Chapter 11

Chromatographic techniques

11.1 PRINCIPLES OF CHROMATOGRAPHY

11.1.1 Distribution coefficients

The Russian botanist Mikhail Tswett is credited with the original development of a separation technique that we now recognise as a form of chromatography. In 1903 he reported the successful separation of a mixture of plant pigments using a column of calcium carbonate. In the process he became the first scientist to recognise that chlorophyll was not a single chemical compound. Modern chromatographic techniques take multiple forms, the majority of which can be automated and adapted to deal with large or very small amounts of the substances to be separated and purified.

The basis of all forms of chromatography is the distribution or partition coefficient (K_d), which describes the way in which a compound (the analyte) distributes between two immiscible phases. For two such phases A and B, the value for this coefficient is a constant at a given temperature and is given by the expression:

$$\frac{\text{concentration in phase A}}{\text{concentration in phase B}} = K_d \qquad (11.1)$$

The term effective distribution coefficient is defined as the total amount, as distinct from the concentration, of analyte present in one phase divided by the total amount present in the other phase. It is in fact the distribution coefficient multiplied by the ratio of the volumes of the two phases present. If the distribution coefficient of an analyte between two phases A and B is 1, and if this analyte is distributed between 10 cm^3 of A and 1 cm^3 of B, the concentration in the two phases will be the same, but the total amount of the analyte in phase A will be 10 times the amount in phase B.

All chromatographic systems consist of the stationary phase, which may be a solid, gel, liquid or a solid/liquid mixture that is immobilised, and the mobile phase, which may be liquid or gaseous, and which is passed over or through the stationary phase after the mixture of analytes to be separated has been applied to the stationary phase. During the chromatographic separation the analytes continuously pass back and forth between the two phases so that differences in their distribution coefficients result in their separation.

11.1.2 Modes of chromatography

Chromatographic separations may be carried out in one of two modes:

- *Column chromatography:* In this mode the stationary phase is packed into a glass or metal column. The mixture of analytes is then applied and the mobile phase, commonly referred to as the eluent, passed through the column either by gravity feed or by use of a pumping system or applied gas pressure. This is the most commonly used mode of chromatography from an analytical biochemical point of view. The stationary phase is either coated onto discrete small particles (the matrix) and packed into the column or applied as a thin film to the inside wall of the column. As the eluent flows through the column the analytes separate on the basis of their distribution coefficients and emerge individually in the eluate as it leaves the column.
- *Thin-layer or planar chromatography:* In this mode the stationary phase attached to a suitable matrix is coated thinly onto a glass, plastic or metal foil plate. The mixture of analytes is applied as a spot or band near the edge of the coated plate and the mobile liquid phase passed across the plate, held either horizontally or vertically, by capillary action, causing the analytes to migrate at characteristic rates to the opposite end. This mode of chromatography has the practical advantage over column chromatography that a number of samples can be studied simultaneously. Planar chromatography is simple to carry out but has been largely superseded by high performance liquid chromatography (HPLC) for many applications. However, it continues to find extensive use in the fields of peptide mapping and natural products research.

Principles of column chromatography

The principle of a column chromatographic separation may be illustrated by considering a column packed with a solid granular stationary phase to a height of 5 cm, surrounded by the mobile liquid phase of which there is 1 cm^3 per cm of column, as shown in Fig. 11.1. If 32 μg of an analyte is added to the column in 1 cm^3 of mobile phase, then, as this 1 cm^3 moves on to the column to occupy position A, 1 cm^3 of mobile phase will leave the base of the column. If the analyte has an effective distribution coefficient of 1, it will distribute itself equally between the solid and liquid phases (stage 1). If a further 1 cm^3 of mobile phase is introduced on to the column, the mobile phase in section A will move down to B, taking 16 μg of the analyte with it, leaving 16 μg at A (stage 2). At both A and B, a redistribution of the analyte will occur so that there is 8 μg in the mobile phase and 8 μg in the solid phase. The addition of a further 1 cm^3 of mobile phase to the column displaces the mobile phase in A to B and that in B to C, giving the distribution of the analyte as shown in stage 3. Addition of a further 1 cm^3 of mobile phase leads to the distribution shown at stage 4, and a further 1 cm^3 of mobile phase leads to the distribution shown at stage 5. It is apparent that, after a relatively small number of equilibrations, the analyte distributes itself symmetrically within a band. It is equally apparent that if a mixture of two analytes, one having a

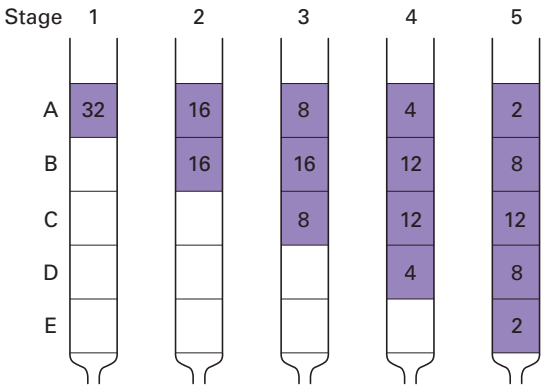

Fig. 11.1. Principle of a column chromatographic separation.

distribution coefficient of 1, the other a distribution coefficient of 100, was added to the column it would separate rapidly into distinct bands. In a real chromatographic column a very large number of equilibrations occur as the mobile phase passes down the column as a result of more mobile phase being added constantly to the top of the column. The outcome is that each analyte emerges in a distinct band from the column.

Basic column chromatographic components

A typical column chromatographic system using a gas or liquid mobile phase consists of the following components:

- *A stationary phase:* Chosen to be appropriate for the analytes to be separated.
- *A column:* This may be either of the conventional type, filled with the matrix coated with the stationary phase, or of the microbore type, in which the stationary phase is coated directly on the inside wall.
- *A mobile phase and delivery system:* Chosen to complement the stationary phase and hence to discriminate between the sample analytes and to deliver a constant rate of flow into the column.
- *An injector system:* To deliver test samples to the top of the column in a reproducible manner.
- *A detector and chart recorder:* To give a continuous record of the presence of the analytes in the eluate as it emerges from the column. Detection is usually based on the measurement of a physical parameter such as visible or ultraviolet absorption or fluorescence. A peak on the chart recorder represents each separated analyte.
- *A fraction collector:* For collecting the separated analytes for further biochemical studies.

Column liquid chromatography can be subdivided according to the back-pressure generated within the column during the separation process. Low pressure liquid chromatography (LPLC) generates pressures of less than 5 bar

(1 bar = 14.5 lbf in^{-2} = 0.1 MPa), since there is little resistance to eluent flow owing to the physical nature of the stationary phase. Gas–liquid chromatography also falls into this category. Medium pressure liquid chromatography (MPLC) generates pressures of between 6 and 50 bar and high pressure liquid chromatography (HPLC) pressures in excess of 50 bar. In practice the distinctions between MPLC and HPLC are often blurred and their equipment and procedures are virtually identical. Both give excellent resolutions and hence the term high performance liquid chromatography is preferred for both of them, since it better describes the chromatographic characteristics of the techniques and avoids the misconception that it is the high pressure that is fundamentally responsible for the high performance chromatography.

11.1.3 Selection of stationary and mobile phases

Successful chromatographic separations depend upon the correct choice of stationary and mobile phases so that the analytes to be separated have different distribution coefficients. This may be achieved by setting up one of the following:

- *Adsorption equilibrium:* This is between a stationary solid phase and a mobile liquid phase (adsorption chromatography; hydrophobic interaction chromatography).
- *Partition equilibrium:* This is between a stationary liquid phase and a mobile liquid or gas phase (partition chromatography; perfusion chromatography; ion-pair chromatography; chiral chromatography; gas–liquid chromatography).
- *Ion-exchange equilibrium:* This is between a stationary, solid ion-exchanger and mobile, liquid electrolyte phase (ion-exchange chromatography; chromatofocusing; membrane chromatography).
- *Exclusion equilibrium:* This is between a liquid phase trapped inside the pores of a stationary porous structure and the same mobile liquid phase (molecular exclusion or gel filtration).
- *Binding equilibrium:* This is between a stationary immobilised ligand and a mobile liquid phase (affinity chromatography; immunoaffinity chromatography; lectin affinity chromatography; metal chelate affinity chromatography; dye–ligand chromatography; covalent chromatography).

In practice it is quite common for two or more of these equilibria to be involved simultaneously in a particular chromatographic separation.

11.1.4 Analyte development and elution

Analyte development and elution relates to the separation of the mixture of analytes applied to the stationary phase by the mobile phase and their elution from the column. Column chromatographic techniques can be subdivided on the basis of the development and elution modes.

- In zonal development, the analytes in the sample are separated on the basis of their distribution coefficients between the stationary and mobile phases. The

sample is dissolved in a suitable solvent and applied to the stationary phase as a narrow, discrete band. Mobile phase, normally consisting of an organic solvent or a mixture of solvents often incorporating a buffered aqueous system, is then allowed to flow continuously over the stationary phase, resulting in the progressive separation and elution of the sample analytes. If the composition of the mobile phase is constant, the process is said to be isocratic elution. To facilitate separation, however, the composition of the mobile phase may be gradually changed, for example with respect to pH, salt concentration or polarity. This is referred to as gradient elution. The composition of the mobile phase may be changed continuously or in a stepwise manner. Successful zonal development results in the elution of pure samples of all the analytes. It is the most common form of chromatography.

- In displacement or affinity development, the analytes in the sample are separated on the basis of their affinity for the stationary phase. The sample of analytes dissolved in a suitable solvent is applied to the stationary phase as a discrete band. The analytes bind to the stationary phase with a strength determined by their affinity constant for the phase. The analytes are then selectively eluted by using a mobile phase containing a specific solute that has a higher affinity for the stationary phase than have the analytes in the sample. Thus, as the mobile phase is added, this agent displaces the analytes from the stationary phase in a competitive fashion, resulting in their repetitive binding and displacement along the stationary phase and eventual elution from the column in the order of their affinity for the stationary phase, the one with the lowest affinity being eluted first.
- In frontal development, the sample is continuously added to the stationary phase, thereby forcing the analytes along the stationary phase in the order of their affinity for it. The analyte with the lowest affinity accumulates at the front of the moving sample band and, whilst a pure sample of it can be isolated, pure samples of the other analytes cannot. In practice, the technique is effectively restricted to the analysis of a single trace impurity in an otherwise pure sample.

11.2 CHROMATOGRAPHIC PERFORMANCE PARAMETERS

11.2.1 Introduction

The successful chromatographic separation of analytes in a mixture depends upon the selection of the most appropriate process of chromatography followed by the optimisation of the experimental conditions associated with the separation. Optimisation requires an understanding of the processes that are occurring during the development and elution, and of the calculation of a number of experimental parameters characterising the behaviour of each analyte in the mixture.

In any chromatographic separation two processes occur concurrently to affect the behaviour of each analyte and hence the success of the separation of the analytes from each other. The first involves the basic mechanisms defining the

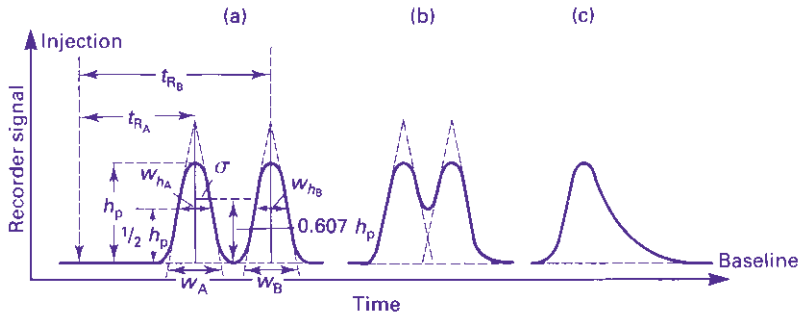

Fig. 11.2. (a) Chromatograph of two analytes showing complete resolution and the calculation of retention times; (b) chromatograph of two analytes showing incomplete resolution (fused peaks); (c) chromatograph of an analyte showing excessive tailing.

chromatographic process such as adsorption, partition, ion exchange, ion pairing and molecular exclusion. These mechanisms involve the unique kinetic and thermodynamic processes that characterise the interaction of each analyte with the stationary phase. The second general process defines the other processes, such as diffusion, which tend to oppose the separation and which result in non-ideal behaviour of each analyte. These processes are manifest as a broadening and tailing of each analyte band. The analytical challenge is to minimise these secondary processes.

11.2.2 Retention time and elution volume

A chromatograph is the pictorial record of the detector response as a function of elution volume or retention time. It consists of a series of peaks or bands, ideally symmetrical in shape, representing the elution of individual analytes, as shown in Fig. 11.2. The retention time t_R for each analyte has two components. The first is the time it takes the analyte molecules to pass through the free spaces between the particles of the matrix coated with the stationary phase. This time is referred to as the dead time, t_M. The volume of the free space is referred to as the column void volume, V_0. The value of t_M will be the same for all analytes and can be measured by using an analyte that does not interact with the stationary phase but simply spends all of the elution time in the mobile phase travelling through the void volume. The second component is the time the stationary phase retains the analyte, referred to as the adjusted retention time, t'_R. This time is characteristic of the analyte and is the difference between the observed retention time and the dead time:

$$t'_R = t_R - t_M \tag{11.2}$$

It is common practice to relate the retention time t_R or t'_R for an analyte to a reference internal or external standard (Section 11.2.5). In such cases the relative retention time is often calculated. It is simply the retention time for the analyte divided by that for the standard.

11.2.3 Capacity factor

One of the most important parameters in chromatography is the capacity factor, k' (also called retention factor and capacity ratio). It is simply the additional time that the analyte takes to elute from the column relative to an unretained or excluded analyte that does not interact with the stationary phase and which, by definition, has a k' value of 0. Thus:

$$k' = \frac{t_R - t_M}{t_M} = \frac{t'_R}{t_M} \tag{11.3}$$

It is apparent from this equation that if the analyte spends an equal time in the stationary and mobile phases, its t_R would equal $2t_M$ and its k' would be 1, whilst if it spent four times as long in the stationary phase as the mobile phase t_R would equal $5t_M$ so that k' would equal $(5t_M - t_M)/t_M = 4$. Note that k' has no units.

If an analyte has a k' of 4 it follows that there will be four times the amount of analyte in the stationary phase than in the mobile phase at any point in the column at any time. It is evident, therefore, that k' is related to the distribution coefficient of the analyte (equation 11.1), which was defined as the relative concentrations of the analyte between the two phases. Since amount and concentration are related by volume, we can write:

$$k' = \frac{t'_R}{t_M} = \frac{M_S}{M_M} = K_d \times \frac{V_S}{V_M} \tag{11.4}$$

where M_S is the mass of analyte in the stationary phase, M_M is the mass of analyte in the mobile phase, V_S is the volume of stationary phase, and V_M is the volume of mobile phase.

The ratio V_S/V_M is referred to as the volumetric phase ratio, β. Hence:

$$k' = K_d\beta \tag{11.5}$$

Thus the capacity factor for an analyte will increase with both the distribution coefficient between the two phases and the volume of the stationary phase. k' values normally range from 1 to 10. Capacity factors are important because they are independent of the physical dimensions of the column and the rate of flow of mobile phase through it. They can therefore be used to compare the behaviour of an analyte in different chromatographic systems. They are also a reflection of the selectivity of the system that in turn is a measure of its inherent ability to discriminate between two analytes. Such selectivity is expressed by the selectivity or separation factor, α, which can also be viewed as simply the relative retention ratio for the two analytes:

$$\alpha = \frac{k'_A}{k'_B} = \frac{K_{d_A}}{K_{d_B}} = \frac{t'_{R_A}}{t'_{R_B}} \tag{11.6}$$

The selectivity factor is influenced by the chemical nature of the stationary and mobile phases. Some chromatographic mechanisms are inherently highly selective. Good examples are affinity chromatography (Section 11.8) and chiral chromatography (Section 11.5.5).

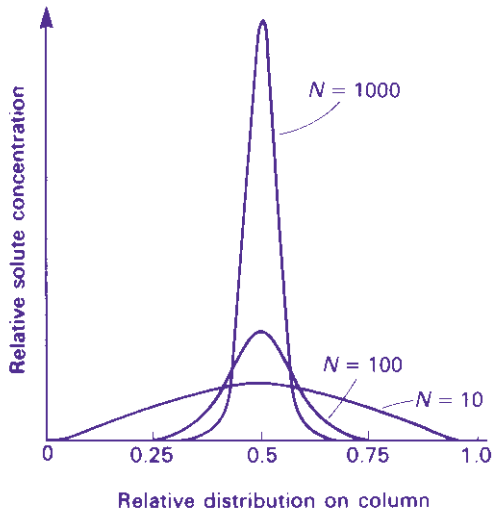

Fig. 11.3. Relationship between the number of theoretical plates (N) and the shape of the analyte peak.

11.2.4 Plate height and resolution

Plate height

Chromatography columns are considered to consist of a number of adjacent zones in each of which there is sufficient space for an analyte to completely equilibrate between the two phases. Each zone is called a theoretical plate (of which there are N in total in the column). The length of column containing one theoretical plate is referred to as the plate height, H, which has units of length normally in micrometres. The numerical value of both N and H for a particular column is expressed by reference to a particular analyte. Plate height is simply related to the width of the analyte peak (Fig. 11.3), expressed in terms of its standard deviation σ, and the distance it travelled within the column, x. Specifically:

$$H = \frac{\sigma^2}{x} \tag{11.7}$$

For symmetrical Gaussian peaks, the base width is equal to 4σ and the peak width at the point of inflection, w_i is equal to 2σ. Hence the value of H can be calculated from the chromatograph by measuring the peak width. The number of theoretical plates in the whole column of length L is equal to L divided by the plate height:

$$N = \frac{L}{H} = \frac{Lx}{\sigma^2} \tag{11.8}$$

If the position of a peak emerging from the column is such that $x = L$, from knowledge of the fact that the width of the peak at its base, w, obtained from tangents

drawn to the two steepest parts of the peak, is equal to 4σ (this is a basic property of all Gaussian peaks) hence $\sigma = w/4$ and equation 11.8 can therefore be converted to:

$$N = \frac{L^2}{\sigma^2} = \frac{16L^2}{w^2} \tag{11.9}$$

If both L and w are measured in units of time rather than length, then equation 11.9 becomes:

$$N = 16(t_R/w)^2 \tag{11.10a}$$

Rather than expressing N in terms of the peak base width, it is possible to express it in terms of the peak width at half height ($w_{\frac{1}{2}}$) and this has the practical advantage that this is more easily measured:

$$N = 5.54(t_R/w_{\frac{1}{2}})^2 \tag{11.10b}$$

Equations 11.9 and 11.10a,b represent alternative ways to calculate the column efficiency in theoretical plates. The value of N, which has no units, can be as high as 50 000 to 100 000 per metre for efficient columns and the corresponding value of H can be as little as a few micrometres. The smaller the plate height (the larger the value of N), the narrower is the analyte peak (Fig. 11.3).

Peak broadening

A number of processes oppose the formation of a narrow analyte peak thereby increasing the plate height:

- *Application of the sample to the column:* It takes a finite time to apply the analyte mixture to the column, so that the part of the sample applied first will already be moving along the column by the time the final part is applied. The part of the sample applied first will elute at the front of the peak.
- *Longitudinal diffusion:* Fick's law of diffusion states that an analyte will diffuse from a region of high concentration to one of low concentration at a rate determined by the concentration gradient between the two regions and the diffusion coefficient (P) of the analyte. Thus the analyte within a narrow band will tend to diffuse outwards from the centre of the band, resulting in band broadening.
- *Multiple pathways:* The random packing of the particles in the column results in many routes between the particles for both mobile phase and analytes. These pathways will vary in length and hence elution time. The smaller the particle size the less serious is this problem and in open tubular columns the phenomenon is totally absent, which is one of the reasons why they give shorter elution times and better resolution than packed columns.
- *Equilibration time between the two phases:* It takes a finite time for each analyte in the test sample to equilibrate between the stationary and mobile phases as it passes down the column. As a direct consequence of the distribution coefficient, K_d, some of each analyte is retained by the stationary

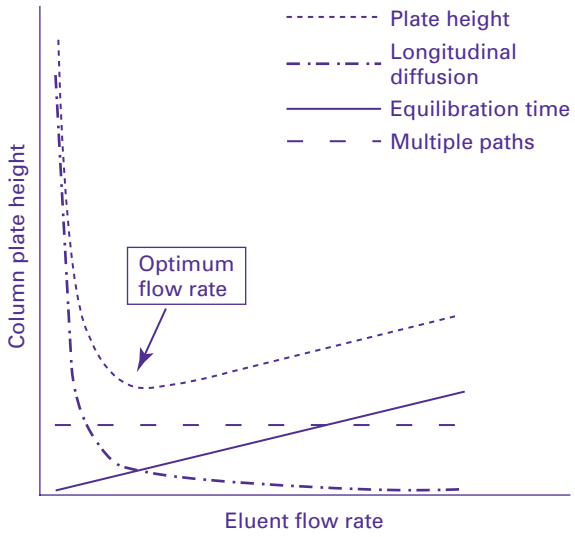

Fig. 11.4. van Deemter plot showing that the influence of flow rate on plate height is the net result of its influence on longitudinal diffusion, equilibration time and multiple pathways.

phase, whilst the remainder stays in the mobile phase and continues its passage down the column. This partitioning automatically results in some spreading of each analyte band. Equilibration time, and hence band broadening, is also influenced by the particle size of the stationary phase. The smaller the size, the less time it takes to establish equilibration. This is one of the reasons why HPLC gives better resolution than conventional LPLC.

