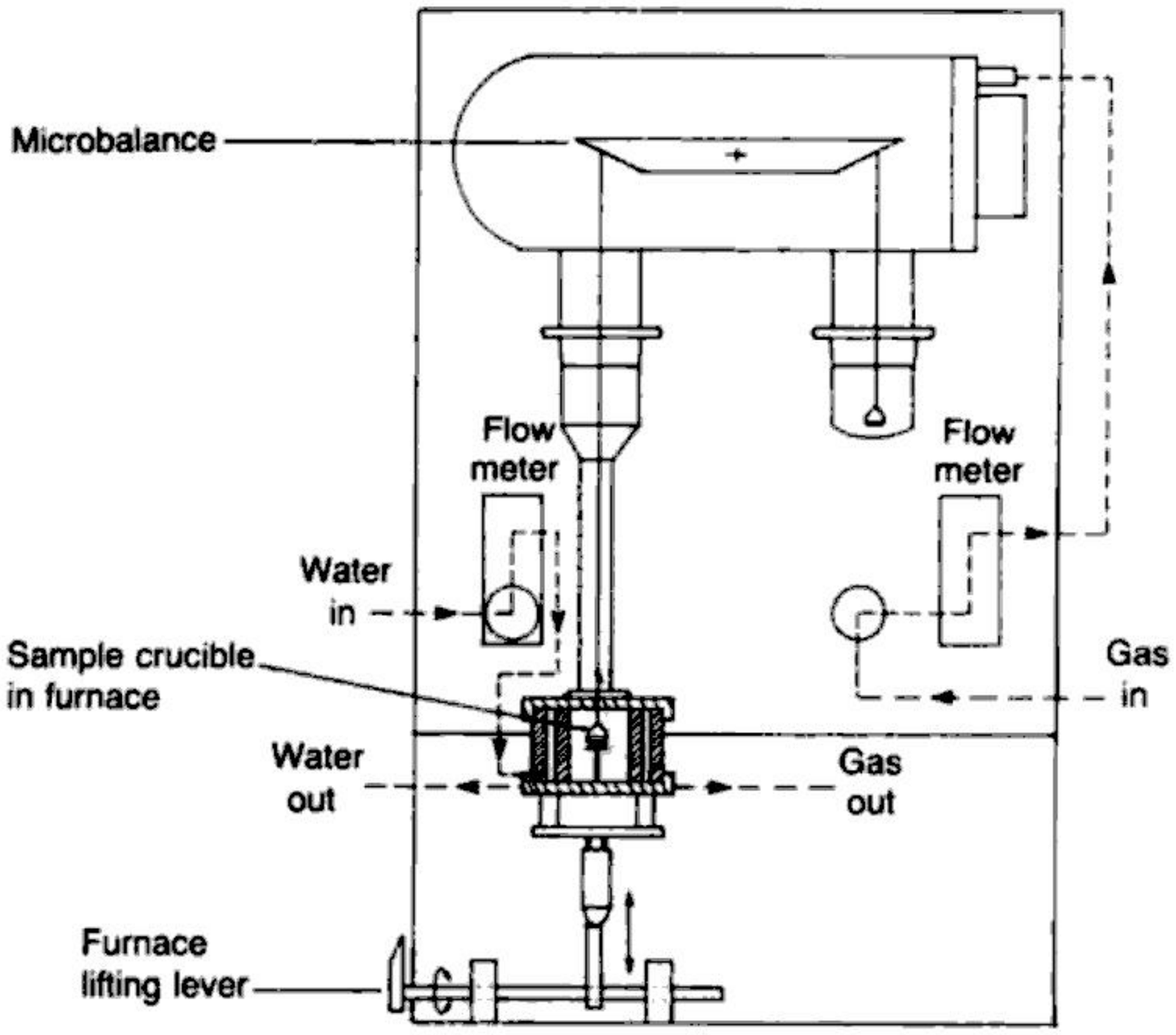

Fig. 3.17. Schematic diagram of a thermobalance (courtesy of Stanton-Redcroft Ltd)

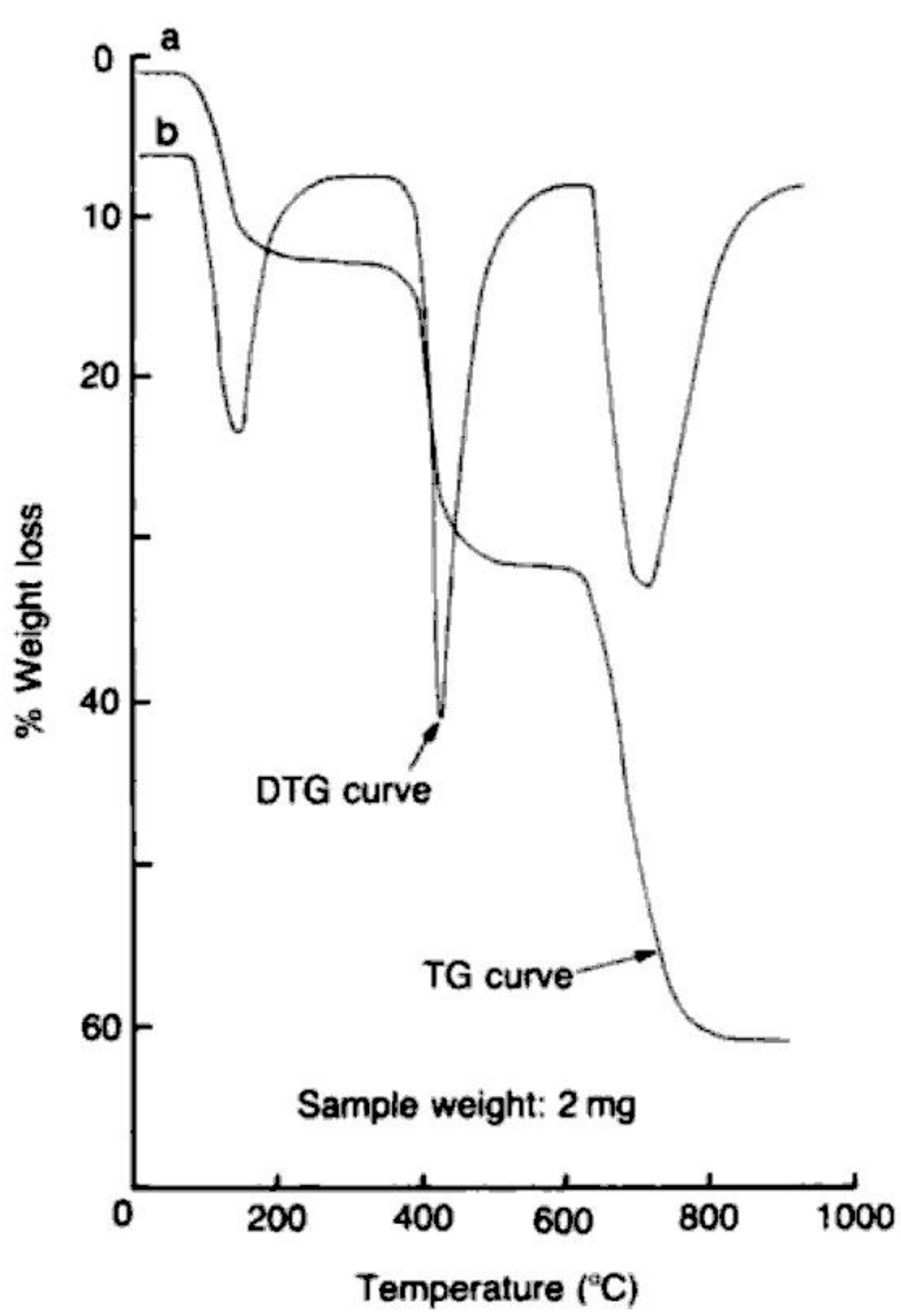

Fig. 3.18. Thermogravimetric analysis of calcium oxalate monohydrate: (a) TG curve; (b) derivative TG curve (courtesy of Stanton-Redcroft Ltd)

relatively complicated behaviour of hydrated calcium oxalate may also be represented by plotting the first derivative of the TG curve (i.e. rate of mass loss against temperature, Fig. 3.18b), a facility available with modern instruments. The advantages of DTG curves are that they highlight the rate and extent of mass loss and often closely resemble the DTA curves for specimens.

Practical experiments

Experiment 5 *Demonstration of a pre-melting crystalline transition by DSC/DTA*

Method Run a sample of potassium nitrate (unground) in static air at $10° \, min^{-1}$ over the temperature range ambient to 350°. After the temperature has reached about 350°, allow the sample chamber to cool to about 100° by passing liquid nitrogen through the cooling assembly and repeat the run up to 400°.

Note the peak melting and crystalline transition temperatures and comment on the effects of the cooling and reheating cycles.

Experiments 6 and 7 *Detection of polymorphism and pseudo-polymorphism in pharmaceuticals by DSC or DTA*

Triamcinolone Forms A and B. A large number of pharmaceuticals exhibit polymorphism and most can be studied by DSC or DTA techniques. Triamcinolone exists in two forms, A and B, obtained by recrystallisation from 60% aqueous propan-2-ol and from dimethylacetamide-water mixtures respectively.

Method Run samples of triamcinolone Form A and Form B from ambient to 350°. With a new sample of Form A, programme the instrument to stop the heating cycle at 270° and cool to ambient. Start the heating cycle to 350° and compare the curves with those of Form A and B. The favoured form of the compound is the form to which the melt reverts on heating and cooling.

7 *Ampicillin trihydrate*

Method Run a sample of ampicillin trihydrate from ambient to 200°. Endotherms due to the desolvation of water and to the melting transition should be observed.

Experiment 8 *Determination of the purity of a sample of testosterone by DSC*

Method Run the drug sample (about 1.5 mg) from 150° to 156° at a slow heating rate ($1.25° \, min^{-1}$). Make a Xerox copy of the chart paper. The complete curve can be cut out to the base line (obtained in the absence of sample) and the cut-out weighed (x mg). Cut out the area under the curve from the start of the trace up to 153° and weigh this (y mg). The fraction (F) melted is obtained from y/x. Cut out the area between 153° and 153.4°, and weigh this, adding the weight to that of the first portion ($y + y^1$ mg). Repeat this procedure at 153.8, 154.4 and 155°, and calculate successive F values. Plot T_S versus $1/F$. Determine T_1 by extrapolation as shown in Fig. 3.16 and calculate the slope of the line and carry out the calculation of mole fraction of the impurity (x_2) as explained in the text.

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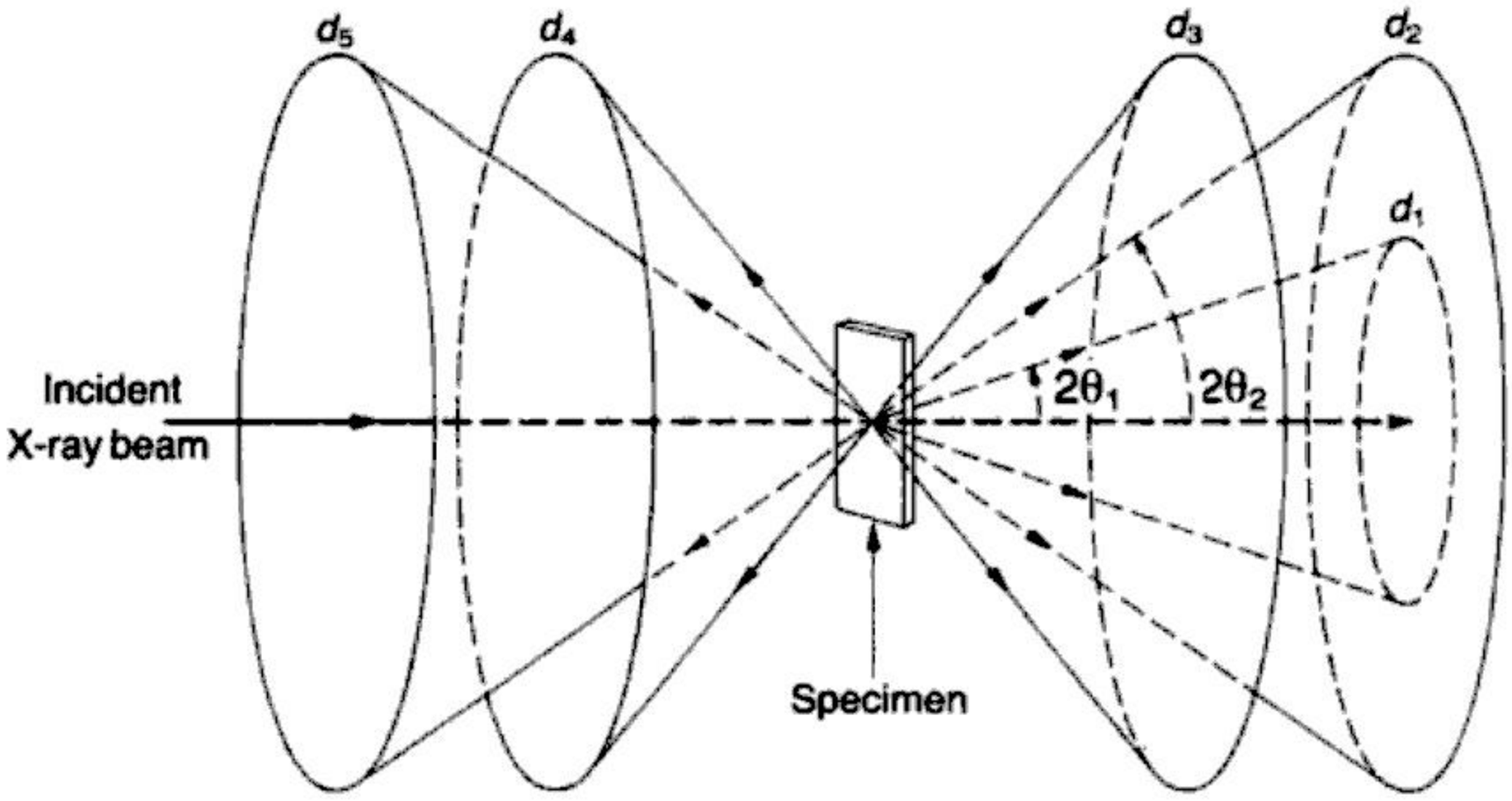

Fig. 3.23. Diffraction of X-rays by a powder sample: in the camera technique the cones, which have a common apex and correspond to particular lattice planes, intercept a photographic film strip to leave a pattern of lines of characteristic spacing and intensity (redrawn from Klug and Alexander, 1974.)

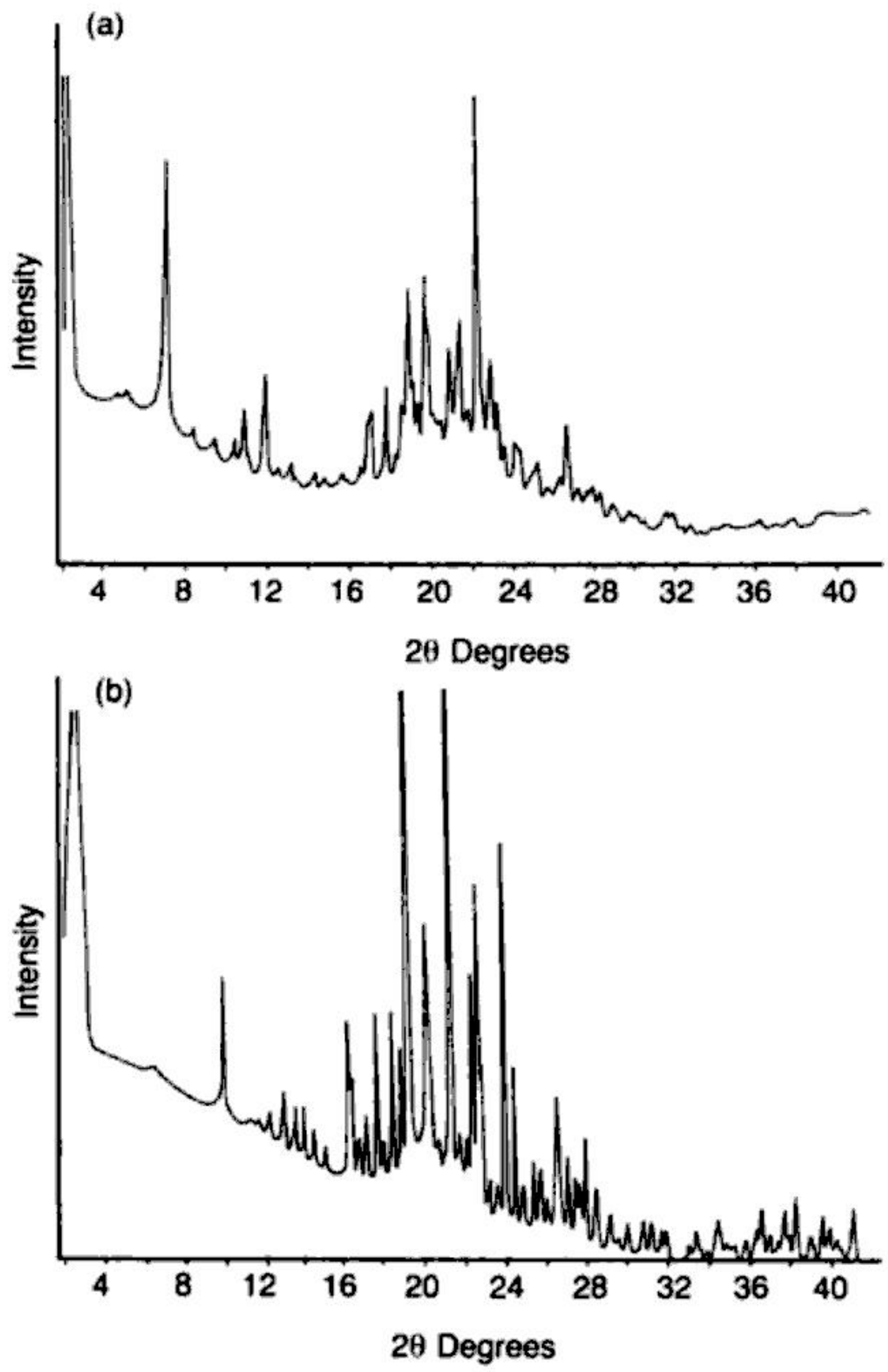

Fig. 3.24. X-ray powder diffraction patterns for chloramphenicol palmitate polymorphs: (a) Form B (or α); (b) Form A (or β) (redrawn from Szulzewsky *et al.*, 1982.)

of a diffraction pattern indicates lack of sufficiently long-range order, i.e. that the solid is essentially amorphous, broad, diffuse reflections suggest specimen particle size $\ll 0.1\ \mu m$ or a strained lattice imposed by, say, rapid precipitation or severe comminution.

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4
Chromatography

A.G. DAVIDSON

Introduction

Chromatography is essentially a group of techniques for the separation of the compounds of mixtures by their continuous distribution between two phases, one of which is moving past the other. The systems associated with this definition are:

(a) a solid stationary phase and a liquid or gaseous mobile phase (adsorption chromatography)
(b) a liquid stationary phase and a liquid or gaseous mobile phase (partition chromatography)
(c) a solid polymeric stationary phase containing replaceable ions, and an ionic liquid mobile phase (ion exchange chromatography)
(d) an inert gel which acts as a molecular sieve, and a liquid mobile phase (gel chromatography).

The basis of the separation of the components of a mixture may be defined in terms of one of these four modes of separation, or by a combination.

Advances in technology since the first simple applications of chromatography were recorded have resulted in a wide range of techniques varying in complexity, separating ability, sensitivity and cost. The modern instrumental techniques of gas–liquid chromatography and high performance liquid chromatography provide excellent separation and allow the accurate assay of very low concentrations of a wide variety of substances in complex mixtures. The older inexpensive chromatographic techniques, such as column chromatography are used in analytical and preparative separations which do not require the resolution and sensitivity or justify the expense of the instrumental techniques.

In this chapter the treatment of the principal chromatographic techniques in pharmaceutical chemistry is based primarily on the equipment used. Where appropriate, the basis of the separation is discussed with reference to the different chromatographic materials available for use in each of the techniques. To illustrate the improvements in sensitivity and resolution which have resulted from technological progress, the techniques are discussed in approximately the order of their historical development.

Column chromatography

Adsorption chromatography

The technique was originally developed by the Russian botanist Tswett in 1906 during the course of an investigation into the nature of leaf pigments. He found that leaf pigments extracted with light petroleum were adsorbed on the top of a column of calcium carbonate supported in a glass tube. As more solvent was allowed to percolate through the column the region of pigmentation became broader and finally separated into distinct and differently coloured bands. Prolonged washing with solvent caused complete separation of the bands, which could be eluted separately. It is one of the simplest laboratory exercises to illustrate the use of column chromatography. Tswett's work attracted little attention and it was not until 1931, when polyene pigments were investigated by Kuhn and Lederer, that interest in chromatography was renewed.

The principle underlying the separation of the compounds is adsorption at the solid-liquid interface. For successful separation, the compounds of a mixture must show different degrees of affinity for the solid support (or adsorbent) and the interaction between adsorbent and component must be reversible. As the adsorbent is washed with fresh solvent the various components will therefore move down the column until, ultimately, they are arranged in order of their affinity for the adsorbent. Those with least affinity move down the column at a faster rate than, and are eluted from the end of the column before, those with the greatest affinity for the adsorbent. The technique in which the individual components of a mixture are separated by eluting the column with fresh solvent is **elution analysis** (Fig 4.1).

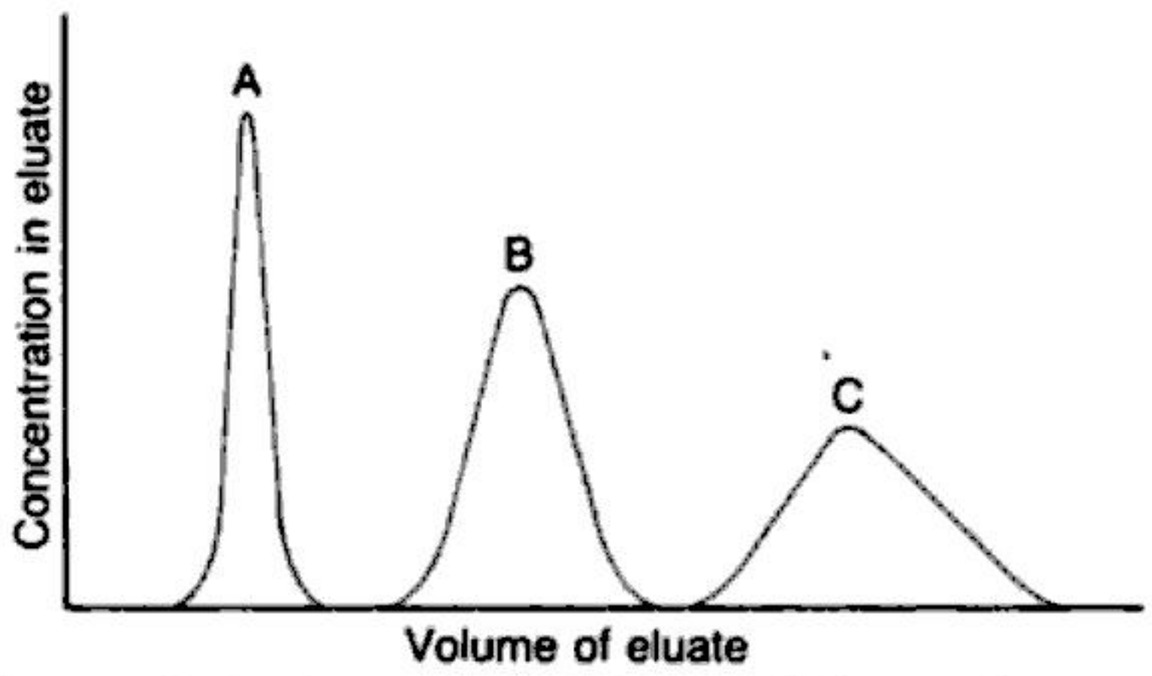

Fig 4.1. Elution analysis: the separate elution of three substances A, B and C

An adsorbent which is already saturated with respect to one substance may take up a small quantity of a second. The latter displaces the former and consequently, if a solution of a mixture is percolated continuously throught the column and the eluate is examined for the presence of substances, a plot of amount of substance (per ml of eluate) against volume of eluate will appear as in Fig. 4.2. This technique was

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Although the adsorption forces involved in chromatography are weak, cognisance must be taken of undesirable chemical changes that might occur because of the properties of the column material itself. Thus, an alkaline grade of alumina may cause hydrolysis of esters or lactones. Other changes associated with a poor choice of column are isomerisation, neutralisation of acids or bases and decomposition of compounds. The last may be put to good use in certain preparations, e.g. cadalene from oil of cade forms a crystalline picrate which is decomposed on a short column of alumina. The pure cadalene is eluted, leaving the picric acid fixed on the column.

The strongest adsorbents are silica and alumina activated by heating to about 200° to remove water. Alumina may be rendered acidic or basic prior to activation. Careful addition of water to the treated alumina allows different degrees of activity to be obtained and reproduced from batch to batch. Preliminary treatment of the material in this way often overcomes the property which leads to the undesirable effects noted above. It may, indeed, introduce increased specificity of the column for certain compounds, e.g. when silica gel is freshly prepared in the presence of propyl orange and the dye is finally removed by elution, the column has a greater affinity for propyl orange than it has for the methyl, ethyl and butyl analogues. A similar situation obtains when the silica gel is prepared in the presence of one enantiomorph of an optically active compound, e.g. laevorotatory quinine.

Adsorption is most powerful from non-polar solvents such as petroleum ether or benzene and a single solvent may often be effective in developing the chromatogram. The rate of movement of the compounds down the column can be increased by the addition of a second solvent to the mobile phase; the second solvent is usually more polar than the first. Strain (1942) has arranged both adsorbents and solvents in order of adsorptive and eluting power respectively and Table 4.1 lists the series. It is usual to redistil all solvents before use, so that traces of non-volatile matter, e.g. grease, are completely absent. The change from one solvent to another should be gradual, e.g. the change-over from petroleum ether to toluene should be done in proportions such as the following (petroleum ether first) 100:0, 95:5, 90:10, 80:20, 60:40, 40:60, 10:90, 0:100. Such a procedure is time-consuming and a more rapid elution may be achieved by the addition of about 0.5 to 1.0% of ethanol to the first non-polar solvent used.

Alteration in the composition of the eluting solvent may also be achieved by adding the second solvent gradually to a reservoir of the first with efficient mixing; the solvent entering the column therefore becomes gradually and continuously richer in the second solvent. This technique is known as **gradient elution** which, with proper choice of adsorbent and gradient, often reduces tailing of the compounds on the column.

Preparation of the column

A typical arrangement for column chromatography is shown in Fig.4.5.

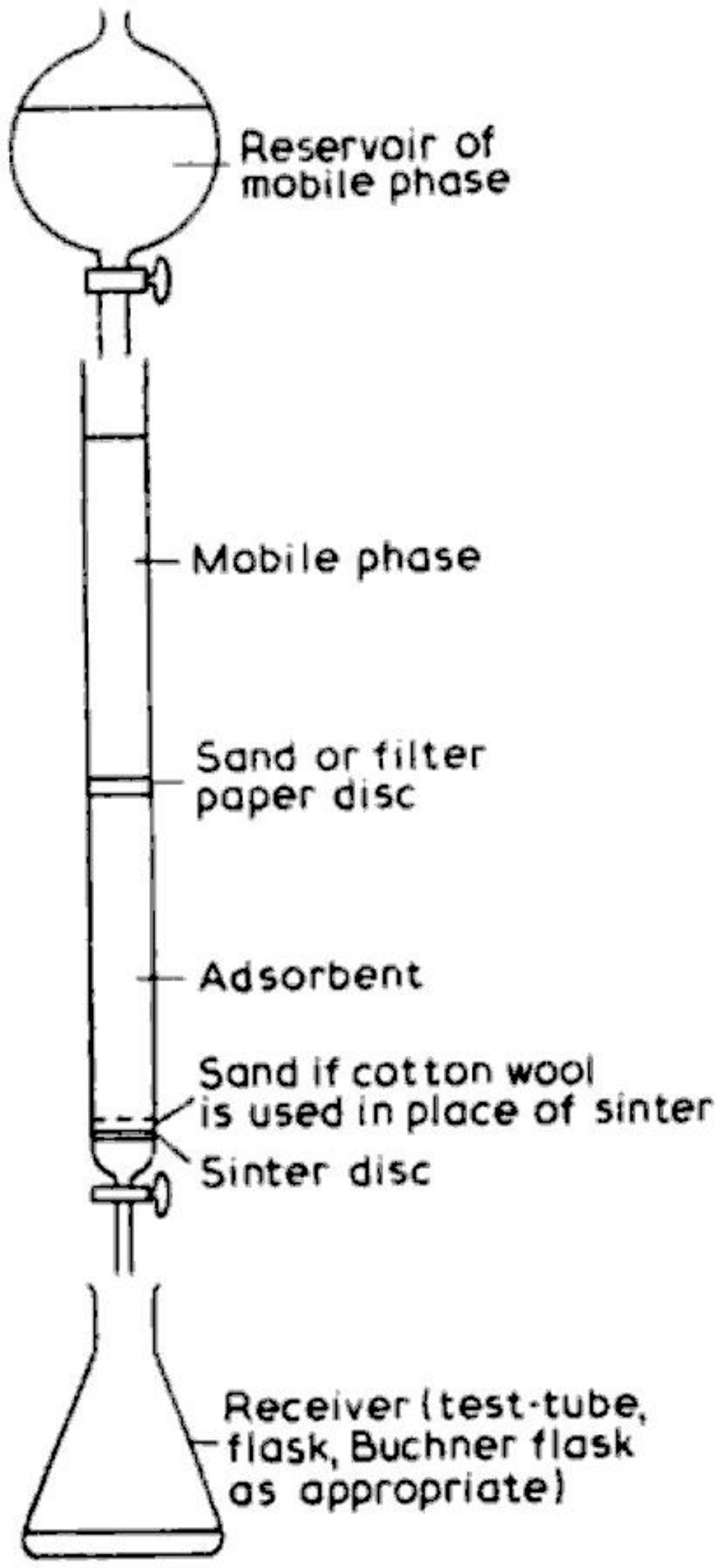

Fig. 4.5. Apparatus for column chromatography

Method Prepare the chromatographic column by mixing the adsorbent into a slurry with the solvent and pouring the mixture into the glass tube which contains solvent. The sand serves to give a flat base to the column of adsorbent when cotton wool is used instead of a sinter disc. After the adsorbent has settled, add a filter paper disc and sand then run off the supernatant liquid until the level falls to about 1 cm above the top layer of sand. The filter paper disc and sand is one means of avoiding disturbance of the adsorbent as fresh mobile phase is added to the column in the initial stages of development. The level of solvent must never be allowed to fall below the level of adsorbent, otherwise the latter develops cracks and becomes useless for chromatography because the solvent runs through the cracks rather than between the particles of adsorbent.

The preparation of the slurry may prove difficult with dense adsorbents and it is convenient to pour the powder directly into the solvent in the tube. Frequent tapping of the tube and stirring of the mixture assists in even packing and removal of air bubbles or pockets. Alternatively, the tube may be packed with the dry powder and the solvent allowed to percolate through with the stopcock open until the level falls to about 1 cm above the adsorbent.

The dimensions of the column and quantity of adsorbent depend upon the nature and amount of the substance to be chromatographed but a rough guide is given in Table 4.2.

Use of column Wash the column with about 50 ml of the mobile phase used to prepare it, which should be the least polar solvent in which the mixture will dissolve. Add the mixture dissolved in a small volume of solvent and carefully allow it to run into the sandy layer by opening the stopcock. Add a small volume of solvent and wash in the mixture. Repeat with gradually increasing quantities of solvent and develop the chromatogram, collecting the eluate in appropriate receivers if the components are to be eluted from the column.

Table 4.2 Column characteristics

Adsorbent/adsorbate weight ratio*	30:1
Length/diameter ratio†	10–15:1
Column length	
(a) multi-component system	long column
(b) components with similar affinities for adsorbent	long column
(c) components with different affinities for adsorbent	short column

*The 30:1 weight ratio is suitable for preparative separations. For analytical purposes the ratio (30:1) is much too small, and often mg quantities of substance are chromatographed on 20 g or more of adsorbent (see Experiment 3).

†In general, narrow columns give better separations than wide columns.

Detection and recovery of components

For those mixtures which are coloured, visual examination of the column is usually sufficient to locate the coloured components. Colourless components may also be detected visually if they fluoresce, e.g. quinine and ergometrine. Recovery of the components after detection on the column requires extrusion of the column of adsorbent and isolation of each zone for extraction with solvents. If plastic tubing is used instead of glass tubes the zones are conveniently isolated by cutting the tubing into sections.

It is however, more convenient to complete the chromatogram by eluting the various components with solvents. For colourless compounds the eluate is collected as a large number of fractions, each of small volume.

Automatic fraction collectors enable large numbers of fractions to be obtained without the tedium associated with manual collection. The large number of fractions also assists in obtaining better separation of components, providing attention is directed to correct choice of flow rate. Each fraction is examined appropriately for the presence of a compound. The examination may be by evaporation of the solvent from each fraction and weighing the residue, by simple spot tests, by examination of the fraction by paper or thin layer chromatography or by spectrophotometry, either directly or after addition of reagents.

Partition chromatography

All partition chromatographic separations are based upon the differences in partition characteristics (partition coefficients) of the individual components of a mixture between a liquid stationary phase and a gaseous or liquid mobile phase. In column partition chromatography, the mobile phase is a liquid.

The theoretical principles of partition chromatography may be readily understood by considering the partitioning behaviour of substances between two immiscible liquids. Few substances, when shaken with two immiscible liquids, partition completely into one or other of the liquids. Instead, most distribute themselves between the liquids such that the partition coefficient (the ratio of **concentrations** of the substance in each phase) is a constant value independent of the total amount, provided neither phase is saturated with the substance.

Substances with large differences in their partition coefficients may be completely separated by simple solvent extraction techniques involving few (one to three) extractions (Part 1, Chapter 9). As the differences in partition coefficients of a mixture of substances decrease, the number of solvent extractions necessary to achieve complete separation increases. In theory, it is possible to exploit even small differences in partition coefficients to separate chemically similar substances by carrying out a sufficiently large number of extractions. The discontinuous

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rapidly. The components whose partition coefficients favour the moving liquid travel down the column faster than those whose coefficients favour the sorbed stationary phase. The components thus emerge from the column in the order of their partition coefficients.

Tailing of the bands, which is commonly encountered in adsorption chromatography (Fig. 4.4), is rarely seen in partition chromatography (because the partition coefficients of substances do not vary with concentration) unless adsorption effects are also present. Consequently, the narrower bands obtained in partition chromatography permit the separation of closely related chemical substances, whereas this is often not possible in adsorption chromatography, which in general is used to separate compounds in different chemical classes.

Plate theory of chromatography

Martin and Synge in 1941 developed the concept of the 'theoretical plate' in order to establish a satisfactory theory for partition chromatography. The column is considered as being made up of a large number of parallel layers or 'theoretical plates', and when the mobile phase passes down the column the components of a mixture on the column distribute themselves between the stationary and mobile phases in accordance with their partition coefficients. The rate of movement of the mobile phase is assumed to be such that equilibrium is established within each plate. The equilibrium, however, is dynamic and the components move down the column at a definite rate depending on the rate of movement of the mobile phase. The R value of a component is

$$R = \frac{\text{rate of movement of component}}{\text{rate of movement of mobile phase}} = \frac{\text{distance moved by component}}{\text{distance moved by front of mobile phase}} \quad (1)$$

The interrelationship between R and the partition coefficient K can be shown to be

$$R = \frac{A_m}{A_m + KA_s} \quad (2)$$

where

A_m = average area of cross-section of mobile phase

A_s = average area of cross-section of stationary phase

$$K = \frac{\text{concentration of component in stationary phase}}{\text{concentration of component in mobile phase}}$$

Although the formula is of potential value in devising optimum conditions for chromatography, these are more frequently determined empirically.

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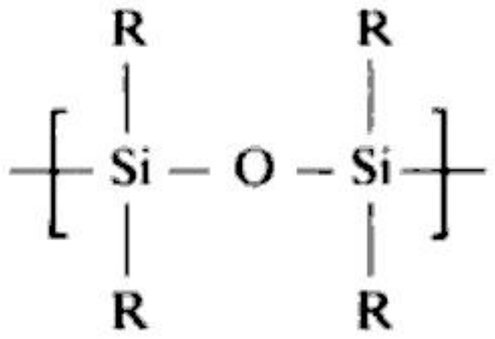
the types of compound to be separated and the packed columns available.

The selection of a packed column depends upon the type of analysis to be performed, and gas–solid chromatography is the method of choice for gases. The low solubility of gases such as oxygen, nitrogen, carbon dioxide, hydrogen and methane in liquids makes partition columns of little use. Reliance must be placed on adsorption effects and typical adsorbents are silica gel, alumina, charcoal and molecular sieves. They are affected by moisture and adsorbed gases, and must therefore be prepared for the column by heating and cooling in an appropriate carrier gas before use.

The appearance of the chromatograms often reflects the non-linearity of the adsorption isotherms (p.88, Fig. 4.4), but the disadvantage of tailing peaks can be overcome to a certain extent by 'poisoning' the adsorbent with a small amount of liquid stationary phase. **Active sites**, which are those local parts of a packed column where adsorption effects are more pronounced than elsewhere in the column, are reduced in activity. The columns prepared in this way may appear to be somewhat similar to partition columns (below), but the latter differ in that great care is taken to reduce the natural adsorption effect of the support by the method of manufacture and by preliminary treatment with a silicone compound. Further, with gases, as in this context, partition plays little or no part in the separation procedure.

Columns packed with highly porous organic polymer beads have proved very suitable for the analysis of water, alcohols and low molecular weight gas and liquid mixtures. Hollis (1966) suggests that adsorption plays little part in the separation of the components of the mixtures. The polymers in the Porapak series are based on ethylvinylbenzene–styrene–divinylbenzene (Porapak P), ethylvinylbenzene–divinylbenzene (Porapak Q) and ethylvinylbenzene–divinylbenzene modified with polar monomers (Porapak R, S and T). Janâk (1967) has pointed out the possibilities when these materials are applied as lipophilic stationary phases in thin-layer and column chromatography.

Gas–liquid chromatography offers considerably more scope for the analysis of mixtures than does gas–solid chromatography, because many stationary phases are now available. In fact, the number of liquid stationary phases in the catalogues of some suppliers of chromatographic materials exceeds 200 and this can make the task of selecting a stationary phase very difficult. The practice of many chromatographers now is to restrict the number of liquid phases used in their laboratories to approximately six. These preferred stationary phases provide the full range of polarity, from the non-polar Apiezon L grease to the polar polyethene glycol (Carbowax) 20M, and are considered to be adequate for the vast majority of separations that are required to be made by GLC (Table 4.11). The stationary phases that are used most extensively for drugs, particularly basic substances, are the low-polarity polysiloxane phases OV-1 (SE-30) and OV-17, whose structures are represented by



OV-1 R = CH_3

OV-17 R = 50% CH_3
50% C_6H_5

Moffat (1975) has recommended the use of 2–3% SE-30 as the preferred stationary phase for drugs and has published the retention indices (p.140) obtained with 480 drugs and commonly used chemicals using this packing material.

Table 4.11 Preferred stationary phases for gas–liquid chromatography

Stationary phase	Solvent for stationary phase	Temperature maximum (°C)	Uses
Apiezon L ½%	Light petroleum	245	Hydrocarbons, steroids and esters
Carbowax 1000 20%	Chloroform	150	Alcohols, chloroform, camphor, essential oils
Carbowax 20M 10% with KOH 5%	Methanol	225	Volatile bases, e.g. amphetamines
Diethylene glycol succinate (DEGS) 10%	Chloroform	190	Methyl esters of fatty acids, nitriles, essential oils
SE-30 2.5%	Chloroform	350	Hydrocarbons, methyl derivatives of barbiturates, general purposes
OV-17 3%	Chloroform	375	General purposes

The support for the liquid phase in partition columns is generally based on silica, e.g. diatomaceous earth or glass beads of suitable mesh size. Diatomaceous earth is most useful because of its porosity and because liquid phases may be incorporated to the extent of 0.5% to 25%. It is a fine powder obtained by grinding the silicaceous skeletons of marine algae (diatoms) and calcining with a small amount of sodium carbonate. The best grades (denoted by AW-DMCS) are deactivated by acid washing and treatment with dimethyldichlorosilane to reduce adsorption on the active sites. Supports should be of a uniform particle size e.g. 80–100 mesh, 100–120 mesh.

The preparation of the packed column is conveniently described by reference to a column of 10% diethlyene glycol succinate (DEGS) on Gas-Chrom Q (80—100 mesh).

Method

Coating of support Dissolve DEGS (1 g) in chloroform (40 ml) in a 250 ml round-bottom flask and add Gas Chrom Q (80–100 mesh; 9 g) to the solution, slowly, and with gentle mixing. Place the flask on a warm water-bath and apply a moderate vacuum to assist evaporation of the solvent. Rotate the flask continually and gently during this stage to avoid depositing the DEGS as a film on the glass and to avoid production of fine particles

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Typical therapeutic levels in plasma are 10–20 $\mu g\,ml^{-1}$. The following procedure is based on that of O'Connell and Zurzola (1982).

Standards Plasma from untreated subjects containing 5, 10, 15, 20 and 25 $\mu g\,ml^{-1}$ of paracetamol (Note).
Samples Plasma obtained from subjects 0.5 to 2 h after ingesting 0.5–1.0 g of paracetamol.
Method To 1 ml of plasma samples and standards in small glass vials add 1 ml of 0.6M barium hydroxide solution to denature the proteins. Vortex for 2 min to ensure thorough mixing and then add 1 ml of zinc sulphate solution (50 $mg\,ml^{-1}$) to precipitate the proteins and vortex for 1 min. Spin at a high speed for 10 min using a bench-top centrifuge. Filter through glass wool contained in the neck of a Pasteur pipette.
Chromatographic conditions
Column: μBondapak C_{18} (300 × 4.6 mm) and a silica-ODS guard column (50 × 4.6 mm).
Mobile phase: Methanol : *water* (15 : 85).
Flow rate: 1 $ml\,min^{-1}$.
Detection: Photometric detection at 240 nm.
Volume injected: 20 μl (valve or syringe).

Note The authors showed that the recovery of paracetamol from plasma was 94% of that from water. If blank plasma is not available for the standards use aqueous solutions of paracetamol as standards and multiply the sample peak heights by 1.06.

Experiment 36 *Assay of adrenaline in Adrenaline Injection by reversed-phase ion-pair HPLC*

Selection of internal standard Prepare standard solutions of the following catecholamines in 0.001M hydrochloric acid (Note 1) and determine their retention times: noradrenaline hydrochloride (0.2 $mg\,ml^{-1}$) adrenaline hydrogen tartrate (0.2 $mg\,ml^{-1}$), α-methylnoradrenaline (0.05 $mg\,ml^{-1}$), 6-hydroxydopamine HBr (0.05 $mg\,ml^{-1}$), dopamine (0.05 $mg\,ml^{-1}$), methyldopamine (0.1 $mg\,ml^{-1}$), isoprenaline hydrochloride (1 $mg\,ml^{-1}$). If necessary dilute the solutions with 0.001M hydrochloric acid to obtain suitable peak heights.
Calibration standards Prepare a series of standard solutions in 0.001M hydrochloric acid of adrenaline hydrogen tartrate containing 0.04 to 0.24 $mg\,ml^{-1}$ and a suitable internal standard from the list above, at a concentration giving a peak height similar to that of the 0.2 $mg\,ml^{-1}$ solution of adrenaline hydrogen tartrate.
Sample solution Dilute a suitable volume of the injection (Note 2) with 0.001M hydrochloric acid to give a concentration of adrenaline hydrogen tartrate of 0.15 to 0.2 $mg\,ml^{-1}$ and include the internal standard at the same concentration that is present in the standard solutions.
Chromatographic conditions
Column: Hypersil (or Spherisorb) 5-ODS (250 × 4.6 mm).
Mobile phase: Methanol : sulphonic acid buffer (below), (10 : 90) (Note 3).
Flow rate: 1.2 to 1.4 $ml\,ml^{-1}$.
Detection: Ultraviolet (285 nm) (Note 4).
Volume injected: 20 μl
Sulphonic acid buffer Dissolve citric acid (2.802 g), anhydrous disodium hydrogen orthophosphate (0.9464 g), ethylenediaminetetraacetic acid disodium salt (0.0233 g) in 820 ml of 0.005M 1-heptane sulphonic acid and dilute to 1 l with *water*.

Note 1 0.001M hydrochloric acid gives a pH of about 3 and prevents the oxidation of catecholamines which occurs rapidly at pH values in excess of 7.

Note 2 The British Pharmacopoeia formulation contains 1.8 $mg\,ml^{-1}$adrenaline hydrogen tartrate.

Note 3 The effect of varying the composition of the mobile phase from 7.5 : 92.5 to 20 : 80 methanol : sulphonic acid buffer should be investigated. The theory of reversed-phase ion-pair HPLC is discussed on p.164.

Note 4 If an electrochemical detector is available repeat the assay using more dilute solutions than those given above for photometric detection and use a potential of +0.72 V.

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5
Electrochemical methods

J.R. JOHNSON

Introduction

In electrochemical methods of analysis one or more electrically related parameters, e.g. voltage, current or charge, are measured and related to the state of the system generating or carrying the charge. In addition these methods can be divided into those relating to systems in equilibrium, e.g. measurement of pH potentiometrically, and those dependent on a transient perturbation being applied to the system before the measurement is made, e.g. voltammetry.

Conductimetric titrations

Theory

The simplest electrochemical method by which electrolyte solutions may be investigated is conductimetry, the theory of which is based simply on Ohm's Law:

$$V = iR$$

where V, i and R represent the applied electromotive force (e.m.f.), the current, and the resistance of the solution respectively. Units of e.m.f. are volts (V; $J\,A^{-1}\,s^{-1}$), of current amps (A), and of resistance ohms (Ω; $kg\,m^2\,s^{-3}\,A^{-2}$). The conductance, G, of the solution is the variable which is measured in conductivity experiments, and is defined as the reciprocal of the resistance and expressed in siemens (S; Ω^{-1}). Thus it can be seen that:

$$G = \frac{1}{R} = \frac{\kappa}{l/a} = \frac{1}{\rho(l/a)}$$

where κ, a and l are conductivity ($\Omega^{-1}\,m^{-1}$; reciprocal of resistivity, ρ), cross-sectional area, and length of the conductor respectively. The conductivity represents the current flowing across unit area of conductor per unit potential gradient. Conductivity depends on ionic concentration and tends to zero as the solution is diluted, whereas the molar conductivity (Λ; $\Omega^{-1}\,m^2\,mol^{-1}$), which represents the conductivity of a solution at a concentation of $1\,mol\,m^{-3}$, reaches its maximum value,

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Experiments 1 to 10

A representative selection of titrations is given in Table 5.2. The equations representing each reaction should be written down and reference made to Table 5.1. This should enable you to explain the reason for the shape obtained for each graph.

Table 5.2 Conductimetric titration experiments

Experiment	Solution	Strength	Volume (ml)	Titrant	Strength	Titration increments (ml)
1	Hydrochloric acid	0.01M	50	Sodium hydroxide	0.1M	1.0
2	Hydrochloric acid	0.0001M	50	Sodium hydroxide	0.001M	1.0
3	Acetic acid	0.001M	50	Sodium hydroxide	0.1M	0.1
4	Acetic acid	0.1M	25	Piperidine	0.5M	1.0
5	Phosphoric acid	0.5M	50	Sodium hydroxide	2.0M	0.2
6	Acetic acid	0.1M	50	Sodium hydroxide	2.0M	0.2
	+ ammonium hydroxide	1.0M	4			
7	Hydrochloric acid	0.1M	10	Sodium hydroxide	1.0M	0.2
	+ acetic acid	0.1M	40			
8	Sodium acetate	0.1M	50	Hydrochloric acid	2.0M	0.2
9	Strychnine chloride	0.01M	25	Sodium hydroxide	0.1M	0.2
	+ hydrochloric acid	0.01M	25			
	+ ethanol	–	50			
10	Silver nitrate	0.001M	50	Potassium chloride	0.1M	0.1

High-frequency titrations

Introduction

Many difficulties are encountered in electrical methods of titration. Conductimetric measurements are complicated by polarisation, difficulty in wetting electrodes with small amounts of liquid, and corrosion and adsorption at electrode surfaces. Potentiometric and other galvanic methods are usually restricted to ionised solutions and are often impossible where non-aqueous solvents are used, especially if these are poor ionisation media.

By using the field of a high frequency (1–300 MHz) oscillator it is possible to produce ionic or dipole motion without introducing electrodes into the solution. If such an oscillator is placed in an insulated titration vessel, coupling takes place across the walls. The energy required to produce this ionic or dipole motion causes changes in loading of the oscillator. It is also possible to rectify the radiofrequency current bypassed by the solution and measure the resulting direct

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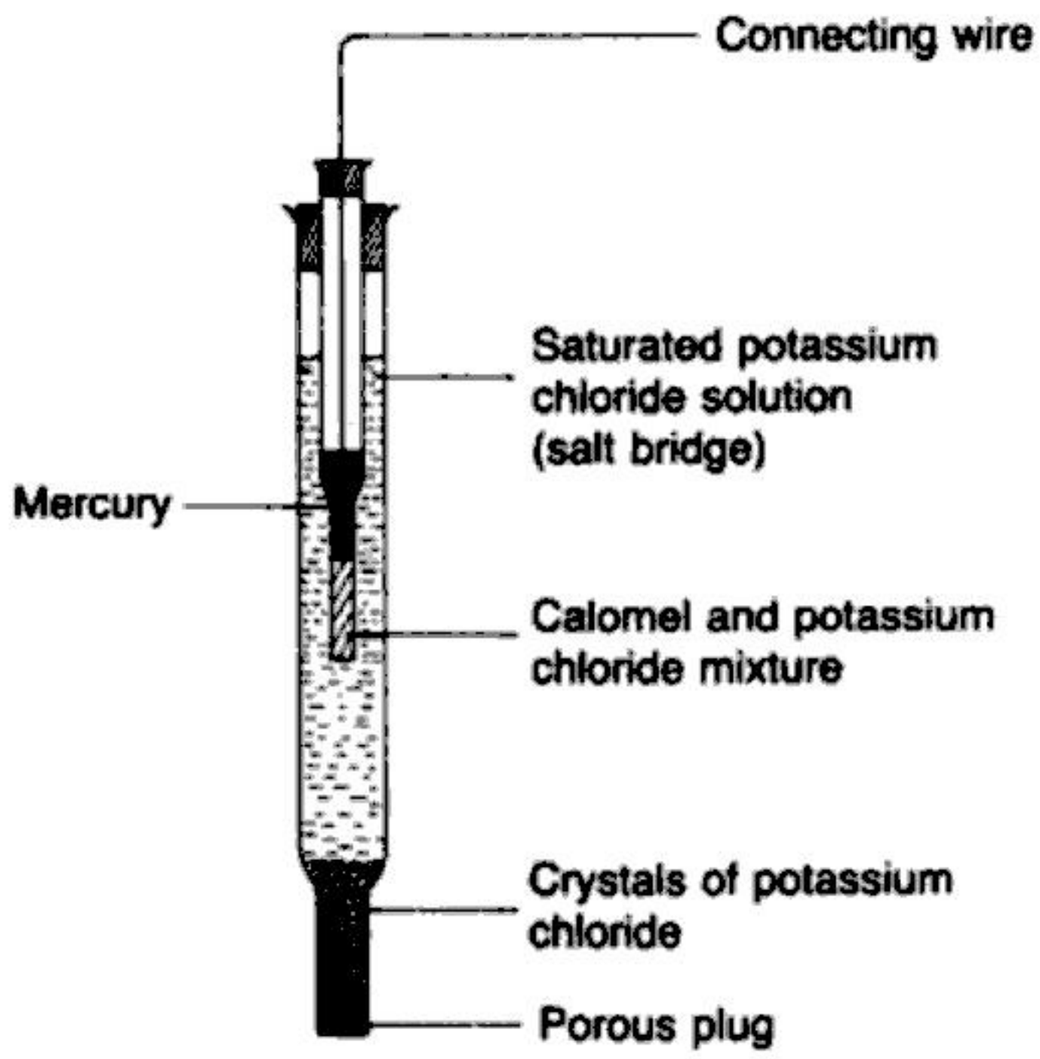

Fig. 5.6 Saturated calomel electrode

Mercury (I) sulphate electrode

This electrode is similar in construction to the calomel electrode but utilises sulphuric acid (0.05M) saturated with mercury (I) sulphate. It is used, for example, in solutions where silver or lead ions are present, and has a potential of 682 mV.

Salt bridges

A salt bridge of saturated potassium chloride, potassium nitrate or ammonium nitrate is used to prevent possible contamination of the reference electrodes with the test solutions. Sometimes the salt bridges are designed as a part of the reference electrode (Fig. 5.6) but are often solidified with a small quantity (3%) of agar. In general, when two solutions of electrolyte are brought into contact, a potential difference is set up betyween them due to the transference of ions across the boundary. This potential difference is known as a diffusion or liquid junction potential. The salt bridge reduces these potentials almost to zero and they become insignificant.

Indicator electrodes

Hydrogen electrode

The hydrogen electrode consists of a small piece of platinum foil, coated electrolytically with platinum black, over which hydrogen gas is passing. The platinum black surface exhibits a strong adsorptive power towards hydrogen and, provided that the metal surface remains in continuous contact with the gas, the electrode will act as if it were an electrode of metallic hydrogen. In use, therefore, only a part of the

foil is immersed in the solution, the remainder being surrounded by pure hydrogen. The potential of the hydrogen electrode is used as a reference zero in the electrochemical series as indicated above.

Glass electrode

A glass electrode consists of a very thin bulb or membrane of specially prepared, pH-responsive glass fused on to a piece of comparatively thick, high resistance glass tube (Fig. 5.7). In contact with the thin membrane is a suitable solution such as 0.1M hydrochloric acid. Electrical contact with this solution is usually made with a silver wire coated with silver chloride, which acts as an internal reference electrode (i.e. is unresponsive to pH change).

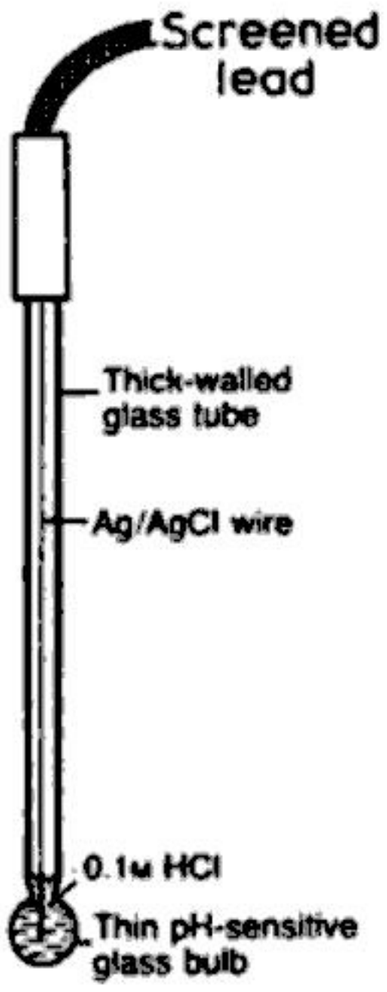

Fig. 5.7 Glass pH electrode

The potential of the glass electrode, when immersed in a solution, is given by the expression:

$$E = K + 0.0592\ (\mathrm{pH}_1 - \mathrm{pH}_2)\ (\text{at } 25^\circ)$$

where K is a constant, pH_1 is the pH of the solution in the bulb and pH_2 is the pH of the test solution. Now, pH_1 is constant for a given electrode, hence:

$$E = k - 0.0592\ \mathrm{pH}_2$$

where k is a constant, known as the asymmetry potential, which depends on several factors such as the existence of strains in the glass, the thickness of the glass bulb, and the composition of the solution within.

The advantages of a glass electrode are its rapid response and the fact that it is unaffected by the presence of oxidising or reducing agents, dissolved gases, highly coloured liquids, or moderate concentrations of many salts, with the main exception of sodium salts. The use of modern lithium silica glasses enables pH measurements to be valid over practically the entire pH range, but high concentrations of alkali can cause errors.

The main disadvantage of the glass electrode is its fragility, although modern devices are available which are especially rugged or resistant to boiling. Small imperfections on the glass bulb, such as scratches, and the presence of dehydrating agents, colloids, and surface deposits can cause interference in the measurements. Glass electrodes generally have a very high internal resistance and thus would never be used with simple potentiometers. The availability of glass electrodes combined with a reference electrode to produce a single unit in a variety of sizes and designs has ensured that the glass electrode remains the most versatile indicator electrode for pH measurements.

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solutions. However, under the same conditions, a potentiometric method for the detection of the end point can yield accurate results without difficulty. The electrical apparatus required consists of a potentiometer or pH meter with a suitable indicator and reference electrode. The other apparatus consists of a burette, beaker and stirrer.

The potential of the reference electrode need not be known accurately for most purposes and usually any electrode may be used provided its potential remains constant throughout the titration. The indicator electrode must be suitable for the particular type of titration (i.e. a glass electrode for acid-base reactions and a platinum electrode for redox titrations), and should reach equilibrium rapidly. The electrodes are immersed in the solution to be titrated and the potential difference between the electrodes is measured. Measured volumes of titrant are added, with thorough stirring, and the corresponding values of e.m.f. or pH recorded. Small increments in volume should be added near the equivalence point, which is found graphically by noting the burette reading corresponding to the maximum change of e.m.f. or pH per unit change of volume (Fig. 5.13). When the slope of the curve is more gradual it is not always easy to locate the equivalence point by this method. However, if small increments (0.1 ml or less) of titrant are added near the end point of the titration and a curve of change of e.m.f. or pH per unit volume against volume of titrant is plotted, a differential curve is obtained in which the equivalence point is indicated by a peak (Fig. 5.14).

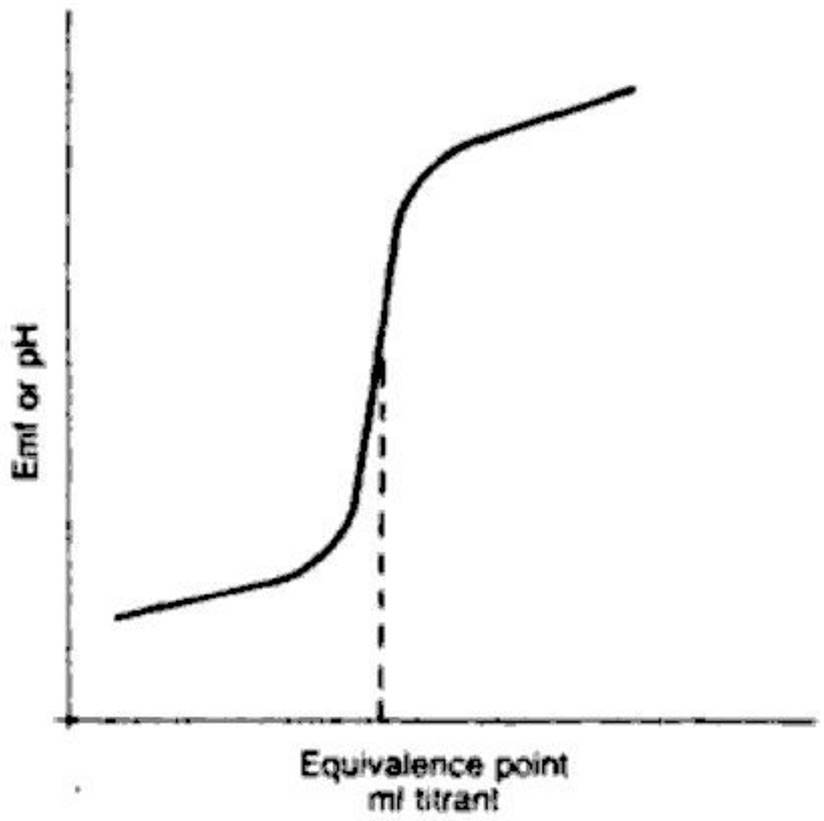

Fig. 5.13 Typical titration curve

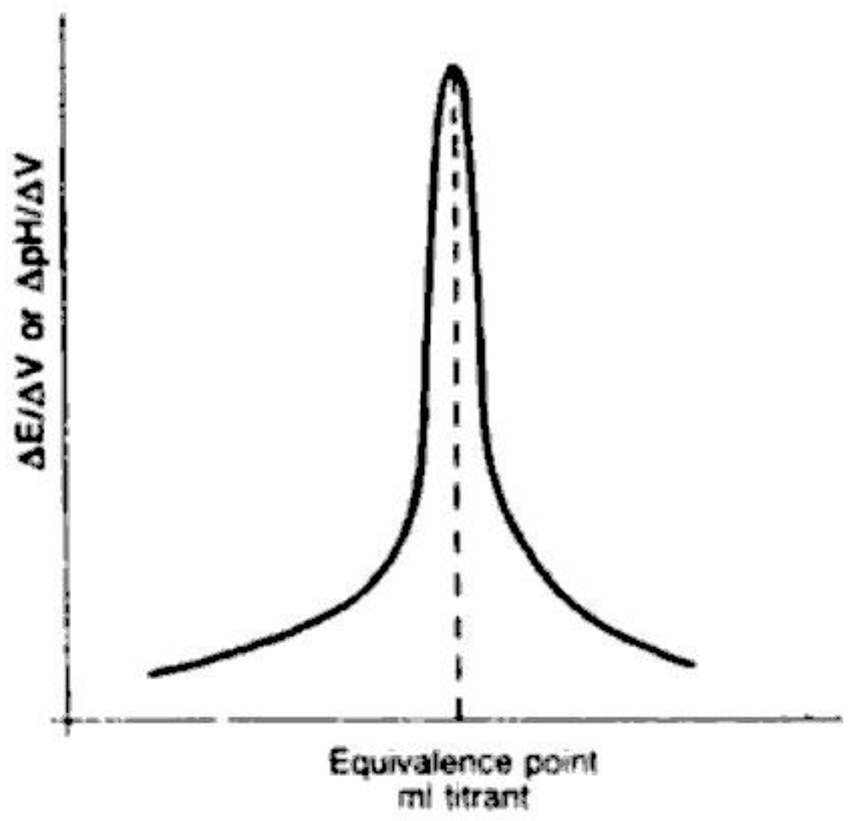

Fig. 5.14 Typical first derivative titration curve

Application of ISEs other than the pH-responsive glass electrode is limited in potentiometric titrations because of the relatively long response times of the electrodes. As previously mentioned, response time depends on electrode type, but concentration of analyte will also have an effect, with readings taking perhaps 15 s at the start of a titration when concentrations are high, but several minutes near the end point, the titration as a whole taking an unacceptably long time. The Gran's

plot technique is more satisfactory in these cases, as fewer data need be obtained for a reliable result and the emphasis that potentiometric titrations make on the experimentally most difficult readings around the end point does not apply because of the linearisation of the data.