Two of these four factors promoting the broadening of the analyte band are influenced by the flow rate of the eluent through the column. Longitudinal diffusion, defined by Fick's law, is inversely proportional to flow rate, whilst equilibration time due to the partitioning of the analyte is directly proportional to flow rate. These two factors together with that of the multiple pathways factor determine the value of the plate height for a particular column and, as previously stated, plate height determines the width of the analyte peak. The precise relationship between the three factors and plate height is expressed by the van Deemter equation (equation 11.11), which is shown graphically in Fig. 11.4.

$$H = A + \frac{B}{u_x} + Cu_x \tag{11.11}$$

where u_x is the flow rate of the eluent and A, B and C are constants for a particular column and stationary phase relating to multiple paths, longitudinal diffusion and equilibration time, respectively.

Fig. 11.4 gives a clear demonstration of the importance of establishing the optimum flow rate for a particular column. Longitudinal diffusion is much faster

in a gas than in a liquid and as a consequence flow rates are higher in gas chromatography than in liquid chromatography.

As previously stated, the width of an analyte peak is expressed in terms of the standard deviation σ, which is half the peak width at the point of inflexion ($0.607h_p$, where h_p is the peak height; Fig. 11.2). It can be shown that $\sigma = \sqrt{2Pt_R}$, where P is the diffusion coefficient of the analyte, i.e. a measure of the rate at which the analyte moves randomly in the mobile phase from a region of high concentration to one of lower concentration. It has units of $m^2 s^{-1}$. Since the value of σ is proportional to the square root of t_R it follows that if the elution time increases by a factor of 4 the width of the peak will double. Thus the longer it takes a given analyte to elute the wider will be its peak. For this reason, increasing the column length is not the preferred way to improve resolution.

Asymmetric peaks

In some chromatographic separations, the ideal Gaussian-shaped peaks are not obtained, but rather asymmetrical peaks are produced. In cases where there is a gradual rise at the front of the peak and a sharp fall after the peak, the phenomenon is known as fronting. The most common cause of fronting is overloading the column so that reducing the amount of mixture applied to the column often resolves the problem. In cases where the rise in the peak is normal but the tail is protracted, the phenomenon is known as tailing (Fig. 11.2). The probable explanation for tailing is the retention of analyte by a few active sites on the stationary phase, commonly on the inert support matrix. Such sites strongly adsorb molecules of the analyte and only slowly release them. This problem can be overcome by chemically removing the sites, frequently hydroxyl groups, by treating the matrix with a silanising reagent such as hexamethyldisilazine. This process is sometimes referred to as capping. Peak asymmetry is usually expressed as the ratio of the width of the peak from the centre of the peak at $0.1h_p$.

Resolution

The success of a chromatographic separation is judged by the ability of the system to resolve one analyte peak from another. Resolution (R_S) is defined as the ratio of the difference in retention time (Δt_R) between the two peaks (t_{RA} and t_{RB}) to the mean (w_{av}) of their base widths (w_A and w_B):

$$R_S = \frac{\Delta t_R}{w_{av}} = \frac{2(t_{R_A} - t_{R_B})}{w_A + w_B} \tag{11.12}$$

When $R_S = 1.0$, the separation of the two peaks is 97.7% complete (thus the overlap is 2.3%). When $R_S = 1.5$ the overlap is reduced to 0.2%. Unresolved peaks are referred to as fused peaks (Fig. 11.2). Provided the overlap is not excessive, the analysis of the individual peaks can be made on the assumption that their characteristics are not affected by the incomplete resolution.

Resolution is influenced by column efficiency, selectivity factor and capacity factors according to the equation:

$$R_S = \left[\frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\right]\left[\frac{k'_2}{1 + k'_{av}}\right] \quad (11.13)$$

where k'_2 is the capacity factor for the longest retained peak and k'_{av} is the mean capacity factor for the two analytes.

Equation 11.13 is one of the most important in chromatography as it enables a rationale approach to be taken to the improvement of the resolution between the analytes. For example, it can be seen that resolution increases with $\sqrt{N}$. Since N is linked to the length of the column, doubling the length of the column will increase resolution by $\sqrt{2}$, i.e. by a factor of 1.4. Since both capacity factors and selectivity factors are linked to retention times and retention volumes, altering the nature of the two phases or their relative volumes will impact on resolution. Capacity factors are also dependent upon distribution coefficients, which in turn are temperature dependent; hence altering the column temperature may improve resolution.

The capacity of a particular chromatographic separation is a measure of the amount of material that can be resolved into its components without causing peak overlap or fronting. Ion-exchange chromatography (Section 11.6) and chromatofocusing (Section 11.6.3) have a high capacity, which is why they are often used in the earlier stages of a purification process.

11.2.5 Qualitative and quantitative analysis

Chromatographic analysis can be carried out on either a qualitative or a quantitative basis.

Qualitative analysis The objective of this approach is to confirm the presence of a specific analyte in a test sample. This is achieved on the evidence of:

- A comparison of the retention time (R_f in thin-layer chromatography mode) of the peaks in the chromatograph with that of an authentic reference sample of the test analyte obtained under identical chromatographic conditions. Confirmation of the presence of the analyte in the sample can be obtained by spiking a second portion of the test sample with a known amount of the authentic compound. This should result in a single peak with the predicted increase in area.
- The use of either a mass spectrometer or nuclear magnetic resonance (NMR) spectrometer as a detector so that structural evidence for the identity of the analyte responsible for the peak can be obtained.

Quantitative analysis The objective of this approach is to confirm the presence of a specific analyte in a test sample and to quantify its amount. Quantification is achieved on the basis of peak area coupled with an appropriate calibration graph. The area of each peak in a chromatograph can be shown to be proportional to the amount of the analyte producing the peak. The area of the peak may be determined by measuring the height of the peak (h_p) and its width at half the

Example 1 CALCULATION OF RESOLUTION OF TWO ANALYTES

Question

Two analytes A and B were separated on a 25 cm long column. The observed retention times were 7 min 20 s and 8 min 20 s, respectively. The base peak width for analyte B was 10 s. When a reference compound, which was completely excluded from the stationary phase under the same elution conditions, was studied, its retention time was 1 min 20 s. What was the resolution of the two analytes?

Answer

In order to calculate the required resolution, it is first necessary to calculate other chromatographic parameters.

(i) The adjusted retention time for A and B based on equation 11.2: $t'_R = t_R - t_M$

For analyte A

$t'_R = 440 - 80 = 360$ s

For analyte B

$t'_R = 500 - 80 = 420$ s

(ii) The capacity factor for A and B based on equation 11.3: $k' = t_R/t_M$

For analyte A

$k'_A = 360/80 = 4.5$

For analyte B

$k'_B = 420/80 = 5.25$

(iii) The selectivity factor for the two analytes based on equation 11.5: $\alpha = k'_B/k'_A$

$\alpha = 5.25/4.5 = 1.167$

(iv) The number of theoretical plates in the column; based on equation 11.10: $N = (t_R/w)^2$

For analyte B

$N = (420/10)^2 = 1764$

(v) The resolution of the two analytes based on equation 11.13:

$R_S = (\sqrt{N}/4)\,[(\alpha - 1)/\alpha)]\,[k'_B/(1 + k'_{av})]$ gives

$R_s = (\sqrt{1764}/4)(0.167/1.167)(5.25/1 + 4.875) = 1.34$

Discussion

From the earlier discussion on resolution, it is evident that a resolution of 1.34 is such as to give a peak separation of greater than 99%. If there were an analytical need to increase this separation it would be possible to calculate the length of column required to double the resolution. Since resolution is proportional to the square root of N, to double the resolution the number of theoretical plates in the column must be increased four-fold, i.e. to $4 \times 1764 = 7056$. The plate height in the column $H = L/N$, i.e. $250/1764 = 0.14$ mm. Hence, to get 7056 plates in the column, its length must be increased to $0.14 \times 7056 = 987.84$ mm or 98.78 cm.

height (w_h) (Fig. 11.2). The product of these dimensions is taken to be equal to the area of the peak. This procedure is time consuming when complex and/or a large number of analyses are involved and dedicated integrators or microcomputers best perform the calculations. These can be programmed to compute retention

time and peak area and to relate them to those of a reference standard, enabling relative retention ratios and relative peak area ratios to be calculated. These may be used to identify a particular analyte and to quantify it using previously obtained and stored calibration data. The data system can also be used to correct problems inherent in the chromatographic system. Such problems can arise either from the characteristics of the detector or from the efficiency of the separation process. Problems that are attributable to the detector are baseline drift, where the detector signal gradually changes with time, and baseline noise, which is a series of rapid minor fluctuations in detector signal, commonly the result of the operator using too high a detector sensitivity or possibly an electronic fault.

Internal standard

Quantification of a given analyte is based on the construction of a calibration curve obtained using a pure, authentic sample of the analyte. The construction of the calibration curve is carried out using the general principles discussed in Section 1.6.6. Most commonly the calibration curve is based on the use of relative peak areas obtained using an internal standard that has been subject to any preliminary extraction procedures adopted for the test samples. The standard must be carefully chosen to have similar physical and structural characteristics to those of the test analyte, and in practice is frequently an isomer or structural analogue of the analyte. Ideally, it should have a retention time close to that of the analyte but such that the resolution is greater than 99.5%.

External standard

An alternative approach to the use of an internal standard is the use of an external standard. In this method the standard is added to the test sample immediately before the sample is applied to the chromatographic column. It is therefore not taken through any preliminary extraction procedure and cannot compensate for variations in the efficiency of the extraction procedure. This method is valid only in those cases where the recovery of the analyte from the test sample is virtually quantitative and in those cases where there are no short-term fluctuations in detector response.

11.2.6 Sample preparation

Solvent extraction

Whilst chromatographic techniques are designed to separate mixtures of analytes this does not mean that attention need not be paid to the preliminary purification (clean up) of the test sample before it is applied to the column. On the contrary, it is clear that, for quantitative work using HPLC techniques in particular, such preliminary action is essential, particularly if the test analyte(s) is in a complex matrix such as plasma, urine, cell homogenate or microbiological culture medium. The extraction and purification of the components from a cell homogenate is often a complex multistage process. The associated principles for protein purification

are discussed in Section 8.3. For some forms of analysis, for example the analysis of drugs in biological fluids, sample preparation is relatively easy. The most common clean-up technique is solvent extraction. This is based on the extraction of the analytes from aqueous mixtures using a low boiling water-immiscible solvent such as diethylether or dichloromethane. The technique is another example of the application of the principle of partition coefficients. Since organic compounds that are weak electrolytes, such as acids and bases, can exist in ionised or unionised forms depending upon their pK_a and the prevailing pH, the pH of the test sample must be adjusted to the appropriate value to permit the extraction of the unionised species. Organic solvents such as diethylether and dichloromethane also extract a significant quantity of water and, in general, this should be removed from the extract, for example by the addition of an anhydrous salt such as sodium sulphate or magnesium sulphate, before it is evaporated to dryness (often under nitrogen or *in vacuo*), dissolved in the minimum volume of an appropriate solvent such as methanol or acetonitrile, and applied to the column. This solvent extraction procedure tends to lack selectivity and is often unsatisfactory for the HPLC analysis of compounds in the range of ng cm^{-3} or less. It can sometimes be improved by the technique of ion-pairing (Section 11.3.2).

Solid-phase extraction

The alternative to solvent extraction is solid-phase extraction. Its advantage over simple solvent extraction is that it exhibits greater selectivity, mainly because it is a form of chromatography. The test solution is passed through a small (few millimetres in length) disposable column (cartridge) packed with relatively large particles of a bonded silica similar to those used for HPLC (Section 11.3.2). These selectively adsorb the analyte(s) under investigation and ideally allow interfering compounds to pass through. Preliminary thought has to be given to the particular bonded silica selected and the test sample should be treated with agents such as trichloroacetic acid, perchloric acid or organic solvents such as acetonitrile to deproteinise it so that the opportunity for protein binding of the analyte(s) is minimised. The pH of the test solution should also be adjusted to maximise the retention of the analyte. Once the test solution has been passed through the column, either by simple gravity feed or by the application of a slight vacuum to the receiver vessel, the column is washed with water to remove final traces of contaminants and the adsorbed analyte(s) recovered by elution with a small volume of an organic solvent such as methanol or acetonitrile. The extract is evaporated to dryness (under nitrogen or *in vacuo*) and the residue dissolved in the minimum volume of an appropriate solvent prior to chromatographic analysis. Several commercial forms of this solid-phase extraction technique are available that facilitate the simultaneous treatment of a large number of test samples and the term pipette tip chromatography coined to describe it.

Column switching

A more sophisticated procedure for sample preparation, particularly suited to the analysis of analytes in very low concentrations in complex mixtures by HPLC, is

the technique of column switching. In this technique, the test solution is applied to a preliminary short column similar to the type used in solid-phase extraction. Once the test analyte has been adsorbed and impurities washed through the column, the analyte is eluted with a suitable organic solvent and the column eluate transferred directly to an analytical HPLC column. Technically this is not easy to achieve and requires several pumps and switching valves and is therefore expensive. One of the main problems with the technique is that, unless all interfering compounds are eluted from the preliminary column before the adsorbed analyte is switched to the analytical column, they will eventually accumulate in the analytical column and reduce its resolving power. Nevertheless, the technique has achieved many very difficult resolutions.

Supercritical fluid extraction

Supercritical fluid extraction (SFE) exploits the fact that gases such as carbon dioxide exist as a liquid under certain critical conditions. In the case of carbon dioxide, these conditions are 31.1 °C and 7.38 MPa and the resultant liquid carbon dioxide can be used as the extraction solvent, behaving as a low polarity solvent comparable to hexane. By altering the physical conditions of the extract, the carbon dioxide can be made to revert to a gas, thus simplifying the recovery of the extracted analytes.

Sample derivatisation

Some functional groups, especially hydroxyl, present in a test analyte may compromise the quality of its behaviour in a chromatographic system. The technique of analyte pre- or post-column derivatisation may facilitate better chromatographic separation and detection by masking these functional groups. Common derivatisation reagents are shown in Table 11.1.

11.3 LIQUID CHROMATOGRAPHY (LPLC AND HPLC)

11.3.1 Low pressure liquid chromatography (LPLC)

Columns

The column is invariably made of glass and is of a length and diameter appropriate to the amount of material to be separated. It should have a means of supporting the stationary phase as near to the base of the column as possible in order to minimise the dead space below the column support in which post-column mixing of separated analytes could occur. Commercial columns possess either a porous glass plate fused onto the base of the column or a suitable device for supporting a replaceable nylon net, which in turn supports the stationary phase. Capillary tubing normally leads the eluate from the column to the detector and/or fraction collection system. For some chromatographic separations, it is necessary for the temperature of the column to be raised or lowered during the separation. This is most simply achieved by jacketing the column so that water from a thermostatically controlled

Table 11.1 Examples of derivatising agents

Analyte	Reagent
A. Pre-column	
Ultraviolet detection	
Alcohols, amines, phenols	3,5-Dinitrobenzoyl chloride
Amino acids, peptides	Phenylisothiocyanate, dansyl chloride
Carbohydrates	Benzoyl chloride
Carboxylic acids	1-*p*-Nitrobenzyl-*N*,*N*′-diisopropylisourea
Fatty acids, phospholipids	Phenacyl bromide, naphthacyl bromide
Electrochemical detection	
Aldehydes, ketones	2,4-Dinitrophenylhydrazine
Amines, amino acids	*o*-Phthalaldehyde, fluorodinitrobenzene
Carboxylic acids	*p*-Aminophenol
Fluorescent detection	
Amino acids, amines, peptides	Dansyl chloride, dabsyl chloride, fluoroescamine, *o*-phthalaldehyde
Carboxylic acids	4-Bromomethyl-7-methoxycoumarin
Carbonyl compounds	Dansylhydrazine
B. Post-column	
Ultraviolet detection	
Amino acids	Phenylisothiocyanate
Carbohydrates	Orcinol and sulphuric acid
Penicillins	Imidazole and mercuric chloride
Fluorescent detection	
Amino acids	*o*-Phthalaldehyde, fluorescamine, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate

bath, set at the required working temperature, may be pumped around the outside of the column. More sophisticated methods include placing the column in a heating block or in a thermostatically controlled oven.

Matrix materials

The selection of a matrix for a particular stationary phase is vital to the successful chromatographic use of the phase. Generally speaking, a matrix needs to have:

- high mechanical stability to encourage good flow rates and to minimise pressure drop along the column;
- good chemical stability;
- functional groups to facilitate the attachment of the stationary phase;
- high capacity, i.e. density of functional groups to minimise bed volume;
- availability in a range of particle sizes; in addition some forms of chromatography require a matrix with a porous structure, in which case the pores need to be of the correct size and shape;
- an inert surface to minimise the non-selective adsorption of analytes and hence peak tailing.

In practice, the six most commonly used types of matrix are:

- *Agarose:* A polysaccharide made up of D-galactose and 3,6-anhydrogalactose units. The unbranched polysaccharide chains are cross-linked with agents such as 2,3-dibromopropanol to give gels that are stable in the pH range 3–14. They have good flow properties and are hydrophilic but they should never be allowed to dry out otherwise they undergo irreversible change. Commercial examples include Sepharose and Bio-Gel A.
- *Cellulose:* A polysaccharide of β-1–4-linked D-glucose units. For matrix use it is cross-linked with epichlorohydrin, the extent of cross-linking dictating the pore size. It is available in bead, microgranular and fibrous forms, has good pH stability and flow properties, and is highly hydrophilic. It is commonly used in ion-exchange chromatography.
- *Dextran:* A polysaccharide consisting of α-1–6-linked D-glucose units. For matrix use it is cross-linked with epichlorohydrin but is less stable to acid hydrolysis than are cellulose matrices. It is stable up to pH 12 and is hydrophilic. Commercial examples include Sephadex.
- *Polyacrylamide:* A polymer of acrylamide cross-linked with *N,N′*-methylene-bis-acrylamide. It is stable in the pH range 2–11. Commercial examples include Bio-Gel P.
- *Polystyrene:* A polymer of styrene cross-linked with divinylbenzene. Polystyrene matrices have good stability over all pH ranges and are most commonly used for exclusion and ion-exchange chromatography. They have relatively low hydrophilicities.
- *Silica:* A polymeric material produced from orthosilicic acid. The numerous silanol (Si—OH) groups make it hydrophilic. When derivatised, excess silanol groups can be capped by treatment with trichloromethylsilane. The stability of silica matrices is confined to the pH range 3–8. It is commonly used in perfusion chromatography. Closely related to the silica matrices is controlled pore glass. It is chemically inert but, like the silicas, tends to dissolve when the pH is above 8.

Stationary phases

The chemical nature of the stationary phase depends upon the particular form of chromatography to be carried out. Full details are given in later sections of this chapter. Most stationary phases are available attached to the matrices in a range of sizes and shapes. Both properties are important because they influence the flow rate and resolution characteristics. The larger the particle, the faster the flow rate but, conversely, the smaller the particle the larger the surface area-to-volume ratio and potentially the greater the resolving power. In practice a balance has to be struck. The best packing characteristics are given by spherical particles and most stationary phases now have a spherical or approximately spherical shape. Particle size is commonly expressed by a mesh size, which is a measure of the openings per square inch in a sieve; hence the larger the mesh size, the smaller is the particle. An 80–100 mesh (0.18–0.15 mm) or 100–120 mesh (0.075–0.038 mm) is most common

for routine use, whereas a 200–400 mesh (0.075–0.38 mm) is used for higher resolution work.

Column packing

Packing a column is normally carried out by gently pouring a slurry of the stationary phase attached to its matrix in the mobile phase into a column that has its outlet closed, whilst the upper part of the slurry in the column is gently tapped to ensure that no air bubbles are trapped and that the packing settles evenly. Poor column packing gives rise to uneven flow (channelling) and reduced resolution. The slurry is added until the required height is obtained. Once the required column height has been obtained, the flow of mobile phase through the packed column is started by opening the outlet, and continued until the packing has completely settled. This whole process generally requires considerable practice to achieve reproducible results. To prevent the surface of the packed material from being disturbed by the addition of mobile phase and the sample to the column, it is normal to place a suitable protection device, such as nylon or rayon gauze, on the top surface of the column. Some commercial columns possess an adaptor and plunger, which serve the dual purpose of protecting the surface of the column and providing an inlet (often capillary tubing) to carry the mobile phase to the column surface. Once a column has been prepared, it is imperative that no part of it should be allowed to run dry; hence a layer of mobile phase should always be maintained above the column surface.