Automatic equipment, using a constant flow burette and the pH meter connected to a suitable chart recorder, can be made quite easily and is also commercially available.

Neutralisation reactions

Any pH-responsive indicator electrode may be used, but a glass electrode is usually preferable. The potential at the equivalence point is given by the expression:

$$E = k - 0.0592 \text{ pH } (25°)$$

where k, the asymmetry potential, depends on the electrode system used.

Dibasic and tribasic acids may be titrated with alkali to the intermediate equivalence points, provided the dissociation constants of each stage are sufficiently far apart; similarly, mixtures of acids may be titrated satisfactorily (e.g. acetic and sulphuric acids). A sufficiently large inflection is obtained when the difference in pK values exceeds 2.7 pK units. In the titration of a mixture of acids, the first inflection in the titration curve occurs when the stronger acid has been neutralised, and the second when neutralisation is complete.

Redox titrations

The indicator electrode most commonly used is a platinum wire or foil. The potential of the indicator electrode is a function of the ratio of the concentrations of oxidised and reduced forms of an ion as previously discussed. As before, the equivalence point in a redox titration is indicated by a marked inflection in the titration curve, if $E^{\ominus}_{\text{ox,red}}$ of the two chemical systems are sufficiently far apart.

Precipitation reactions

The solubility product of the almost insoluble material formed during a precipitation reaction determines the ionic concentration at the equivalence point. The indicator electrode must readily come into equilibrium with one of the ions. For example, a silver electrode is used for the titration of halides with silver nitrate, and its potential is given by the expression for cationic electrodes discussed earlier.

Experiment 11 *Determination of pH using a glass electrode*

It will be assumed that a commercial potentiometric type pH meter is used, set to read pH units.

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the steeply rising portion of the curve, is characteristic of the particular system being reduced and thus enables qualitative analyses to be performed.

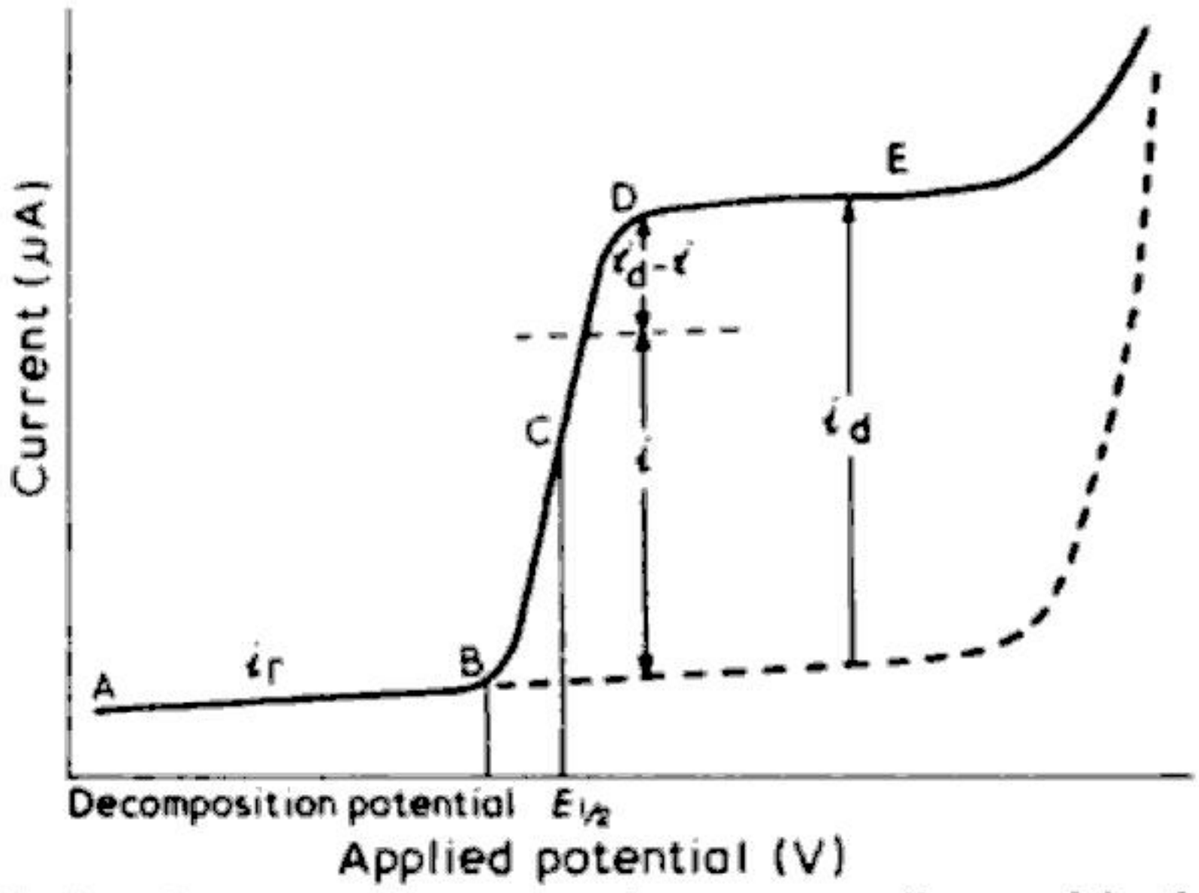

Fig. 5.18 A polarogram illustrating the currents discussed in the text

The gradual rise in current, known as the residual current i_r, is the sum of a relatively large capacitance current i_c and a very small faradaic current i_f. The capacitance current is produced when mercury drops, from the dme, become charged at the mercury–solution interface due to the formation of a Helmholtz double layer of positively and negatively charged ions. This is the cause of the oscillations, superimposed on a d.c. polarogram, which follow the mercury drop growth cycle, showing a steep rise as the drop area rapidly increases at the start of its lifetime but becoming less steep towards the end of the drop life. The residual faradaic current is due to traces of impurities in the solution being reduced. For example, it is very difficult to remove the last traces of oxygen even after bubbling nitrogen through a solution: ordinary distilled water often contains traces of copper and, when solutions are deoxygenated in the presence of the pool anode, mercury ions sometimes go into solution with the formation of hydrogen peroxide.

The residual current should always be subtracted from the total observed current, as shown in Fig. 5.18, in order to obtain the diffusion current i_d. The minimum detectable concentration of electroreducible ions depends, to a very large extent, on the accuracy with which this correction is measured. During an experiment a suitable solution of the electroactive substance under examination is freed from dissolved oxygen by bubbling O_2-free nitrogen through for several minutes and blanketing the solution with nitrogen, because oxygen is electroreducible and this could interfere with the electroreaction being observed. After the voltage scan has been made and the polarogram obtained, a further polarogram should be recorded in the absence of the electroreducible species to give a residual current curve (broken line in Fig. 5.18).

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be cleaned by immersing periodically in 50% v/v nitric acid with the mercury flowing and then washing thoroughly with a jet of water. A disassembled capillary can be cleaned by aspirating concentrated hydrochloric acid through it for half a minute, following this with methanol and then air until dry.

Advantages of the dropping mercury electrode

(1) It has a smooth and continually renewable surface exposed to the solution being analysed.
(2) Each drop formed is unaffected by the reactions which occurred at the surface of earlier drops.
(3) Mercury amalgamates readily with most metals.
(4) The high hydrogen overvoltage of mercury enables analyses to be carried out in acid solutions.
(5) The diffusion equilibrium at the mercury–solution interface is rapidly attained.

Disadvantages of the dropping mercury electrode

(1) Mercury has a limited application in the more positive potential range (i.e. when used for anodic polarography), since anodic dissolution of mercury takes place at about +0.5 V.
(2) The surface area of the drop is never constant.
(3) Changes in the applied voltage produce changes in the surface tension of mercury and, therefore, changes in drop size.
(4) The addition of surface active agents produces changes in drop size, and adsorption of surface active agents can interfere with electrode reactions.
(5) Mercury may be toxic in certain biological studies.

Basic principles of polarographic instrumentation

Potentiometric manual polarograph

A means of measuring current used by Kolthoff and Lingane was to pass it through a standard resistance and measure the potential difference set up. Figure 5.22 shows the simplified circuit of such a polarograph which is also capable of measuring accurately the potential difference across the cell electrodes. The primary circuit consists of a battery, switch, and uncalibrated potentiometer.

The resistance R is at least 10 k Ω and its actual value should be known accurately to within ±0.1%. The calibrated potentiometer P_2 is shown schematically. Any type of potentiometer is suitable provided it can measure potentials correctly to ±1 mV. The galvanometer associated with P_2 should have a period of about 10–20 s so as to minimise

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be fully investigated. Care must be taken to ensure that no ions in the electrolyte interact with the substance being determined and that pH changes do not affect its stability, and that the use of aqueous/alcoholic solvents does not cause changes in the $E_{1/2}$ of the electroactive species, the limiting current values, or the reduction mechanism involved, which might lead to erroneous conclusions in an analysis.

Table 5.4 Typical solvents for inorganic polarographic analyses. (Adapted from Application Note 151, Princeton Applied Research Corporation.)

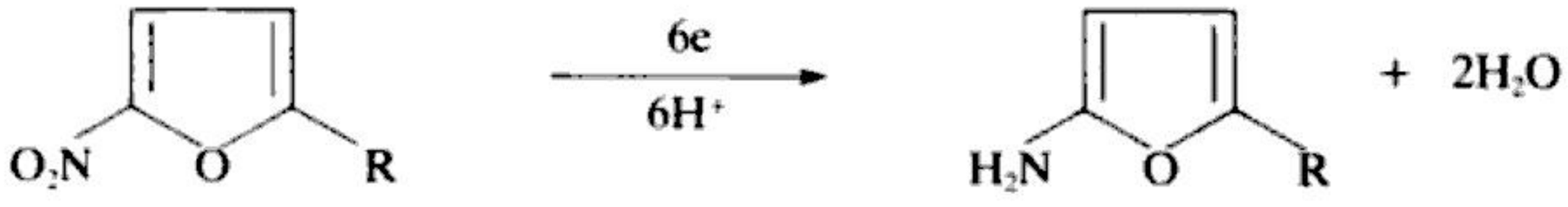
Metal	Supporting electrolyte	$E_{1/2}/V$
As(III)	1M HCl	−0.43/−0.67
Bi(III)	1M HCl	−0.09
Cd(II)	0.2M NH_4 citrate, pH 3	−0.62
Co(II)	1M NH_3–1M NH_4Cl	−1.22
Cr(III)	0.2M KSCN, pH 3 with HOAc	−0.85
Cr(VI	1M NaOH	−0.85
Cu(II)	0.2M NH_4 citrate, pH 3	−0.07
Fe(III)	0.2M TEA–0.2M NaOH	−1.0
Mn(II)	1M NH_3–1M NH_4Cl	−1.66
Ni(II)	1M NH_3–1M NH_4Cl	−1.0
Pb(II)	0.2M NH_4 citrate, pH 3	−0.45
Sb(II)	6M HCl	−0.18
Sn(II)	0.2M HOAc–0.2M NaOAc	−0.20/−0.53
Sn(IV)	1M HCl–4M NH_4Cl	−0.25/−0.52
Tl(I)	1M HNO_3	−0.48
Zn(II)	0.2M NH_4 citrate, pH 3	−1.04

The wider negative working voltage range for the dme compared with the positive inevitably means that reduction reactions are more frequently encountered in organic analysis than oxidations. A wide range of functional groups undergo electroreduction, but the products of such processes may not be the same as if the reduction were attempted chemically, as the electrode kinetics limit the progress of multi-stage reactions. Some organic functional groups which can be reduced at the dme are shown in Table 5.5.

Table 5.5 Some functional groups reducible in polarography. (Adapted from Application note 151, Princeton Applied Research Corporation.)

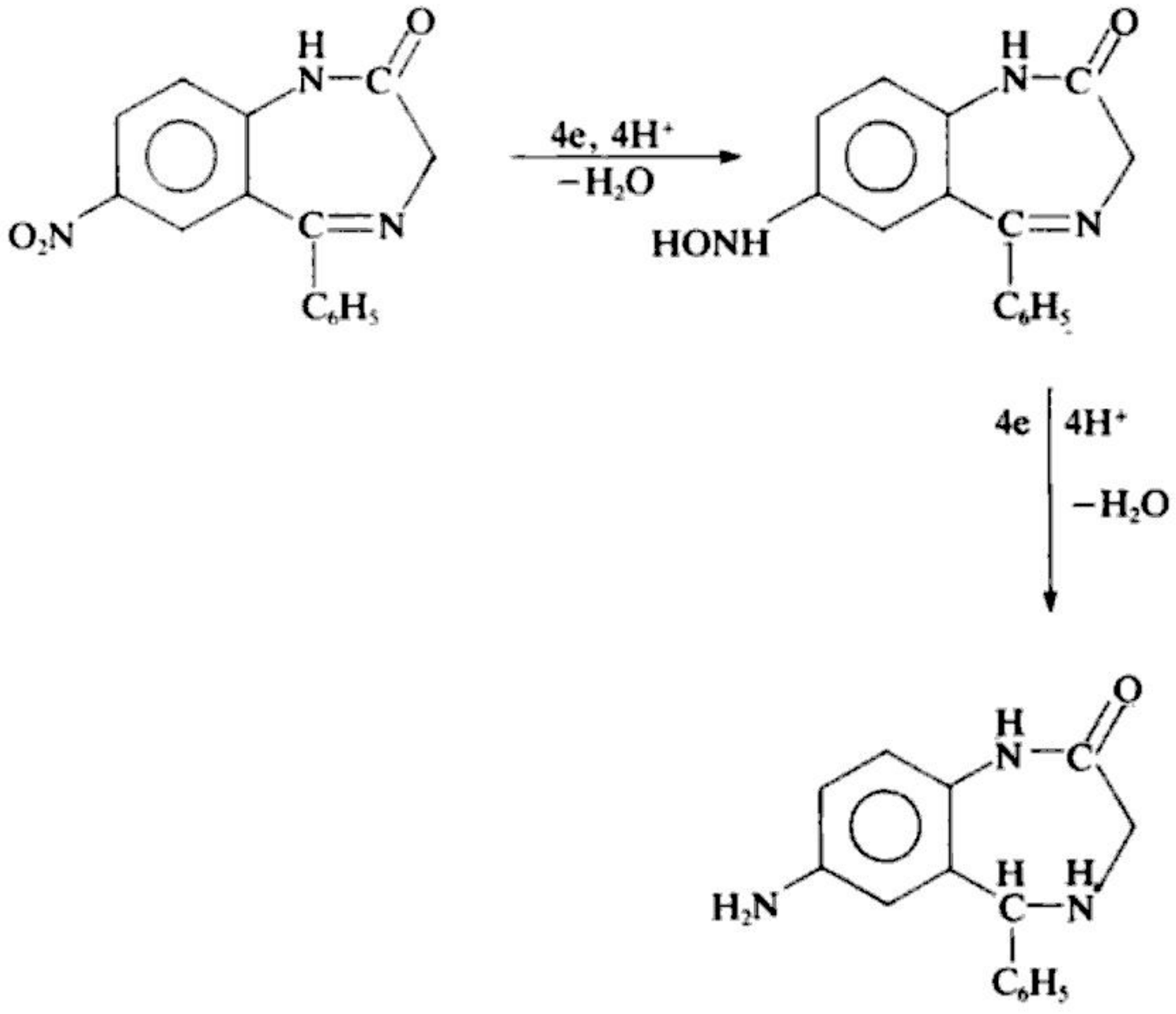
Imines	Dienes
Oximes	Alkynes
Nitriles	Ketones
Diazo compounds	Aldehydes
Diazonium salts	Aromatic carboxylic acids
Nitroso compounds	Halides
Sulphones	Thiocyanates
Sulphonium salts	Heterocycles
Nitro compounds	Organometallics

One of the easiest and most frequently encountered organic reductions is that of the nitro group. In nitrofurans and nitroimidazoles, for example, the reaction is



Nitrofurantoin, *Metronidazole* and *Tinidazole* are examples whose reductions have been shown to follow this route.

The benzodiazepines constitute another class of compounds frequently analysed by polarography. A typical reduction is that of *Nitrazepam* in 20% methanol–0.1M hydrochloric acid, which proceeds by the following mechanism:



This gives peaks at −0.11 V and −0.67 V *vs* silver–silver chloride. To illustrate further the applicability of polarography in organic analysis and the value of derivitisation, the analysis of atropine by d.c. polarography may be quoted, in which 0.1M tetrabutylammonium perchlorate in acetonitrile can be used as solvent. Nitration of atropine can be effected by reaction at room temperature with 10% potassium nitrate in concentrated sulphuric acid, and a sensitivity of 200 ng atropine ml^{-1} of 1M sodium hydroxide supporting electrolyte with dpp has been claimed by Brooks *et al* (1974).

Polarographic methods of analysis

Direct comparison method

In this method, the diffusion current obtained for the 'test' solution is compared, under identical conditions, with that of a solution of known concentration. Maximum accuracy is obtained when the diffusion currents of both solutions are about equal.

The most important conditions which must be kept constant in the comparison are temperature, concentration of maximum suppressor (if any), composition of the supporting electrolyte and the characteristics of the dme (i.e. constant m and t values).

For complex mixtures it is advisable to keep standard comparison samples of known composition. These comparison samples must approximate closely in content to the samples being analysed.

Use of empirical calibration curves

The dme is calibrated empirically with various known concentrations of the substance in question, and a graph of diffusion current *vs* concentration is plotted. The concentration of the test solution can be read from the graph. It is essential to control the temperature accurately and to check that the capillary characteristics do not vary. This is the most frequently used method, and is satisfactory if standards and test samples are analysed under identical conditions.

Internal standard or pilot ion method

This method is based on the fact that the relative diffusion current constants I are independent of the particular capillary used, provided the nature and concentration of the supporting electrolyte and the temperature are kept constant.

From the Ilkovic equation:

$$i_d = ICm^{2/3}t^{1/6}$$

where

$$I = 607nD^{1/2}$$

For the 'pilot' ion

$$i_{d_1} = I_1C_1m^{2/3}t^{1/6}$$

and the 'test' ion

$$i_{d_2} = I_2C_2m^{2/3}t^{1/6}$$

hence

$$\frac{i_{d_1}}{i_{d_2}} = \frac{I_1C_1}{I_2C_2}$$

The ratio I_1/I_2 is known as the pilot ion ratio (symbol R) and is

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mercury in a small glass container. Wash the mercury with water and dry by rinsing with acetone followed by a jet of air. Weigh the mercury on an analytical balance and hence calculate m. Determine P by correcting the reservoir height for back pressure. Conclude whether or not the relationships (a), (b) and (c) above are correct.

Experiment 52 *Analysis of a polarographic wave*

Method

(a) Prepare a solution containing cadmium sulphate (0.01M), potassium chloride (0.1M), and gelatin (0.005%). Place some solution in a polarographic cell which is immersed in a thermostatic bath set at 25° ± 0.1°. Deoxygenate and obtain the CV curve from −0.4 to −0.8 *vs* SCE. Use the same current sensitivity throughout this experiment.

(b) Prepare a solution of potassium chloride (0.1M) and gelatin (0.005%) and obtain, at 25°, the residual current curve over the same voltage range. Correct the values of current obtained in (a) by subtraction.

(c) Calculate suitable individual log $(i_d - i)/i$ values and plot against the corresponding applied potentials. Determine the number of electrons involved in the reduction by measuring the slope of the graph (see Fig. 5.21).

Experiment 53 *Limit test for trivalent antimony in Sodium Stibogluconate*

Sodium Stibogluconate contains not more than 0.2% trivalent antimony when determined by the following method.

Method To 0.2 g sample, accurately weighed, in 10 ml of water, add 2 ml 0.1% w/v aqueous solution of gelatin, and 2 ml concentrated hydrochloric acid, and dilute to 20 ml with water. Transfer an aliquot portion to a polarographic cell and bubble nitrogen through the solution for 10 min. Record a polarogram over the range 0 to +0.5 V (i.e. an anodic wave) and compare with a standard trivalent antimony calibration curve using, in the above procedure, 0.25 ml of a 0.8% w/v aqueous solution of a trivalent organo-antimony compound. The height of the step at a potential of approximately 0.15 V *vs* SCE is a measure of Sb^{3+}. (A potential of about 0.4 V *vs* SCE is probably better since $E_{1/2} \triangleq +0.15$ V.) The solutions must be examined within 30 min. of preparation as they are unstable.

Experiment 54 *The multimode polarograph in the analysis of Metronidazole*

A multimode polarograph with synchronised dme which can perform sampled d.c., pulse, and differential pulse polarography should be used. The analysis should be performed in each of the three modes and the results compared.

Method Prepare 50 μg ml^{-1} solution of metronidazole in 0.1M hydrochloric acid, and prepare from this six solutions of appropriate concentrations for a calibration graph. Deoxygenate the test solutions for 4 min before each measurement. Choose a suitable voltage scan range advancing at 2 mV s^{-1} to include 0 to −0.5 V w.r.t. silver–silver chloride and start with 10 μA full scale current range, adjusting as necessary. Use a 1 s drop time with a small mercury drop if this adjustment is available. In differential pulse polarography use a 25 mV pulse amplitude. Run the polarograms in any or all of the available modes, measure the response height and plot a calibration graph. Analyse the data statistically as if it were a straight line.

Compare the data obtained above with those from an identical experiment except for

the addition of 10^{-4} mol l^{-1} polyethylene glycol 4000 (PEG 4000) in each of the test solutions. Analyse the calibration graphs similarly and comment constructively on the results.

Amperometric titrations

Introduction

This technique uses a polarographic or other voltammetric electrode to detect the end point in a titration, thus extending the application of titrimetry to lower concentrations of analyte than would be possible with visual indicators. An abrupt change in limiting current will occur when all the analyte has been removed from the solution by the titrant (Fig. 5.29). No potential scanning is used: a fixed voltage of magnitude sufficient to cause electroreaction of the analyte is applied across the electrodes and the current is monitored as titrant is added. A similar principle is applied in the electrometric end point method for the titration of sulphonamides and in the conventional determination of water by titration with Karl Fischer reagent (q.v.).

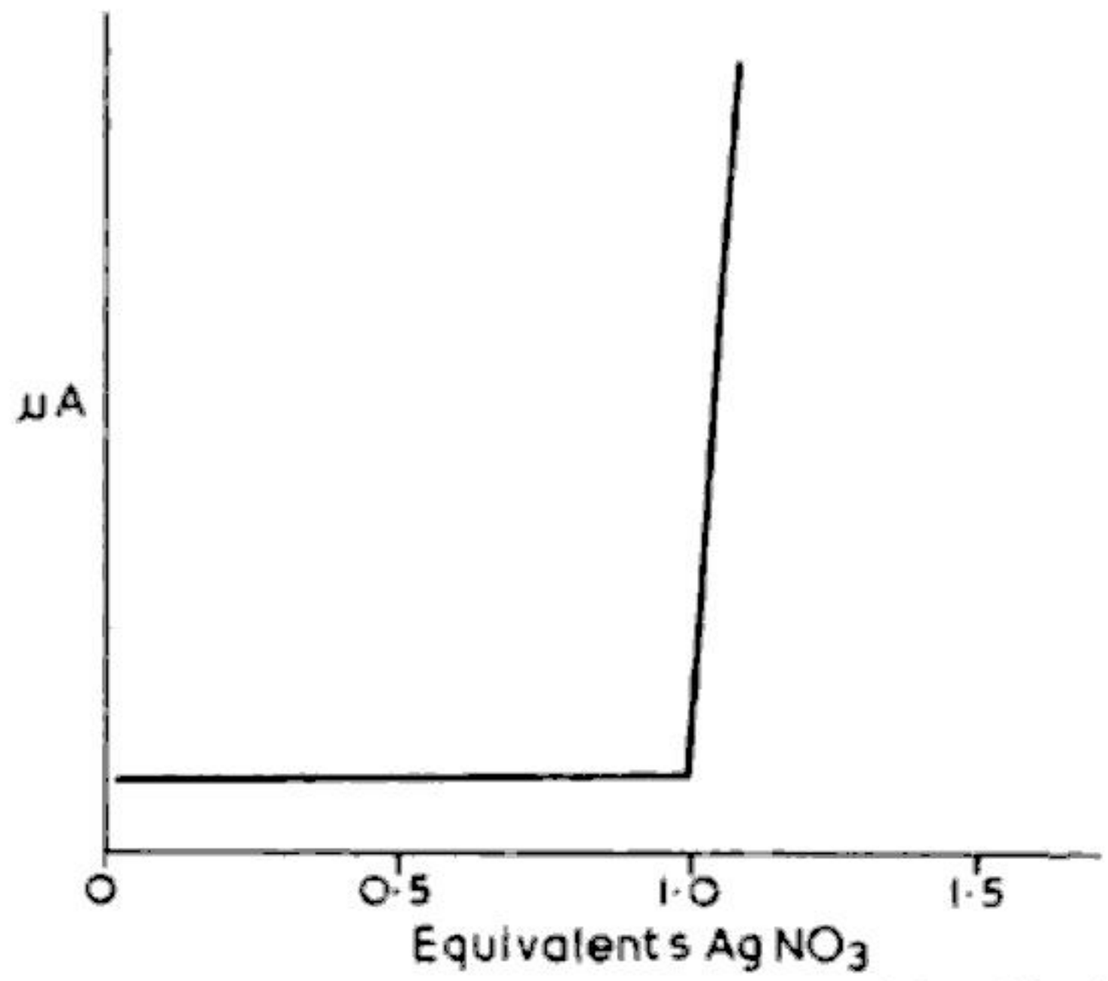

Fig. 5.29 Amperometric titration of potassium chloride with silver nitrate

Amperometric titrations using a dropping mercury electrode

Very dilute solutions may be titrated accurately (ca. ±0.3%) and rapidly. Amperometric titration results are independent of the capillary characteristics and temperature, provided that there is no change during a titration; the reaction need not be reversible, and substances which are not oxidised or reduced may be titrated if the reagent gives a diffusion current. Amperometric titrations involving precipitation may be carried out when the solubility is appreciable and under conditions where potentiometric and indicator methods are inaccurate. Titrations may also be carried out in the presence of large amounts of electrolyte (e.g. potassium chloride) without interference (contrast conductimetric titrations).

Apart from the normal sources of error in volumetric determinations, impurities which give diffusion currents may have to be removed by a preliminary chemical separation, or the conditions must be so chosen that foreign constituents do not contribute to the current. Several types of titration curve may be obtained, as follows:

(1) Consider a lead solution containing an excess of an indifferent electrolyte. The polarogram (Fig. 5.30) has a plateau between points A and B, where the current is practically constant. This solution may therefore be titrated with a solution of sodium oxalate at any fixed applied e.m.f. between the values A and B by measuring the current until the end point is reached, when only a small residual current flows (Fig. 5.31). The slight curvature is due to dilution of the solution with the reagent and is minimised by using a relatively strong titrant (compare conductimetric titrations). In any case, it can be corrected by multiplying the observed values of current by the ratio total volume to initial volume.