Application of sample

Several methods are available for the application of the sample to the top of the prepared column:

- *Direct application to top of column bed:* A simple way is to drain the mobile phase from above the column into the column bed by opening the flow outlet. The sample is then carefully applied by pipette and it too is allowed to run into the column. A small volume of mobile phase is then applied in a similar manner to wash remaining traces of the sample into the bed. Mobile phase is then carefully added to the column to a height of 2–5 cm and its flow started so that the height of the phase above the packed column is maintained at 2–5 cm.
- *Use of a sucrose density:* An alternative procedure, which avoids the necessity to drain the column to the surface of the bed, is to increase the density of the sample by the addition of sucrose to a concentration of about 1%. When this solution is layered onto the liquid above the column bed, it will automatically sink to the surface of the column and hence be quickly passed into the column. This method of sample application is satisfactory, provided that the presence of sucrose does not interfere with the separation and subsequent analysis of the sample.
- *Use of a peristaltic pump:* A third method involves the use of capillary tubing and/or syringe or peristaltic pump to pass the sample directly to the column surface. This method is the most satisfactory of the three possibilities.

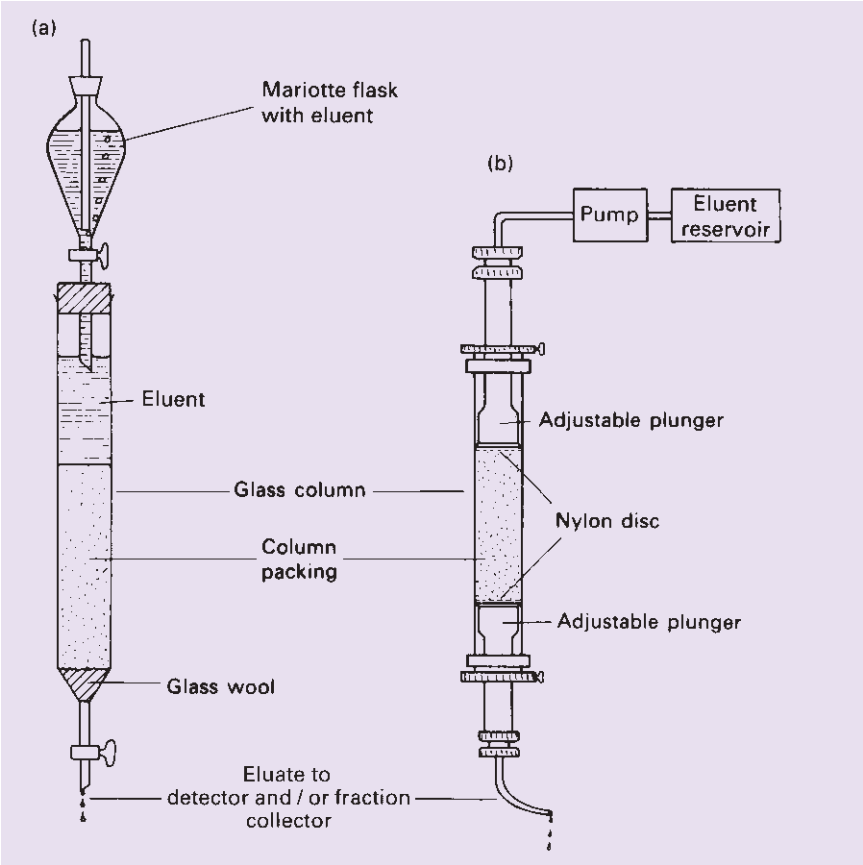

Fig. 11.5. Forms of columns for low pressure liquid chromatography.

In all cases, care must be taken to avoid overloading the column with sample, otherwise irregular separation and fronting will occur. It is also advantageous to apply the sample in as small a volume of solvent as possible because this ensures an initial tight band of material when the separation commences.

Column development and analyte elution

The components of the applied sample are separated by the continuous passage of the mobile phase through the column. This is referred to as column development. The separated analytes are then removed from the column by elution. During the development and elution process it is essential that the flow of mobile phase is maintained at a constant rate. This is best achieved by the use of either a Mariotte flask (Fig. 11.5) or a peristaltic pump, the most common form of which is the roller type (Fig. 11.6). Isocratic elution, using a single liquid as the mobile phase, or gradient elution may be used. In order to produce a suitable gradient, two or more eluents have to be mixed in the correct proportions prior to their entering the column. This may be achieved by the use of either gradient mixers (Fig. 11.7) or

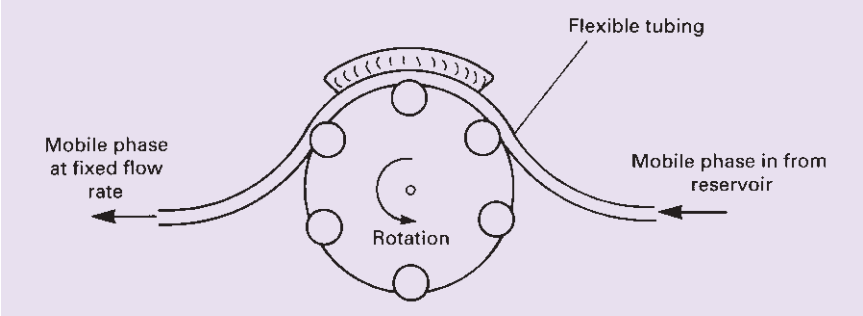

Fig. 11.6. Simple peristaltic pump commonly used in low pressure liquid chromatography.

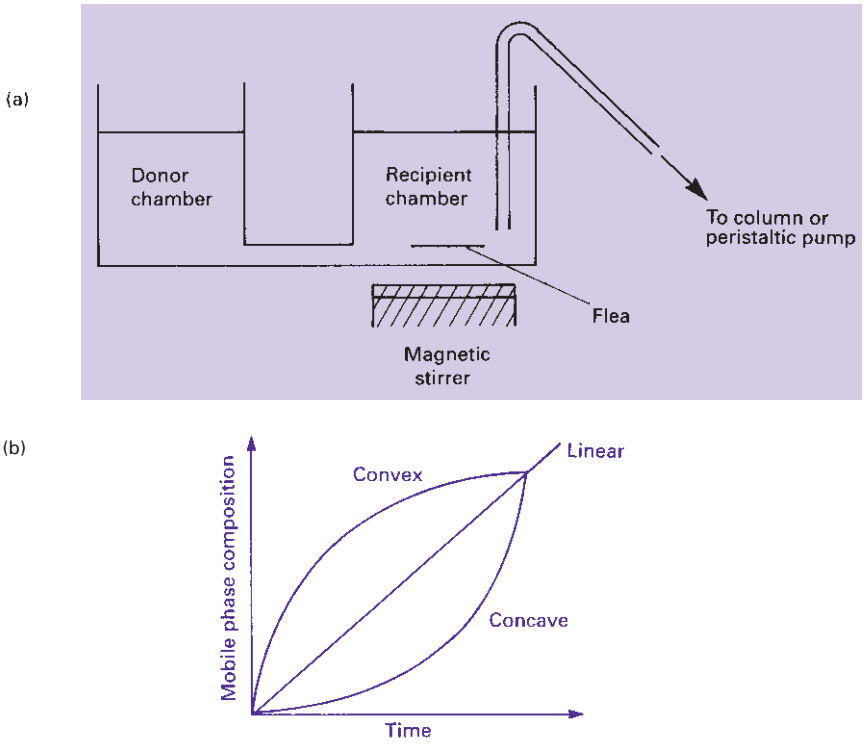

Fig. 11.7. (a) Simple apparatus for producing gradient elution; (b) common gradient shapes.

two or more peristaltic pumps programmed to deliver the separate eluents at predetermined rates into a mixing area before application to the column. Convex gradients give better resolution initially whereas concave gradients give better resolution at the end.

The methods for the detection and collection of the individual analytes as they emerge in the eluate are similar to those employed for HPLC (Section 11.3.2).

11.3.2 High performance liquid chromatography (HPLC)

It is evident from equations 11.1 to 11.12 that the resolving power of a chromatographic column increases with column length and the number of theoretical plates per unit length. However, there are practical limits to the length of a column owing to the problem of peak broadening (Section 11.2.4). As the number of theoretical plates in the column is related to the surface area of the stationary phase, it follows that the smaller the particle size of the stationary phase, the better the resolution, in part because it reduces the equilibration time of the analyte between the stationary and mobile phases (Section 11.2.4). Unfortunately, the smaller the particle size, the greater is the resistance to the flow of the mobile phase for a given flow rate. This resistance creates a back-pressure in the column that may be sufficient to cause the structure of the matrix to collapse, thereby actually reducing eluent flow and impairing resolution. This problem has been solved by the development of small particle size stationary phases that can withstand these pressures. This development, which has occurred in adsorption, partition, ion-exchange, exclusion and affinity chromatography, has resulted in faster and better resolution and explains why HPLC has emerged as the most popular, powerful and versatile form of chromatography. Many commercially available HPLC systems are microprocessor controlled to allow dedicated, continuous chromatographic separations.

Columns

Conventional columns (Fig. 11.8) used for HPLC are generally made of stainless steel and are manufactured so that they can withstand pressures of up to 5.5×10^7 Pa. The columns are generally 3–50 cm long and approximately 4 mm internal diameter, with flow rates of 1–3 $cm^3\ min^{-1}$. Microbore or open tubular columns have an internal diameter of 1–2 mm and are generally 25–50 cm long. They can sustain flow rates of 5–20 $mm^3\ min^{-1}$. Microbore columns have three important advantages over conventional columns:

- reduced eluent consumption owing to the slower flow rates,
- ideal for interfacing with a mass spectrometer owing to the reduced flow rate,
- increased sensitivity owing to the higher concentration of analytes that can be used.

Matrices and stationary phases

Three forms of matrix/stationary phase material are available, based on a rigid solid, as opposed to gel, structure as the materials need to withstand the high pressures generated in the column. All forms involve approximately spherical particles of a uniform size to minimize space for diffusion and hence band broadening to occur. The three forms are:

- *Microporous supports:* In which micropores ramify through the particles, which are generally 5–10 mm in diameter.
- *Pellicular (superficially porous) supports:* In which porous particles are coated onto an inert solid core such as a glass bead of about 40 mm in diameter.

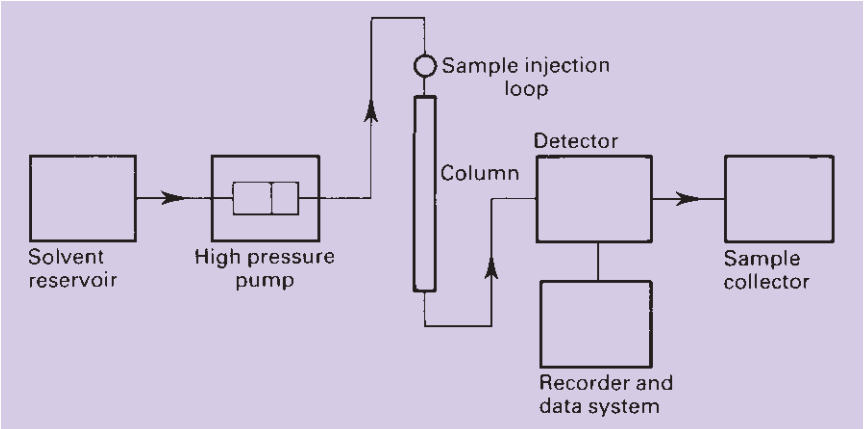

Fig. 11.8. Components of an isocratic HPLC system. For gradient elution two reservoirs and two pumps are used with liquid phase mixing before entry to the sample injection loop.

- *Bonded phases:* In which the stationary phase is chemically bonded onto an inert support such as silica.

For adsorption chromatography, adsorbents such as silica and alumina are available as microporous or pellicular forms with a range of particle sizes. Pellicular systems generally have a high efficiency but low sample capacity and therefore microporous supports are preferred.

In partition chromatographic systems, the stationary phase may be coated onto the inert microporous or pellicular support. One disadvantage of supports coated with liquid stationary phases is that the mobile phase may gradually wash off the liquid phase. To overcome this problem, bonded phases have been developed in which the supporting material is silica.

Many different types of ion exchanger suitable for HPLC are available. The cross-linked microporous polystyrene resins are widely used. Pellicular resin forms are also available, as are bonded-phase exchangers covalently bonded to a cross-linked silicone network. These resins are classed as hard gels and readily withstand the pressures generated during analysis.

The stationary phases for exclusion separations are generally based on silica, polymethacrylate, polyvinylacetate, polyvinylchloride or on cross-linked dextran or agarose. All are available in a range of pore sizes. They are generally used where the eluent is an organic system. The supports for affinity separations are similar to those for exclusion separations.

Application of sample

The application of the sample onto a HPLC column in the correct way is a particularly important factor in achieving successful separations. The most common technique is the use of a loop injector (Fig. 11.9). This consists of a metal loop, of fixed small volume, that can be filled with the sample. The eluent from the pump is then channelled through the loop by means of a valve switching system and the

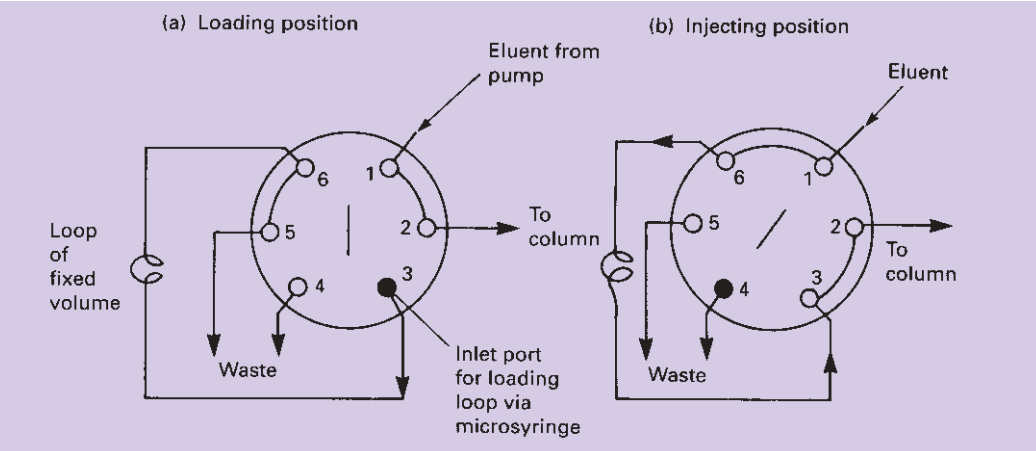

Fig. 11.9. HPLC loop injector: the loop is loaded (a) via port 3, with excess sample going to waste via port 5. In this position the eluent from the pump passes to the column via ports 1 and 2. In the injecting position (b), eluent flow is directed through the loop via ports 1 and 6 and then onto the column.

sample flushed onto the column via the loop outlet without interruption of the flow of eluent to the column.

Repeated application of highly impure samples such as sera, urine, plasma or whole blood, which have preferably been deproteinated, may eventually cause the column to lose its resolving power. To prevent this occurrence, a guard column is often installed between the injector and the column. This guard column is a short (1–2 cm) column of the same internal diameter and packed with material similar to that present in the analytical column. The packing in the guard column preferentially retains contaminating material and can be replaced at regular intervals.

Mobile phases

The choice of mobile phase to be used in any separation depends on the type of separation to be achieved. Isocratic elution may be made with a single pump, using a single eluent or two or more eluents pre-mixed in fixed proportions. Gradient elution generally uses separate pumps to deliver two eluents in proportions predetermined by a gradient programmer. All eluents for use in HPLC systems must be specially purified because traces of impurities can affect the column and interfere with the detection system. This is particularly the case if the detection system is based on the measurement of absorbance changes below 200 nm. Pure eluents for use in HPLC systems are available commercially, but even with these a 1–5 mm microfilter is generally introduced into the system prior to the pump. It is also essential that all eluents be degassed before use otherwise gassing (the presence of air bubbles in the eluent) tends to occur in most pumps. Gassing, which tends to be particularly bad for eluents containing aqueous methanol and ethanol, can alter column resolution and interfere with the continuous monitoring of the eluate. Degassing of the eluent may be carried out in several ways: by warming, by stirring

vigorously with a magnetic stirrer, by applying a vacuum, by ultrasonication, and by bubbling helium gas through the eluent reservoir.

Pumps

Pumping systems for delivery of the eluent are one of the most important features of HPLC systems. The main features of a good pumping system are that it is capable of outputs of at least 5×10^7 Pa and ideally there must be no pulses (i.e. cyclical variations in pressure) as this may affect the detector response. There must be a flow capability of at least 10 cm^3 min^{-1} and up to 100 cm^3 min^{-1} for preparative separations.

Constant displacement pumps maintain a constant flow rate through the column irrespective of changing conditions within the column. One form of constant displacement pump is a motor-driven syringe-type pump that delivers a fixed volume of eluent onto the column by a piston driven by a motor. The constant volume syringe pump contains a screw-jack driven by a stepper motor. On the delivery stroke, the piston is driven at a constant rate, displacing eluent onto the column at the same rate. Two one-way valves control eluent flow in the chamber (Fig. 11.10a). The reciprocating pump is the most commonly used form of constant displacement pump. A motorized crank drives the piston, and check valves regulate entry of eluent to the column. On the compression stroke, eluent is forced from the pump chamber onto the column. During the return stroke, the exit check valve closes and eluent is drawn in via the entry valve to the pump chamber ready to be pumped onto the column on the next compression stroke (Fig. 11.10b). Such pumps produce small pulses of flow and pulse dampeners are usually incorporated into the system to minimise this pulsing effect. All constant displacement pumps have in-built safety cut-out mechanisms so that if the pressure within the column changes from pre-set limits the pump is inactivated automatically.

11.3.3 Detectors

Since the quantity of material applied to a LPLC or HPLC column is normally very small, it is imperative that the sensitivity of the detector system is sufficiently high and stable to respond to the low concentrations of each analyte in the eluate. The most commonly used detectors are as follows:

- *Variable wavelength detectors:* These are based upon ultraviolet–visible spectrophotometry. These types of detector are capable of measuring absorbances down to 190 nm and can give full-scale deflection (AUFS) for as little as 0.001 absorbance units. They have a sensitivity of the order of 5×10^{-10} g cm^{-3} and a linear range of 10^5. All spectrophotometric detectors use continuous flow cells with a small internal volume (typically 8 mm^3) and optical path length of 10 mm that allow the continuous monitoring of the column eluate.
- *Scanning wavelength detectors:* These have the facility to record the complete absorption spectrum of each analyte, thus aiding identification. Such

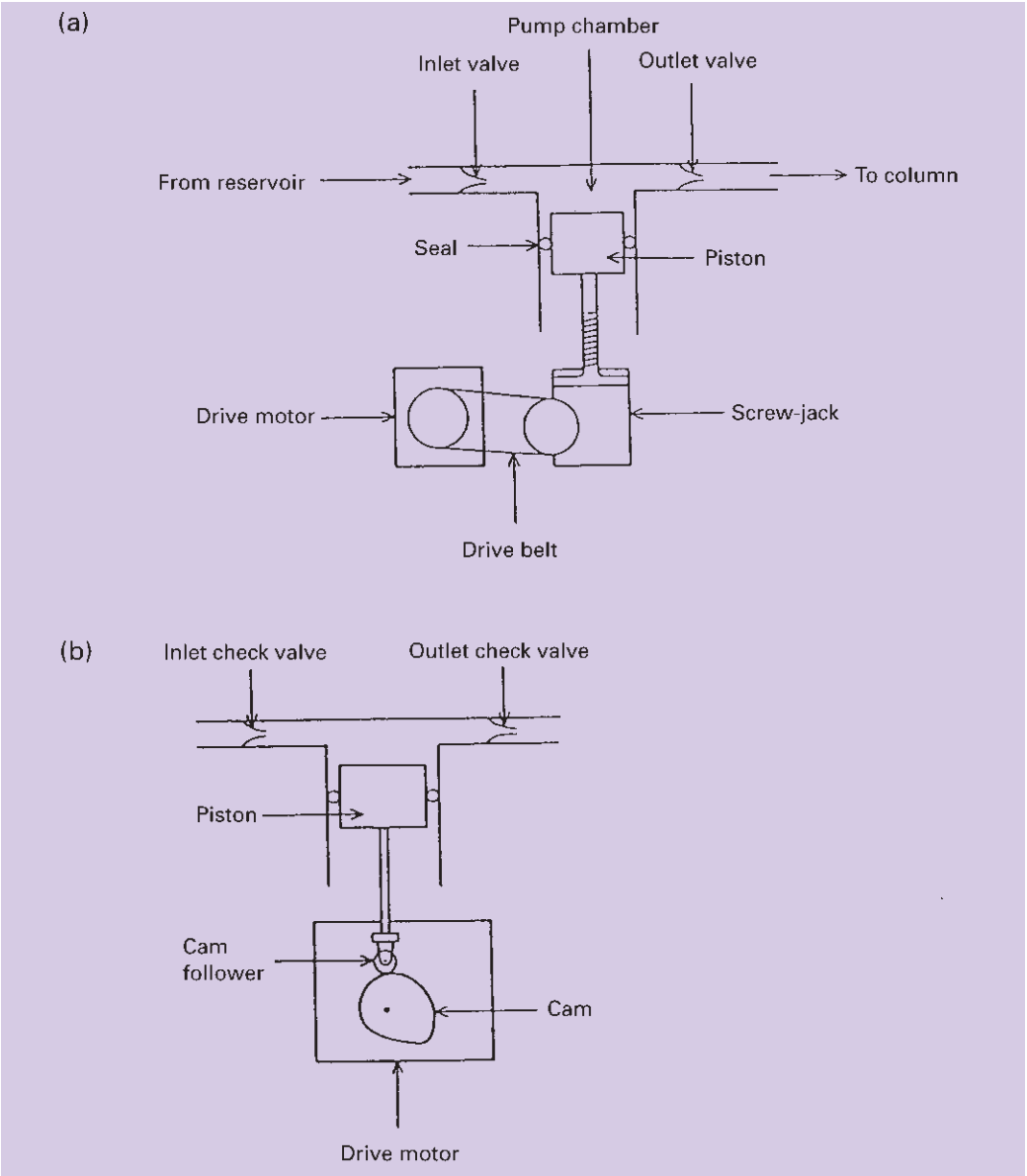

Fig. 11.10. Commonly used constant displacement pumps: (a) constant volume; (b) reciprocating. In both types, the down stroke of the piston closes the outlet valve and opens the inlet valve to release eluent into the pump chamber. The upstroke of the piston closes the inlet valve and opens the outlet valve to release the eluent onto the column. (Reproduced with permission from R. Newton (1982), Instrumentation for HPLC, in *HPLC in Food Analysis*, R. Macrae (ed.), Academic Press, London.)

opportunities are possible either by temporarily stopping the eluent flow or by the use of diode array techniques, which allow a scan of the complete spectrum of the eluate within 0.01 s and its display as a three-dimensional plot on a computer screen in real time (Fig. 11.11).