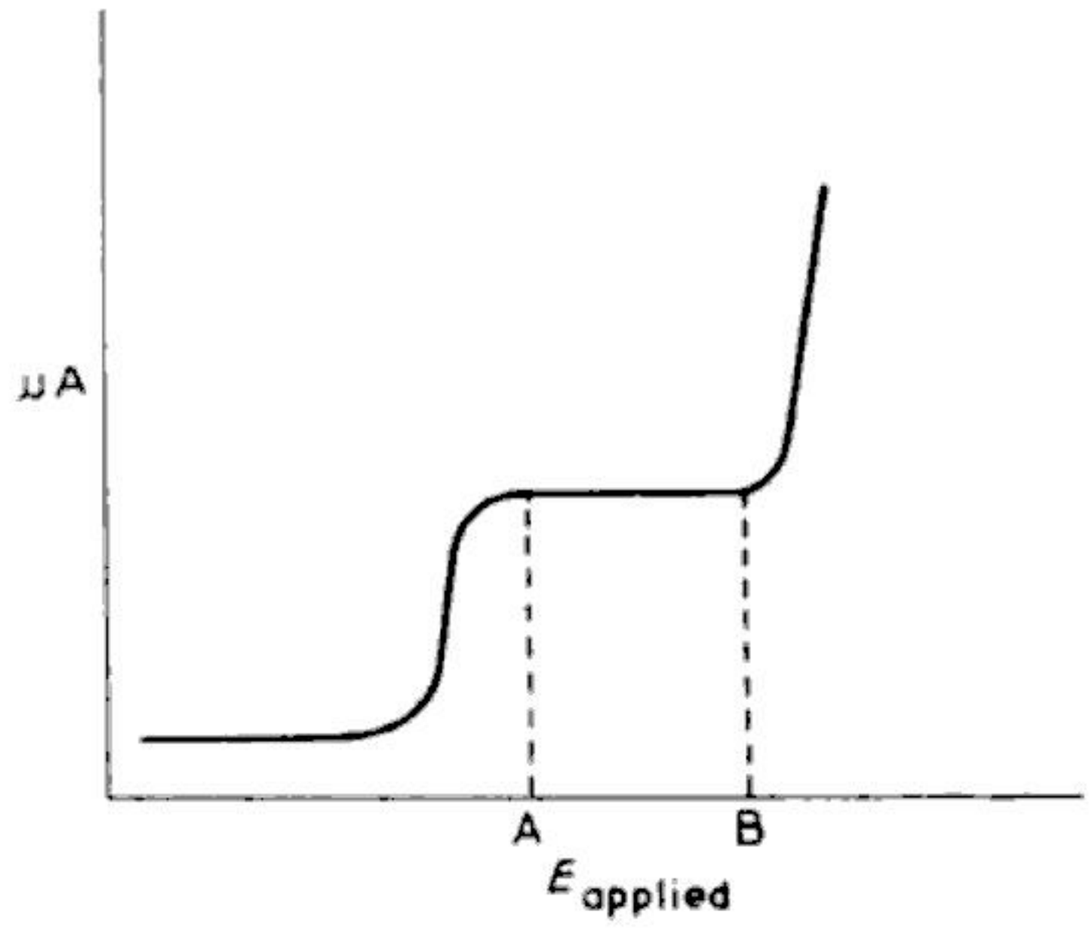

Fig. 5.30 Polarogram of lead nitrate solution

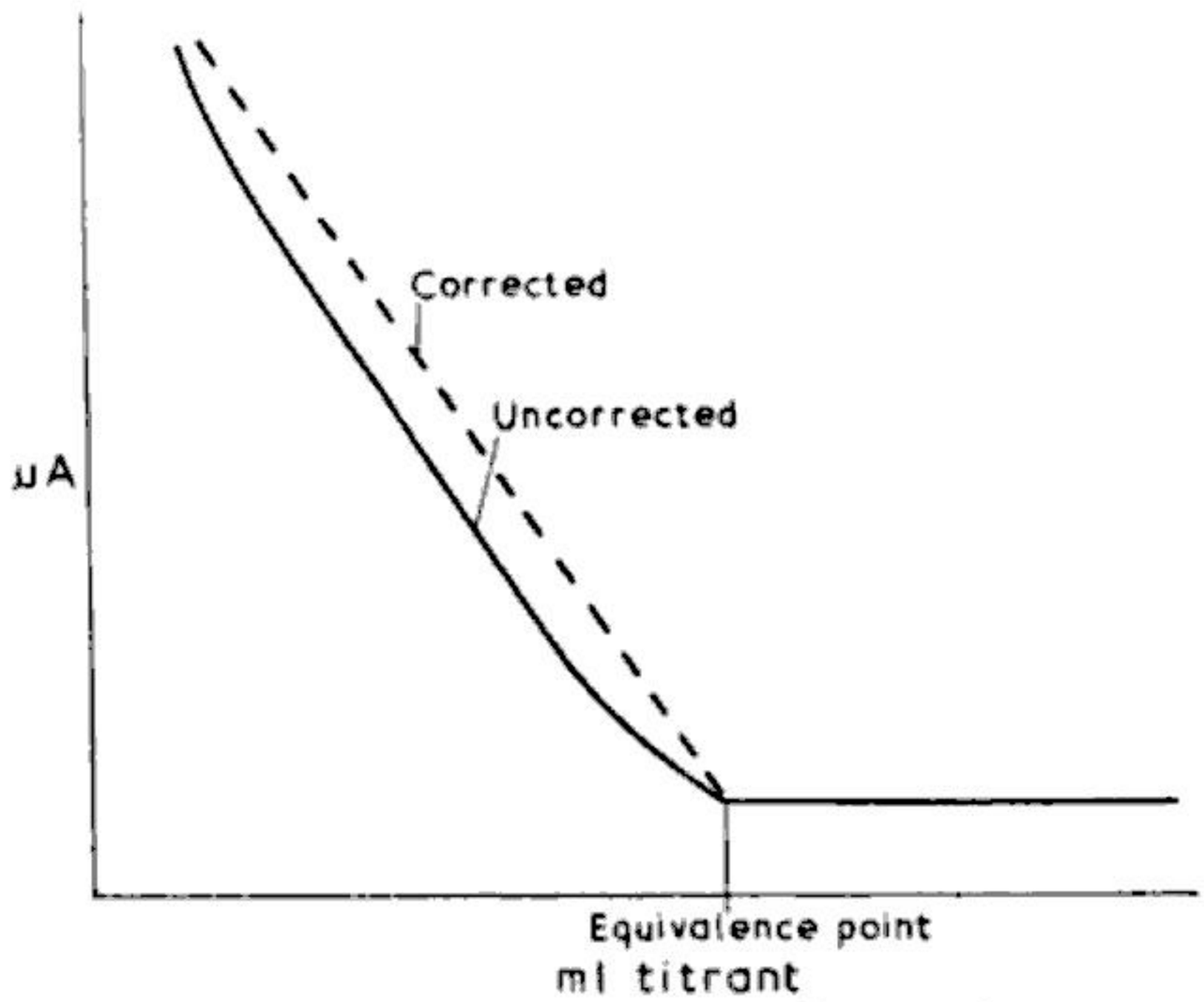

Fig. 5.31 Amperometric titration of lead with sodium oxalate at 1.0 V *vs* SCE

(2) It is possible to titrate a non-reducible substance with a reagent which is electroreducible. Lead nitrate, for example, may be titrated with potassium dichromate, in acetate buffer, at zero applied volts. (Pb^{2+} is not reduced at this voltage.) When the end point is reached, the dichromate ion ($Cr_2O_7^{2-}$) yields a diffusion current (Fig. 5.32).

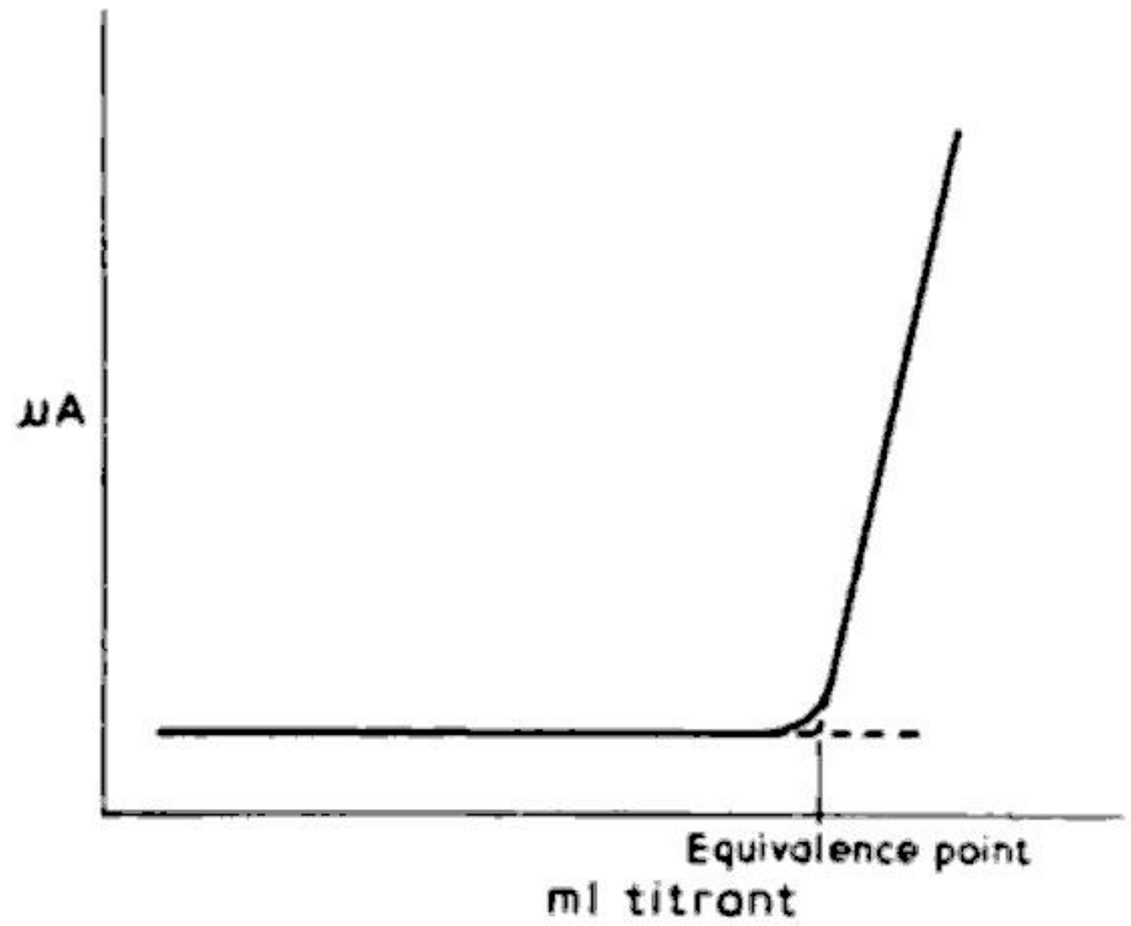

Fig. 5.32 Amperometric titration of lead with potassium dichromate at zero volts *vs* SCE

(3) The substance to be titrated and the reagent used may both be electroreducible. Lead nitrate and potassium dichromate, as above, may be titrated at an applied potential of −1.0 V *vs* SCE. The diffusion current, due to the lead ions, first falls as lead is precipitated out and rises when excess dichromate ions are present (Fig. 5.33).

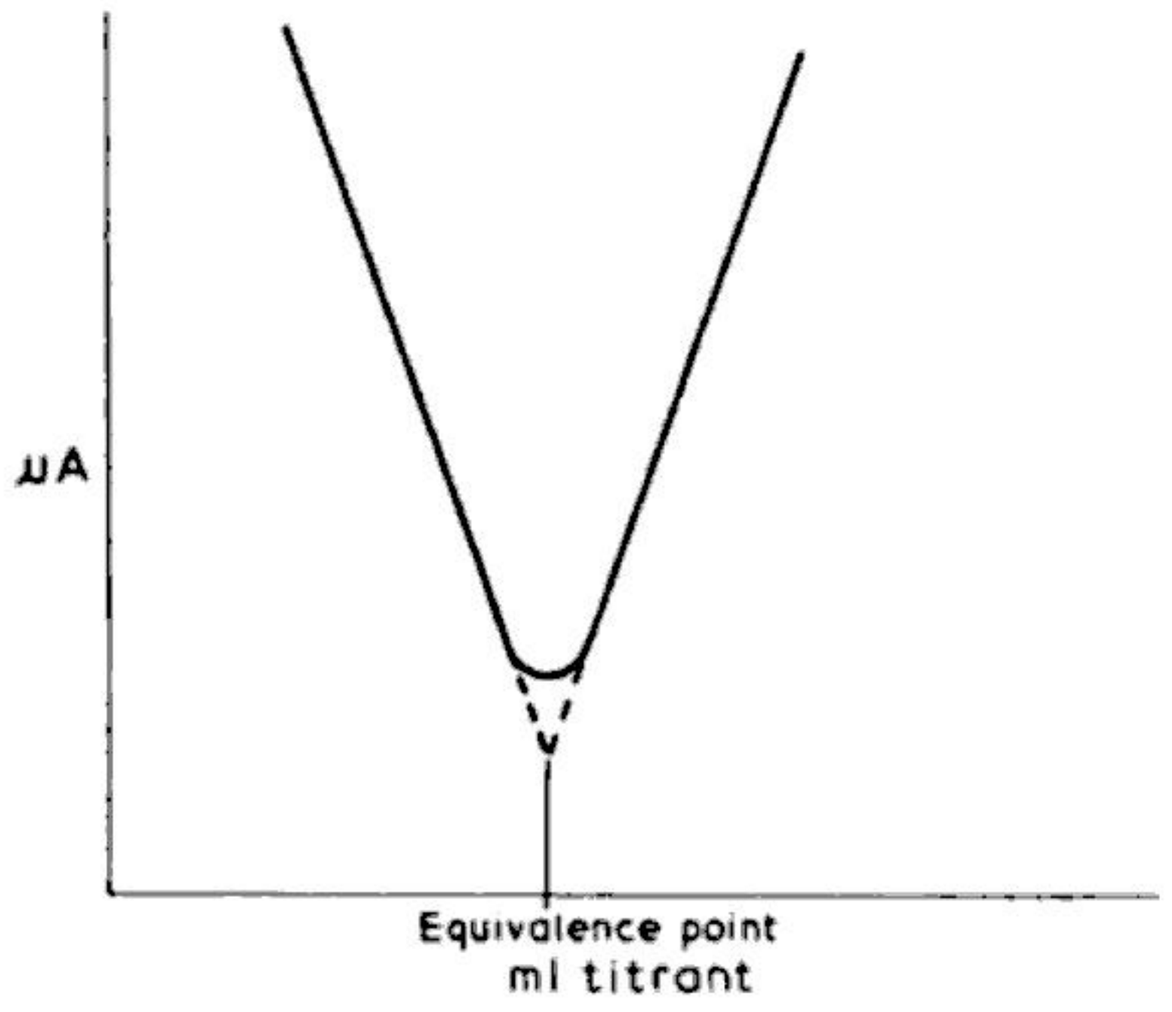

Fig. 5.33 Amperometric titration of lead with potassium dichromate at −1.0 V *vs* SCE

(4) Amperometric titrations may also be applied to electro-oxidisable substances, provided a suitable electrode is used. Potassium iodide may, for example, be titrated with mercury(II) nitrate:

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current arising from oxidation or reduction of analyte as it emerges from the HPLC column after the appropriate elution time. In principle LCEC operates in the same way as the equivalent electrochemical method described earlier in this section. Detectors can be polarographic but are more commonly amperometric or coulometric, with potentiostat, electrode and output requirements essentially identical to those for operation in the conventional way. The cell design must obviously be different, however, to allow for the flow-through requirement of HPLC, and much attention has been given to optimising background noise, sensitivity, electrode accessibility, and to reducing cell volumes. In the familiar three electrode cell arrangement the reference electrode is usually silver–silver chloride in 3M potassium chloride and the auxiliary electrode is usually either glassy carbon, platinum wire, or of a proprietary carbon construction.

Materials used for the working electrode are much more diverse, each possessing virtues for particular applications. The most popular for oxidative detection are carbon paste and glassy carbon, the latter being more robust but giving a higher background current. Carbon paste electrodes prepared by mixing graphite with paraffin oil, ceresin wax or silicon grease, are generally unsuitable for use in non-aqueous solvents, so mixtures containing, for example, >25% v/v methanol or >5% v/v acetonitrile, would be prohibited. Glassy carbon can be readily repolished and carbon paste electrodes readily remade so, if damaged by excessive voltage application, current flow, or solvent degradation, they are easily recoverable. The criteria by which electrodes should be judged are the acceptable range of applied potential, chemical and physical compatibility with the solvent, signal-to-noise performance, long-term stability, and requirement for preconditioning.

Metal working electrodes include platinum or mercury deposited on gold in which the advantage of the high hydrogen overpotential of mercury can be exploited as in polarography in that a wider range in the reductive direction is possible than on many other surfaces. Polarographic mercury drop electrodes are commercially available but lack stability in the flowing stream of eluent from the HPLC column. However, it has been noted that the detection limits (usually in the low ng/ml range) of amperometric detectors made of glassy carbon, carbon paste, and mercury are reduction potential dependent for certain benzodiazepines, and this can in general be expected as many organic compounds react at different rates according to the electrode being used. The highest possible reaction rate is the most desirable condition, because the current is then transport limited rather than rate limited. One of the advantages of the mercury drop in polarography is that contamination is irrelevant because of the renewable electrode surface; however, contamination is reduced in LCEC in comparison with non-chromatographic electrochemistry because the contact time of the contaminant with the solid electrode is minimised by the narrow elution band created by the chromatographic system.

Cell design

Cell design is dependent on the intended mode of operation of the detector, of which there are two. The coulometric mode converts 100% of the electroactive compound passing through the cell and therefore requires a large surface area for the reaction to take place. The amperometric mode converts <10% of the material passing through the cell. The three different cell designs, intended to improve sensitivity or selectivity, are known as tubular, channel, and wall-jet types. In the first type the solution flows through an annulus which is the working electrode, in the second it flows over an electrode plate, and in the third it emerges from a nozzle perpendicularly onto the electrode surface. Each has advantages in mass transfer, resistance to poisoning, ease of replacement of electrode substance, or signal-to-noise ratio.

In coulometric operation a large surface area is required to achieve 100% electroreaction. Two types of cell are available: one consists of an open tube electrode through which the solution passes directly, and in the other the solution passes over a reticulated, cloth or gauze electrode. It can be shown that the smaller the electrode area the higher is the current density, and coulometric cells show that additional current due to area increase is a decreasing function of increased electrode area. In addition, noise and background current are in general linearly related to area, so coulometric cells usually have poorer detection limits than amperometric ones.

Theory

Faraday's Law relates the charge Q in coulombs transferred in the electroreaction to the number of moles N of reactant being processed; thus

$$Q = zFN$$

where z is the number of electrons involved in the reaction of one mole of reactant, and F is Faraday's constant (96485 coulombs per mole of electrons). In coulometric detection the current flowing is measured and integrated over time to give the charge consumed, which is therefore represented by the peak area on the recorder trace. As 100% conversion occurs in coulometric operation, the peak area will be independent of flow rate u, and thus one calibration curve will apply for all flow rates, but it can be shown that the current i is directly dependent on flow rate as follows:

$$i = zFcu$$

where c is the analyte concentration in the bulk of solution prior to entry into the coulometric detector.

Analogous equations can be written for each geometry of amperometric detector; that for a channel cell in which the eluent passes over the electrode is

$$i = 1.467zFAc\ (D/h)^{2/3}(u/d)^{1/3}$$

where A is the electrode area (cm^2), D is the diffusion coefficient (cm^2s^{-1}), h is the thickness of the channel and d is its width. Both these equations show direct dependence of current on analyte concentration, but the amperometric design is less dependent on volume flow rate. The current, which both detection methods display, describes the rate of analyte conversion while the integrated, coulometric, response reflects the total amount of analyte reacted; a cell design which permits rapid transfer rates is therefore important in both methods of detection.

Operating potential

The choice of applied potential is dependent on the functional groups on the molecule to be analysed. Oxidation or reduction can be undertaken but the voltage range in each direction will be dependent on choice of electrode, as discussed above and on pp. 225–230. Reducible groups are of course the same as described there, and the most frequently encountered oxidisable groups are the aromatic hydroxyl and amine. Less frequently encountered are indoles, phenothiazines, mercaptans and miscellaneous compounds such as ascorbic acid and vitamin A. With LCEC reduction, as in polarography, all traces of oxygen must be excluded from the solvents and no permeable tubing should be used.

Selection of a suitable applied voltage is often done by generating a hydrodynamic voltammogram for the compound after a rough assessment of likely potentials has been made. This allows the potential for maximum signal and selectivity to be found by repetition of the chromatographic run with the detector set at a constant, but successively altered, potential for each run. When insufficient voltage is applied, no response will occur; alteration by 0.1 V steps will eventually lead to a response which is very sensitive to voltage changes. Finally a plateau region will be obtained where diffusion is the rate determining factor for current generation. Further increase in voltage will only reduce selectivity by possibly including more electroactive compounds and functional groups within the voltage range, so an operating potential should be chosen which is sufficiently within the plateau region for maximum sensitivity without decreasing selectivity. A typical hydrodynamic voltammogram is shown in Fig. 5.39. All measurements should be done under controlled and recorded conditions of flow rate, temperature, amplifier gain etc.

Some LCEC coulometric systems have two or more cells operating in series on the eluent. By allowing the application of different potentials at each cell, selective conversion (screening) and analysis of

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Voltammetry

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6
The basis of spectrophotometry

A. G. DAVIDSON

Introduction

Photometric techniques are among the most important instrumental techniques available to the pharmaceutical analyst. Instrumentation ranges from the simple flame photometers, which are used to determine the concentration of certain metallic elements, to the much more expensive spectrometers such as ultraviolet–visible and nuclear magnetic resonance spectrometers which are used in structural and quantitative analysis of molecules.

The basis of all these instrumental techniques is that they measure the interaction of electromagnetic radiation with matter in quantised, i.e. specific, energy levels. The purpose of this chapter is to introduce the various spectrophotometric techniques which are discussed in greater detail in the subsequent chapters, to show their relationship with each other and to describe the theoretical principles involved.

Electromagnetic radiation

White light from an incandescent solid such as the filament of an electric lamp is made up of a large number of individual waves of varying wavelength. This is readily shown by passing a beam of light through a prism when a band of colour, or so-called continuous spectrum, is formed, in which each colour corresponds to waves of a particular wavelength (Fig. 6.1).

The visible spectrum, however, forms only a small part of the complete spectrum of electromagnetic radiation, which extends, as shown in Fig. 6.1, from the ultra-short wave region of the cosmic rays at one end to that of radio waves at the other.

Wavelength is defined as the distance between any two consecutive parts of the wave whose vibrations are in phase, for example from the crest of one wave to that of the next, (A to B or B to C in Fig. 6.2). Its symbol is λ, the Greek letter lambda. The units in which wavelength is commonly expressed are recorded in Table 6.1. By adapting the unit to the appropriate region, the use of cumbersome figures can be avoided.

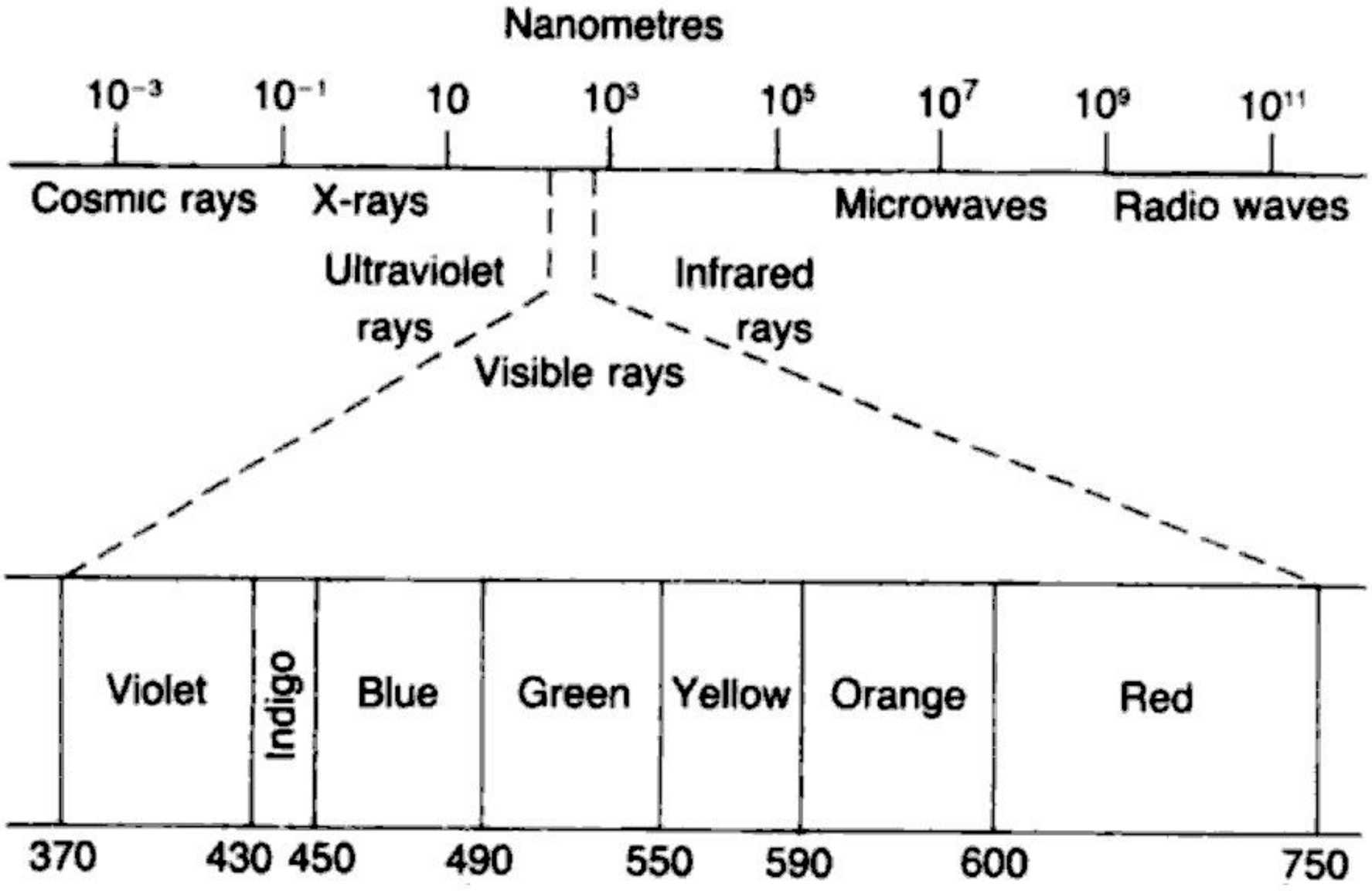

Fig. 6.1. Visible light and electromagnetic spectrum

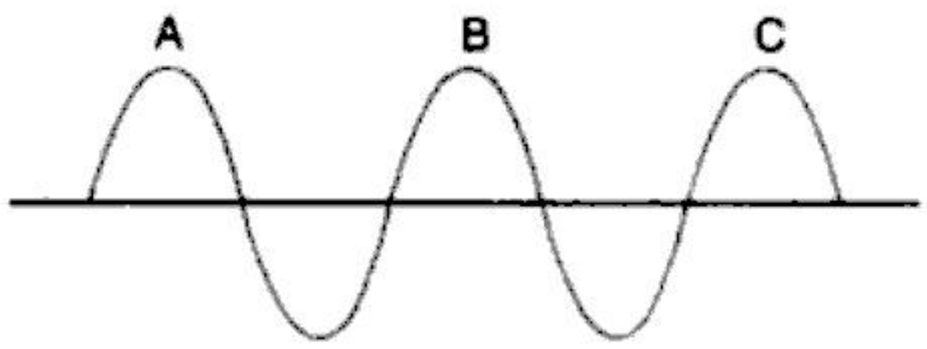

Fig. 6.2. Wavelength

Table 6.1 Wavelength units

Unit	Abbreviation	Metre	Region where used
Angstrom	Å	10^{-10}	Visible and ultraviolet
Nanometre	nm	10^{-9}	Visible and ultraviolet
Micrometre	μm	10^{-6}	Infrared

Older units for micrometre (micron) and for nanometre (millimicron) are now obsolete.

Wavenumber is defined as the reciprocal of the wavelength expressed in cm, i.e. the number of waves per cm. Its units are cm^{-1}.

Frequency is the number of waves passing a point in one second, i.e. the number of cycles per second. Its symbol is ν, the Greek letter nu, and the units are s^{-1} or hertz (Hz).

The interrelationships of these units can be expressed as follows:

$$\frac{1}{\text{wavelength in vacuo (in cm)}} = \text{wavenumber} = \frac{\text{frequency}}{\text{speed of light in vacuo (cm s}^{-1})}$$

Atomic spectra

Atomic emission

It has long been known that, when certain compounds are heated, light of characteristic colours is emitted. For example, sodium salts emit yellow light and potassium salts emit lilac light. The work of Kirchhoff and Bunsen in the middle of the 19th century showed that this is due to the emission of light at wavelengths characteristic of the metallic elements in the sample. Flame tests, in which the metallic elements are quickly identified by the characteristic colours imparted to a premixed combustible gas–air flame, remain one of the simplest qualitative analytical procedures.

When a sample containing metallic atoms is heated above 2000°, it first undergoes partial or complete dissociation into free atoms and then volatilises to free gaseous atoms. The electrons of the gaseous atoms exist in discrete quantised energy levels, i.e. they are in orbitals which have specific energy levels that are characteristic of the element. The electrons in the outer orbitals of the atoms may absorb thermal energy and be promoted to one or more higher energy states. The gain in energy of each electron during a transition is a specific quantity corresponding to the difference between the energy levels after and before excitation. Deactivation of the thermally excited atoms to lower energy states occurs very rapidly and photons of light are emitted which have energy (ΔE) equal to the difference between the upper (E_u) and lower (E_l) energy states. The energy of the emitted light is directly proportional to the frequency (ν) and inversely proportional to the wavelength (λ). Hence,

$$\Delta E = E_u - E_l = \frac{hc}{\lambda}$$

where h = Planck's constant = 4.132×10^{-15} eV s, and c = speed of light = 3×10^8 m s^{-1} (in vacuo).

$$\Delta E = \frac{1240}{\lambda \text{ (in nm)}}$$

As the atomic energy levels are characteristic of the element, the energies and wavelengths of light emitted are also characteristic of the element. The electronic energy transitions during the thermal excitation of and the emission of light from sodium atoms is shown in Fig. 6.3. The energy levels of the electronic orbitals (on the ordinate) are conventionally given in electronvolts greater than the lowest electronic energy level of the atom, which is given the energy eV = 0.

The wavelengths of light emitted from the sample as a result of thermal excitation may be viewed through a **spectroscope**, which is a simple instrument containing an entrance slit and a dispersing device, e.g. a prism. Alternatively, they may be recorded on photographic film using a **spectrograph**. The emission spectrum of sodium is seen to

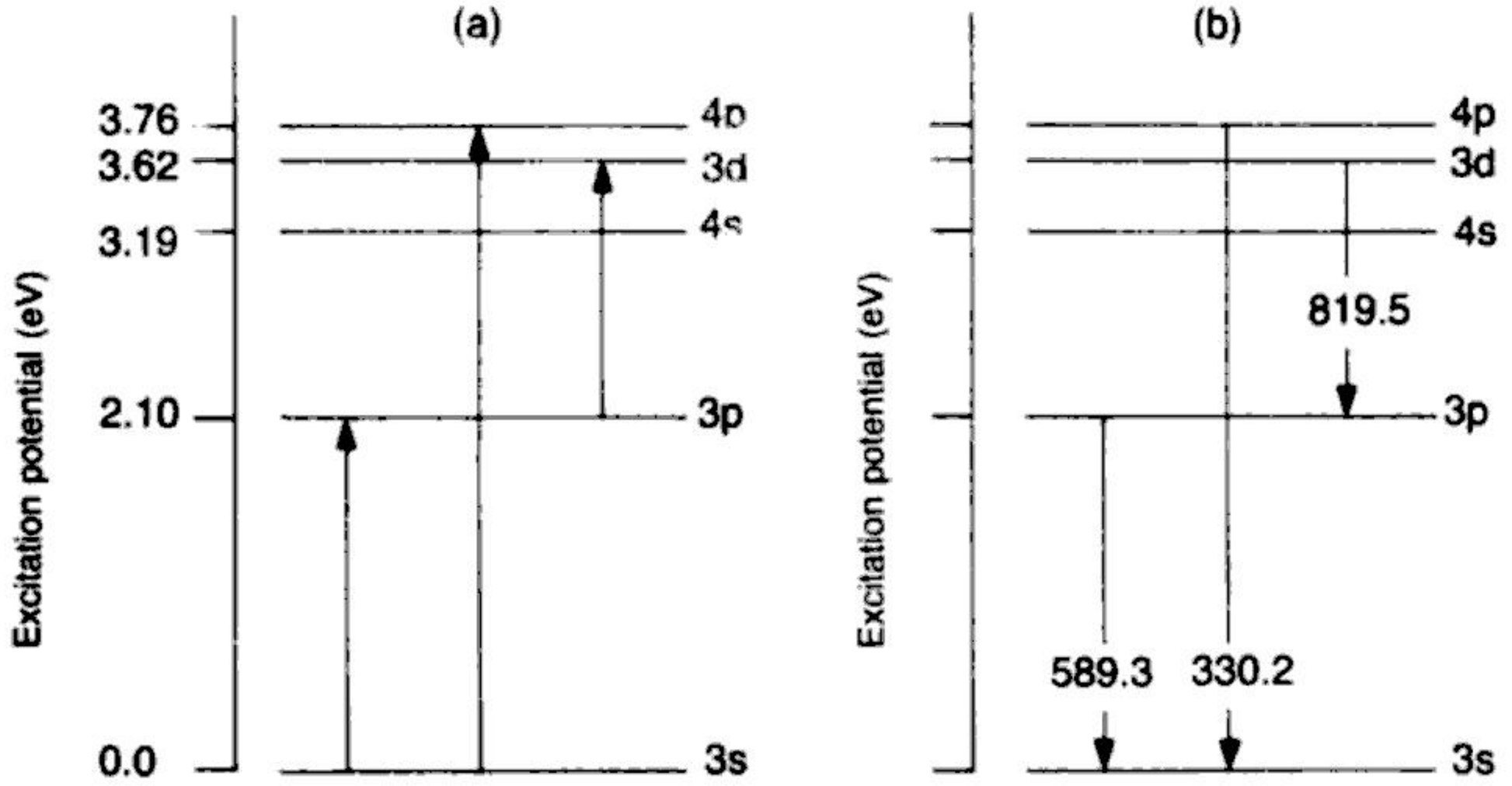

Fig. 6.3. Partial energy levels of sodium (a) during the absorption of thermal energy or electromagnetic radiation and (b) during deactivation with the emission of resonance wavelengths

comprise approximately 20 lines, the most intense of which occurs at 589.3 nm (consisting of a doublet at 589.0 nm and 589.6 nm), with less intense lines at 330.2 nm and 819.5 nm (Fig. 6.4). The line at 589.3 nm (the sodium D-line) is emitted when valence electrons thermally excited from the 3s orbital to the 3p orbital return to the ground state. The difference between the energy levels of the 3p (2.10 eV) and 3s (0 eV) orbitals corresponds with a wavelength of

$$\lambda = \frac{1240}{2.10 - 0} = 589\,\text{nm}$$

The less intense lines at 330.2 nm and 819.5 nm are due to 4p → 3s and 3d → 3p transitions respectively, which are given by fewer atoms. As an exercise you should identify the transition which accounts for the line at 1139 nm in the near infrared region. The intense line at 589.3 nm is yellow light (Fig. 6.1) and accounts for the observation of the yellow colour imparted to a Bunsen flame when sodium salts are introduced. The lilac coloration of a flame in the presence of potassium salts is due to the emission of two lines in the red region of the spectrum at 767 nm (4p → 4s) and 694 nm (4d → 4p) and one line in the violet region at 404 nm (5p → 4s).

The atomic emission techniques of **flame emission spectrometry (flame photometry)** and **emission spectrography** are based on the **measurement of light emitted from thermally excited atoms.**

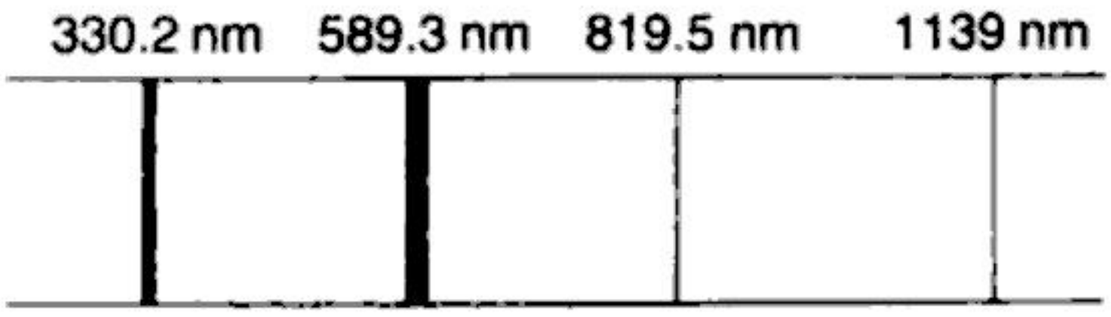

Fig. 6.4. Partial emission spectrum of sodium

Atomic absorption

Eight dark lines (lines A–H) in the continuous solar spectrum were explained by Fraunhofer in 1823 by the phenomenon of atomic absorption. Certain elements in the outer core of the sun absorb radiation at wavelengths characteristic of the elements. For example, absorption by sodium atoms gives rise to the line at 589.3 nm (the sodium D-line, the only line which is still commonly referred to by its original letter).

The excitation of electrons in atomic orbitals to higher energy states may be induced by electromagnetic radiation if the energy of the radiation exactly matches that corresponding to the difference between the upper and lower energy states (Fig. 6.3). Thus, ground state atoms absorb light of exactly the same characteristic resonance wavelengths that they emit after thermal excitation. For example, if a beam of light at 589 nm is passed through a vapour of sodium atoms, a portion of the ground state atoms will absorb radiation promoting the valence electrons to higher energy states.

Atomic absorption spectrophotometry is a technique for the quantitative determination of metallic elements and metalloids, which is based on the **measurement of the absorption of monochromatic light by ground state atoms**.

Molecular spectra

Molecular spectra are characterised by the absorption or emission of light over a much wider range of wavelengths (called spectral bands) than atomic spectra, which consist of sharp resonance lines (Fig. 6.4). This is due to the very large number of transitions which molecules can undergo, in comparison to the relatively few electronic transitions of atoms.

Molecular absorption

The absorption by molecules of electromagnetic radiation of a suitable wavelength can promote:

(a) The energy of electrons to one or more higher energy states (as in atomic absorption). The types of electrons that are responsible for ultraviolet–visible absorption are discussed in Chapter 7.
(b) An increase in the internuclear vibrational energies of the constituent atoms.
(c) An increase in the energy of rotation of the atoms round the bonds joining the atoms.

The total energy of a molecule is the sum of its electronic, vibrational and rotational energies, which are each quantised, i.e. they have specific energies characteristic of the molecular species. The relative energies required to induce electronic, vibrational and rotational transitions

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(bandwidth) is very wide and may exceed 150 nm. Gelatin filters consisting of a mixture of dyes incorporated in gelatin and sandwiched between glass plates are more selective, with bandwidths about 25 nm.

Interferometric filters have an even narrower bandwidth (about 15 nm) and consist of two parallel glass plates, silvered internally and separated by a thin film of cryolite or other dielectric material. Such filters make use of the interference of light waves rather than absorption to eliminate undesired radiation, and serve as relatively inexpensive monochromators for a specific purpose, e.g. the isolation of calcium radiation at 626 nm from that of sodium at 589.3 nm in the flame photometric method for Na^+ and Ca^{2+} in the same solution. Ordinary filters are incapable of this and cause large errors in measurement of the calcium content.

Prisms

When a beam of monochromatic light passes through a prism, it is bent or refracted. The amount of deviation is dependent on the wavelength, blue light being refracted more than red. If white polychromatic light is substituted for monochromatic radiation, a separation of the different wavelengths leads to the formation of a spectrum (Fig. 6.9) from which the required wavelength may be selected for the spectrometric measurement.

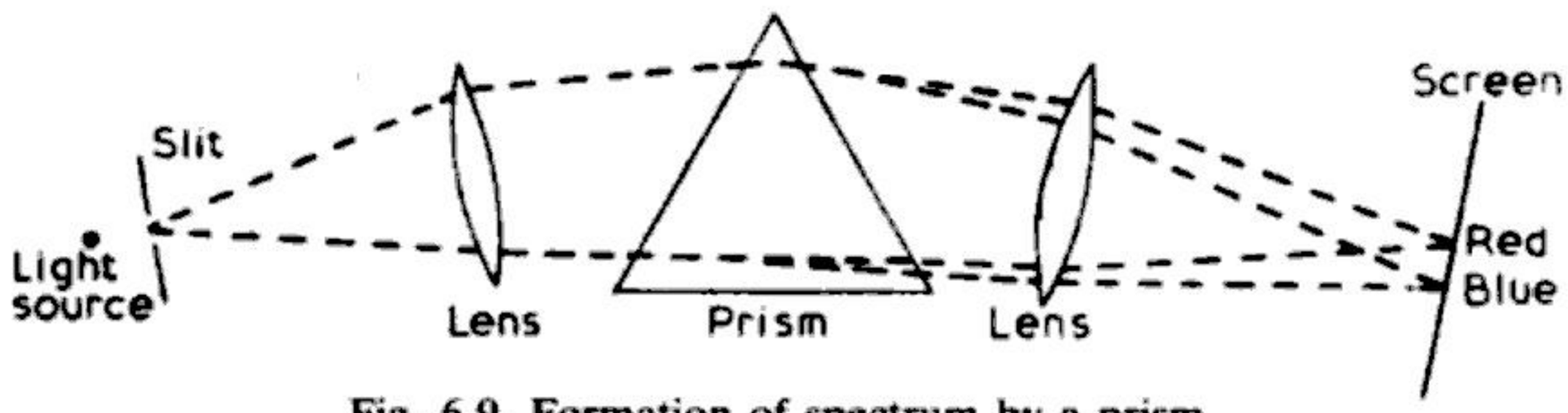

Fig. 6.9. Formation of spectrum by a prism

Prisms are made of quartz for use in the ultraviolet region, since glass absorbs wavelengths shorter than about 330 nm. Glass prisms are preferable for the visible region of the spectrum, as the dispersion is much greater than that obtained with quartz. For the infrared region, the transparent substances usually used for prisms are sodium chloride (2–15 μm), potassium bromide (12–25 μm), lithium fluoride (0.2–6 μm) and caesium bromide (15–38 μm).

Prisms produce a non-linear dispersion, with long wavelengths being less efficiently separated than short wavelengths. The wavelength scales of some (usually older) ultraviolet–visible spectrophotometers show larger divisions between each nm in the ultraviolet region than in the visible region.

Gratings

The dispersing element in the monochromator of most modern ultraviolet, visible and infrared spectrophotometers is the diffraction grating. It consists of a very large number of equispaced lines (200–2000 per mm) ruled on a glass blank coated with a thin film of aluminium. Gratings are now produced by modern holographic techniques. Parallel lines or grooves are chemically etched on a thin layer of photoresist coated on a blank after exposure to the interference fringes produced by the intersection of two beams of light from a laser. They can be used either as transmission gratings, or, when aluminised, as reflection gratings. Rotation of the grating permits appropriate wavelengths of the spectrum to emerge from the exit slit of the monochromator.

The theory of the plane transmission gratings is given below. Fig 6.10(a) shows part of a diffraction grating in which the gaps represent

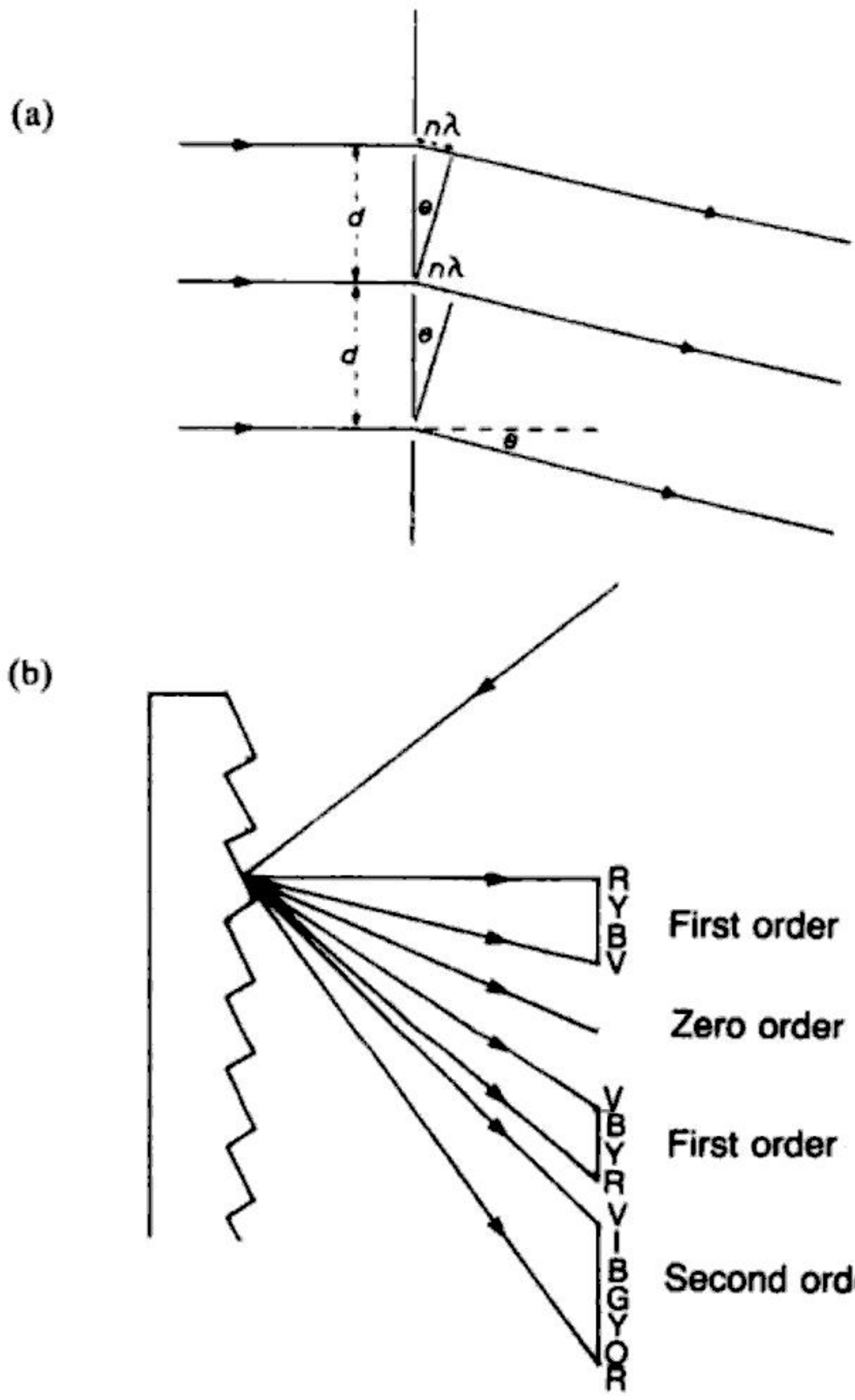

Fig. 6.10. (a) Formation of nth order spectrum: $d \sin \theta = n\lambda$. (b) Dispersion of visible light by a reflection diffraction grating. R, O, Y, G, B, I and V denote the colours of the dispersed light, red, orange, yellow, green, blue, indigo and violet respectively

the transparent spaces. The distance d between consecutive corresponding elements is called the grating space. A parallel beam of monochromatic light falls upon the grating at normal incidence. If the path difference for light diffracted through an angle θ from consecutive elements is $n\lambda$, then the various rays will reinforce each other. The values of θ in the equation $d \sin \theta = n\lambda$ ($n = 0, 1, 2, 3$) correspond to the angles of diffraction of different orders. When white light is used instead of monochromatic light, first and successive order images give rise to spectra (Fig 6.10(b)) due to the variation of θ with λ for a given n but the zero order is undispersed. The rulings of a grating can be shaped to concentrate the light in certain orders to give greater efficiency than when the light is spread over many spectra, and this is especially important in the infrared. A grating can be plane or concave, the latter being capable of focussing its own spectrum without the use of lenses or mirrors. However, for reasons of convenience in scanning, plane gratings are most frequently used in monochromators which can be made to cover all spectral regions from 180 nm to 15 μm. To eliminate the effect of overlapping orders, filters have sometimes to be used.

Cells

Samples presented for spectrometric analysis may be in the solid, liquid or gaseous state. The material that contains the sample ideally should be transparent at the wavelength(s) of measurement. For the analysis of liquids and gases in the ultraviolet–visible region above 320 nm, cells (or cuvettes) constructed with optically flat fused glass may be used. Measurements below the ultraviolet cutoff of glass at about 320 nm require the use of more expensive fused silica cells, which are transparent to below 180 nm. The standard pathlength of cells for measurements of molecular absorption or fluorescence in the ultraviolet–visible range is 10 mm, although cells of pathlength 1–50 mm are also available for special applications. The most common sample-containing materials in infrared spectrometry (Chapter 10) are sodium chloride (2.5–17 μm) and potassium bromide (2.5–30 μm). In the flame spectroscopic techniques of atomic absorption spectrophotometry and flame photometry (Chapter 8), the flame contains the absorbing or emitting atoms of the sample and functions as the sample cell.

Detectors

For the accurate determination of substances by spectrophotometric techniques, precise determinations of the light intensities are necessary. Photoelectric detectors are most frequently used for this purpose. They must be employed in such a way that they give a response linearly proportional to the light input, and they must not suffer from drift or fatigue.

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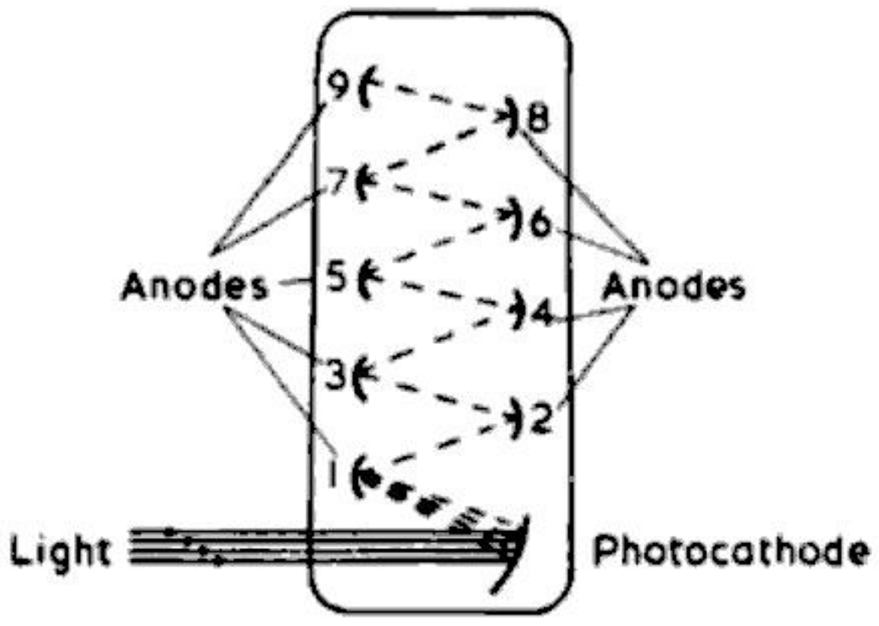

Fig. 6.13. Diagram of photomultiplier tube

impinges on the diode, the charge is depleted in proportion to the intensity of the radiation. When the electron beam scans again, a current in proportion to the light intensity flows to recharge the diode to the pre-set voltage.

Photodiodes, like vacuum phototubes and photomultiplier tubes, are single-channel detectors, i.e. they monitor the total intensity of light and cannot distinguish between different wavelengths. A spectrum is obtained with single-channel detectors by varying the monochromatic light passing through the sample. Recent advances in integrated circuitry and silicon wafer technology have led to the development of linear photodiode arrays containing 256, 512 or 1024 diodes which permit multi-channel detection. When used in combination with a dispersing system (e.g. a grating) each diode can monitor the light intensity at a different wavelength, and the array provides an almost instantaneous spectrum. The multi-channel detector based upon linear diode arrays is of particular advantage in fast reaction kinetics and in monitoring the eluate in HPLC.

Thermocouples

A thermocouple consists of elements of two different metals joined together at one end, the other end being attached to a sensitive galvanometer. When radiant energy impinges on the junction of the metals, a thermoelectromotive force is set up which causes a current to flow. Thermocouples are used in the infrared region, and to assist in the complete absorption of the available energy the 'hot' junction or receiver is usually blackened.

Bolometers

These make use of the increase in resistance of a metal with increase in temperature. For example, if two platinum foils are suitably incorporated into a Wheatstone Bridge, and radiation is allowed to fall on one foil, a change in resistance is produced. This results in an out-of-balance current which is proportional to the incident radiation. Like thermocouples, they are used in the infrared region.

Thermistors

The principle of operation is similar to that described under bolometers but thermistors are constructed of semi-conducting material which has a high negative coefficient of resistivity, i.e. the resistance decreases with increase in temperature.

Golay detector

In this detector, the absorption of infrared radiation causes expansion of an inert gas in a cell chamber. One wall of the chamber consists of a flexible mirror and the resulting distortion varies the intensity of illumination falling on a photocell from a reflected beam of light. The current from the photocell is proportional to the incident radiation.

Readout systems

The signal from the detector is normally proportional to the intensity of light incident on the detector, and after amplification may be displayed as % transmittance (%T) or, after passage through a logarithmic conversion circuit, as absorbance (log $1/T$). There are three common systems for displaying the %T or absorbance, i.e. (a) moving coil meter, (b) digital display or (c) strip-chart recorder.

Spectrophotometers

The brief description of the design and operation of spectrophotometers that follows is restricted to absorption spectrophotometers which measure in the ultraviolet and visible regions of the spectrum. The design of other spectrophotometers is considered in the relevant chapters on these techniques.

Single-beam spectrophotometers

The arrangement of the components in a commercially available single-beam ultraviolet–visible spectrophotometer is shown in Fig. 6.14. The essential characteristic is that the light travels in a single continuous optical path between the light source and the detector.

To make a measurement of absorbance using a manually controlled single-beam instrument, the monochromator is adjusted to the required wavelength and the appropriate lamp and photocell are selected by means of levers or switches. The first step is to close a shutter in the path and adjust a control labelled 'dark current' or 'zero' to offset the dark current from the detector to zero. This sets the scale to 0%T or infinite absorbance. The second step is to open the shutter and place the cell containing only the solvent in the light beam. The scale is set to read 100%T (equivalent to zero absorbance) by a control labelled '100%T' or 'zero absorbance'. The third step is to place the sample cell

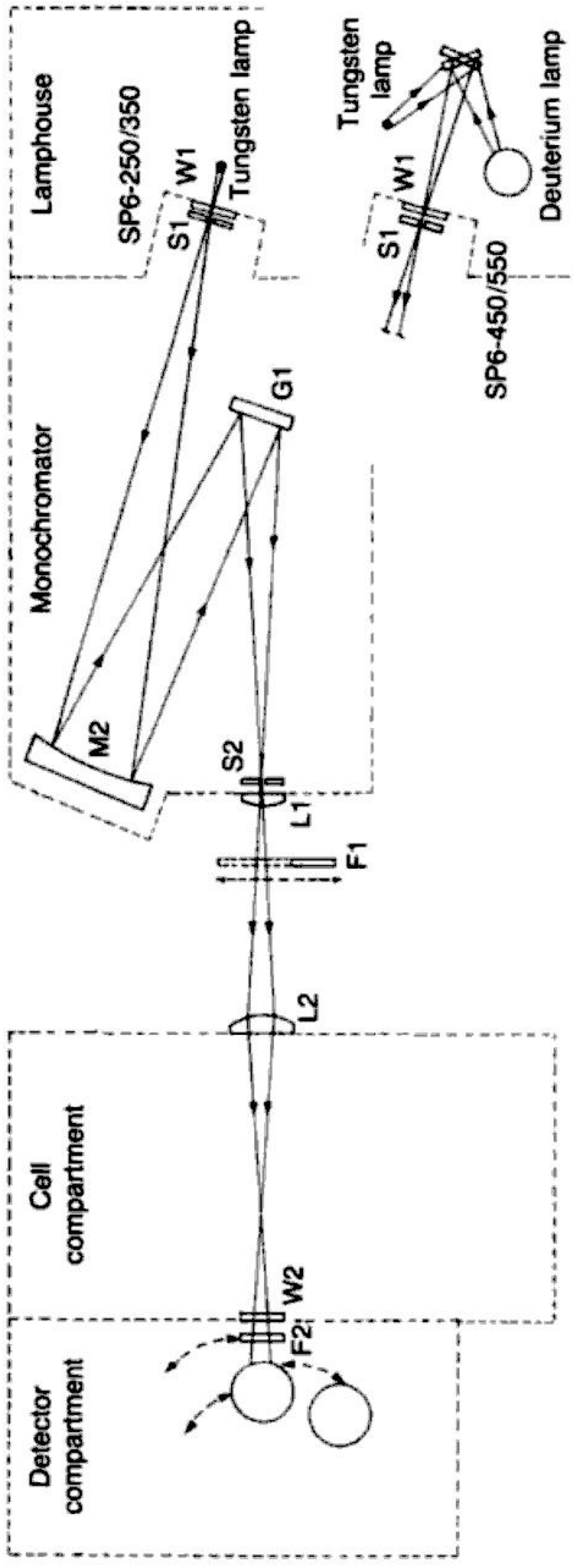

Fig. 6.14. Optical diagram of a single-beam ultraviolet-visible spectrophotometer (F = filter; G = grating; L = lens; M = mirror; S = slit; W = window). Reproduced with permission of Pye Unicam Ltd

in the light path and to measure the intensity I_T, or its equivalent absorbance, on the scale.

Single-beam instruments are relatively inexpensive and are satisfactory when many samples are being assayed by a simple measurement of absorbance at the same wavelength. A major disadvantage is the need to reset the 100%T at each wavelength to compensate for the large variation of intensity of light from the lamp with wavelength.

Double-beam spectrophotometers

In this type of instrument, the monochromatic light is split by a rapidly rotating beam chopper into two beams which are directed alternately in rapid succession through a cell containing the sample and one containing the solvent only (Fig. 6.15). If there is greater absorption of light in the sample cell than in the reference cell, the recombination of the beams at the detector produces a pulsating current which is converted into two direct current voltages proportional to the light intensities I_0 and I_T transmitted by the reference solution and the sample solution respectively. The ratio of voltages is recorded as a %T ($100I_T/I_0$) or, after logarithmic conversion, as absorbance ($\log I_o/I_T$). Double–beam optics therefore automatically compensate for variation of I_0 with wavelength. Recording spectrophotometers are double-beam instruments equipped with a wavelength scanning device which allows the rapid automatic scanning of spectra.

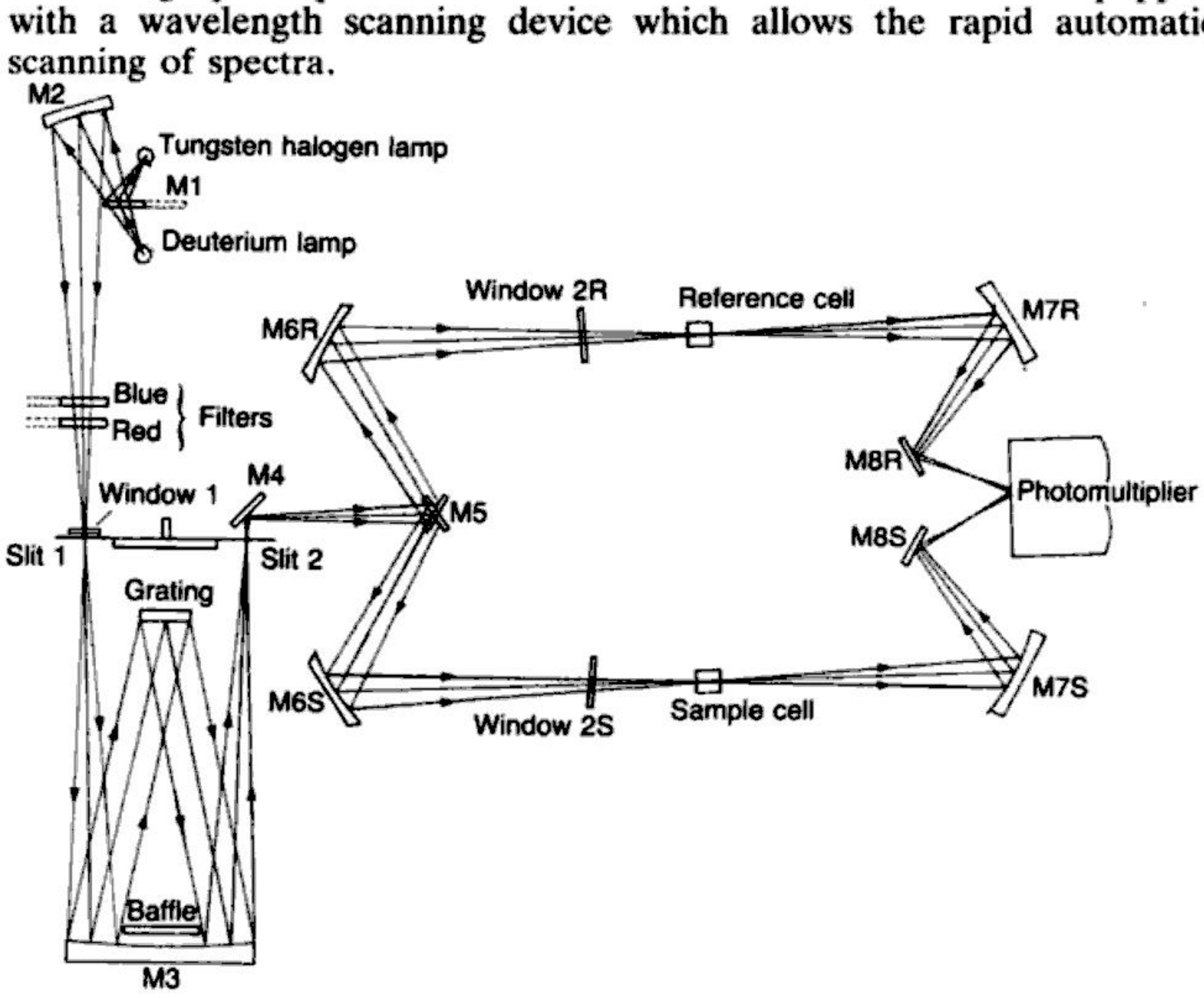

Fig. 6.15. Optical diagram of a double-beam ultraviolet–visible spectrophotometer (M = mirror). Reproduced with the permission of Pye Unicam Ltd

7

Ultraviolet-visible absorption spectrophotometry

A.G.DAVIDSON

Introduction

The technique of ultraviolet–visible spectrophotometry is one of the most frequently employed in pharmaceutical analysis. It involves the measurement of the amount of ultraviolet (190–380 nm) or visible (380–800 nm) radiation absorbed by a substance in solution. Instruments which measure the ratio, or a function of the ratio, of the intensity of two beams of light in the ultraviolet–visible region are called **ultraviolet–visible spectrophotometers**. Absorption of light in both the ultraviolet and visible regions of the electromagnetic spectrum occurs when the energy of the light matches that required to induce in the molecule an electronic transition and its associated vibrational and rotational transitions (p.260). It is thus convenient to consider the techniques of ultraviolet spectrophotometry and visible spectrophotometry together.