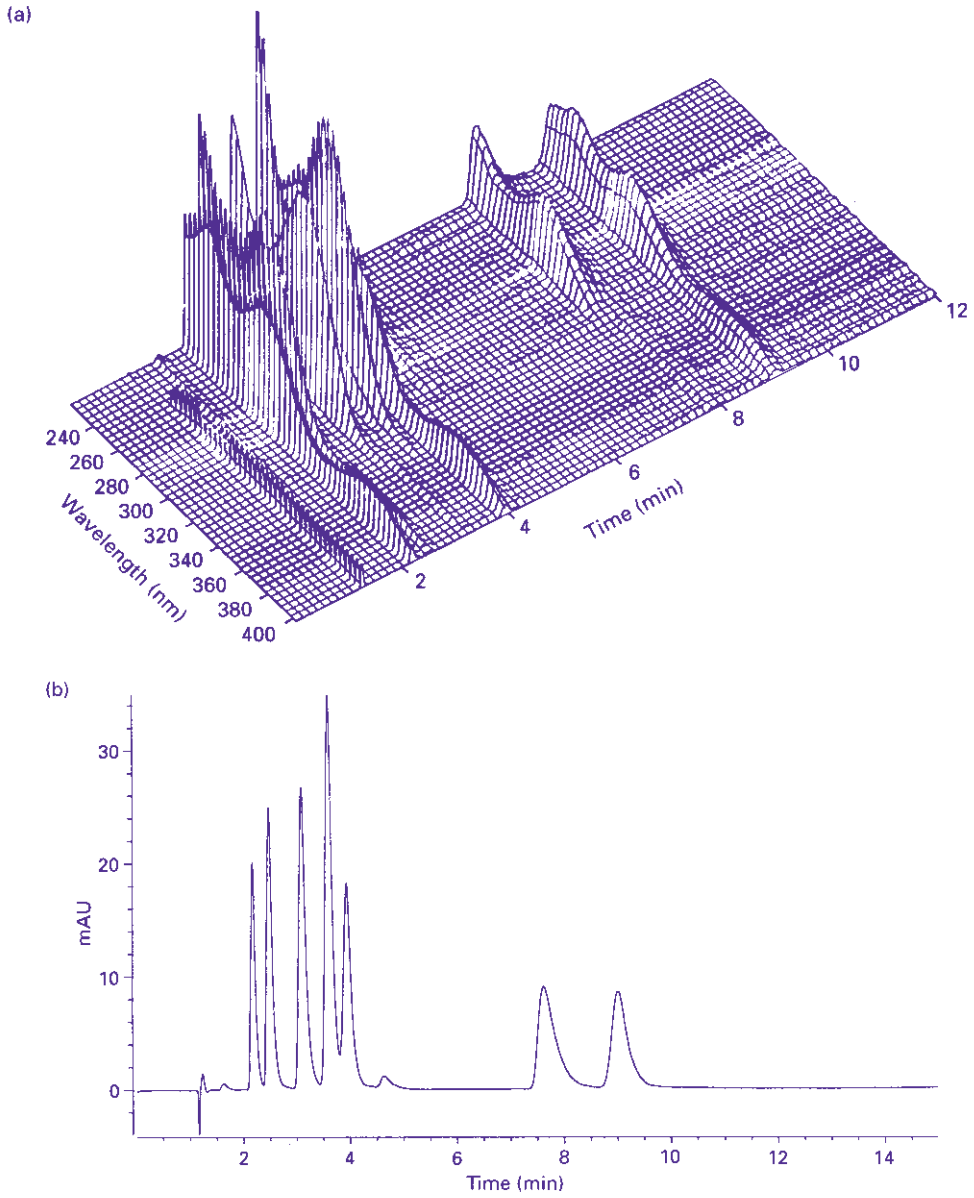

Fig. 11.11. Separation by HPLC of the dihydropyridine calcium channel blocker lacidipine and its metabolites. Column: ODS Hypercil. Eluent: methanol/acetonitrile/water (66%, 5%, 29%, by vol.) acidified to pH 3.5 with 1% formic acid. Flow rate: 1 $cm^3\ min^{-1}$. Column temperature: 40 °C. As recorded (a) by a diode array detector and (b) by an ultraviolet detector. (Reproduced by permission of GlaxoSmithKline, Stevenage.)

- *Fluorescence detectors:* These are extremely valuable for HPLC because of their greater light sensitivity (10^{-12} g cm^{-3}) than ultraviolet detectors but they have a slightly reduced linear range (10^4). However, the technique is limited by the fact that relatively few analytes fluoresce. Pre-derivatisation of the test sample can broaden the applications of the technique.

- *Electrochemical detectors:* These are selective for electroactive analytes and are potentially highly sensitive. Two types are available, amperometric and coulometric, the principles of which are similar. A flow cell is fitted with two electrodes: a stable counter electrode (Ag/AgCl or calomel, see Section 1.5.1) and a working electrode. A constant potential is applied to the working electrode at such a value that, as an analyte flows through the flow cell, molecules of the analyte at the electrode surface undergo either an oxidation or a reduction reaction, resulting in a current flow between the two electrodes (Section 1.5.1). The size of the current is recorded to give the chromatograph. The potential applied to the counter electrode is sufficient to ensure that the current detected gives a full-scale deflection on the recorder within the working analyte range. The two types of detector differ in the extent of conversion of the analyte at the detector surface and on balance amperometric detectors are preferred, since they have a higher sensitivity (10^{-12} g cm^{-3} as opposed to 10^{-8} g cm^{-3}) and greater linear range (10^5 as opposed to 10^4). For reduction reactions the working electrode is normally mercury, and for oxidative reactions carbon or a carbon composite. Analytes capable of undergoing oxidation include hydrocarbons, amines, amides, phenols, di- and triazines, phenothiazines, catecholamines and quinolines. Analytes capable of undergoing reduction include alkenes, esters, ketones, aldehydes, ethers, azo and nitro compounds. The eluent should of course be free from traces of compounds capable of responding to the detector.
- *Mass spectrometer detector:* This enables the analyte to be detected and its structure determined simultaneously. The technical problems associated with the logistics of removing the bulk of the mobile phase before the sample is introduced into the mass spectrometer have been resolved in a number of ways that are discussed in detail in Chapter 9. Analytes may be detected by total ion current (TIC) (Section 9.4.1) or selected ion monitoring (SIM) (Section 9.5.7). An advantage of mass spectrometry detection is that it affords a mechanism for the identification of overlapping peaks. If there is a suspicion that a large peak is masking a smaller peak then the presence of a minor analyte can be confirmed by SIM, provided that the minor and major analytes have a unique molecular ion or fragment ion.
- *NMR spectrometer detector:* This gives structural information about the analyte that is complementary to that obtained via HPLC–MS.
- *Refractive index detector:* This relies on a change in the refractive index of the eluate as analytes emerge from the column. Its great advantage is that it will respond to any analyte in any eluent, changes in refractive index being either positive or negative. Its limitation is its relatively modest sensitivity (10^{-7} g cm^{-3}) but it is commonly used in the analysis of carbohydrates.
- *Evaporative light-scattering detector (ELSD):* This relies on the vaporisation of the eluate, evaporation of the eluent and the quantification of the analyte by light scattering. The eluate emerging from the column is combined with a flow of air or nitrogen to form an aerosol; the eluent is then evaporated

from the aerosol by passage through an evaporator and the emerging dry particles of analyte irradiated with a light source and the scattered light detected by a photodiode. The intensity of the scattered light is determined by the quantity of analyte present and its particle size. It is independent of the analyte's spectroscopic properties and hence does not require the presence of a chromophoric group or any prior derivatisation of the analyte. It can quantify analytes in flow rates of up to 5 cm^3 min^{-1}. Appropriate calibration gives good, stable quantification of the analyte with no baseline drift. It is an attractive method for the detection of fatty acids, lipids and carbohydrates.

The sensitivity of ultraviolet absorption, fluorescence and electrochemical detectors can often be increased significantly by the process of derivatisation, whereby the analyte is converted pre- or post-column to a chemical derivative. Examples are given in Table 11.1.

Fraction collectors

For studies in which the analyte in the eluate is to be collected and studied further, the eluate has to be divided into fractions. Two approaches are available to achieve this objective: either the eluate can be continuously monitored and the fraction containing a particular analyte collected, or the eluate can be divided into small (1–10 cm^3) fractions, which are subsequently analysed and those containing a particular analyte bulked together. Automatic fraction collectors are designed either to collect a selected volume of eluate or to collect the eluate for a predetermined period of time before a new collection tube is placed in position automatically. The volume of eluate in each fraction may be determined in one of several ways. There may be a siphoning or similar system to deliver a predetermined volume into each tube, or there may be an electronic means of allowing a predetermined number of drops of eluate to enter each tube. This latter method has the slight disadvantage that if the composition of the eluate changes (e.g. during gradient elution), so too may its surface tension and hence droplet size, so that the actual volume collected also changes. A further possibility is that the eluate is allowed to enter each tube for a fixed time interval. In this case, if the flow rate through the column varies, so too will the volume of each fraction, but this is unusual and, in practice, fixed-time collectors are the most common.

11.3.4 Fast protein liquid chromatography (FPLC)

The wide applicability, speed and sensitivity of HPLC has resulted in it becoming the most popular form of chromatography and virtually all types of biological molecules have been assayed or purified using the technique. HPLC has had a particular impact on the separation of amino acids, peptides and proteins. Instruments dedicated to the separation of proteins have given rise to the

technique of fast protein liquid chromatography (FPLC). There are no unique principles associated with FPLC, it is simply based on reversed-phase, affinity, exclusion, hydrophobic interaction and ion-exchange chromatography, and chromatofocusing. Mainly aqueous-based elution systems are used with special high capacity stationary phases of similar diameter to those used in conventional HPLC. However, the operating pressure (1–2 MPa) is lower than conventional HPLC. Microbore glass-lined stainless steel columns enable very small amounts of sample to be used, with separation taking as little as a few minutes. The technique enables such complex mixtures as tryptic digests of proteins and the culture supernatant of microorganisms to be applied directly to the column, but protein mixtures from cell extracts still need some form of preliminary fractionation (Section 8.3.3) prior to study.

11.3.5 Capillary electrochromatography

Capillary electrochromatography (CEC) is effectively a hybrid of HPLC and capillary electrophoresis (CE). As its name implies, it is carried out in capillary columns in which the stationary phase is either attached to an inert support and packed in the capillary or is coated directly onto the walls of the capillary. As in capillary electrophoresis, a potential is applied across the walls of the capillary, generating solvent flow by electroosmosis (Section 10.1). This electroosmotic flow (EOF) drives the solvent and the analytes in the applied sample through the capillary column. As the analytes move along the capillary they are subject to the opposing forces of EOF and distribution between the mobile and stationary phases. They will therefore be separated partly on the basis of differences in their distribution coefficients, K_d, between the two phases exactly as in all other forms of chromatography, and partly on the basis of differences in their electrophoretic mobility as in capillary electrophoresis. The consequence is that the capacity factor, k', characteristic of chromatography, is not valid in CEC.

The stationary phases used are similar to those of HPLC based on silica matrices. The mobile phase is generally an organic–aqueous system containing an electrolyte. The EOF is generated at the solid/liquid interface. The silica surface is negatively charged owing to deprotonisation of silanol groups and hence the mobile phase molecules carry a net positive charge, thereby forming an electrical double layer. The positively charged molecules near the surface of the silica induce similar changes in the nearby molecules. These molecules migrate towards the negative electrode under the influence of the applied field, dragging the bulk of the mobile phase with them. The velocity of the EOF is determined by a number of factors, including the size of the applied field and the viscosity and dielectric constant of the mobile phase. It is significantly slower in packed capillary columns than in the open tube variety.

The instrumentation for CEC is similar to that for CE except that both ends of the column are pressurised to ensure no pressure drop. Columns measuring 50 cm × 100 μm are commonly used and, because flow is produced by electroosmosis rather than applied pressure, smaller sized particles (1.5–5 μm) can be used than

with HPLC, with the result that the column efficiency is much higher in CEC and the resolution time shorter than in HPLC.

11.3.6 Perfusion chromatography

The high resolution achieved by HPLC is based on the use of small diameter particles for the stationary phase. However, this high resolution is achieved at the cost of the generation of high pressures, relatively low flow rates and the constraints the high pressure imposes on the instrumentation. Perfusion chromatography overcomes some of these limitations by the use of small particles (10–50 μm diameter) that have channels of approximately 1 μm diameter running through them that allow the use of high flow rates without the generation of high pressures. The high flow rates result in small plate heights (Section 11.2.4) and hence high resolution in very short separation times. The particles are made of polystyrene-divinylbenzene and are available under the trade name POROS. Two types of pore are available: through pores that are long (up to 8000 Å (800 nm)) and diffusive pores that are shorter (up to 1000 Å (100 nm)). The stationary phase is coated to the particles, including the surface of the pores. The eluent perfuses through the pores, allowing the analyte to equilibrate rapidly with the stationary phase. By comparison, the microporous particles used in HPLC have a much smaller diameter pore, hence the greater back-pressure. All the forms of stationary phase used for the various form of chromatography are available for perfusion chromatography. The technique uses the same type of instrumentation as HPLC and FPLC. Protein separations in as short a time as 1 min can be achieved.

11.3.7 Membrane chromatography

An alternative to perfusion chromatography as a solution to the inherent limitations of HPLC is membrane chromatography. This avoids the use of small particles by using porous membranes stacked on top of each other and onto which the stationary phase is coated. The pores in the membrane are large, allowing free passage to the eluent and analyte. Equilibration is fast owing to the large surface area of each membrane, allowing high flow rates (up to 10 $cm^3\ min^{-1}$) to be used. The membranes are contained in a cartridge that substitutes for the column in a conventional HPLC system.

11.4 ADSORPTION CHROMATOGRAPHY

11.4.1 Principle

This is the classic form of chromatography, which is based upon the principle that certain solid materials, collectively known as adsorbents, have the ability to hold molecules at their surface. This adsorption process, which involves weak,

non-ionic attractive forces of the van der Waals and hydrogen-bonding type, occur at specific adsorption sites. These sites have the ability to discriminate between types of molecules and are occupied by molecules of either the eluent or of the analytes in proportions determined by their relative strength of interaction. As eluent is constantly passed down the column, differences in these binding strengths eventually lead to the separation of the analytes. The strength of interaction of a particular analyte with the binding sites depends upon the functional groups present in its structure. Hydroxyl and aromatic groups, for example, tend to increase interaction with the adsorption surface. In general, the strength of adsorption is influenced more by the presence of specific functional groups than by the overall molecular size of the analyte because only a specific group rather than the whole molecule can interact with the adsorption site.

Silica is a typical adsorbent. It has silanol (Si—OH) groups on its surface, which are slightly acidic and can interact with polar functional groups of the analyte or eluent. The topology (arrangement) of these silanol groups in different commercial preparations of silica explains their different separation properties. Other commonly used adsorbents are alumina and carbon. Adsorbents based on carbon, alumina or silica are available for low pressure chromatography and for HPLC. The silicas are acidic and good for the separation of basic materials whereas the aluminas are more basic and better suited for the resolution of acidic materials.

In general, an eluent with a polarity comparable to that of the most polar analyte in the mixture is chosen. Thus alcohols would be selected if the analytes contained hydroxyl groups, acetone or esters would be selected for analytes containing carbonyl groups, and hydrocarbons such as hexane, heptane and toluene for analytes that are predominantly non-polar. Mixtures of solvents are commonly used in the context of gradient elution. The presence of small amounts of water in the mobile phase is often beneficial when silica is used as the stationary phase, as the water molecules selectively block the more active silanol groups, leaving a more selective population of weaker binding sites.

Adsorption chromatography is most commonly used to separate non-ionic, water-insoluble compounds such as triglycerides, vitamins and many drugs.

11.4.2 Hydroxylapatite chromatography

Crystalline hydroxylapatite ($Ca_{10}(PO_4)_6(OH)_2$) is an adsorbent used to separate mixtures of proteins or nucleic acids. The mechanism of adsorption is not fully understood but is thought to involve both the calcium ions and phosphate ions on the surface and to involve dipole–dipole interactions and possibly electrostatic attractions. One of the most important applications of hydroxylapatite chromatography is the separation of single-stranded DNA from double-stranded DNA. Both forms of DNA bind at low phosphate buffer concentrations but, as the buffer concentration is increased, single-stranded DNA is selectively desorbed. As the buffer concentration is increased further, double-stranded DNA is released. This behaviour is exploited in the technique of Cot analysis (Section 5.3.4).

The affinity of double-stranded DNA for hydroxylapatite is so high that it can be selectively removed from RNA and proteins in cell extracts by use of this type of chromatography.

Hydroxylapatite is available commercially in a range of forms suitable for LPLC and HPLC. These include crystalline or spheroidal hydroxylapatite and forms bonded to an agarose matrix. The adsorption capacity of all these forms is maximal around neutral pH and the conditions usually include 20 mM phosphate buffer for the adsorption process. Elution is achieved by increasing the phosphate buffer concentration to 500 mM.

11.4.3 Hydrophobic interaction chromatography

This type of chromatography was developed to purify proteins by exploiting their surface hydrophobicity, which is related to the presence of non-polar amino acid residues. Groups of hydrophobic residues are scattered over the surface of proteins in a way that gives characteristic properties to each protein. In aqueous solution, these hydrophobic regions on the protein are covered with an ordered layer of water molecules that effectively mask the hydrophobic groups. These groups can, however, be exposed by the addition of salt ions, which preferentially take up the ordered water molecules. The exposed hydrophobic regions can then interact with each other. In hydrophobic interaction chromatography (HIC), the presence of hydrophobic groups attached to a suitable matrix facilitates protein–matrix interaction rather than facilitating protein–protein interaction. The most commonly used stationary phases are alkyl (ethyl to octyl) or phenyl groups attached to either polyamide-coated silicas or an agarose matrix. Commercial materials include: Phenyl Sepharose and Phenyl SPW, both for low pressure HIC; and Poly PROPYL Aspartamide, Bio-Gel TSK Phenyl and Spherogel TSK Phenyl for HPLC HIC.