Beer–Lambert law

When a beam of light is passed through a transparent cell containing a solution of an absorbing substance, reduction of the intensity of the light may occur (Fig 7.1).

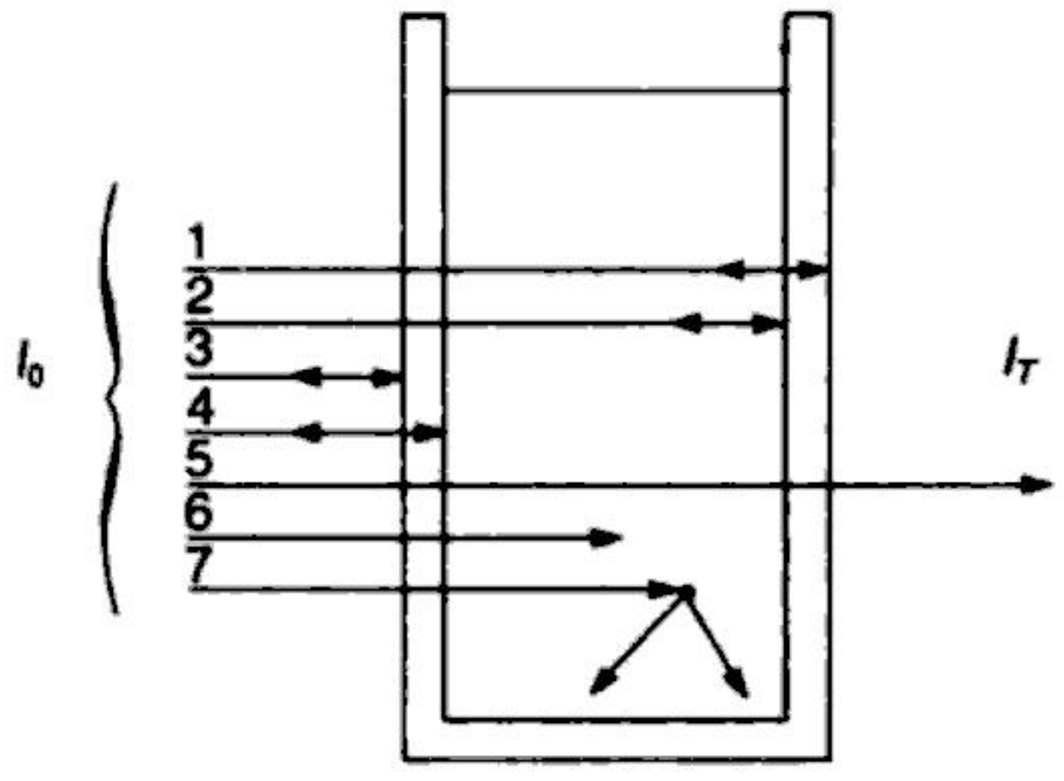

Fig. 7.1 The reduction of the intensity of light by reflection at cell faces (rays 1–4), absorption (ray 6) and scattering by particles (ray 7)

This is due to:

(a) reflections at the inner and outer surfaces of the cell
(b) scatter by particles in the solution
(c) absorption of light by molecules in the solution.

The reflections at the cell surfaces can be compensated by a reference cell containing the solvent only, and scatter may be eliminated by filtration of the solution. The intensity of light absorbed is then given by

$$I_{\text{absorbed}} = I_0 - I_T$$

where I_0 is the original intensity incident on the cell and I_T is the reduced intensity transmitted from the cell.

The **transmittance** (T) is the ratio I_T/I_0 and the % **transmittance** ($\%T$) is given by

$$\%T = \frac{100I_T}{I_0}$$

In 1760, Lambert investigated the relationship between I_0 and I_T for various thicknesses of substance and found that the rate of decrease in the intensity of light with the thickness, b, of the medium is proportional to the intensity of incident light. Expressed mathematically

$$-\frac{dI}{db} \propto I \text{ or } -\frac{dI}{db} = k' I$$

where k' is a proportionality constant. Therefore,

$$-\frac{db}{dI} = \frac{1}{k'I}$$

Integrating

$$-b = \frac{1}{k'} \ln I_T + C$$

where I_T is the intensity transmitted at thickness b.

When b = 0,

$$C = -\frac{1}{k'} \ln I_0$$

$$\therefore \quad -b = \frac{1}{k'} \ln I_T - \frac{1}{k'} \ln I_0$$

$$\therefore \quad \ln \frac{I_0}{I_T} = k'b$$

On conversion to a common logarithm, the expression becomes

$$\log \frac{I_0}{I_T} = \frac{k'b}{2.303}$$

The quantity $\log I_0/I_T$ is called **absorbance** (A) and is equal to the reciprocal of the common logarithm of transmittance.

$$A = \log_{10}\frac{I_0}{I_T} = \log_{10}\left(\frac{1}{T}\right) = -\log T = 2 - \log(\%T)$$

Older terms for absorbance such as **extinction, optical density** and **absorbancy** are now obsolete.

Lambert's Law is defined as follows: the intensity of a beam of parallel monochromatic radiation decreases exponentially as it passes through a medium of homogeneous thickness. More simply it is stated that the absorbance is proportional to the thickness (pathlength) of the solution.

Beer showed in 1852 that a similar relationship exists between the absorbance and the concentration.

$$\log\frac{I_0}{I_T} = \frac{k''c}{2.303}$$

where k'' is a proportionality constant and c is the concentration.

Beer's Law is defined as follows: the intensity of a beam of parallel monochromatic radiation decreases exponentially with the number of absorbing molecules. More simply it is stated that the absorbance is proportional to the concentration.

A combination of the two laws yields the Beer–Lambert Law:

$$A = \log\frac{I_0}{I_T} = abc$$

in which the proportionality constants $k'/2.303$ and $k''/2.303$ are combined as a single constant called the **absorptivity** (a).

The name and value of a depend on the units of concentration. When c is in moles per litre, the constant is called **molar absorptivity** (formerly the **molar extinction coefficient**) and has the symbol ϵ (the Greek letter epsilon). The equation therefore takes the form

$$A = \epsilon bc$$

The molar absorptivity at a specified wavelength of a substance in solution is the absorbance at that wavelength of a $1\,\text{mol}\,l^{-1}$ solution in a 1 cm cell. The units of ϵ are therefore $1\,\text{mol}^{-1}\,\text{cm}^{-1}$. Expressing the absorptivity in terms of a $1\,\text{mol}\,l^{-1}$ solution facilitates the comparison of the light-absorbing abilities of compounds with widely differing molecular weights. Substances that have ϵ values less than 100 are weakly absorbing; those with ϵ values above 10 000 are intensely absorbing. Many absorbing drugs have an ϵ value at their wavelength of maximum absorption of $10^{3.5}$–$10^{4.5}$.

Another form of the Beer–Lambert proportionality constant is the **specific absorbance**, which is the absorbance of a specified concentration in a cell of specified pathlength. The most common form in pharmaceutical analysis is the $A(1\%, 1\,\text{cm})$, which is the absorbance of a 1 g/100 ml (1% w/v) solution in a 1 cm cell. The Beer–Lambert equation therefore takes the form

$$A = A^{1\%}_{1\,\text{cm}}bc$$

where c is in g/100 ml and b is in cm. The units of $A(1\%, 1\,\text{cm})$ are $\text{dl g}^{-1}\,\text{cm}^{-1}$. Occasionally, the concentration of liquids in solution is given as % v/v (e.g. in the British Pharmacopoeial assay of methyl salicylate and diethyl phthalate in Surgical Spirit) in which case the specific absorbance is the absorbance of a 1 ml/100 ml solution in a 1 cm cell (see Experiment 12).

A simple easily derived equation allows interconversion of ϵ and $A(1\%, 1\,\text{cm})$ values

$$\epsilon = \frac{A^{1\%}_{1\,\text{cm}} \times \text{molecular weight}}{10}$$

The majority of applications in which spectrophotometric measurements are made rely on the compliance of the absorbing substance in solution with the Beer–Lambert Law at the wavelength of measurement. The absorbance of most substances when correctly measured in a calibrated spectrophotometer (p.325) is normally found to have a proportional relationship with the concentration and pathlength of the solution. Nevertheless, it is essential in the development of new spectrophotometric procedures to confirm that Beer's Law in particular obtains over the range in which the absorbance of the sample is found. A number of instrumental or chemical effects (p.312) may be responsible for deviations from the Beer–Lambert Law.

Quantitative spectrophotometric assay of medicinal substances

The assay of an absorbing substance may be quickly carried out by preparing a solution in a transparent solvent and measuring its absorbance at a suitable wavelength. The wavelength normally selected is a wavelength of maximum absorption (λ_{max}) where small errors in setting the wavelength scale have little effect on the measured absorbance. Ideally, the concentration should be adjusted to give an absorbance of approximately 0.9, around which the accuracy and precision of the measurement are optimal (p.309). The preferred method is to read the absorbance from the instrument display under non-scanning conditions, i.e. with the monochromator set at the analytical wavelength. Alternatively, the absorbance may be read from a recording of the spectrum obtained by using a recording double-beam spectrophotometer. The latter procedure is particularly useful for qualitative purposes (pp.315–325) and in certain assays in which absorbances at more than one wavelength are required. The concentration of the absorbing substance is then calculated from the measured absorbance using one of three principal procedures.

Use of a standard absorptivity value

This procedure is adopted by official compendia, e.g. British Pharmacopoeia, for stable substances such as *Methyltestosterone* that have reason-

ably broad absorption bands and which are practically unaffected by variation of instrumental parameters, e.g. slit width, scan speed (pp.309–312). The use of standard $A(1\%, 1\,cm)$ or ϵ values avoids the need to prepare a standard solution of the reference substance in order to determine its absorptivity, and is of advantage in situations where it is difficult or expensive to obtain a sample of the reference substance.

Example 1

Calculate the concentration of methyltestosterone in an ethanolic solution of which the absorbance in a 1 cm cell at its λ_{max}, 241 nm, was found to be 0.890. The $A(1\%, 1\,cm)$ in the B.P. monograph of *Methyltestosterone* is given as 540 at 241 nm.

Substituting in the appropriate form of the Beer–Lambert equation:

$$A = A^{1\%}_{1\,cm}\, bc$$

gives

$$0.890 = 540 \times 1 \times c$$
$$\therefore \quad c = 0.00165\ g/100\ ml$$

Example 2

Calculate the concentration in $\mu g\,ml^{-1}$ of a solution of tryptophan (molecular weight 204.2) in 0.1M hydrochloric acid, giving an absorbance at its λ_{max}, 277 nm, of 0.613 in a 4 cm cell. The molar absorptivity at 277 nm is 5432.

Substituting in the appropriate form of the Beer–Lambert equation:

$$A = \epsilon bc$$

gives

$$0.613 = 5432 \times 4 \times c$$
$$\therefore \quad c = 2.82 \times 10^{-5}\ mol\ \ell^{-1}$$
$$= 2.82 \times 10^{-5} \times 204.2\ g\ \ell^{-1}$$
$$= 0.00576\ g\ \ell^{-1}$$
$$= 5.76\ \mu g\ ml^{-1}$$

Use of a calibration graph

In this procedure the absorbances of a number (typically 4–6) of standard solutions of the reference substance at concentrations encompassing the sample concentrations are measured and a calibration graph is constructed. The concentration of the analyte in the sample solution is read from the graph as the concentration corresponding to the absorbance of the solution. Calibration data are essential if the absorbance has a non-linear relationship with concentration, if it is necessary to confirm the proportionality of absorbance as a function of concentration, or if the absorbance or linearity is dependent on the assay conditions. In

certain visible spectrophotometric assays of colourless substances, based upon conversion to coloured derivatives by heating the substance with one or more reagents, slight variation of assay conditions, e.g. pH, temperature and time of heating, may give rise to a significant variation of absorbance, and experimentally derived calibration data are required for each set of samples.

Statistical treatment of the calibration data, facilitated by micro-computers or pre-programmable calculators, provides a more elegant and accurate determination of the relationship between absorbance and concentration than manually constructed graphs. If the absorbance values and concentrations bear a linear relationship, the regression line $y = \alpha + \beta x$ may be estimated by the **method of least squares**.

$$\alpha = \frac{(\Sigma y)\ (\Sigma x^2) - (\Sigma x)(\Sigma xy)}{N\Sigma x^2 - (\Sigma x)^2}$$

$$\beta = \frac{N\Sigma xy - (\Sigma x)(\Sigma y)}{N\Sigma x^2 - (\Sigma x)^2}$$

where y is the absorbance value at concentration x and N is the number of pairs of values.

Example 3

The absorbance values at 250 nm of five standard solutions, a blank solution and a sample solution of a drug are given in Table 7.1. Calculate, using linear regression analysis, the line of best fit and the concentration of the sample.

Table 7.1 Absorbance Values of Five Standard Solutions, a Blank Solution and a Sample Solution of a Drug

Concentration (μg ml^{-1}) x	A_{250} y
0	0.002
10	0.168
20	0.329
30	0.508
40	0.660
50	0.846
Sample	0.611

Calculation of the slope (β) and intercept (α) using the equations above gives

$$y = 0.01679x - 0.0008$$

$$\therefore \quad \text{Concentration of the sample} = 36.5\mu\text{g ml}^{-1}$$

Note The data may be further evaluated statistically to confirm that a linear relationship between x and y exists and to provide confidence

limits for the slope, intercept and estimated concentration of the sample. For this the reader is referred to standard texts on statistical analysis (e.g. Colquhoun, 1971).

Single-or double-point standardisation

The single-point procedure involves the measurement of the absorbance of a sample solution and of a standard solution of the reference substance. The standard and sample solutions are prepared in a similar manner; ideally, the concentration of the standard solution should be close to that of the sample solution. The concentration of the substance in the sample is calculated from the proportional relationship that exists between absorbance and concentration.

$$c_{\text{test}} = \frac{A_{\text{test}} \times c_{\text{std}}}{A_{\text{std}}}$$

where c_{test} and c_{std} are the concentrations in the sample and standard solutions respectively, and A_{test} and A_{std} are the absorbances of the sample and standard solutions respectively. Since sample and standard solutions are measured under identical conditions, this procedure is the preferred method of assay of substances that obey Beer's Law and for which a reference standard of adequate purity is available. It is the procedure adopted in many spectrophotometric assays of the British Pharmacopoeia and for the majority of spectrophotometric assays of the United States Pharmacopeia.

Occasionally a linear but non-proportional relationship between concentration and absorbance occurs, which is indicated by a significant positive or negative intercept in a Beer's Law plot. A 'two-point bracketing' standardisation is therefore required to determine the concentration of the sample solutions. The concentration of one of the standard solutions is greater than that of the sample while the other standard solution has a lower concentration than the sample. The concentration of the substance in the sample solution is given by the equation (the derivation of which can be attempted as an exercise):

$$c_{\text{test}} = \frac{(A_{\text{test}} - A_{\text{std}_1})(c_{\text{std}_1} - c_{\text{std}_2}) + c_{\text{std}_1}(A_{\text{std}_1} - A_{\text{std}_2})}{A_{\text{std}_1} - A_{\text{std}_2}}$$

where the subscripts std_1 and std_2 refer to the more concentrated standard and less concentrated standard respectively.

Assay of substances in multicomponent samples

The spectrophotometric assay of drugs rarely involves the measurement of absorbance of samples containing only one absorbing component. The pharmaceutical analyst frequently encounters the situation where the concentration of one or more substances is required in samples known to contain other absorbing substances which potentially interfere in the assay. If the recipe of the sample formulation is available to the

analyst, the identity and concentration of the interferents are known and the extent of interference in the assay may be determined. Alternatively, interference which is difficult to quantify may arise in the analysis of formulations from manufacturing impurities, decomposition products and formulation excipients. Unwanted absorption from these sources is termed **irrelevant absorption** and, if not removed, imparts a systematic error to the assay of the drug in the sample.

A number of modifications to the simple spectrophotometric procedure described above for single-component samples is available to the analyst, which may eliminate certain sources of interference and permit the accurate determination of one or all of the absorbing components. Each modification of the basic procedure may be applied if certain criteria are satisfied. The correct choice of procedure for a particular analytical problem provides the analyst with an opportunity to demonstrate his/her analytical expertise.

The basis of all the spectrophotometric techniques for multicomponent samples is the property that at all wavelengths:

(a) the absorbance of a solution is the sum of absorbances of the individual components; or
(b) the measured absorbance is the difference between the total absorbance of the solution in the sample cell and that of the solution in the reference (blank) cell.

Assay as a single-component sample

The concentration of a component in a sample which contains other absorbing substances may be determined by a simple spectrophotometric measurement of absorbance as described above, provided that the other components have a sufficiently small absorbance at the wavelength of measurement. This condition is satisfied if the concentration of the interfering substances, their absorptivity or the pathlength of the solution are sufficiently small that their product (i.e. the absorbance) can be ignored. A systematic error of less than 1% would normally be considered to be acceptable. For example, if the contribution to a total absorbance of 1.00 from the interferents is less than 0.01, and if there is no chemical interaction between the components, the sample may be analysed for its principal absorbing component by a simple direct measurement of absorbance at its λ_{max}. An example of this approach is the assay of paracetamol in *Paediatric Paracetamol Elixir*. At the large overall dilution (approximately 3250 times) of the sample the absorbance of the other ultraviolet-absorbing components is negligible.

Assay using absorbance corrected for interference

If the identity, concentration and absorptivity of the absorbing interferents are known, it is possible to calculate their contribution to the total

absorbance of a mixture. The concentration of the absorbing component of interest is then calculated from the corrected absorbance (total absorbance minus the absorbance of the interfering substances) in the usual way.

Example 4

The λ_{max} of ephedrine hydrochloride and chlorocresol are 257 nm and 279 nm respectively and the $A(1\%,1\,cm)$ values in 0.1M hydrochloric acid solution are:

ephedrine hydrochloride at 257 nm	= 9.0
ephedrine hydrochloride at 279 nm	= 0
chlorocresol at 257 nm	= 20.0
chlorocresol at 279 nm	= 105.0

Calculate the concentrations of ephedrine hydrochloride and chlorocresol in a batch of *Ephedrine Hydrochloride Injection*, diluted 1 to 25 with *water*, giving the following absorbance values in 1 cm cells.

$$A_{279} = 0.424 \qquad A_{257} = 0.972$$

(a) Since ephedrine does not absorb at 279 nm, calculate the concentration of chlorocresol from the A_{279} of the diluted injection.

$$0.424 = 105 \times 1 \times c$$
$$\therefore \quad c = 0.00404 \text{ g/100 ml}$$
$$\therefore \quad \text{concentration of chlorocresol in the injection}$$
$$= 0.00404 \times 25$$
$$= 0.1010 \text{ g/100 ml}$$
$$= 1.010 \text{ mg/ml}$$

(b) Calculate the absorbance of chlorocresol at 257 nm in the diluted injection.

$$A = 20 \times 1 \times 0.00404$$
$$= 0.081$$

(c) Calculate the concentration of ephedrine hydrochloride from the corrected absorbance at 257 nm

$$\text{Corrected absorbance at 257 nm} = 0.972 - 0.081$$
$$= 0.891$$
$$0.891 = 9.0 \times 1 \times c$$
$$\therefore \quad c = 0.0990 \text{ g/100 ml}$$
$$\therefore \quad \text{concentration of ephedrine hydrochloride in the injection}$$
$$= 0.0990 \times 25$$
$$= 2.475 \text{ g/100 ml}$$
$$= 24.75 \text{ mg/ml}$$

Assay after solvent extraction of the sample

If the interference from other absorbing substances is large or if its contribution to the total absorbance cannot be calculated, it may be possible to separate the absorbing interferents from the analyte by solvent extraction procedures. These are particularly appropriate for acidic or basic drugs whose state of ionisation determines their solvent partitioning behaviour (Part 1, Chapter 9). The judicious choice of pH of the aqueous medium and of immiscible solvent may effect the complete separation of the interferents from the analyte, the concentration of which may be obtained by a simple measurement of absorbance of the extract containing the analyte. An example of this type of assay is the B.P. assay of caffeine in *Aspirin and Caffeine Tablets*.

Simultaneous equations method

If a sample contains two absorbing drugs (X and Y) each of which absorbs at the λ_{max} of the other (Fig.7.2, λ_1 and λ_2), it may be possible to determine both drugs by the technique of simultaneous equations (Vierodt's method) provided that certain criteria apply (see later).

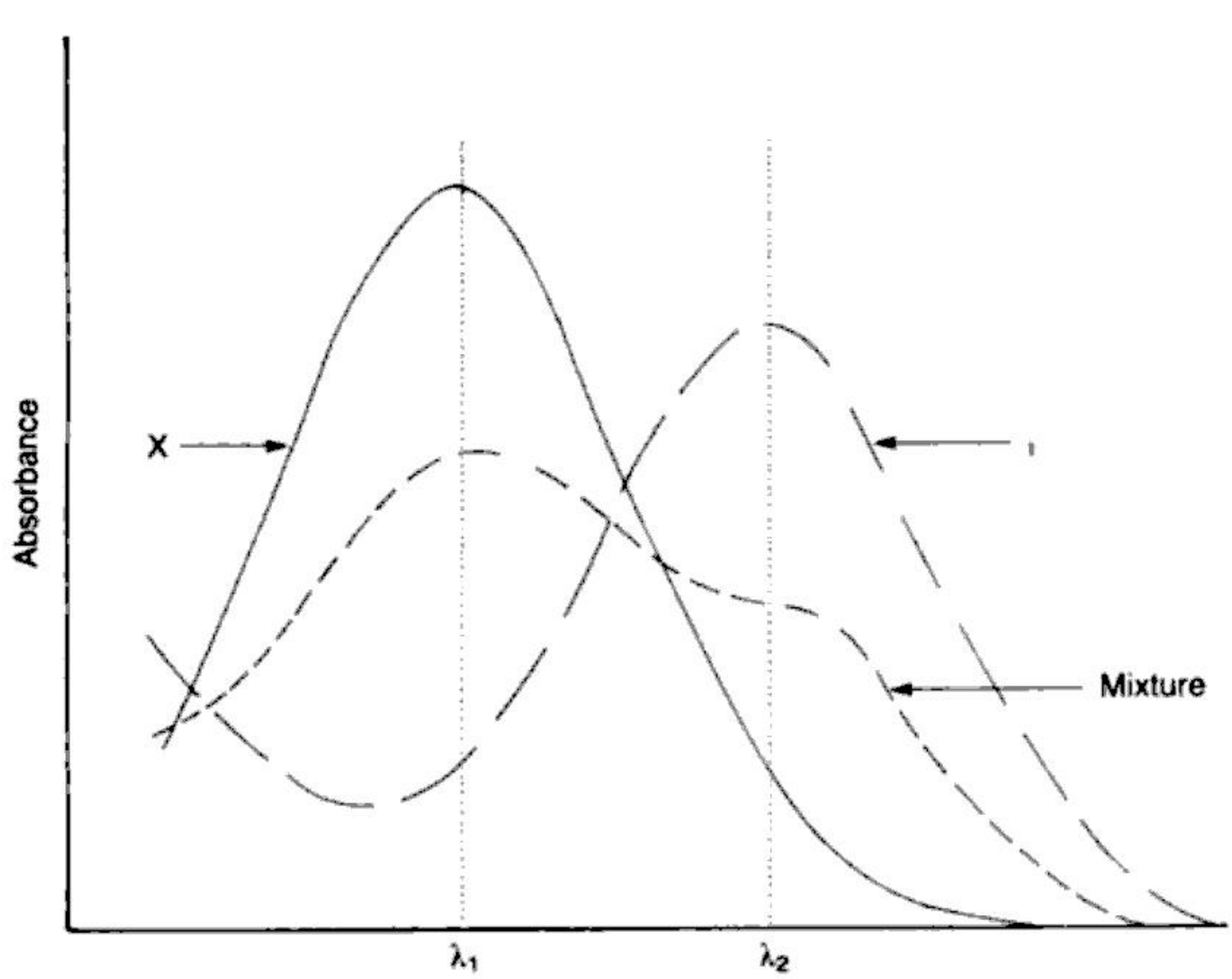

Fig. 7.2 The individual absorption spectra of substances X and Y, showing the wavelengths for the assay of X and Y in admixture by the method of simultaneous equations

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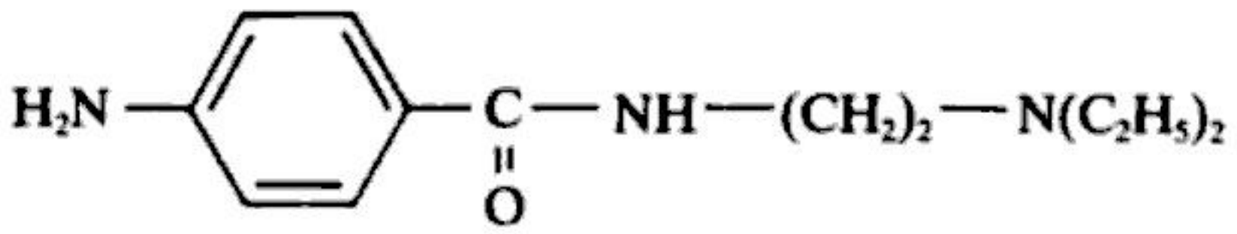
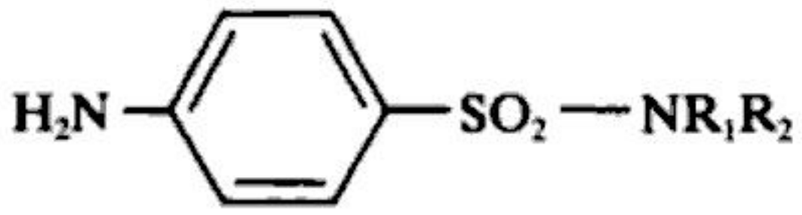
When phenolic hydroxyl groups are present, a marked bathochromic displacement of the absorption to about 280 nm occurs, and the effect of solvent on the absorption is considerable because, whereas in neutral or acid media the auxochrome is —OH, in alkaline media it becomes $—O^-$. For example, the absorption maximum for chlorocresol in acid solution occurs at 279 nm, but in alkaline media the peak is found at 296 nm. Typical examples of phenols are *Morphine Hydrochloride*, *Nalorphine Hydrobromide* and *Ethinyloestradiol*, whilst *Adrenaline* and *Isoprenaline* are examples of *o*-dihydroxyphenols.

The amino group is a powerful auxochrome when attached directly to a benzene system, and aniline exhibits high intensity absorption at about 230 nm and typical low intensity benzenoid absorption at 280 nm. If additional conjugation is present, a cumulative effect is obtained. Thus in *Procainamide* the 200 nm high intenstiy absorption of benzene suffers a bathochromic displacement to 280 nm.



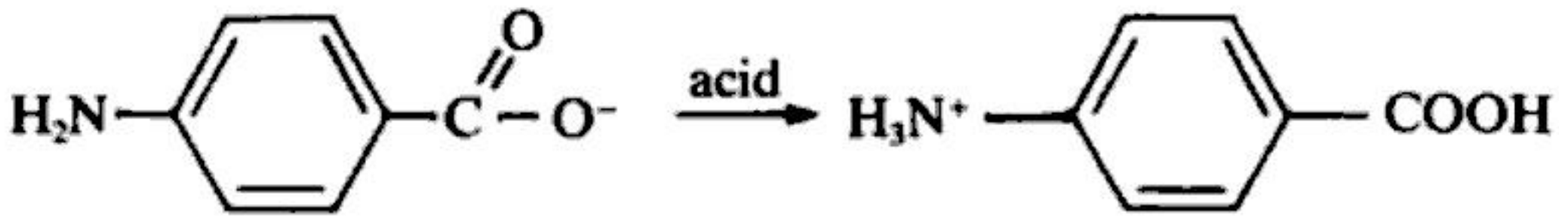
Procainamide

Similarly, the sulphonamide drugs of general formula

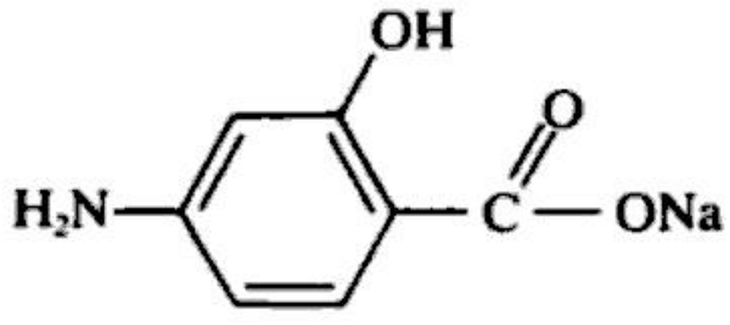


show high intensity absorption at about 251 nm. The effect of change in pH in the solvent used for the ultraviolet measurements is very striking (Fig.7.14). In alkaline solution, the absorbing system is as given above, but in acidic solution the amino $—NH_2$ group is replaced by $—\overset{+}{N}H_3$ which is considerably less efficient as an auxochrome.

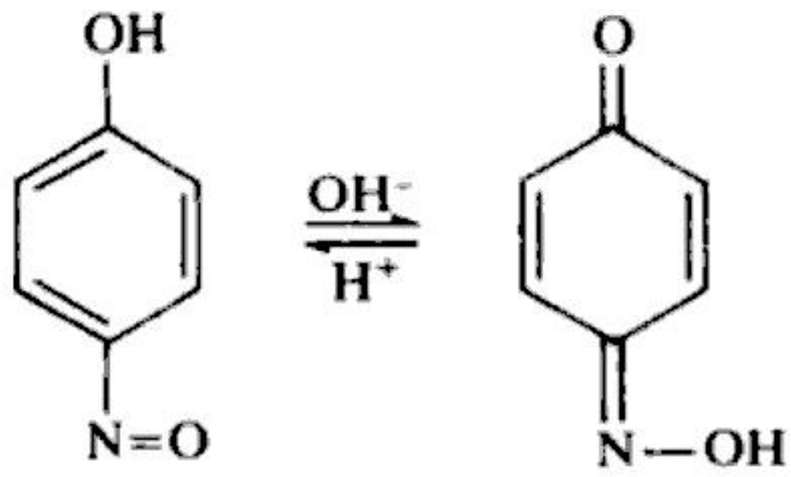
p-Aminobenzoic acid is similar in absorption characteristics to *Procainamide* and *Procaine* in neutral or alkaline media, but in acid solution the absorption curves of all three approach that of benzoic acid.



Sodium aminosalicylate in alkaline solution has an additional auxochrome the phenate ion, so that the high intensity absorption noted in



Sodium Aminosalicylate



This is actually done in the colorimetric assay for morphine in *Camphorated Opium Tincture*. The sensitivity of the method is increased by measuring the absorption of the nitroso-morphine in alkaline solution.

The subject could be expanded greatly but enough has been said to indicate that inspection of the formula of a substance will reveal whether or not interesting features of absorption in the ultraviolet region can be expected.

Ultraviolet–visible spectra rarely provide a complete identification of a substance, owing to the lack of characteristic spectral features. Structural elucidation of new compounds is usually carried out by the examination of infrared, nuclear magnetic reasonance and mass spectrometric data in conjunction with ultraviolet data (Chapter 14). The ultraviolet–visible absorption spectra and spectral characteristics (λ_{max} and $A\,^{1\%}_{1\,cm}$ or ϵ values) of many drug substances may be obtained from a number of sources (Sharkey et al, 1968; Clarke, 1985; Sunshine, 1981).

Experiments in ultraviolet–visible spectrophotometry

The following exercises are designed to illustrate principles of method and theory and to provide practical examples of spectrophotometric techniques in the assays of drugs in medicines and in body fluids. The variation in the design of spectrophotometers makes it inadvisable to relate experiments to any one particular instrument.

Instrument performance

Experiment 1 *Calibration of absorbance scale*

Prepare a standard solution of potassium dichromate (Note 1) approximately 60 mg (Note 2) diluted to 1 l with 0.005M sulphuric acid (Note 3). Measure the absorbance of the solution in a 1 cm cell using 0.005M sulphuric acid in the reference cell (Note 4) at the two wavelengths of maximum absorption (257 nm and 350 nm) and at the two wavelengths of minimum absorption (235 nm and 313 nm). Alternatively, record the absorption spectrum on the 0–1 absorbance range between 400 nm and 220 nm using a *slow* scan speed (Note 5) and read the absorbance at the two λ_{max} and two λ_{min} values. Calculate the $A(1\%, 1\,cm)$ values using the exact concentration of the standard solution and compare the results with the standard values in Table 7.9 (Note 6).

Note 1 Analytical reagent quality; dried at 110° for 1 h.
Note 2 Accurately weighed ±0.1 mg.
Note 3 The effect of pH on the dissociation of dichromate is discussed on p.314.
Note 4 Before using the solution, a check should be carried out on the cells in use.

Normally these are matched, but it is of interest to note how often the following will indicate differences in the cells. Fill both cells with the solvent being used. Dry the outside of the cells with paper tissues. Do not touch the polished faces with the fingers but handle the cells by the ground glass sides only. Check the absorbance of one cell against the other as blank at the selected wavelength. Note the reading, reject the solvent in one cell and fill again with solvent. Check the reading and repeat the emptying and filling until consistent readings are obtained. Now reject the solvent in the other cell and repeat the above procedure. Any residual difference at this stage can be used to correct absorbances. When the difference becomes large (0.005–0.010) then cleaning of cells must be undertaken.

Note 5 The effect of scan speed is discussed on p.311.

Note 6 The A(1%, 1 cm) values are the mean values obtained in a study by the UV Spectrometry Group and the range is based on the variation found in the literature values.

Table 7.9 Recommended A(1%, 1 cm) values for acidic potassium dichromate solutions

Wavelength (nm)	A(1%, 1 cm)
235 (min)	125.1 ± 1.9
257 (max)	145.4 ± 1.5
313 (min)	48.8 ± 0.7
350 (max)	107.1 ± 1.1

Experiment 2 *Calibration of wavelength*

(a) *Holmium filter* For the routine calibration of instruments, a holmium filter is satisfactory. Record the absorption spectrum (0–2 absorbance range) from 500 to 230 nm using the slowest scan speed and the narrowest slit setting. Identify the three fused absorption bands centred around 452.2 nm and the single band at around 360.9 nm. Instruments with accurately calibrated wavelength scales will show λ_{max} at 453.2, 418.4, 360.9, 287.5, 279.4 and 241.5 nm.

(b) *Holmium perchlorate solution* Prepare a solution of holmium(III) perchlorate by dissolving 0.5 g of holmium oxide in 2.4 ml perchloric acid (72%; AR) by warming gently and diluting to 10 ml with *water*. Record the absorption spectrum (0–1 absorbance) as described for the holmium filter. The wavelengths of the principal bands (A >0.4) should be 485.8, 450.8, 416.3, 361.5, 287.1, 278.7 and 241.1 nm.

(c) *Discharge lamps* A low pressure mercury discharge lamp is the most suitable for the high-accuracy calibration of instruments. Record the **transmission** spectrum from 600 nm to 240 nm (i.e. with the instrument in the 'single-beam' or 'energy' mode) of the mercury lamp placed near the entrance to the monochromator, using the minimum slit setting and slowest scan speed. The principal emission lines of mercury are at 579.0, 576.9, 546.1, 435.8, 404.5, 364.9 and 253.7 nm.

Experiment 3 *Detection of stray light*

The effects of stray light on an absorption spectrum are discussed on p.312. Stray light at a particular wavelength may be detected using a cut-off filter which absorbs intensely at that wavelength.

Method Measure the absorbance at 200 nm of a 1.2% solution of potassium chloride in *water* against *water* in the reference cell. Alternatively, measure the absorbance in the range 200–210 nm of a Vycor glass filter against air. Any absorbance less than 2 indicates the presence of stray light in this region.

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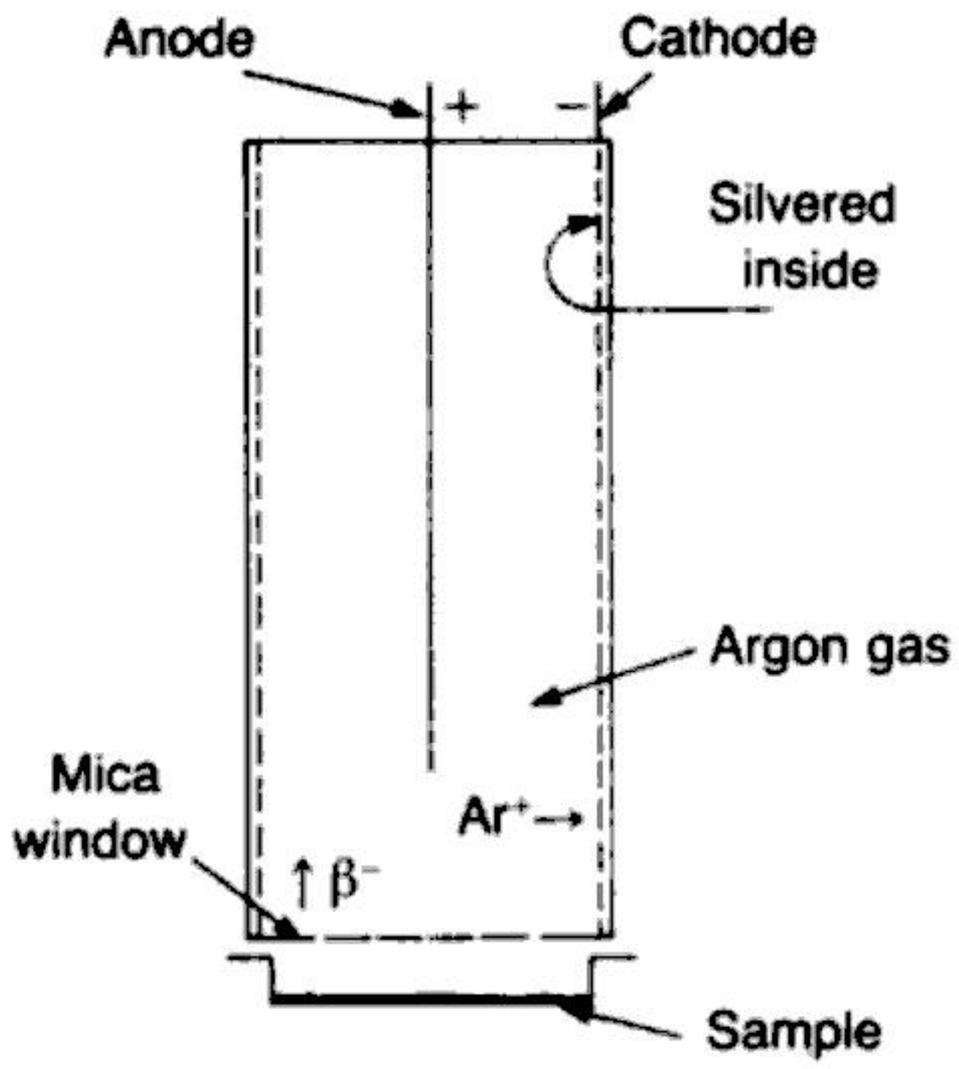

Fig. 13.5. Geiger–Müller counter

effect is to ionise the whole volume of the detector tube; this is equivalent to the flow of a pulse of current which can be amplified readily and presented as an analog or digital output and/or used to give an audio output. Detailed aspects of operation such as recovery and quenching will not be considered here.

The Geiger–Müller tube has the advantage of providing a high output. However, all pulses produced are of the same value irrespective of the energy of the β-particle initiating the avalanche, i.e. it is a **non-proportional** counter. Efficiencies are not high for low energy β-emitters, and it may not be possible to detect ^{3}H. Although the efficiency for γ-radiation is very low (< 1%), it is sufficient, for the use of Geiger–Müller detectors to be practical in, for example, contamination monitoring.

Liquid scintillation counting

The measurement of low energy β-emitters such as ^{3}H and ^{14}C, which have wide application in tracer work, e.g. in drug metabolism, was greatly improved by the introduction of liquid scintillation counting.

In this method the sample is dissolved in a solvent (e.g. toluene or dioxane, but not water) together with some scintillant material; this will be a compound such as diphenyloxazole (PPO). Such a molecule has the property that it can be raised to an excited electronic state from which it can fall to the ground state, emitting in the process visible (or near ultraviolet) light, i.e. fluorescence can occur. The term 'scintillation' is used to cover the whole sequence of events whereby the energy of the fast-moving electron (i.e. the emitted β-particle) is transferred via the solvent to the scintillant molecules, which are thereby raised to the excited electronic level and which then fluoresce with the emission of

light which is detected by photomultipliers (very sensitive light detectors). The experimental arrangement is shown in Fig. 13.6.

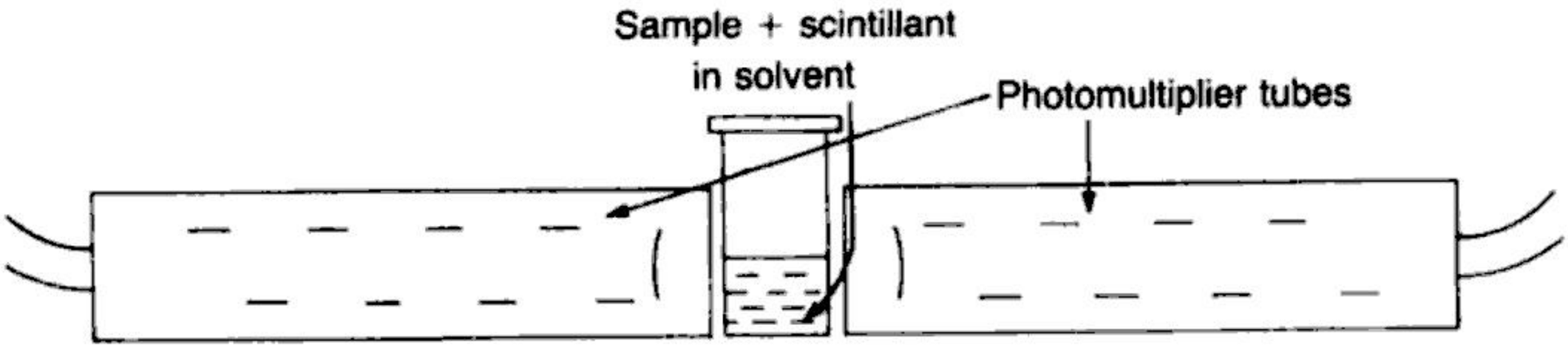

Fig. 13.6. Liquid scintillation counting

Because of the very low levels of light intensity involved, the photomultiliers need to be operated at high sensitivity, with consequent increase in electronic noise generated within the photomultiplier tubes. To reduce this background electronic noise, two photomultipliers are used in the 'coincidence counting' mode, i.e. only output pulses coincident in time are counted. Scintillation events will give coincident output pulses in both photomultipliers, but the random noise pulses developed independently in each photomultiplier will not, in general, be coincident.

The main problems associated with liquid scintillation counting are:

(1) sample preparation
(2) variable quenching.

Sample preparation can be a problem due to the requirement of having a clear (or at least translucent) solution or dispersion in a non-aqueous solvent such as toulene and dioxane. Many methods are used to overcome this problem (e.g. use of detergents); the best source of advice on this matter is probably the booklet produced by Amersham International plc on 'Preparation of Samples for Liquid Scintillation Counting'. Quenching and quench correction are discussed in Experiment 3.

Measurement of gamma-radiation

A solid scintillation process is used here: a single crystal of sodium iodide with a trace impurity level of thallium (about 0.1%) is used, NaI(Tl). Fig. 13.7 shows a cross-sectional diagram of a typical γ-detector. The hygroscopic sodium iodide needs to be coated with a thin film of aluminium for protection against atmospheric moisture.

The sample, in a counting vial, sits in a 'well', a hole drilled into the sodium iodide which will typically be about 2 inches diameter by 2 inches in height. This 'well' minimises the fraction of radiation from the sample which does not pass through the detecting crystal. Interaction of a γ-photon with the high density sodium iodide crystal results in the

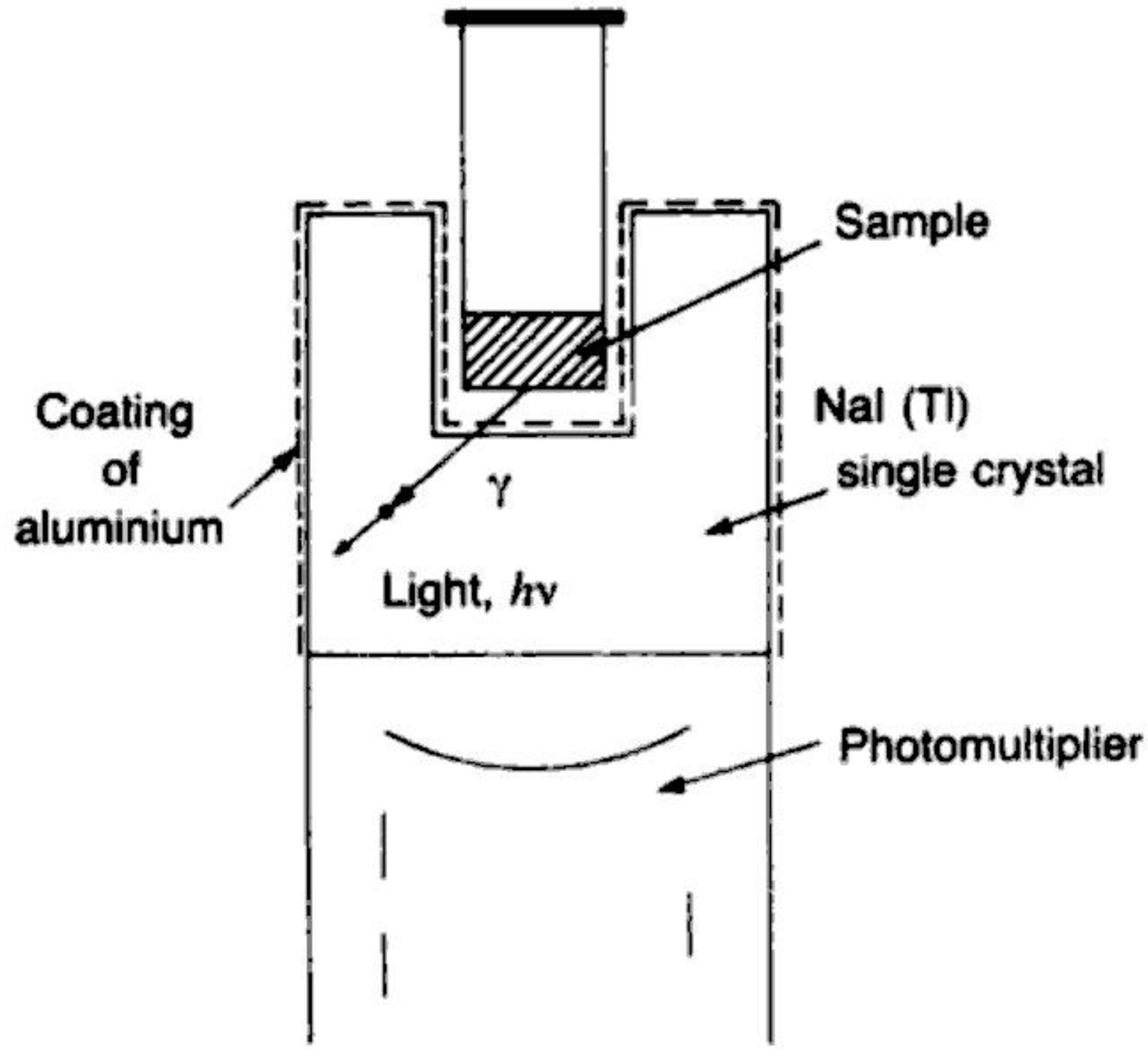

Fig. 13.7. Gamma scintillation detector

production of a 'centre' in an excited electronic level (the thallium plays a role in this process but will not be considered in detail here). Decay back to the ground state results in the emission of visible light which travels through the transparent or translucent single crystal and is detected by the photomultiplier as in liquid scintillation counting. The process is a proportional counting method, and hence gamma energies and gamma spectra can be determined by the process of pulse height analysis (PHA).

Statistics of counting

Radioactive decay is a random process, and so there is a variation in the number of nuclei disintegrating in a unit time interval. If a large number of replicated counts are made on a given sample (with a sufficiently long half-life so that there is no change in the activity over the time scale of the measurements), there will be a variation about the mean. This distribution of counts is strictly a Poisson distribution, which is asymmetric when the mean count, $\bar{x}$, is small (e.g. $\bar{x}<20$) but which rapidly tends to a normal distribution at high values of $\bar{x}$. For large values of $\bar{x}$ the standard deviation, σ, is given by $\sqrt{\bar{x}}$, i.e.

$$\sigma = \sqrt{\bar{x}}$$

Thus, 68.4% of observations should lie between $\bar{x} \pm \sqrt{\bar{x}}$, 95.5% should lie between $\bar{x} \pm 2\sqrt{\bar{x}}$, and 99.7% should lie between $\bar{x} \pm 3\sqrt{\bar{x}}$. In general, a mean of 10000 counts should be collected, as this results in the coefficient of variation, $\sigma/\bar{x}$ or $\sqrt{\bar{x}}/\bar{x}$ or 100/10000 or 0.01, being not greater than 1%.

The count rate for the sample will include a contribution from the

background, and this, or course, must be subtracted. The standard deviation of the resultant count will be given by

$$\sigma = (\sigma_s^2 + \sigma_b^2)^{1/2}$$
$$\sigma = (\bar{x}_s + \bar{x}_b)^{1/2}$$

where the subscripts s and b refer to sample and background respectively. It is only when the sample count rate is of the same order of magnitude as the background count rate that σ is affected by the background count. When this is the case, the most efficient distribution of counting time (t) in order to reduce the overall error is given by

$$\frac{t_s}{t_b} = \left(\frac{\bar{x}_s}{\bar{x}_b}\right)^{1/2}$$

This gives the best precision on the estimate of the required $(\bar{x}_s - \bar{x}_b)$.

Paralysis time

When measurements are made at high count rates, there may be a reduction in the indicated count rate as compared to the true rate due to loss by coincidence of pulses due to the finite resolving time of the detector and its associated electronic equipment. With Geiger–Müller detectors the paralysis time (the time during which the detector is effectively not operative following a detection event) may be of the order of 400 μs. Corrections can be made using the following equation:

$$N = \frac{N_0}{1 - N_0 T}$$

where N is true count rate per second; and N_0 is observed count rate per second; and T is paralysis time (in seconds)

Such a correction equation (based on evenly spaced pulses) is only approximate and should only be used when the product N_0T is small; count rates greater than 10 000 cpm should not be used with Geiger–Müller detectors.

Radiopharmaceuticals and radionuclide generators

Radiopharmaceuticals

The requirements for a radiopharmaceutical can be described under three headings,

1. Properties of the radionuclide

(a) Suitable half-life , i.e. a few hours, such that a reasonable dose can be administered to the patient, counting and/or imaging can be performed within an hour or so with good counting statistics, and the radioactivity will then decay away within a day so that the radiation

dose to the patient is minimised. The total radiation dose to the patient is of course determined by a combination of both the physical (nuclear) half-life of the radionuclide and of the biological half-life of the imaging agent. In general very little is known of the metabolism and excretion of technetium scanning agents, and calculations of patient dose are based on the physical half-life.

(b) Pure gamma emitter of suitable energy . There must be no α and β emissions and the γ-energy must be sufficiently high for the radiation to penetrate out from internal organs, e.g. liver, but not too high to prevent efficient detection. The range 100–200 KeV is optimal.

(c) The radionuclide must be capable of being produced daily, on a routine basis, in the hospital radiopharmacy (the requirement of a short half-life precludes supply from any distance).

(d) The radionuclide must be capable of being converted to a range of chemical entities for imaging a range of organs.

The radionuclide ^{99m}Tc fulfils all of the above criteria and is the radionuclide of choice for most radiopharmaceuticals. The half-life of 6 h, and γ-energy of 140 KeV, are optimum; the ^{99}Mo/^{99m}Tc generator allows production in the radiopharmacy, and technetium chemistry is sufficiently versatile (if not fully understood) to allow for a range of imaging agents to be produced.

2. Pharmaceutical properties

Normal requirements for an injection must be applied, e.g. sterility, apyrogeneity, pH, and absence of particulates (or particle size of a particulate preparation).

3. Chemical properties

The correct chemical (or physico-chemical) form is needed to target to the required organ. Pharmacological and toxicological aspects are important, but because of the extremely small masses injected are generally not a problem.

Radionuclide generators

The system which is widely used in hospital radiopharmacies will be used to illustrate the principle radionuclide generators. An understanding of the operation of the ^{99m}Tc generator is essential to the understanding of the quality control and quality assurance of radiopharmaceuticals.

Generators are based on the concept of radioactive equilibrium . If one radionuclide decays to give another radioactive material, then a radioactive equilibrium can be established when the ratio of the two

half-lives is appropriate. Consider the following scheme:

$$^{99}\text{Mo} \xrightarrow[66\text{ h}]{t_{1/2}} {}^{99m}\text{Tc} \xrightarrow[6\text{ h}]{t_{1/2}} {}^{99}\text{Tc}$$

Table 13.2 Some radiopharmaceutical preparations in use

Preparation	*Application*
$Na^{99m}Tc0_4$	Thyroid uptake studies
	Brain scanning (block thyroid with perchlorate)
^{99m}Tc-Colloid (50–1000 nm) e.g. tin colloid	Liver and spleen scanning
^{99m}Tc-DTPA (diethylenetetramine pentacetic acid)	Kidney function
	Brain scanning
^{99m}Tc-HIDA	Biliary tract imaging
^{99m}Tc-Macroaggregated Albumin (20–50 μm)	Lung imaging (capillary bed entrappment)
^{99m}Tc-phosphate complexes	Bone imaging
e.g. —MDP (methyl diphosphonate) —HMDP (hydroxymethylene diphosphonate) —PYP (pyrophosphate)	^{99m}Tc-PYP also used for myocardial infraction imaging, 1–4 days after occurrence
^{201}Thallous Chloride	Myocardial infarction imaging
^{67}Gallium Citrate	Localises in abscesses in kidney, liver
^{169}Yb-DTPA	Cisternography
^{111}In-DTPA	Cisternography

A radioactive equilibrium will be established whereby the rate of formation of ^{99m}Tc (from the decay of ^{99}Mo) will be equal to the rate of decay of ^{99m}Tc (by isomeric transition to ^{99}Tc).

Initially (with pure ^{99}Mo) there is no ^{99m}Tc, but this is formed from the decay of ^{99}Mo and consequently builds up; at the same time, ^{99m}Tc is decaying to ^{99}Tc via γ-emission. After about 24 h an equilibrium is established and the activities of both ^{99}Mo (the 'parent') and ^{99m}Tc (the 'daughter') appear to decay with the decay constant of the parent ^{99}Mo.

The fact that the parent ^{99}Mo and daughter ^{99m}Tc have different chemistry can be utilised to produce the 'generator' which allows the short-lived ^{99m}Tc ($t_{1/2}$ 6 h) to be produced daily in the hospital radiopharmacy, a procedure which has revolutionised the practice of nuclear medicine in the last 15 years and has led to the close involvement of pharmacists in the preparation and quality assurance of radiopharmaceuticals.

The ^{99}Mo parent can be irreversibly adsorbed onto an alumina (Al_20_3) column as the $^{99}Mo0_4^=$, molybdate ion. The daughter product, in the form of the $^{99m}Tc0_4^-$, pertechnetate ion, is not adsorbed strongly to the alumina column. Thus, when radioactive equilibrium has been established, elution of the column with a suitable eluent, such as sterile, isotonic saline (i.e. suitable for intravenous injection) causes the ^{99m}Tc (as pertechnetate) to be eluted from the column, while the parent ^{99}Mo

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low field, are in the ratio 3 : 3 : 2 and therefore the resonance signals are assigned as follows:

A B

δ 1.06 (3H, t, J = 8) $\underline{CH_3}CH_2-C(=O)-CH_3$

δ 2.14 (3H, s) $CH_3CH_2-C(=O)-\underline{CH_3}$

δ 2.43 (2H, q, J = 8) $CH_3\underline{CH_2}-C(=O)-CH_3$

Methyl group **A** and the methylene group appear respectively as the expected triplet and quartet. Methyl group **B** and the methylene group are both deshielded (moved to lower field) by the adjacent carbonyl group and their proton chemical shifts equate well with the range for such protons given in Chapter 11 (Fig. 11.9).

The principal ions in the mass spectrum of butan-2-one are $CH_3-C\equiv O^+$ (m/z 43) and $CH_3CH_2C\equiv O^+$ (m/z 57) the former appearing in greater abundance (Chapter 12, p.483).

Compound 3

The IR band at 1740 cm^{-1} points to a carbonyl group. and further consideration of the absorption at 1240 cm^{-1} and the molecular formula and description indicates that the compound is an aliphatic ester. The band at 1240 cm^{-1} strongly suggests an acetate (Chapter 10, Fig. 10.4).

The PMR spectrum confirms the structure as ethyl ethanoate (ethyl acetate) and the resonance signals are assigned as follows:

δ 1.25 (3H, t, J = 8) $CH_3-C(=O)-O-CH_2\underline{CH_3}$

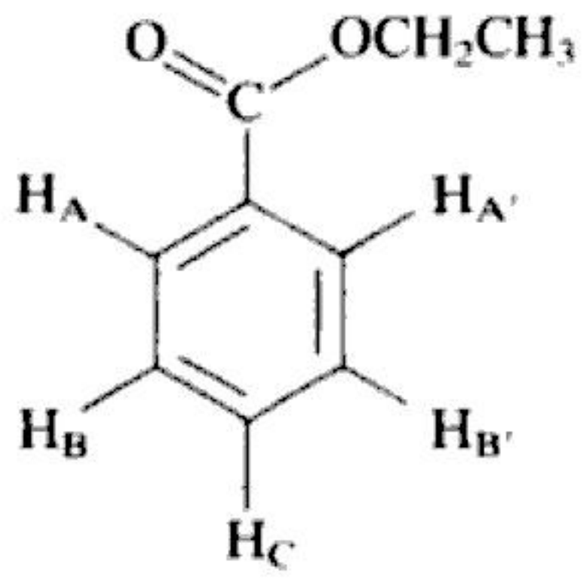
δ 2.03 (3H, s) $\underline{CH_3}-C(=O)-O-CH_2CH_3$

δ 4.12 (2H, q, J = 8) $CH_3-C(=O)-O-\underline{CH_2}CH_3$

Compound 5

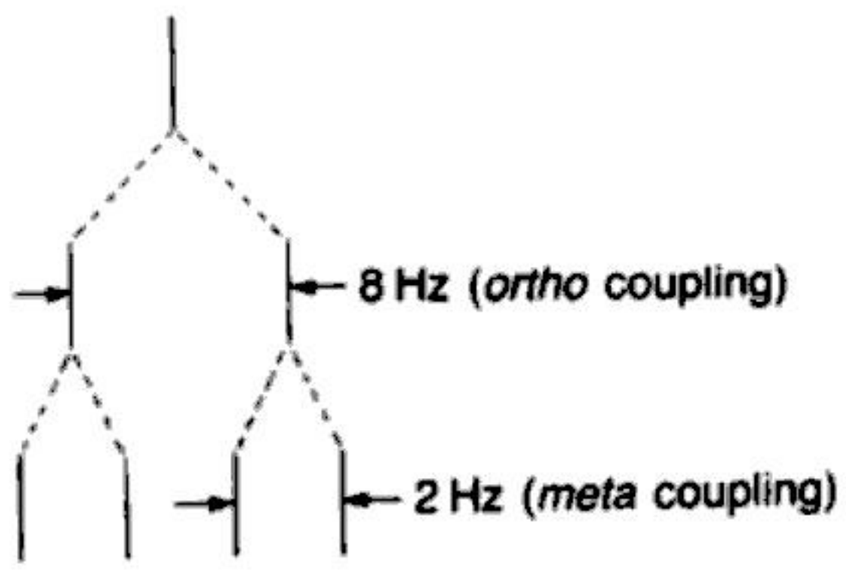
The carbonyl absorption at 1720 cm^{-1} in the IR spectrum of compound 5 and the bands at 1270 and 1105 cm^{-1} are compatible with an aromatic ester, the bands at 1602 and 1581 cm^{-1} confirming the presence of an aromatic ring.

The integrations in the PMR spectrum are in the ratio 3 : 2 : 3 : 2 (≡ 10H). The triplet at δ 1.29 (3H, $J = 8$) and the quartet at δ 4.35 (2H, $J = 8$) clearly indicate (compare compound 3) the moiety —O—CH_2—CH_3, and the compound is therefore ethyl benzoate (5).