Since HIC requires the presence of salting-out compounds such as ammonium sulphate to facilitate the exposure of the hydrophobic regions on the protein molecule, it is commonly used immediately after fractionation with ammonium sulphate as ammonium and sulphate ions are already present in the protein sample. To maximise the process, it is advantageous to adjust the pH of the protein sample to that of its isoelectric point. Once the proteins have been adsorbed onto the stationary phase, selective elution can be achieved in a number of ways, including the use of an eluent of gradually decreasing ionic strength or of increasing pH (this increases the hydrophilicity of the protein) (Fig. 11.12) or by selective displacement by a displacer that has a stronger affinity for the stationary phase than has the protein. Examples include non-ionic detergents such as Tween 20 and Triton X-100, aliphatic alcohols such as 1-butanol and ethylene glycol, and aliphatic amines such as 1-aminobutane. HIC has two advantages over reversed-phase HPLC. The first is that the use of aqueous elution conditions minimises protein denaturation. The second is that it has a high capacity. A limitation is that it gives only moderate resolution.

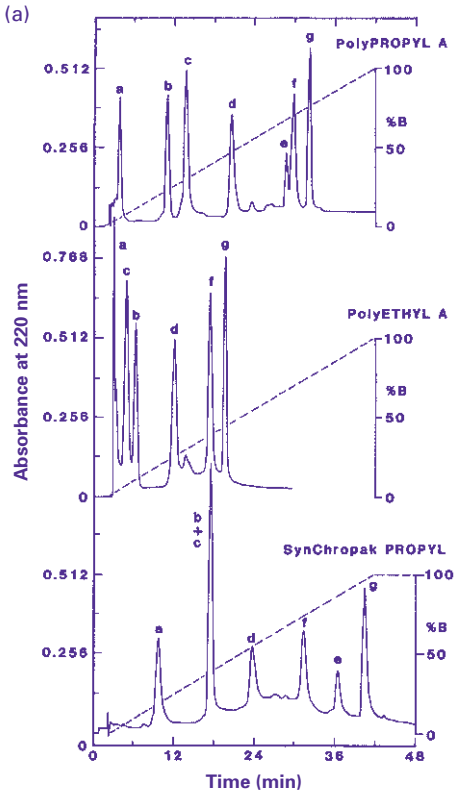

Fig. 11.12. (a) Chromatograph of a mixture of proteins separated by hydrophobic interaction chromatography using different stationary phases. A linear gradient elution program was used changing from 0 to 100% mobile phase B in 40 min. Mobile phase A: 1.8 M ammonium sulphate + 0.1 M potassium phosphate, pH 7.0. Mobile phase B: 0.1 M potassium phosphate, pH 7.0. Elution was monitored at 220 nm. (Reproduced with permission from K. Benedek (2003), High-Performance Interaction Chromatography, in *HPLC of Peptides and Proteins: Methods and Protocols* (Methods in Molecular Biology, **251**), M.-I. Aguilar (ed.), Humana Press, Totowa, NJ.)

11.5 PARTITION CHROMATOGRAPHY

11.5.1 Principle

Like other forms of chromatography, partition chromatography is based on differences in capacity factors, k', and distribution coefficients, K_d, of the analytes using liquid stationary and mobile phases. It can be subdivided into liquid–liquid chromatography, in which the liquid stationary phase is attached to a supporting matrix by purely physical means, and bonded-phase liquid chromatography, in which the stationary phase is covalently attached to the matrix. An example of liquid–liquid chromatography is one in which a water stationary phase is supported by a cellulose, starch or silica matrix, all of which have the ability to physically bind as much as 50% (w/v) water and remain free-flowing powders.

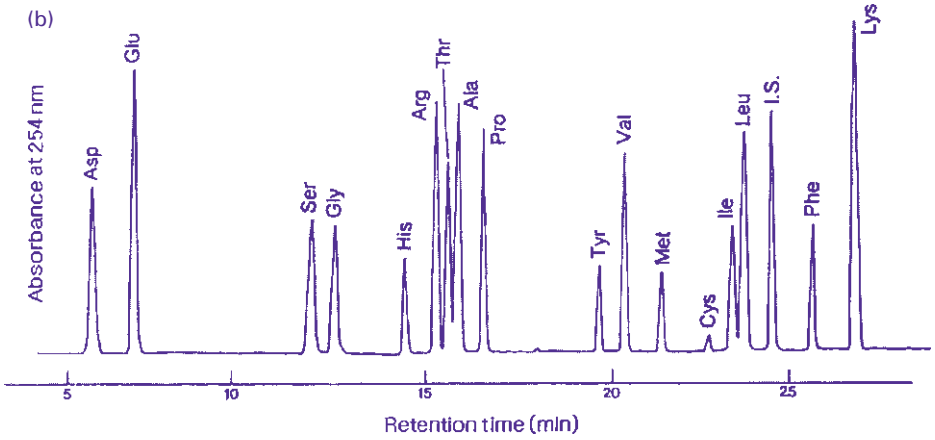

Fig. 11.12. (b) Chromatogram of the amino acids present in a hydrolysate of whole egg separated by reversed-phase chromatography. Egg proteins were hydrolysed with 6 M HCl at 145 °C for 4 h and then derivatised with phenylisothiocyanate to give the phenylthiocarbamyl derivatives (PTC). The derivatives were separated on a Nova-pak C_{18} column (300 mm × 3.9 mm internal diameter, 4 μm dimethyloctadecylsilyl-bonded amorphous silica, Waters) heated to 40 °C. A gradient elution program was used: 0–15 min solvent A (0.02 M phosphate buffer containing 5% methanol, 1.5% tetrahydrofuran, pH 6.8); 15–20 min 76% solvent A, 20% solvent B (solvent A: acetonitrile, 50:50, v/v), 4% solvent C (acetonitrile–water, 70:30, v/v); 20–30 min 70% solvent A, 20% solvent B, 10% solvent C with a flow rate of 1.2 $cm^3\ min^{-1}$ and detection at 254 nm. IS, internal standard (nor-leucine). (Reproduced with permission from H.-L. Woo (2001), Determination of amino acids in foods by reversed-phase high-performance liquid chromatography with new precolumn derivatives, butylthiocarbamyl, and benzylthiocarbamyl derivatives compared to the phenylthiocarbamyl derivative and ion-exchange chromatography, in *Amino Acid Analysis Protocols* (Methods in Molecular Biology, **159**), C. Cooper, N. Packer and K. Williams (eds.), Humana Press, Totowa, NJ.)

The advantages of this form of chromatography are that it is cheap, has a high capacity and has broad selectivity. Its disadvantage is that the elution process may gradually remove the stationary phase, thereby altering the chromatographic conditions. This problem is overcome by the bonded phases and this explains their more widespread use. Most bonded phases use silica as the matrix, which is derivatised to immobilise the stationary phase by reaction with an organochlorosilane:

$$\underset{\text{silica}}{\text{-Si-OH}} + \underset{\substack{\text{organo}\\ \text{chlorosilane}}}{\text{Cl-Si-(CH}_3)_2\text{R}} \rightarrow \underset{\substack{\text{derivatised silanol}\\ \text{group}}}{\text{Si-O-Si-(CH}_3)_2\text{R}} + \text{HCl}$$

Surplus silanol groups are removed by capping with chlorotrimethylsilane to improve the quality of the chromatography by decreasing tailing. There are two commonly used modes of partition chromatography that differ in the relative polarities of the stationary and mobile phases.

11.5.2 Normal-phase liquid chromatography

In this form of partition chromatography, the stationary phase is polar and the mobile phase relatively non-polar. The most popular stationary phase is an alkylamine bonded to silica. The mobile phase is generally an organic solvent such as hexane, heptane, dichloromethane or ethylacetate. These solvents form an elutropic series based on their polarity. Such a series in order of increasing polarity is as follows:

n-hexane < cyclohexane < trichloromethane < dichloromethane < tetrahydrofuran < acetonitrile < ethanol < methanol < ethanoic acid < water

The mechanism of separation exploits the ability of the analyte to displace molecules of the mobile phase adsorbed as a monolayer on the surface of the stationary phase, as well as the ability of the analyte to compete with mobile phase molecules in the formation of a bilayer on the stationary phase surface. The order of elution of analytes is such that the least polar is eluted first and the most polar last. Indeed, polar analytes generally require gradient elution with a mobile phase of increasing polarity, generally achieved by the use of methanol or dioxane. The main applications of normal-phase liquid chromatography are its use to separate analytes that have low water solubility and those that are not amenable to reversed-phase liquid chromatography.

11.5.3 Reversed-phase liquid chromatography

In this form of liquid chromatography, the stationary phase is non-polar and the mobile phase relatively polar, hence the name 'reversed phase'. By far the most commonly used type is the bonded-phase form, in which alkylsilane groups are chemically attached to silica. Butyl (C_4), octyl (C_8) and octadecyl (C_{18}) silane groups are most commonly used (Table 11.2). The mobile phase is commonly water or aqueous buffers, methanol, acetonitrile, or tetrahydrofuran or mixtures of them. The organic solvent is referred to as an organic modifier. Reversed-phase liquid chromatography differs from most other forms of chromatography in that the stationary phase is essentially inert and only non-polar (hydrophobic) interactions with analytes are possible.

Reversed-phase separation of analytes is determined principally by the characteristics of the mobile phase and probably involves a combination of adsorption and partition mechanisms. It is believed to have many similarities to hydrophobic interaction chromatography. No simple model has been described to explain reversed-phase chromatography but the solvophobic theory is the one most widely considered. It is based on the consideration of the balance of free energy and entropy changes associated with bonding of the analyte with the stationary phase and with the mobile phase. The attraction of the reversed-phase technique is that small changes in the mobile phase composition such as the addition of salts, change of pH or the amount of organic solvent, profoundly affect the separation characteristics. Moreover, the technique is sensitive to temperature change such that a 10 deg.C increase approximately halves the capacity factor, k'. In

Table 11.2 Examples of silica-bonded phases for reversed-phase HPLC

Product	Particle size (μm)	Pore size (Å)
μBondapak octadecyl	10	70
μBondapak phenyl	10	125
μBondapak CN	10	125
μBondapak NH_2	10	80
Zorbax octadecyl	6	70
Zorbax octyl	6	70
Zorbax NH_2	6	70
Discovery octyl	5	180
Supelcosil LC-octadecyl	5	120
Supelcosil LC-301 methyl	5	300
Supelcosil LC-308 octyl	5	300

1 Å = 0.1 nm.

reversed-phase chromatography, polar analytes elute first and non-polar analytes last. Non-polar analytes may need gradient elution using increasing proportions of a low polarity solvent such as hexane.

Reversed-phase HPLC is probably the most widely used form of chromatography mainly because of its flexibility and high resolution. It is widely used to analyse drugs and their metabolites, insecticide and pesticide residues, and amino acids and peptides. It is also now widely applied to proteins by using FPLC (Fig. 11.12). Octadecylsilane (ODS) phases bind proteins more tightly than do octyl- or methylsilane phases and are therefore more likely to cause protein denaturation because of the more extreme conditions required for the elution of the protein. In non-aqueous form, reversed-phase chromatography can be used to separate lipophilic compounds such as fats.

11.5.4 Ion-pair reversed-phase liquid chromatography

Although the separation of some highly polar compounds, such as amino acids, peptides, organic acids and the catecholamines, is commonly undertaken by reversed-phase chromatography, it is sometimes possible to achieve improved separation by one of two possible approaches:

- *Ion suppression:* The ionisation of the compound is suppressed by using a mobile phase with an appropriately high or low pH. For weak acid analytes, for example, an acidified mobile phase would be used.
- *Ion-pairing:* A counter ion that has a charge opposite to that of the analytes to be separated is added to the mobile phase so that the resulting ion-pair has sufficient lipophilic character to be retained by the non-polar stationary phase of a reversed-phase system. Thus, to aid the separation of acidic organic compounds (RCOOH), which would be present as their conjugate anions, a quaternary alkylamine ion such as tetrabutylammonium would be used as the

counter ion, whereas for the separation of bases (RNH_2), which would be present as cations, an alkyl sulphonate such as sodium heptanesulphonate would be used:

$$\underset{\text{carboxylic acid anion}}{RCOO^-} + \underset{\text{counter cation}}{R'_4N^+} \rightleftharpoons \underset{\text{ion-pair}}{[RCOO^-N^+R'_4]}$$

$$\underset{\text{conjugate acid of weak base}}{RN^+H_3} + \underset{\text{counter anion}}{R'SO_3^-} \rightleftharpoons \underset{\text{ion-pair}}{RH_3N^+\ {}^-O_3SR']}$$

where R′ is an appropriate aliphatic group. The mechanism by which ion-pairing results in better separation is not clear but two theories have been proposed. The first suggests that the ion-pair behaves as a single neutral species, whilst the second suggests that an active ion-exchange surface is produced in which the counter ion, which has considerable lipophilic properties, and the ions to be separated are adsorbed by the hydrophobic, non-polar stationary phase. In practice, the success of the ion-pairing approach is variable and somewhat empirical. The size of the counter ion, its concentration and the pH of the solution are all factors that may profoundly influence the outcome of the separation.

Octyl- and octadecylsilane-bonded phases are used most commonly in conjunction with a water/methanol or water/acetonitrile mobile phase. One of the advantages of ion-pair reversed-phase chromatography is that, if the sample to be resolved contains a mixture of non-ionic and ionic compounds, the two groups of compounds can be separated simultaneously because the ion-pair reagent does not affect the chromatography of the non-ionic species. This is not true of ion-exchange chromatography.

11.5.5 Chiral chromatography

Chiral compounds either contain at least one asymmetric carbon atom (chiral centre) or are molecularly asymmetric. They exist in two enantiomorphic forms (enantiomers), related as object and mirror images, that have the same physical and chemical properties and differ only in their interaction with plane-polarised light such that one is dextrorotatory (+) and the other laevorotatory (−). There are a number of conventions for indicating the spatial configuration, as opposed to optical properties, of enantiomers. The classical D and L system for monosaccharides and amino acids cannot be applied easily to other structures and the Cahn–Ingold–Prelog system, which assigns R (Latin, *rectus*) or S (Latin, *sinister*) configurations to an enantiomer, is of more general use. Until recently it has not been possible to resolve mixtures of enantiomers and this has created problems for the pharmaceutical industry in its development and clinical use of drugs, many of which are chiral, for although enantiomers have identical chemical and

physical properties they are distinguishable biologically. Thus they differ in their ability to interact with the receptors involved in a range of physiological responses and they are often metabolised and excreted at different rates.

Chromatographic techniques have now been developed that allow mixtures of enantiomers to be resolved. One of these techniques is based on the fact that diastereoisomers, which are optical isomers that do not have an object–image relationship, have different physical properties even though they contain identical functional groups. They can therefore be separated by conventional chromatographic techniques, most commonly reversed-phase chromatography. The diastereoisomer approach requires that the enantiomers contain a function group that can be derivatised by a chemically and optically pure chiral derivatising agent (CDA) that converts them to a mixture of diastereoisomers:

$$\underset{\text{mixture of enantiomers}}{(R + S)} + \underset{\text{chiral derivatising agent}}{R'} \rightleftharpoons \underset{\text{mixture of diastereoisomers}}{RR' + SR'}$$

Examples of CDAs include the R or S form of the following:

For amines	*N*-trifluoroacetyl-1-prolylchloride, α-phenylbutyric anhydride
For alcohols	2-Phenylpropionyl chloride, 1-phenylethylisothiocyanate
For ketones	2,2,2-Trifluoro-1-pentylethylhydrazine
For aliphatic and alicyclic acids	1-Menthol, desoxyephedrine

Although this approach to chiral resolution is relatively simple, it is essential that the derivatisation process is rapid and quantitative. Very often this is not the case and this has restricted its use. An alternative approach to the resolution problem is to use a chiral mobile phase. In this technique a transient diastereomeric complex is formed between the enantiomers and the chiral mobile phase agent. Examples of chiral mobile phase agents include albumin, α_1-acid glycoprotein, α-, β- and γ-cyclodextrins, camphor-10-sulphonic acid and *N*-benzoxycarbonylglycyl-L-proline, all of which are used with a reversed-phase chromatographic system.

The most successful approach to chiral chromatography, however, has been the use of a chiral stationary phase. This is based upon the principle that the need for a three-point interaction between the stationary phase (working as a chiral discriminator) and the enantiomer would allow the resolution of racemic mixtures due to the different spatial arrangement of the functional groups at the chiral centre in the enantiomers. One such successful approach uses Pirkle phases, based on dinitrobenzoyl derivatives of amino acids, such as phenylglycine, that are bonded to silica. These phases are thought to function by allowing transient formation of enantiomer–stationary phase complexes by bonding such as hydrogen bonding

and van der Waals forces. Elution is generally by the reversed-phase technique. Alternative chiral stationary phases include triacetylcellulose and various cyclodextrins bonded to silica. These cyclodextrins are cyclic oligosaccharides that have an open truncated conical structure 6–8 Å (0.6–0.8 nm) wide at their base. Their inner surface is predominantly hydrophobic, but secondary hydroxyl groups are located around the wide rim of the cone. β-Cyclodextrin has 7 glucopyranose units and contains 35 chiral centres and α-cyclodextrin has 6 glucopyranose units, 30 chiral centres and is smaller than β-cyclodextrin. Collectively they are referred to as chiral cavity phases because they rely on the ability of the enantiomer to enter the three-dimensional cyclodextrin cage while at the same time presenting functional groups and hence the chiral centre for interaction with hydroxyl groups on the cone rim. Enantiomers possessing a five-, six- or seven-membered aromatic ring have been resolved by this approach in conjunction with reversed-phase elution. Another approach is the use of the macrocyclic antibiotics vancomycin and teicoplanin as chiral stationary phases. Vancomycin has 18 chiral centres and teicoplanin 23. Both have been used successfully in chiral separations using normal and reversed-phase separations.

Since proteins are optically active, they can in principle be used as a chiral stationary phase. Bovine serum albumin and α_1-acid glycoprotein (AGP) have been evaluated and found to be successful for a wide range of separations, but their mechanism of chiral separation is poorly understood. Both albumin and α_1-acid glycoprotein occur in plasma and have long been known to bind drugs. Albumin has at least two distinct binding sites to which acidic and basic drugs may bind. α_1-Acid glycoprotein has a single drug-binding site restricted to the binding of basic drugs such as propranolol. These protein chiral phases are used in conjunction with aqueous buffers and cannot be used at extremes of pH or in the presence of organic solvents.

11.6 ION-EXCHANGE CHROMATOGRAPHY

11.6.1 Principle

This form of chromatography relies on the attraction between oppositely charged particles. Many biological materials, for example amino acids and proteins, have ionisable groups that may carry a net positive or negative charge. The net charge exhibited by these compounds is dependent on their pK_a values and on the prevailing pH of the solution in accordance with the Henderson–Hasselbalch equation (Section 1.4.2, equations 1.8a,b and 1.9a,b). Ion-exchange chromatography is frequently chosen for the separation and purification of proteins, peptides, nucleic acids, polynucleotides and other charged molecules, mainly because of its high resolving power and high capacity.

There are two types of ion exchanger, namely cation and anion exchangers. Cation exchangers possess negatively charged groups and these will attract positively charged cations. These exchangers are also called acidic ion exchangers because their negative charges result from the ionisation of acidic groups. Anion

exchangers have positively charged groups that will attract negatively charged anions. The term basic ion exchangers is also used to describe these exchangers, as positive charges generally result from the association of protons with basic groups.

The ion-exchange mechanism is thought to be composed of five distinct steps:

- *Diffusion of the ion to the exchanger surface:* This occurs very quickly in homogeneous solutions.
- *Diffusion of the ion through the matrix structure of the exchanger to the exchange site:* This is dependent upon the degree of cross-linkage of the exchanger and the concentration of the solution. This process is thought to be the feature that controls the rate of the whole ion-exchange process.
- *Exchange of ions at the exchange site:* This is thought to occur instantaneously and to be an equilibrium process:

Cation exchanger:

$$\underset{\text{exchanger}}{RSO_3^-\ldots}\quad \underset{\text{counter ion}}{Na^+} + \underset{\substack{\text{charged}\\ \text{ion to be}\\ \text{exchanged}}}{N^+H_3R'} \rightleftharpoons \underset{\substack{\text{bound}\\ \text{ion}}}{RSO_3^-\ldots N^+H_3R'} + \underset{\substack{\text{exchanged}\\ \text{ion}}}{Na^+}$$

Anion exchanger:

$$(R)_4N^+\ldots Cl^- + {}^-OOCR' \rightleftharpoons (R)_4N^+\ldots {}^-OOCR' + Cl^-$$

The more highly charged the ionised molecule to be exchanged, the tighter it binds to the exchanger and the less readily it is displaced by other ions.

- *Diffusion of the exchanged ion through the exchanger to the surface.*
- *Selective desorption by the eluent and diffusion of the molecule into the external eluent:* The selective desorption of the bound ion is achieved by changes in pH and/or ionic concentration or by affinity elution. In the latter case an ion that has a greater affinity for the exchanger than has the bound ion is introduced into the system.