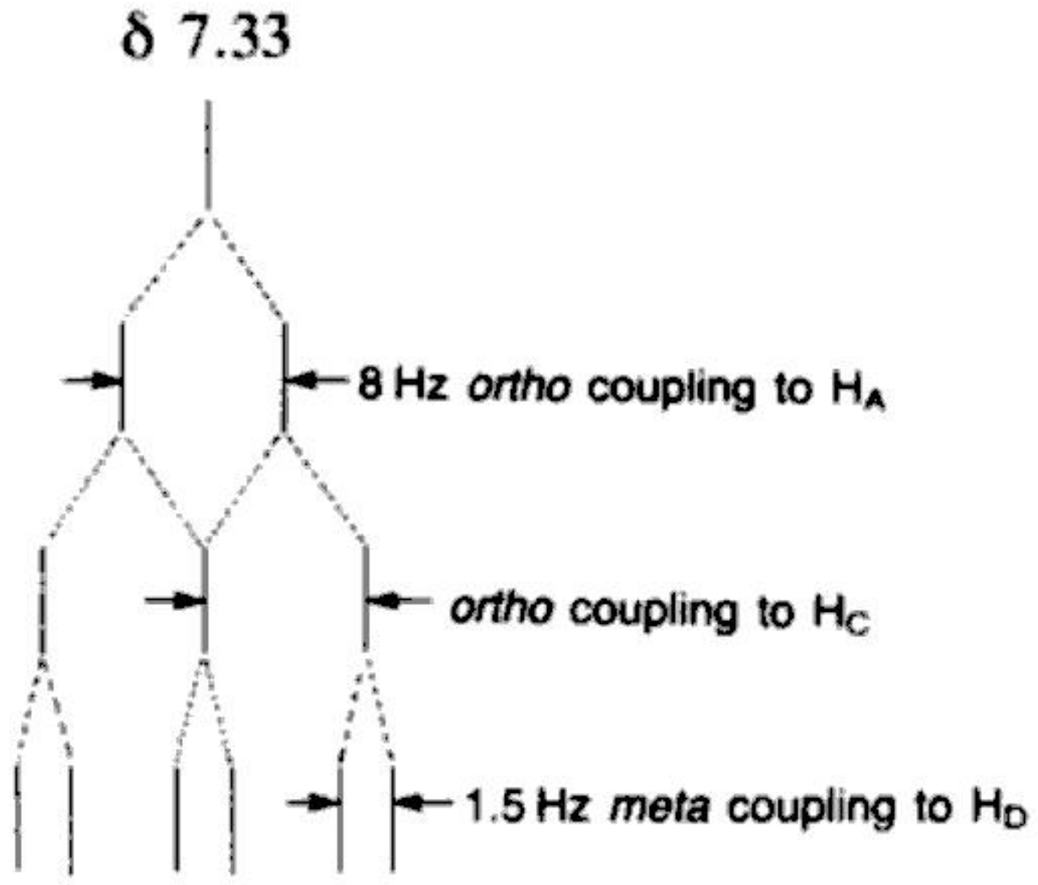


(5)

The aromatic protons appear as two groups of signals centred at δ 7.4 and δ 8.1, which respectively integrate for three and two protons. The lower field group arises from the protons, H_A and $H_{A'}$, *ortho* to the carbonyl group which has the expected deshielding effect on these proximate protons (Chapter 11, Fig. 11.8). The pattern may be approximately interpreted as a double doublet by considering H_A and $H_{A'}$ to be magnetically equivalent and H_B and $H_{B'}$ to be magnetically equivalent but of different chemical shift from the **A** protons. Thus the **A** protons are *ortho* coupled (Chapter 11, p.432) to the **B** protons ($J = 8$) and *meta* coupled to H_C ($J = 2$), giving the following theoretical coupling diagram:



The **B** and **C** protons, however, appear as an unresolved multiplet in this 90 MHz spectrum.



Proton H_C is observed as a similar double triplet centred at δ 7.61 ($J_{BC} = J_{CD} = 8$ and $J_{AC} = 2$). The slight difference in the values of the *meta* coupling constants (J_{AC} and J_{BD}), which permits the unequivocal assignment of protons H_B and H_C, can be observed by simple visual inspection of Fig. 14.9(a).

Interpretation of spectral data

Compound 10

UV. The molar absorptivities can be calculated (Chapter 7, p.277) as being about 6800 at 253 nm in water and about 21 750 at 294 nm in alkali. The bathochromic shift and hyperchromic effect observed on raising the pH is characteristic of phenolic compounds (Chapter 7, p.320).

IR. The broad band at 3250 cm^{-1} is assigned to the hydrogen bonded OH group. The ester carbonyl band at 1670 cm^{-1} is lower than that characteristic for aromatic esters (compare compound 5) but the phenolic hydroxyl group lowers the bond order of the carbonyl group by the mesomeric effect shown below. Similar consideration applies to the

(10) (10a)

carbonyl stretching frequency observed in compound 7. The bands at 1601, 1585 and 1505 cm^{-1} arise from skeletal vibrations of the aromatic ring. The absorption at 1280 cm^{-1} originates from asymmetric C—O stretching of the ester. Neither the symmetrical stretching band nor the bands associated with *para*–substitution can be identified with certainty.

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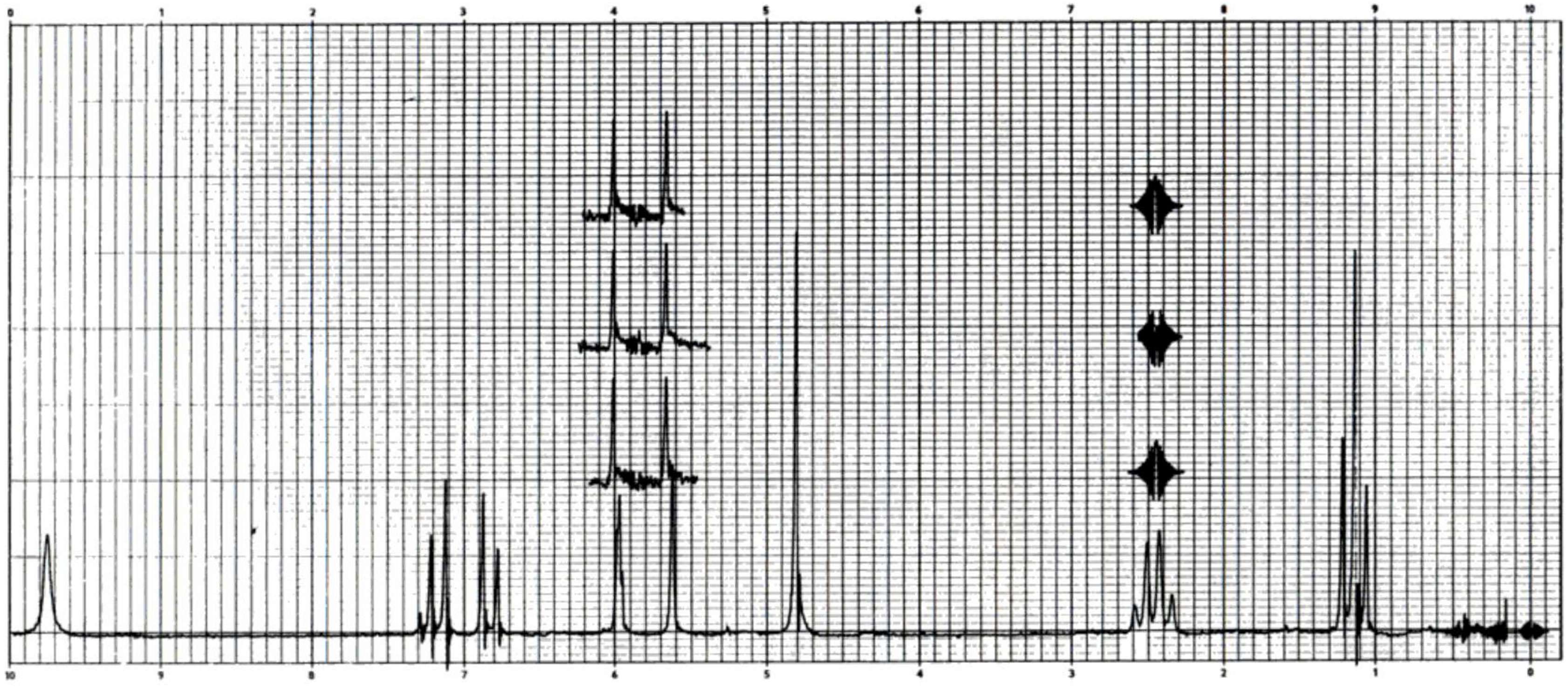
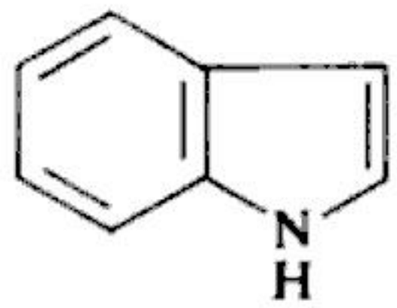


Fig. 14.18(b)

Compound 14

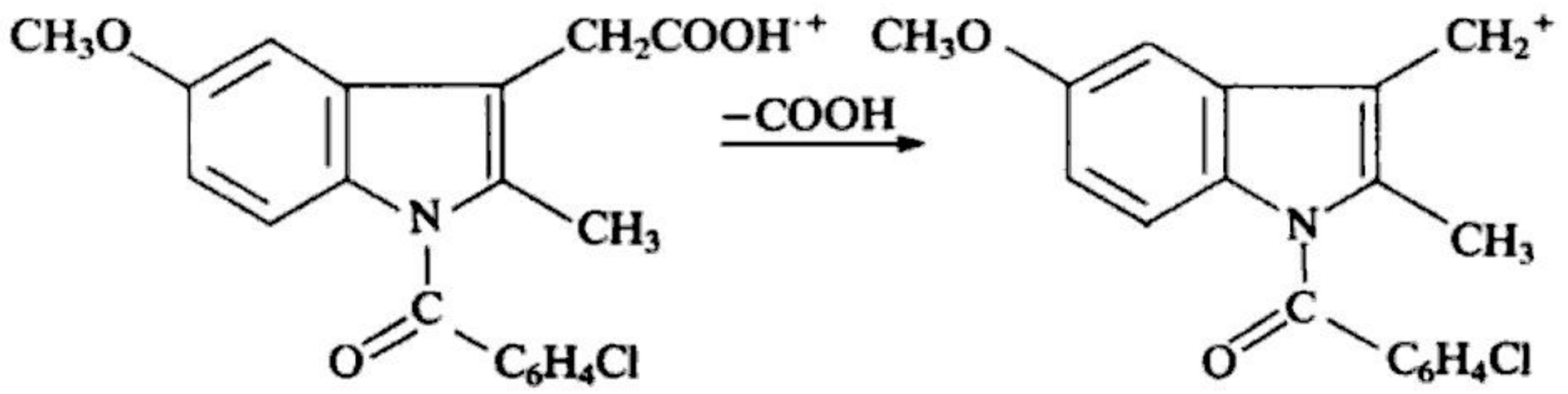
UV. The ultraviolet spectra of heteroaromatic compounds are less amenable than those of substituted benzenes to the simple treatment used in this text. The parent indole (14a) shows $\lambda_{max}^{cyclohexane}$ (nm) 220 (ϵ 6300), 280 (ε 5600) and 288 (ε 4200). Substituents, as in benzenoid compounds, have profound effects on the spectrum of the parent, and in interpretation recourse frequently has to be made to published catalogues of ultraviolet spectra to identify a model chromophoric system.



(14a)

IR. The bands at 3000–2500 cm^{-1} and the band at 1715 cm^{-1} are readily identified with the carboxyl group (compare compounds 11 and 13). The carbonyl stretching vibration of the tertiary amide is observed at 1685 cm^{-1}.

MS. The presence of one chlorine atom in indomethacin gives two molecular ions *m/z* 359 and 357 (compare compound 12). The ions *m/z* 314 and 312 similarly contain one chlorine atom. Accurate mass measurement (peak matching, Chapter 12, p.482) of these ions gives the structures shown in the following fragmentation pathway:



m/z 359.0741 = $C_{19}H_{14}{}^{37}ClNO_4$
357.0728 = $C_{19}H_{14}{}^{35}ClNO_4$

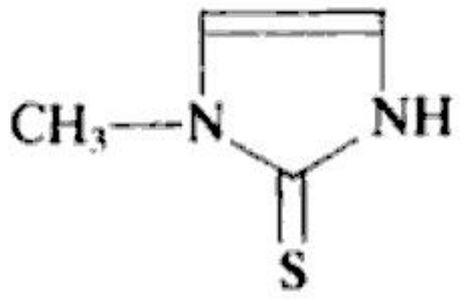
m/z 314.0776 = $C_{18}H_{15}{}^{37}ClNO_2$
312.0784 = $C_{18}H_{15}{}^{35}ClNO_2$

PMR. The interpretation (δ) is 2.38 (3H, s, indole $C\underline{H}_3$); 3.68 (2H, s, $C\underline{H}_2$); 3.81 (3H, s, $OC\underline{H}_3$); 6.67 (1H, dd, J = 8 and 2, H_B); 6.85* (1H, d, J = 8, H_C); 6.95 (1H, d, J = 2, H_A); 7.26 ($C\underline{H}Cl_3$ solvent); 7.45 (2H, d, H_E and $H_{E'}$); 7.65 (2H, d, H_D and $H_{D'}$); 9.1–10.0 (1H, br, COO$\underline{H}$).

*The second peak of this doublet overlaps the doublet of proton H_A as is more readily seen in the inset spectrum obtained at higher sensitivity.

Compound 15

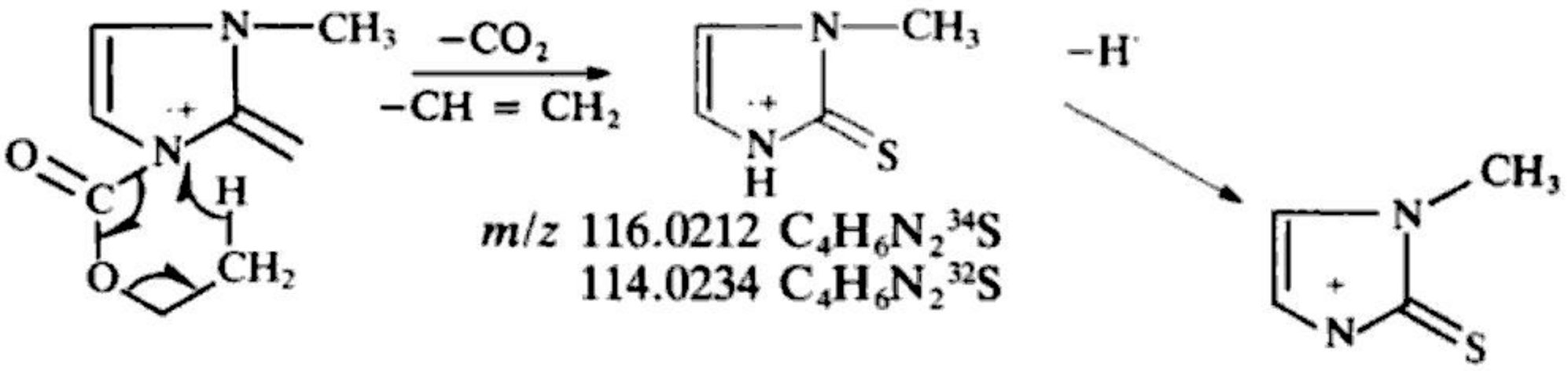
UV. As in the case of compound 14, identification of a model chromophoric system, through either published catalogues of ultraviolet spectra or a literature search, is necessary. For example, 1-methyl-2-thioxo-4-imidazoline (15a) shows maxima at about 210 nm and 250 nm, the longer wavelength band being the more intense. Addition of the chromophore —COOEt to this ring system would be expected to cause a bathochromic shift of the maxima, an observation which is compatible with the UV characteristics of carbimazole.



(15a)

IR. The series of bands at 3190–2490 cm^{-1} are associated with C—H stretching vibrations of the methyl groups and the ethenic CH groups; the higher frequency bands are more likely to arise from the ring CH groups. The carbonyl band is observed at 1750 cm^{-1} and C=C stretching at 1705 cm^{-1}.

MS. The series of ions separated by two mass units and appearing in the ratio 0.04 : 1 within each pair approximately reflects the relative isotopic abundance of ^{34}S and ^{32}S. Peak matching gave the molecular weight of each ion (Chapter 12, p.483) and a computer program (see introduction) its elemental composition. The following fragmentation pathway is proposed:



m/z 188.0430 $C_7H_{10}N_2O_2{}^{34}S$
186.0431 $C_7H_{10}N_2O_2{}^{32}S$

m/z 116.0212 $C_4H_6N_2{}^{34}S$
114.0234 $C_4H_6N_2{}^{32}S$

m/z 115.0134 $C_4H_5N_2{}^{34}S$
113.0169 $C_4H_5N_2{}^{32}S$

PMR. The interpretation (δ) is 1.42 (3H, t, $CH_2C\underline{H_3}$); 3.57 (3H, s, N—$\underline{CH_3}$); 4.46 (2H, q, $OC\underline{H_2}$); 6.7* (1H, d, $J = 2$, H_A); 7.2* (1H, d, $J = 2$, H_B).

*Unequivocal assignment of H_A and H_B is difficult, since both protons are attached to a carbon atom which is adjacent to nitrogen and many canonical forms contribute to the resonance hybrid.

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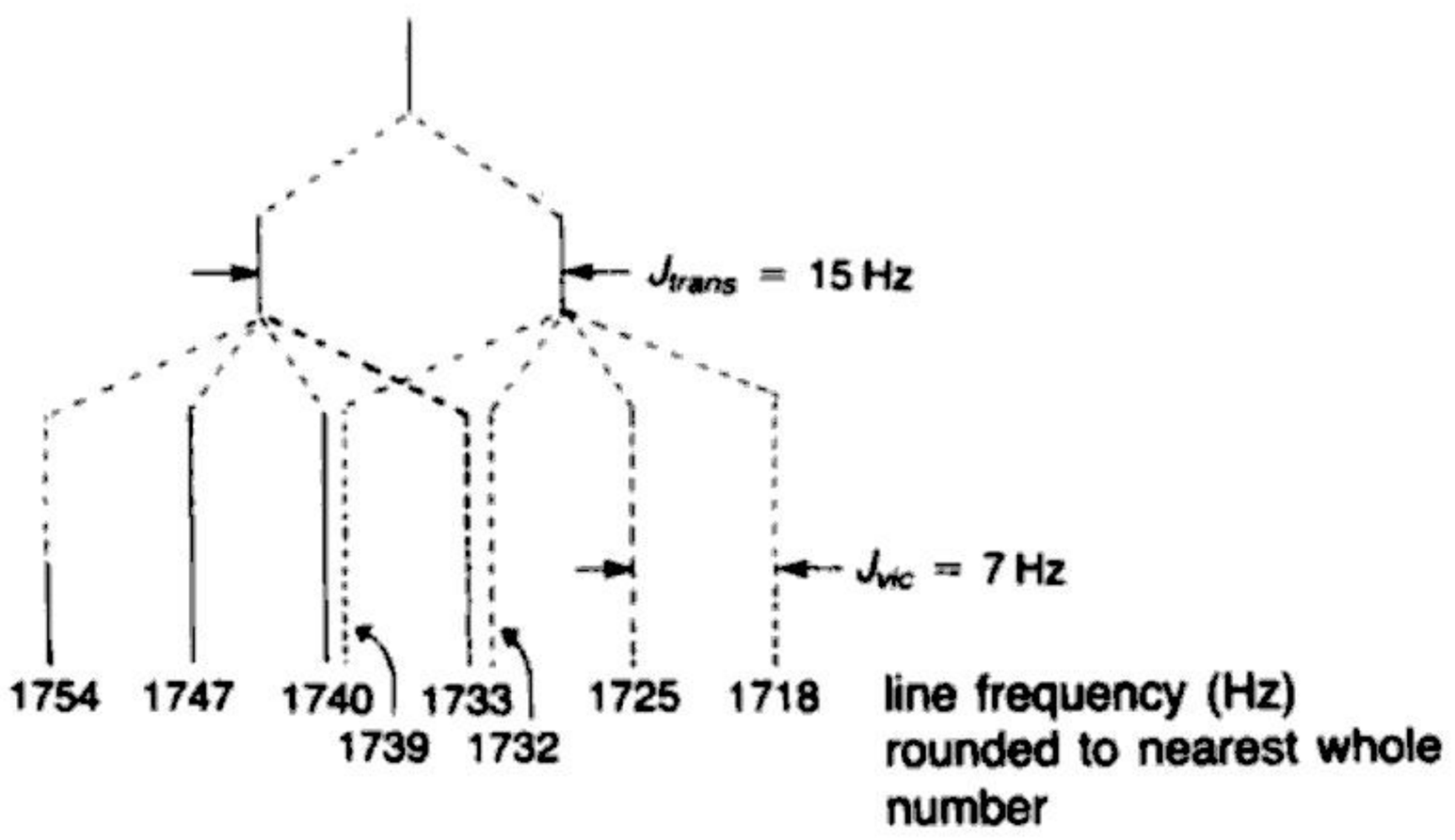
features as the 90 MHz spectrum (Fig. 14.16) but the region δ 6.75–7.4 is now partly resolved to reveal at δ 6.95 the signal from proton H_A (a sextet) and a broad doublet at δ 7.1 from proton H_C. The 250 MHz spectrum also shows an impurity in the sample by the appearance of the small doublet at δ 2.15; the impurity has probably arisen through storage of the sample under inappropriate conditions after receipt (i.e., in a small, well-closed container protected from light).

Scale expansion of regions of interest presents a clearer picture and simultaneously permits accurate measurement of coupling constants. As an illustrative example, 20 Hz cm^{-1} scale expansions of the regions 1360–1420 Hz (δ 5.44–5.63) and 1700–1900 Hz (δ 6.80–7.60) are shown in Figs. 14.16(b) and (c). Proton H_B (1360–1410 Hz) is now readily observed as the expected doublet of quartets in which the *trans*-coupling constant is 15 Hz and the long-range (allylic) coupling constant is 1.6 Hz. High field instruments can print out the frequency of each line in a spectrum, which permits still better accuracy in coupling constant measurement, and in this instance J_{trans} is 14.99 Hz and $J_{allylic}$ is 1.64 Hz. Proton H_A (1710–1760 Hz) is clearly observed as two overlapped quartets in which J_{trans} is 15 Hz (14.99 Hz) as before and J_{vic} is 7 Hz (6.94 Hz accurately). The following coupling diagram accounts for the observed pattern:



Proton H_C appears as a broadened doublet at δ 7.1 (J_{ortho} about 8 Hz, J_{meta} about 2 Hz). The remaining aromatic protons are left unresolved.

Similar treatment of the two sextets arising from the non-equivalent methylene protons reveals these also to be overlapped quartets in which J_{gem} is 13 Hz (13.34 accurate value) and J_{vic} is 7 Hz (6.94 accurate value). Construction of a coupling diagram, drawn to scale and using the values of 13 and 7 Hz, establishes the arrangement of the lines in these overlapping quartets.

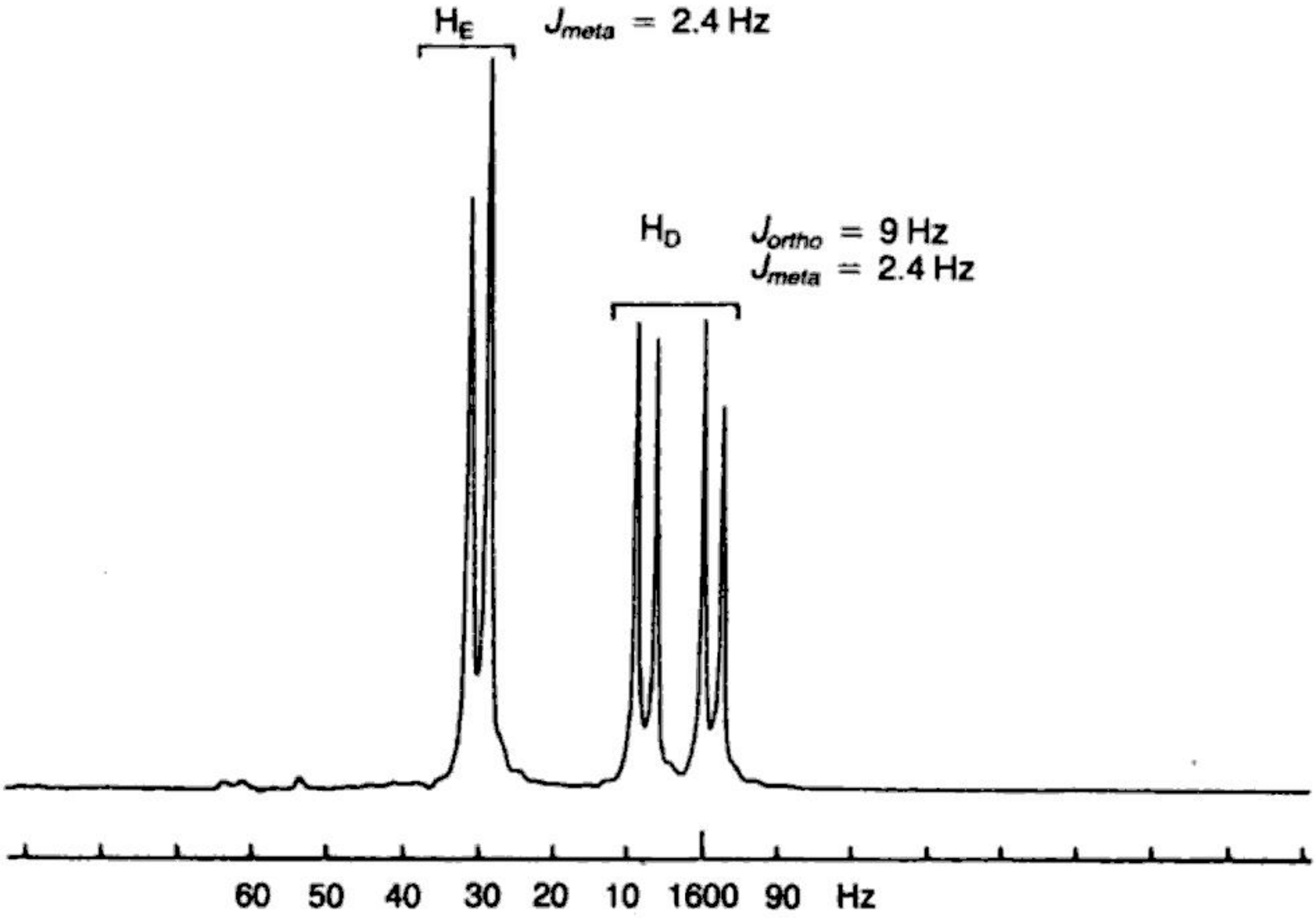


Fig. 14.19(a).

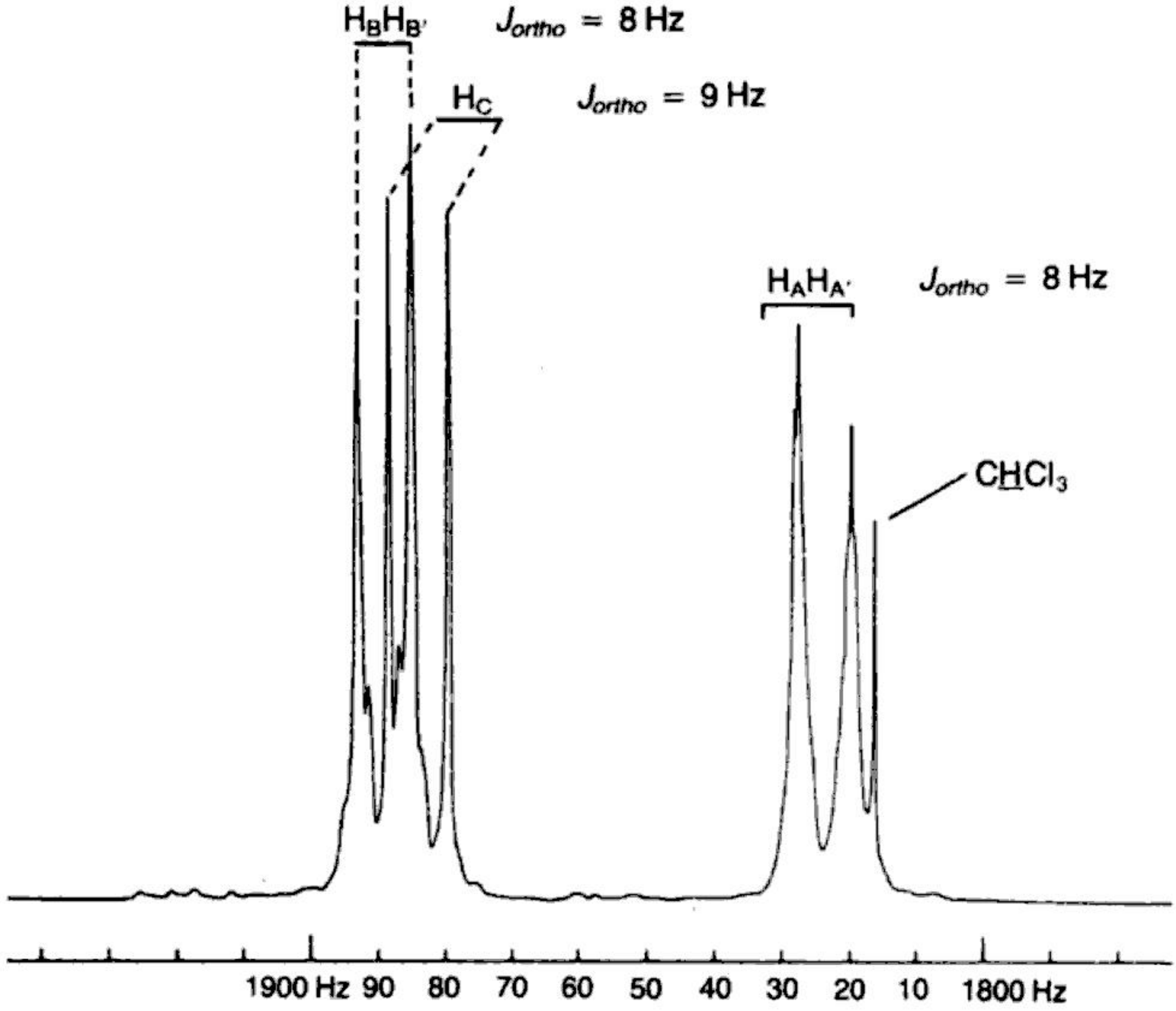


Fig. 14.19(b).

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