11.6.2 Materials and applications

Low pressure ion-exchange chromatography can be carried out using a variety of matrices and ionic groups. Matrices used include polystyrene, cellulose and agarose. Functional ionic groups include sulphonate ($-SO_3^-$) and quaternary ammonium ($-N^+R_3$), both of which are strong exchangers because they are totally ionised at all normal working pH values, and carboxylate ($-COO^-$) and diethylammonium ($-HN^+(CH_2CH_3)_2$), both of which are termed weak exchangers because they are ionised over only a narrow range of pH values. Examples are given in Table 11.3. Bonded-phase ion-exchangers suitable for HPLC, containing a wide range of ionic groups, are available in pellicular and porous forms. The porous

Table 11.3 Examples of commonly used ion exchangers

Type	Functional groups	Functional group name	Matrices
Weakly acidic (cation exchanger)	$-COO^-$ $-CH_2COO^-$	Carboxy Carboxymethyl	Agarose Cellulose Dextran Polyacrylate
Strongly acidic (cation exchanger)	$-SO_3^-$ $-CH_2SO_3^-$ $-CH_2CH_2CH_2SO_3^-$	Sulpho Sulphomethyl Sulphopropyl	Cellulose Dextran Polystyrene Polyacrylate
Weakly basic (anion exchanger)	$-CH_2CH_2N^+H_3$ $-CH_2CH_2N^+H(CH_2CH_3)_2$	Aminoethyl Diethylaminoethyl	Agarose Cellulose Dextran Polystyrene Polyacrylate
Strongly basic (anion exchanger)	$-CH_2N^+(CH_3)_3$ $-CH_2CH_2N^+(CH_2CH_3)_3$ $-CH_2N^+(CH_3)_2$ $\lvert$ CH_2CH_2OH $-CH_2CH_2N^+(CH_2CH_3)_2$ $\lvert$ $CH_2CH(OH)CH_3$	Trimethylaminomethyl Triethylaminoethyl Dimethyl-2-hydroxyethyl-aminomethyl Diethyl-2-hydroxypropyl-aminoethyl	Cellulose Dextran Polystyrene

variety is based on polystyrene, porous silica or hydrophilic polyethers, and is particularly valuable for the separation of proteins. They have a particle diameter of 5–25 μm. Most HPLC ion exchangers are stable up to 60 °C and separations are often carried out at this temperature, owing to the fact that the raised temperature decreases the viscosity of the mobile phase and thereby increases the efficiency of the separation.

Exchange capacity

All exchangers are characterised by a total exchange capacity, which is defined as the number of milliequivalents of exchangeable ions available, either per gram of dried exchanger or per unit volume of hydrated resin. Sometimes available capacity is also used to express the available capacity for an arbitrarily chosen molecule such as haemoglobin. These exchange capacities give an indication of the degree of substitution of the exchanger and are therefore a helpful guide in deciding on the scale of a particular application.

Choice of exchanger

The choice of the ion exchanger depends upon the stability of the test analytes, their relative molecular mass and the specific requirements of the separation. Many biological analytes, especially proteins, are stable within only a fairly narrow pH range so the exchanger selected must operate within this range. Generally, if an

analyte is most stable below its isoionic point (giving it a net positive charge), a cation exchanger should be used, whereas if it is most stable above its isoionic point (giving it a net negative charge) an anion exchanger should be used. Either type of exchanger may be used to separate analytes that are stable over a wide range of pH. The choice between a strong and weak exchanger also depends on analyte stability and the effect of pH on analyte charge. Weak electrolytes requiring a very low or high pH for ionisation can be separated only on strong exchangers, as only they operate over a wide pH range. In contrast, for strong electrolytes, weak exchangers are advantageous for a number of reasons, including a reduced tendency to cause sample denaturation, their inability to bind weakly charged impurities and their enhanced elution characteristics. Although the degree of cross-linking of an exchanger does not influence the ion-exchange mechanism, it does influence its capacity. The relative molecular mass and hence size of the sample component therefore determines which specific exchanger should be used.

Eluent pH

The pH of the buffer selected as eluent should be at least one pH unit above or below the isoionic point of the analytes to be separated. In general, cationic buffers such as Tris, pyridine and alkylamines are used in conjunction with anion exchangers, and anionic buffers such as acetate, barbiturate and phosphate are used with cation exchangers. The precise initial buffer pH and ionic strength should be such as just to allow the binding of the sample components to the exchanger. Equally, a buffer of the lowest ionic strength that effects elution should initially be used for the subsequent elution of the analytes. This ensures that initially the minimum numbers of contaminants bind to the exchanger and that subsequently the maximum number of these impurities remains on the column. The amount of sample that can be applied to a column is dependent upon the size of the column and the capacity of the exchanger. Generally, if isocratic elution is to be used, the sample volume should be 1–5% of the bed volume. If, however, gradient elution is to be used, the initial conditions chosen are such that the exchanger binds all the test analytes at the top of the column. In this case the sample volume is not important and large volumes of dilute solution can be applied, thereby effectively introducing a concentration stage.

Elution

Gradient elution is far more common than isocratic elution. Continuous or stepwise pH and ionic strength gradients may be employed but continuous gradients tend to give better resolution with less peak tailing. Generally, with an anion exchanger, the pH gradient decreases and the ionic strength increases, whereas for cation exchangers both the pH and ionic gradients increase during the elution.

11.6.3 Chromatofocusing

The principle of chromatofocusing is similar to that of isoelectric focusing (Section 10.3.4). A linear pH gradient is generated in the column by initially

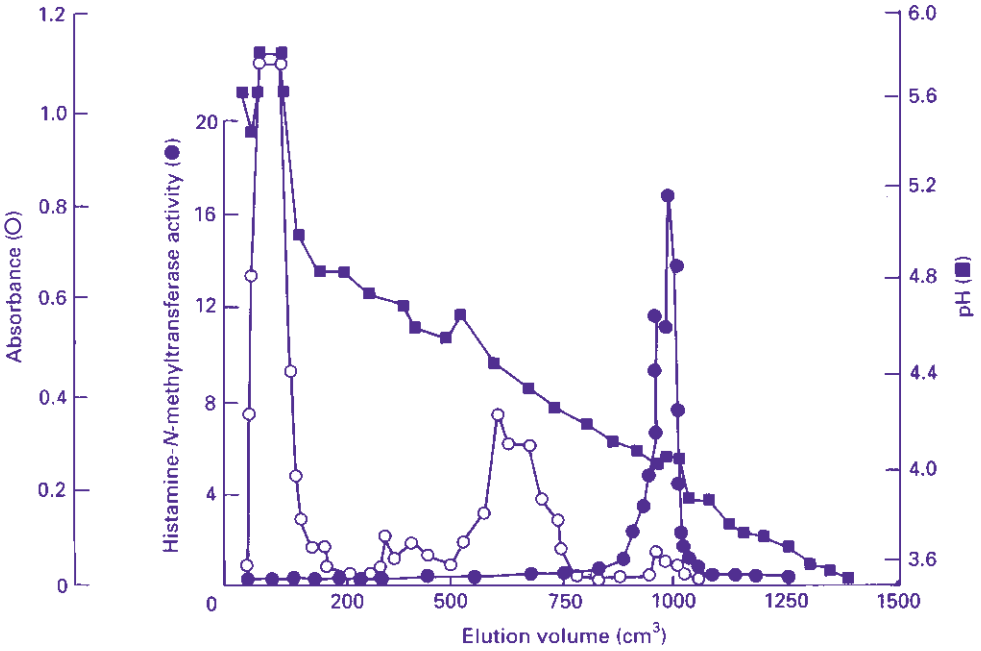

Fig. 11.13. Chromatofocusing elution profile of rat histamine-*N*-methyltransferase. The partially purified sample (approximately 130 mg protein) was applied to a polybuffer exchanger column (62 cm × 1.6 cm), previously equilibrated with 25 mM piperazine-HCl at pH 5.5; 5 cm^3 of the eluent Polybuffer™74, pH 3.5, preceded the sample. Elution was carried out at a flow rate of 20 $cm^3\ h^{-1}$. The fractions (5 cm^3) were assayed for pH (■), histamine-*N*-methyltransferase activity (●) expressed as extracted d.p.m. × 10^{-4} per 2.5 cm^3 chloroform, and absorbance at 600 nm (○) measured after reaction with Coomassie Brilliant Blue. (Reproduced with permission from M. J. York (1982), The purification and kinetic properties of histamine-*N*-methyl transferase, M.Phil. thesis, University of Hertfordshire.)

pre-equilibrating an anion exchanger to a particular pH, and then running an amphoteric buffer, which has even buffering capacity over a range of pH and a starting pH lower than that at which the column was pre-equilibrated, through the column for a predetermined time. The result is the formation of a pH gradient that is 3–4 pH units lower at the top of the column than at the bottom. If a protein in a starting buffer of a pH similar to that prevailing at the top of the column is added to this pH gradient, it will migrate down the column as a cation, encountering an increasing pH, until it reaches a pH corresponding to its isoelectric point. Just beyond this point it will become an anion and will be able to bind to the positive groups of the exchanger. As the elution with the starting buffer continues, the prevailing pH along the column will be gradually lowered, causing the bound protein to become a cation and binding to cease. The protein will continue its movement down the column until once again it encounters a pH slightly above its isoelectric point, when again it will bind. This process is repeated continuously until the protein is eluted at a pH slightly above its isoelectric point (Fig. 11.13).

Proteins in a mixture added to the column would elute in the order of their isoelectric points. If more of the protein mixture were added to the top of the column

during this elution process each protein would automatically catch up with its identical protein in the initial mixture, thereby producing a focusing effect and enabling large volumes to be applied to the column with no deleterious effect to resolution. Thus the technique has a high capacity. Chromatofocusing gives a good resolution of quite complex mixtures of proteins, provided that there are discrete differences in their isoelectric points. Proteins possessing very similar isoelectric points tend to be poorly resolved.

11.7 MOLECULAR EXCLUSION (GEL FILTRATION) CHROMATOGRAPHY

11.7.1 Principle

This chromatographic technique for the separation of molecules on the basis of their molecular size and shape exploits the molecular sieve properties of a variety of porous materials. Probably the most commonly used of such materials is a group of polymeric organic compounds that possess a three-dimensional network of pores that confers gel properties upon them. The term gel filtration is used to describe the separation of molecules of varying molecular size using these gel materials. Porous glass granules have also been used as molecular sieves and the term controlled-pore glass chromatography introduced to describe this separation technique. The terms exclusion or permeation chromatography describe all molecular separation processes using molecular sieves. This section is devoted mainly to gel filtration, as its principles and applications are best documented.

The general principle of exclusion chromatography is quite simple. A column of gel particles or porous glass granules is in equilibrium with a suitable mobile phase for the analytes to be separated. Large analytes that are completely excluded from the pores will pass through the interstitial spaces between the gel particles and will appear in the eluate first. Smaller analytes will be distributed between the mobile phase inside and outside the gel particles and will therefore pass through the column at a slower rate, hence appearing last in the eluate. Three stages in such a column are represented diagrammatically in Fig. 11.14.

The mobile phase trapped by a gel is available to an analyte to an extent that is dependent upon the porosity of the gel particle and the size of the analyte molecule. Thus the distribution of an analyte in a column of a gel is determined solely by the total volume of mobile phase, both inside and outside the gel particles, that is available to it. For a given type of gel, the distribution coefficient, K_d, of a particular analyte between the inner and outer mobile phase is a function of its molecular size. If the analyte is large and completely excluded from the mobile phase within the gel, $K_d = 0$ whereas, if the analyte is sufficiently small to gain complete access to the inner mobile phase, $K_d = 1$. Due to variation in pore size between individual gel particles, there is some inner mobile phase that will be available and some that will not be available to analytes of intermediate size; hence K_d values vary between 0 and 1. It is this complete variation of K_d between these two limits that makes it possible to separate analytes within a narrow molecular size range on a given gel.

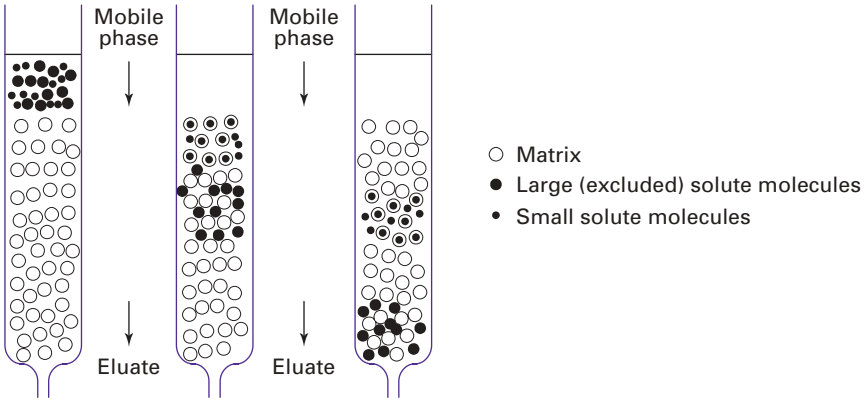

Fig. 11.14. Separation of different size molecules by exclusion chromatography. Large (excluded) molecules are eluted first in the void volume.

For two analytes of different relative molecular mass and K_d values, K'_d and K''_d, the difference in their elution volumes, V_S, can be shown to be:

$$V_S = (K'_d - K''_d)\, V_i \qquad (11.14)$$

where V_i is the inner volume within the gel available to a compound whose $K_d = 1$.

In practice, deviations from ideal behaviour, for example owing to poor packing of the column, make it advisable to reduce the sample volume below the value of V_S because the ratio between sample volume and inside gel volume affects both the sharpness of the separation and the degree of dilution of the sample.

11.7.2 Materials and applications

Gels that are commonly used for LPLC include dextrans, agarose, polyacrylamide, polyacryloylmorphine and polystyrene. The materials for HPLC and FPLC need to be more rigid to withstand the higher working pressures and are based on cross-linked dextrans, agarose, polystyrene, polyvinylchloride, polyvinylalcohol, polymethacrylate or rigid controlled-pore glasses or silicas (Table 11.4). Some of these materials, such as those based on polystyrene, can be used only with non-aqueous systems.

Exclusion chromatography requires a single mobile phase and isocratic elution. It is most commonly used with ultraviolet absorption spectrophotometric detectors. Exclusion chromatography columns tend to be longer than those for other forms of chromatography in order to increase the amount of stationary phase and hence pore volume.

Applications

Purification The main application of exclusion chromatography is in the purification of biological macromolecules by facilitating their separation from larger and smaller molecules. Viruses, enzymes, hormones, antibodies, nucleic acids and

Table 11.4 Stationary phases commonly used for exclusion chromatography

Polymer	Trade name	Fractionation range[a] ($M_r \times 10^{-3}$)
Low pressure liquid chromatography		
Dextran	Sephadex	
	G-10	<0.7
	G-25	1–5
	G-50	1.5–30
	G-100	4–150
	G-200	5–600
Dextran, cross-linked	Sephacryl	
	S-100	1–100
	S-200	5–250
	S-300	10–1500
	S-400	20–8000
Agarose	Sepharose	
	6B	10–4000
	4B	60–20000
	2B	70–40000
Polyacrylamide	Bio-Gel	
	P-2	0.1–1.8
	P-6	1–6
	P-30	2.5–40
	P-100	5–100
	P-300	60–400
High performance liquid chromatography (HPLC and FPLC)		
Polyvinylchloride	Fractogel	
	TSK HW-40	0.1–10
	TSK HW-55	1–700
	TSK HW-65	50–5000
	TSK HW-75	500–50000
Dextran linked to cross-linked agarose	Superdex	
	75	3–70
	200	10–600

[a] Determined for globular proteins. The range is approximately the same for single-stranded nucleic acids and smaller for fibrous proteins and double-stranded DNA.

polysaccharides have all been separated and purified by the use of appropriate gels or glass granules.

Relative molecular mass determination The elution volumes of globular proteins are determined largely by their relative molecular mass (M_r). It has been shown that, over a considerable range of M_r values, the elution volume or K_d is an

Example 2 ESTIMATION OF RELATIVE MOLECULAR MASS

Question

The relative molecular mass (M_r) of a protein was investigated by exclusion chromatography using a Sephacryl S300 column and using aldolase, catalase, ferritin, thyroglobulin and Blue Dextran as standard. The following elution data were obtained.

	Retention volume	
	M_r	V_r (cm^3)
Aldolase	158 000	22.5
Catalase	210 000	21.4
Ferritin	444 000	18.2
Thyroglobulin	669 000	16.4
Blue Dextran	2 000 000	13.6
Unknown		19.5

What is the approximate M_r of the unknown protein?

Answer

A plot of the logarithm of the relative molecular mass of individual proteins versus their retention volume has a linear section from which it can be deduced that the unknown protein with a retention volume of 19.5 cm^3 must have a relative molecular mass of 330 000.

approximately linear function of the logarithm of M_r. Hence the construction of a calibration curve, with proteins of a similar shape and known M_r, enables the M_r values of other proteins, even in crude preparations, to be estimated.

Solution concentration Solutions of high M_r substances can be concentrated by the addition of dry Sephadex G-25 (coarse). The swelling gel absorbs water and low M_r substances, whereas the high M_r substances remain in solution. After 10 min the gel is removed by centrifugation, leaving the high M_r material in a solution whose concentration has increased but whose pH and ionic strength are unaltered.

Desalting By use of a column of Sephadex G-25, solutions of high M_r compounds may be desalted. The high M_r compounds move with the void volume, whereas the low M_r compounds are distributed between the mobile and stationary phases and hence move slowly. This method of desalting is faster and more efficient than dialysis. Applications include removal of phenol from nucleic acid preparations, ammonium sulphate from protein preparations and salt from samples eluted from ion-exchange chromatography columns.

11.7.3 Molecular imprinting

This innovative form of chromatography has similarities with size exclusion chromatography. In molecular imprinting, the stationary phase is prepared by

polymerising a suitable monomer in the presence of a cross-linking reagent and the specific analyte or one closely related to it, for which the phase is required. The chosen compound is referred to as the template. The resulting polymer is finely ground, sieved and washed with an organic solvent to remove the template. Its subsequent use as a stationary phase is based upon the fact that the polymer contains cavities with a molecular shape similar to that of the template and hence will selectively retain the test analyte from a mixture when the sample is eluted with an appropriate mobile phase. The technique has been successfully applied to the separation of a wide range of natural products.

11.8 AFFINITY CHROMATOGRAPHY

11.8.1 Principle

Separation and purification by affinity chromatography is unlike most other forms of chromatography and such techniques as electrophoresis and centrifugation in that it does not rely on differences in the physical properties of the molecules to be separated. Instead, it exploits the unique property of extremely specific biological interactions to achieve separation and purification. As a consequence, affinity chromatography is theoretically capable of giving absolute purification, even from complex mixtures, in a single process. The technique was originally developed for the purification of enzymes, but it has since been extended to nucleotides, nucleic acids, immunoglobulins, membrane receptors and even to whole cells and cell fragments.

The technique requires that the material to be isolated is capable of binding reversibly to a specific ligand that is attached to an insoluble matrix:

$$\underset{\text{macromolecule}}{\text{M}} + \underset{\substack{\text{ligand}\\(\text{attached}\\\text{to matrix})}}{\text{L}} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \underset{\text{complex}}{\text{ML}}$$

Under the correct experimental conditions, when a complex mixture containing the specific compound to be purified is added to the immobilised ligand, generally contained in a conventional chromatography column, only that compound will bind to the ligand. All other compounds can therefore be washed away and the compound subsequently recovered by displacement from the ligand (Fig. 11.15). The method requires a detailed preliminary knowledge of the structure and biological specificity of the compound to be purified so that the separation conditions that are most likely to be successful may be carefully planned. In the case of an enzyme, the ligand may be the substrate, a competitive reversible inhibitor or an allosteric activator. The conditions chosen would normally be those that are optimal for enzyme–ligand binding. Since the success of the method relies on the reversible formation of the complex and on the numerical values of the first-order rate constants k_{+1} and k_{-1}, as the enzyme is added progressively to the

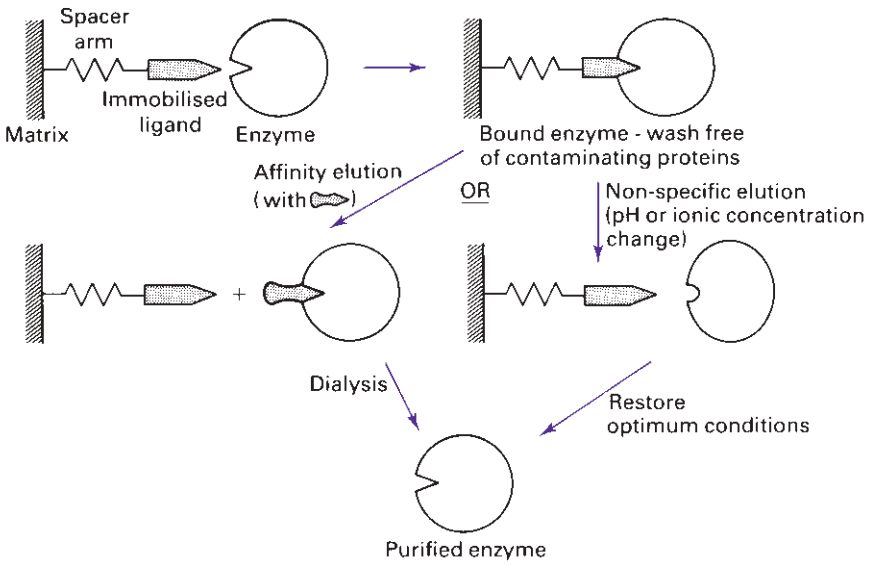

Fig. 11.15. Principle of purification of an enzyme by affinity chromatography.

insolubilised ligand in a column, the enzyme molecules will be stimulated to bind and a dynamic situation develops in which the concentration of the complex and the strength of the binding increase. It is because of this progressive increase in effectiveness during the addition of the sample to the column that column procedures are invariably more successful than batch-type methods. Nevertheless, alternative forms have been developed and are particularly suitable for large-scale work. They include the following:

- *Affinity precipitation:* The ligand is attached to a soluble carrier that can be subsequently precipitated by, for example, a pH change.
- *Affinity partitioning:* The ligand is attached to a water-soluble polymer such as polyethylene glycol, which, with the ligand bound, preferentially partitions into an aqueous polymer phase that is in equilibrium with a pure aqueous phase.

In all cases, for effective chromatography, the association constant, K_a, for the complex should be in the region 10^4 to 10^8 M.

11.8.2 Materials and applications

Matrix

An ideal matrix for affinity chromatography must have the following characteristics:

- possess suitable and sufficient chemical groups to which the ligand may be covalently coupled and it must be stable under the conditions of the attachment,

- be stable during binding of the macromolecule and its subsequent elution,
- interact only weakly with other macromolecules to minimise non-specific adsorption,
- exhibit good flow properties.

In practice, particles that are uniform, spherical and rigid are used. The most common ones are the cross-linked dextrans and agarose, polyacrylamide, polymethacrylate, polystyrene, cellulose and porous glass and silica.

Ligand

The chemical nature of a ligand is dictated by the biological specificity of the compound to be purified. In practice it is sometimes possible to select a ligand that displays absolute specificity in that it will bind exclusively to one particular compound. More commonly, it is possible to select a ligand that displays group selectivity in that it will bind to a closely related group of compounds that possess a similar in-built chemical specificity. An example of the latter type of ligand is 5′-AMP, which can bind reversibly to many NAD^+-dependent dehydrogenases because it is structurally similar to part of the NAD^+ molecule. It is essential that the ligand possesses a suitable chemical group that will not be involved in the reversible binding of the ligand to the macromolecule, but which can be used to attach the ligand to the matrix. The most common of such groups are $-NH_2$, -COOH, -SH and -OH (phenolic and alcoholic).

To prevent the attachment of the ligand to the matrix interfering with its ability to bind the macromolecule, it is generally advantageous to interpose a spacer arm between the ligand and the matrix. The optimum length of this spacer arm is 6–10 carbon atoms or their equivalent. In some cases, the chemical nature of this spacer is critical to the success of separation. Some spacers are purely hydrophobic, most commonly consisting of methylene groups; others are hydrophilic, possessing carbonyl or imido groups. Spacers are most important for small immobilised ligands but generally are not necessary for macromolecular ligands (e.g. in immunoaffinity chromatography, Section 11.8.4) as their binding site for the mobile macromolecule is well displaced from the matrix.

The most common method of attachment of the ligand to the matrix involves the preliminary treatment of the matrix with cyanogen bromide (CNBr) (Fig. 11.16). The reaction conditions and the relative proportion of the reagents will determine the number of ligand molecules that can be attached to each matrix particle. Alternative coupling procedures involve the use of bis-epoxides, *N*,*N*′-disubstituted carbodiimides, sulphonyl chloride, sodium periodate, *N*-hydroxysuccinimide esters and dichlorotriazines. Many pre-activated matrices, prepared using these coupling reagents, are available commercially.

A range of different spacer arms is used. Examples include 1,6-diaminohexane, 6-aminohexanoic acid and 1,4-bis(2,3-epoxypropoxy)butane. Several supports of the agarose, dextran and polyacrylamide type are commercially available with a variety of spacer arms and ligands pre-attached ready for immediate use. Examples of ligands are given in Table 11.5.

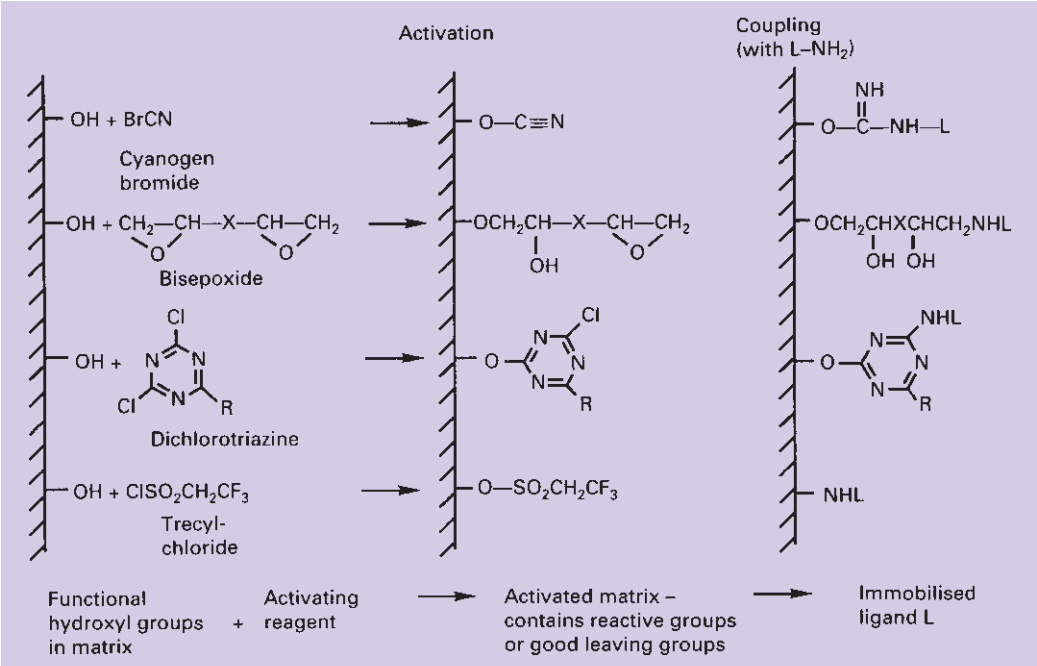

Fig. 11.16. Examples of coupling reactions used to immobilise ligands (L) for affinity chromatography. If a spacer arm is to be introduced between the immobilised ligand and the matrix, the coupling chemistry is similar.

Table 11.5 Examples of group-specific ligands commonly used in affinity chromatography

Ligand	Affinity
Nucleotides	
5′-AMP	NAD^+-dependent dehydrogenases, some kinases
2′,5′-ADP	$NADP^+$-dependent dehydrogenases
Calmodulin	Calmodulin-binding enzymes
Avidin	Biotin-containing enzymes
Fatty acids	Fatty-acid-binding proteins
Heparin	Lipoproteins, lipases, coagulation factors, DNA polymerases, steroid receptor proteins, growth factors, serine protease inhibitors
Proteins A and G	Immunoglobulins
Concanavalin A	Glycoproteins containing α-D-mannopyranosyl and α-D-glucopyranosyl residues
Soybean lectin	Glycoproteins containing *N*-acetyl- α-(or β)-D-galactopyranosyl residues
Phenylboronate	Glycoproteins
Poly(A)	RNA containing poly(U) sequences, some RNA-specific proteins
Lysine	rRNA
Cibacron Blue F3G-A	Nucleotide-requiring enzymes, coagulation factors

Practical procedure

The procedure for affinity chromatography is similar to that used in other forms of liquid chromatography. The ligand-treated matrix suspended in buffer is packed into a column in the normal way for the particular type of support. The buffer used must contain any cofactors, such as metal ions, necessary for ligand–macromolecule interaction. Once the sample has been applied and the macromolecule bound, the column is eluted with more buffer to remove non-specifically bound contaminants.

The purified compound is recovered from the ligand by either specific or non-specific elution. Non-specific elution may be achieved by a change in either pH or ionic strength. pH shift elution using dilute acetic acid or ammonium hydroxide results from a change in the state of ionisation of groups in the ligand and/or the macromolecule that are critical to ligand–macromolecule binding. A change in ionic strength, not necessarily with a concomitant change in pH, also causes elution owing to a disruption of the ligand–macromolecule interaction; 1 M NaCl is frequently used for this purpose. If elution is achieved by a pH change, the pH of the collected fractions must be readjusted to the optimum value to minimise the opportunity for protein denaturation. Specific elution involves the addition of a high concentration of substrate, or reversible inhibitor of the macromolecule if it is an enzyme, or the addition of ligands for which the purified compound has a higher affinity than it has for the immobilised ligand. The purified compound is eventually recovered in a buffered solution that may be contaminated with eluting agents or high concentrations of salt and these must be removed by techniques such as exclusion chromatography before the isolation is complete.

Applications

Many enzymes and other proteins, including receptor proteins and immunoglobulins, have been purified by affinity chromatography. The application of the technique is limited only by the availability of immobilised ligands. The principles have been extended to nucleic acids and have made a considerable contribution to developments in molecular biology. Messenger RNA, for example, is routinely isolated by selective hybridisation on poly(U)-Sepharose 4B by exploiting its poly(A) tail. Immobilised single-stranded DNA can be used to isolate complementary RNA and DNA. Whilst this separation can be achieved on columns, it is usually performed using single-stranded DNA immobilised on nitrocellulose filters. Immobilised nucleotides are useful for the isolation of proteins involved in nucleic acid metabolism.

A valuable development of affinity chromatography is its use for the separation of a mixture of cells into homogeneous populations. The technique relies on the antigenic properties of the cell surface or the chemical nature of exposed carbohydrate residues on the cell surface or on a specific membrane receptor–ligand interaction. The immobilised ligands used include protein A, which binds to the Fc region of IgG (Section 7.1), a lectin or the specific ligand for a membrane receptor.

11.8.3 Lectin affinity chromatography

The lectins are a group of proteins produced by animals, plants and slime moulds that have the ability to bind carbohydrate and hence glycoproteins. They have a polymeric structure, most being tetrameric. Their subunits may be either identical, in which case they recognise a single specific saccharide, or of two types in which case they recognise two different saccharides. They all have a molecular mass in the range 40–400 kDa. Their ability to recognise and bind specific saccharides has made them highly valuable in the purification of glycoproteins, particularly membrane receptor proteins.

The most widely used lectins for lectin chromatography are those from leguminous plants (pea, castor bean, soybean) owing to their abundance. They can be immobilised to agarose matrices by conventional techniques and many are available commercially. If the nature of the saccharide component of a glycoprotein is not known, the lectin of choice is selected by a simple screening procedure. Once the glycoproteins have been bound to the immobilised lectin, elution can be achieved in a number of ways:

- by affinity elution using the simple monosaccharide for which the lectin has an affinity,
- by use of a borate buffer, which forms a complex with glycoproteins,
- by the careful change of pH (not below pH 3 or above pH 10),
- by the addition of a reagent such as ethylene glycol to reduce ligand hydrophobic interaction.

One of the attractions of lectin affinity chromatography is that it can be carried out in the presence of relatively high salt concentrations because it does not rely on ionic interactions. In principle, therefore, it can be applied directly after salt fractionation. It has also been used to separate mixtures of cells by taking advantage of the saccharide components of their outer membranes. Most lectin affinity chromatography has been carried out using conventional LPLC.

11.8.4 Immunoaffinity chromatography

The use of antibodies as the immobilised ligand has been exploited in the isolation and purification of a range of proteins including membrane proteins of viral origin. Monoclonal antibodies may be linked to agarose matrices by the cyanogen bromide technique. Protein binding to the immobilised antibody is achieved in neutral buffer solution containing moderate salt concentrations. Elution of the bound protein quite often requires forceful conditions because of the very tight binding with the antibody ($K_d = 10^{-8}$ to 10^{-12} M) and this may lead to protein denaturation. Examples of elution procedures include the use of high salt concentrations with or without the use of detergent and the use of urea or guanidine hydrochloride, both of which cause denaturation. The use of some other chaotropic agents (ions or small molecules that increase the water solubility of

non-polar substances) such as thiocyanate, perchlorate and trifluoroacetate, or lowering the pH to about 3, may avoid denaturation.

11.8.5 Metal chelate chromatography (immobilised metal affinity chromatography)

This is a special form of affinity chromatography in which an immobilised metal ion such as Cu^{2+}, Zn^{2+}, Hg^{2+} or Cd^{2+} or a transition metal ion such as Co^{2+}, Ni^{2+} or Mn^{2+} is used to bind proteins selectively by reaction with the imidazole groups of histidine residues, thiol groups of cysteine residues and indole groups of tryptophan residues. The immobilisation of the protein involves the formation of a coordinate bond that must be sufficiently stable to allow protein attachment and retention during the elution of non-binding contaminating material. The subsequent release of the protein can be achieved either by simply lowering the pH, therefore destabilising the protein–metal complex, or by the use of complexing agents such as EDTA. Most commonly the metal atom is immobilised by attachment to an iminodiacetate- or tris(carboxymethyl)ethylenediamine-substituted agarose.

11.8.6 Dye–ligand chromatography

A number of triazine dyes that contain both conjugated rings and ionic groups, fortuitously have the ability to bind to some proteins. The term pseudo-ligands has therefore been used to describe the dyes. It is not possible to predict whether a particular protein will bind to a given dye as the interaction is not specific but is thought to involve interaction with ligand-binding domains via both ionic and hydrophobic forces. Dye binding to proteins enhances their binding to materials such as Sepharose 4B and this is exploited in the purification process. The attraction of the technique is that the dyes are cheap, readily coupled to conventional matrices and are very stable. The most widely used dye is Cibacron Blue F3G-A. Dye selection for a particular protein purification is empirical and is made on a trial-and-error basis. Attachment of the protein to the immobilised dye is generally achieved at pH 7–8.5. Elution is most commonly brought about either by a salt gradient or by affinity (displacement) elution.

11.8.7 Covalent chromatography

This form of chromatography has been developed specifically to separate thiol (-SH)-containing proteins by exploiting their interaction with an immobilised ligand containing a disulphide group. The principle is illustrated in Fig. 11.17. The most commonly used ligand is a disulphide 2′-pyridyl group attached to an agarose matrix such as Sepharose 4B. On reaction with the thiol-containing protein, pyridine 2-thione is released. This process can be monitored spectrophotometrically at 343 nm, thereby allowing the adsorption of the protein to

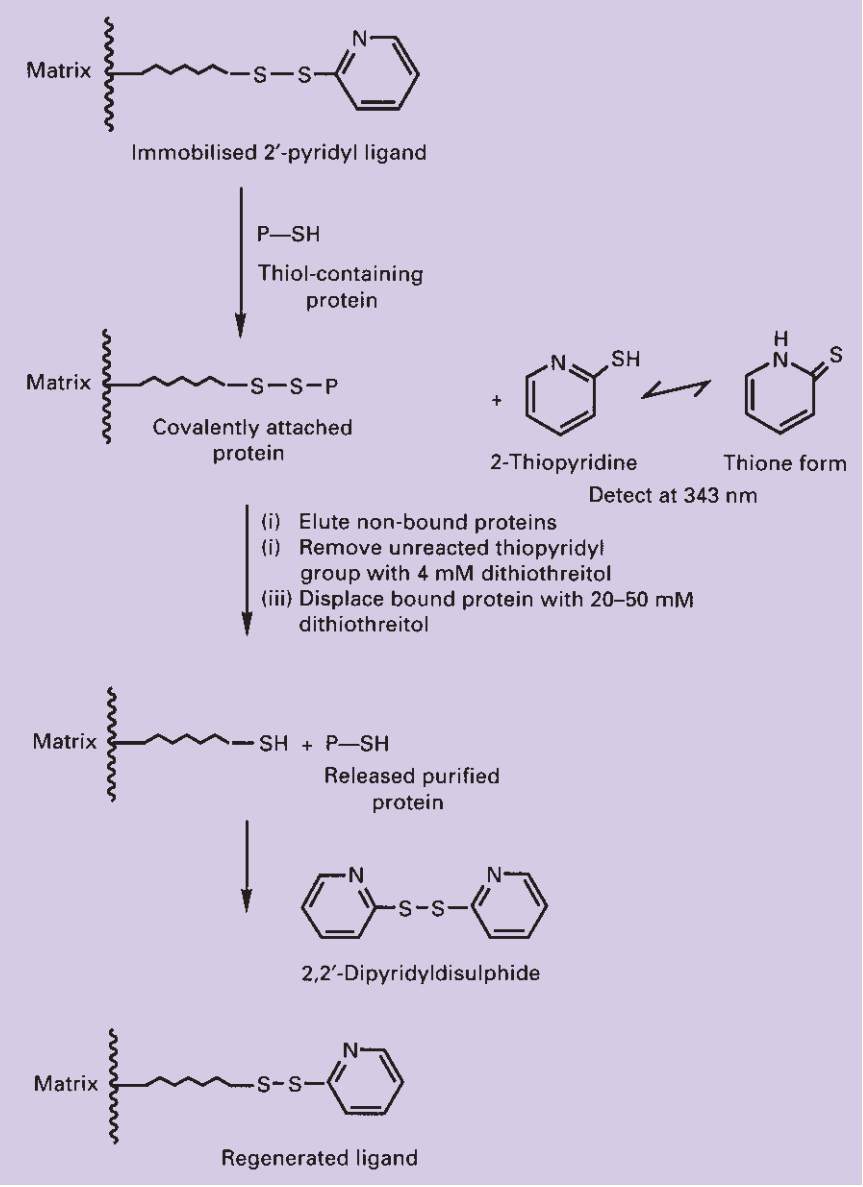

Fig. 11.17. Principle of purification of a protein (P-SH) by covalent chromatography.

be followed. Once the protein has been attached covalently to the matrix, non-thiol-containing contaminants are eluted and unreacted thiopyridyl groups removed by use of 4 mM dithiothreitol or mercaptoethanol. The protein is then released by displacement with a thiol-containing compound such as 20–50 mM dithiothreitol, reduced glutathione or cysteine. The matrix is regenerated by reaction with 2,2′-dipyridyldisulphide. The method has been used successfully for many proteins but its use is limited by its cost and the rather difficult regeneration stage. It can, however, be applied to very impure protein preparations.

11.9 GAS–LIQUID CHROMATOGRAPHY

11.9.1 Principle

The principles of gas–liquid chromatography (GLC or GC) are similar to those of LPLC and HPLC but the apparatus is significantly different. It exploits differences in the partition coefficients between a stationary liquid phase and a mobile gas phase of the volatilised analytes as they are carried through the column by the mobile gas phase. Its use is therefore confined to analytes that are volatile but thermally stable. The partition coefficients are inversely proportional to the volatility of the analytes so that the most volatile elute first. The temperature of the column is raised to 50–300 °C to facilitate analyte volatilisation. The stationary phase consists of a high boiling point liquid material such as silicone grease or wax that is either coated onto the internal wall of the column or supported on an inert granular solid and packed into the column. There is an optimum flow rate of the mobile gas phase for maximum column efficiency (minimum plate height, *H*). Very high resolutions are obtained (equations 11.8 to 11.12), hence the technique is very useful for the analysis of complex mixtures. GLC is a widely used for the qualitative and quantitative analysis of a large number of low polarity compounds because it has high sensitivity, reproducibility and speed of resolution. Analytically, it is a very powerful technique when coupled to mass spectrometry.

11.9.2 Apparatus and experimental procedure

The major components of a GLC system are:

- a column housed in an oven that can be temperature programmed,
- a sample inlet point,
- a carrier gas supply and control,
- a detector, amplifier and data recorder system (Fig. 11.18).

Columns

These are of two types:

- *Packed conventional columns:* These consist of a coiled glass or stainless steel column 1–3 m long and 2–4 mm internal diameter. They are packed with stationary phase coated onto an inert silica support. Commonly used stationary phases include the polyethylene glycols (Carbowax 20M, very polar), methylphenyl- and methylvinylsilicone gums (OV17 and OV101, medium and non-polar respectively), Apiezon L (non-polar) and esters of adipic, succinic and phthalic acids. β-Cyclodextrin-based phases are available for chiral separations (Section 11.5.5). The most commonly used support is Celite (diatomaceous silica), which, because of the problem of support–sample interaction, is often treated so that the hydroxyl groups that occur in the Celite are modified. This is normally achieved by silanisation of the support with such compounds as hexamethyldisilazane. The support particles have an even

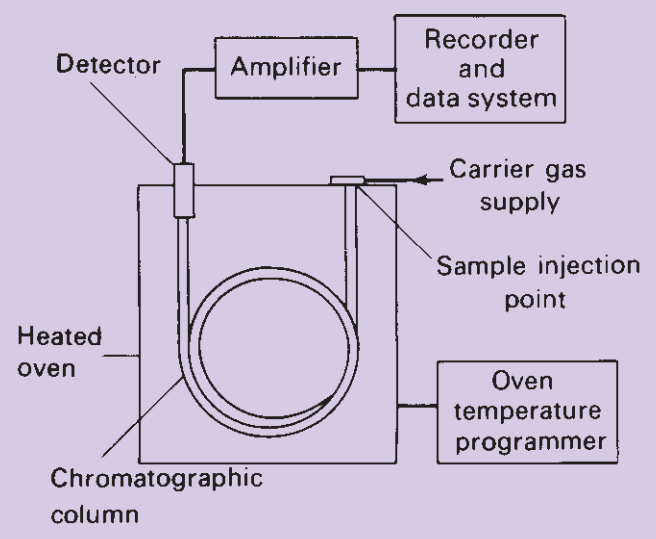

Fig. 11.18. Components of a GLC system.

size, which, for the majority of practical applications, is 60 to 80, 80 to 100, or 100 to 120 mesh (Section 11.3.1). Columns are dry-packed under a slight positive gaseous pressure and after packing must be conditioned for 24–48 h by heating to near the upper working temperature limit, whilst the carrier gas at normal flow rates is passed through the column. During this conditioning, the column should be disconnected from the detector to prevent its contamination. With good-quality liquid phases, column conditioning can be simplified to flushing with carrier gas at 100 °C.

- *Capillary (open tubular) columns:* These are made of high quality fused quartz and are 10–100 m long and 0.1–1.0 mm internal diameter. They are of two types known as wall-coated open tubular (WCOT) and support-coated open tubular (SCOT), also known as porous layer open tubular (PLOT) columns, for adsorption work. In WCOT columns the stationary phase is thinly coated (0.1–5 μm) directly onto the walls of the capillary, whilst in SCOT columns the support matrix is bonded to the walls of the capillary column and the stationary phase coated onto the support. Commonly used stationary phases include polyethylene glycol (CP wax and DB wax, very polar) and methyl and phenyl-polysiloxanes (BP1, non-polar; BP10, medium polar). They are coated onto the supporting matrix to give a 1–25% loading, depending upon the analysis. The capacity of SCOT columns is considerably higher than that of WCOT columns.

The operating temperature for all types of column must be compatible with the stationary phase chosen for use. Too high a temperature results in excessive column bleed owing to the phase being volatilised off, contaminating the detector and giving an unstable recorder baseline. The working temperature range is chosen to give a balance between peak retention time and resolution. Column

temperature is controlled to ±0.1 deg.C. Analyte partition coefficients are particularly sensitive to temperature so that analysis times may be regulated by adjustment of the column oven, which can be operated in one of two modes:

- *Isothermal analysis:* Here a constant temperature is employed.
- *Temperature programming:* The temperature is gradually increased to facilitate the separation of compounds of widely differing polarity or M_r. This, however, sometimes results in excessive bleed of the stationary phase as the temperature is raised, giving rise to baseline variation. Consequently some instruments have two identical columns and detectors, one set of which is used as a reference. The currents from the two detectors are opposed, hence, assuming equal bleed from both columns, the resulting current gives a steady baseline as the column temperature is raised. The choice of phase for analysis depends on the analytes under investigation and is best chosen after reference to the literature.

Application of sample

The majority of low and non-polar compounds are directly amenable to GLC, but other compounds possessing such polar groups as -OH, -NH_2 and -COOH are generally retained on the column for excessive periods of time if they are applied directly. Poor resolution and peak tailing usually accompany this excessive retention (Section 11.2.4). This problem can be overcome by derivatisation of the polar groups. This increases the volatility and effective distribution coefficients of the compounds. Methylation, silanisation and perfluoracylation are common derivatisation methods for fatty acids, carbohydrates and amino acids.

The test sample is dissolved in a suitable solvent such as acetone, heptane or methanol. Chlorinated organic solvents are generally avoided as they contaminate the detector. For packed and SCOT columns the sample is injected onto the column using a microsyringe through a septum in the injection port attached to the top of the column. Normally 0.1–10 mm^3 of solution is injected. It is common practice to maintain the injection region of the column at a slightly higher temperature (+20–50 °C) than the column itself as this helps to ensure rapid and complete volatilisation of the sample. Sample injection is automated in many commercial instruments as this improves the precision of the analysis. As there is only a small amount of stationary phase present in WCOT columns, only very small amounts of sample may be applied to the column. Consequently a splitter system has to be used at the sample injection port so that only a small fraction of the injected sample reaches the column. The remainder of the sample is vented to waste. The design of the splitter is critical in quantitative analyses in order to ensure that the ratio of sample applied to the column to sample vented is always the same.

Mobile phase

The mobile phase consists of an inert gas such as nitrogen for packed columns or helium or argon for capillary columns. The gas from a cylinder is pre-purified by passing through a variety of molecular sieves to remove oxygen, hydrocarbons and water vapour. It is then passed through the chromatography column at a flow

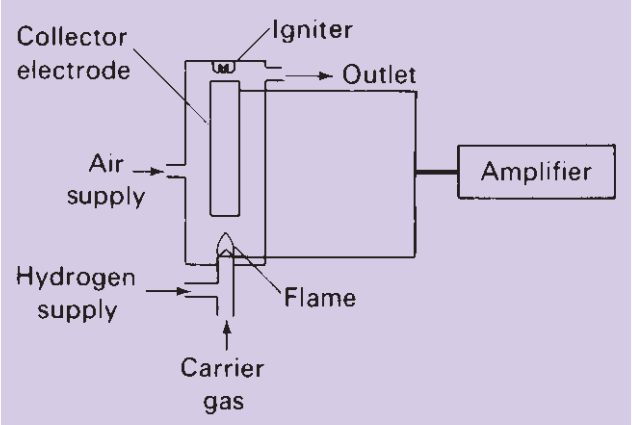

Fig. 11.19. GLC flame ionisation detector. The tip of the flame forms the anode and the collector electrode the cathode.

rate of 40–80 cm^3 min^{-1}. A gas-flow controller is used to ensure a constant flow irrespective of the back-pressure and temperature of the column.

11.9.3 Detectors

Several types of detector are in common use in conjunction with GLC:

- *Flame ionisation detector (FID):* This responds to almost all organic compounds. It has a minimum detection quantity of the order of 5×10^{-12} g s^{-1}, a linear range of 10^7 and an upper temperature limit of 400 °C. A mixture of hydrogen and air is introduced into the detector to give a flame, the jet of which forms one electrode, whilst the other electrode is a brass or platinum wire mounted near the tip of the flame (Fig. 11.19). When the sample analytes emerge from the column they are ionised in the flame, resulting in an increased signal being passed to the recorder. The carrier gas passing through the column and the detector gives a small background signal, which can be offset electronically to give a stable baseline.

$$\text{analyte} + H_2 + O_2 \rightarrow \text{combustion products} + H_2O + \text{ions} + \text{radicals} + \text{electrons}$$
$$\Sigma(\text{ions})^- + \Sigma(\text{electrons})^- \rightarrow \text{current}$$

- *Nitrogen–phosphorus detector (NPD) (also called a thermionic detector):* This is similar in design to an FID but has a crystal of a sodium salt fused onto the electrode system, or a burner tip embedded in a ceramic tube containing a sodium salt or a rubidium chloride tip. The NPD has excellent selectivity towards nitrogen- and phosphorus-containing analytes and shows a poor response to analytes possessing neither of these two elements. Its linearity (10^5) and upper temperature limit (300 °C) are not quite as good as an FID but its detection limits (10^{-14} g s^{-1}) are better. It is widely used in organophosphorus pesticide residue analysis.

- *Electron capture detector (ECD):* This responds only to analytes that capture electrons, particularly halogen-containing compounds. This detector is widely used in the analysis of polychlorinated compounds, such as the pesticides DDT, dieldrin and aldrin. It has a very high sensitivity (10^{-13} g s^{-1}) and an upper temperature limit of 300 °C but its linear range (10^2–10^4) is much lower than that of the FID. The detector works by means of a radioactive source (^{63}Ni) ionising the carrier gas and releasing an electron that gives a current across the electrodes when a suitable voltage is applied. When an electron-capturing analyte (generally one containing a halogen atom) emerges from the column, the ionised electrons are captured, the current drops and this change in current is recorded. The carrier gas most commonly used in conjunction with an ECD is nitrogen or an argon + 5% methane mixture.
- *Flame photometric detector:* This exploits the fact the P- and S-containing analytes emit light when they are burned in a FID-type detector. This light is detected and quantified. The detection limit is of the order of 1.0 pg for P-containing compounds and 20 pg for S-containing compounds.
- *Rapid scanning Fourier transform infrared detector:* This records the infrared spectrum of the emerging analytes and can give structural as well as quantitative information about the analyte. Any analyte with an infrared spectrum can be detected with a detection limit of about 1 ng.
- *Mass spectrometer detector:* This is a universal detector that gives a mass spectrum of the analyte and therefore gives both structural and quantitative data. Its detection limit is less than 1 ng per scan. Analytes may be detected by a total ion current (TIC) (Section 9.4.1) trace that is non-selective, or by selected ion monitoring (SIM) (Section 9.5.7) that can be specific for a selected analyte. In cases where authentic samples of the test compounds are not available for calibration purposes or where the identity of the analytes is not known, a mass spectrometer is the best means of detecting and identifying the analyte. Special separators are available for removing the bulk of the carrier gas from the sample emerging from the column prior to its introduction in the mass spectrometer (Section 9.3).

The volatile solvent used to introduce the test sample onto the column gives rise to a solvent peak at the beginning of the chromatograph. The main forms of detector respond to this solvent with varying sensitivity, thereby affecting the detection and resolution of rapidly eluting analytes.

Most modern GLC systems are controlled by dedicated microcomputers capable of automating and optimising the experimental conditions, recording the calibration and test retention data, carrying out the statistical analysis, and displaying the outputs in colour graphics in real time.

11.9.4 Applications

Until the development of HPLC, GLC was probably the most commonly used form of chromatography. Its use nowadays is confined to volatile, non-polar compounds

that do not need derivatisation. Analytes are characterised by their retention time or preferably by their retention time relative to a standard reference compound. In the analysis of compounds that form a homologous series, for example the methyl esters of the saturated fatty acids, there is a linear relationship between the logarithm of the retention time and the number of carbon atoms. There are similar but parallel calibration lines for mono- and di-unsaturated fatty acids. This can be exploited, for example, to identify an unknown fatty acid ester in a fat hydrolysate.

11.10 THIN-LAYER (PLANAR) CHROMATOGRAPHY

11.10.1 Principle

Although thin-layer chromatography (TLC) shares many theoretical principles with the various forms of column chromatography discussed so far, its practical format is quite different. The stationary phase is coated as a thin (0.25–2.0 mm) layer on a glass or metal foil plate commonly 5–20 cm square or rectangular. The test sample is applied as a spot or thin band near one end of the plate (origin) that is then placed in a reservoir of mobile phase that is allowed to pass over the plate, generally by simple capillary action. As there is little resistance to the flow, the front of the mobile phase moves rapidly across the layer. As it does so it transfers analytes in the test sample with it at a rate determined by their distribution coefficients between the mobile phase and the stationary phase. The stationary phase may be one of a variety of forms so that the separation process may be based on adsorption, partition, chiral, ion-exchange or molecular exclusion principles. Analyte movement ceases when the mobile phase front reaches the end of the layer or when the plate is removed from the mobile phase reservoir. The movement of a given analyte is characterised not by a retention time or volume but by a retardation factor (R_f) defined as follows:

$$R_f = \frac{\text{distance moved by the analyte from the origin}}{\text{distance moved by the mobile phase front from the origin}} \tag{11.15}$$

The performance of a thin-layer separation can be characterised by the number of theoretical plates (N), plate height (H) and capacity factor (k') that may be calculated according to the following equations:

$$N = 16(d_A/w)^2 \tag{11.16}$$

$$H = d_A/N \tag{11.17}$$

$$k' = (1 - R_f)/R_f = (d_f/d_A) - 1 \tag{11.18}$$

where d_A is the distance moved by the analyte from the origin, d_f is the distance moved by the mobile phase front from the origin, and w is the width of the analyte spot.

11.10.2 Apparatus and experimental procedure

Preparation of plates

The stationary phase is applied to the plate as a slurry, generally in water, using a plate spreader that allows a uniform layer of the required thickness to be deposited. Alternatively, pre-prepared plates are available commercially. For analytical separations the layer needs to be of the order of 0.25 mm thick and for preparative purposes about 2 mm. For all stationary phases except those for separations based on molecular exclusion, the coated plates are allowed to dry at room temperature before use. In the case of adsorption phases, this drying is achieved by heating the plate to 100–120 °C, since this temperature also serves to activate the adsorbent.

Application of sample

The sample dissolved in an organic solvent is applied by a micropipette about 2 cm from the edge of the plate. The solvent may be removed from the spot by gentle heating with an air dryer and more sample applied if necessary. Twelve to fifteen different samples can be applied as discrete spots on a single plate. For preparative purposes, the sample is applied as a band across the plate.

Plate development

The separation process takes place in a glass tank containing the mobile phase to a depth of about 1.5 cm. The tank is allowed to equilibrate for about 1 h with a glass lid on top. The plate is then placed vertically in the tank with the edge near the origin standing in the liquid. The lid is replaced and separation allowed to take place, generally within 20–30 min for analytical separations and up to 75–90 min for preparative separations. When the mobile phase front approaches the distant edge of the plate, the plate is removed from the tank and the separated analytes detected. The resolution of the analytes can be increased by the technique of two-dimensional development. In this technique the sample is applied as a single spot in one corner of the plate and the plate developed as previously. It is then dried, turned through 90° and developed in a second direction with a different mobile phase that has different K_d values for the analytes than had the first.

Detection of analytes

The separated analytes may be detected in a number of ways:

- by spraying the plate with a specific reagent that converts the analytes to a coloured product,
- by examining the plate under ultraviolet light, assuming that the analytes absorb in this region,
- by incorporating a fluorescent dye into the stationary phase so that when the plate is examined under ultraviolet light the analytes show as blue, green or black spots against a fluorescent background,

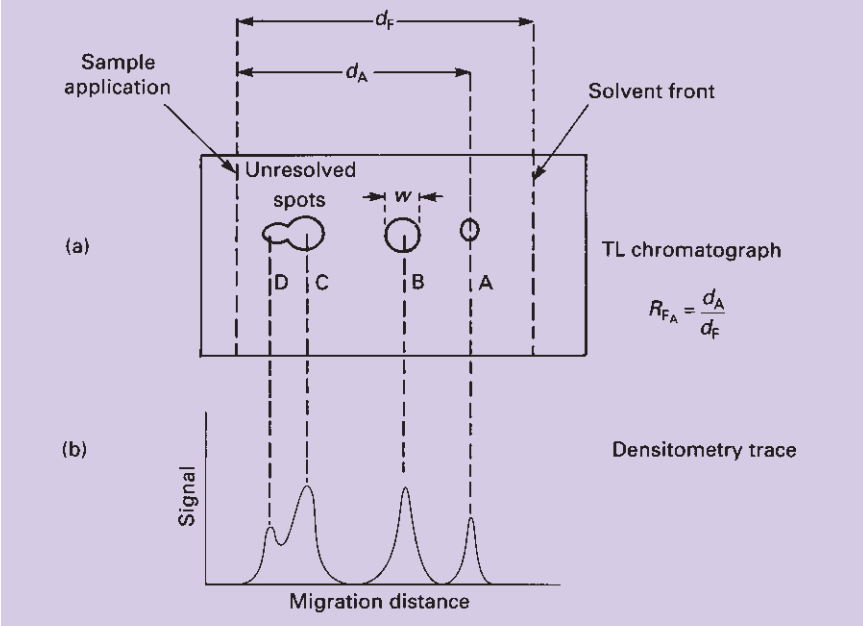

Fig. 11.20. (a) Thin-layer chromatograph of a mixture of analytes A to D; (b) the densitometer trace from which quantitative data can be calculated.

- by using radiolabelled analytes and subjecting the developed plate to autoradiography using an X-ray film or by scanning the plate with a radiochromatograph scanner.

The amount of analyte present in each spot can best be measured using a precision densitometer. This measures the intensity of the spot in the visible or ultraviolet region and may simultaneously give a complete spectrum of the compound for identification purposes. The identification of unknown analytes is based on such data and on the comparison of the measured R_f value with those of reference compounds chromatographed alongside the test sample (Fig. 11.20).

11.10.3 Applications

The great attractions of TLC are its practical simplicity, low cost and ability to separate several test samples simultaneously. Its main disadvantages are the ease with which the layer may be damaged and its lack of good reproducibility. The consequence of these combined characteristics is that its use is currently confined to qualitative studies such as to check for the presence of a particular analyte. Examples include studies on natural products and peptide mapping. Further details can be found in the 'Suggestions for further reading' (Section 11.12).

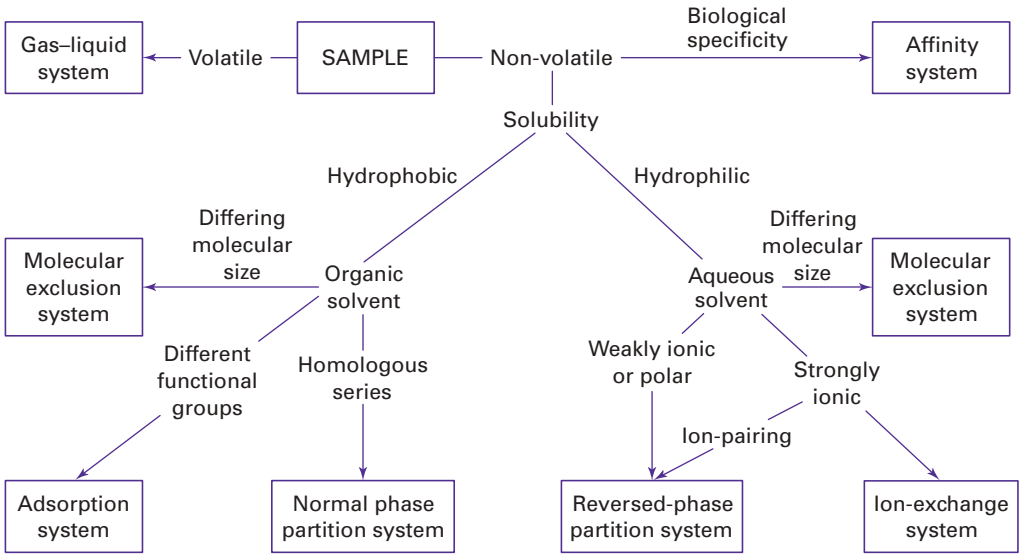

Fig. 11.21. Rationale for the choice of a chromatographic system.

11.11 SELECTION OF A CHROMATOGRAPHIC SYSTEM

It is possible to predict the type of system most likely to be applicable to the separation of compounds for which the physical characteristics are known (Fig. 11.21). The majority of chromatographic procedures exploit differences in physical properties of compounds, the exception being affinity chromatography, which is based on the specific ligand-binding properties of biological macromolecules. If this form of chromatography can be applied, it is the most likely to be successful. Volatile compounds are best separated by GLC, whereas non-volatile compounds that are soluble in organic solvents are generally best separated by either adsorption or normal-phase liquid chromatography. If the compounds have different functional groups, adsorption chromatography on silica with non-polar solvent is probably the better method. To separate low polarity compounds in a homologous series, normal-phase liquid systems are preferred. If water-soluble compounds are non-ionic or weakly ionic, reversed-phase liquid chromatography is preferable where a non-polar stationary phase such as a hydrocarbon is used together with a polar mobile phase such as water/acetonitrile or water/methanol mixtures. Water-soluble compounds that are strongly ionic are best separated by an ion-exchange system, using either an anionic or cationic resin, together with a suitable buffer system for elution. Ionic compounds can, however, be separated by reversed-phase partition systems by the technique of ion-pairing. Compounds differing in molecular size are best separated by molecular exclusion chromatography.

Whatever form of liquid chromatography is chosen for a particular biochemical study, the decision to use LPLC or HPLC depends on many factors including the availability of apparatus, cost, the scale of the separation, and whether the separation is to be qualitative or quantitative. The modern trend is to select HPLC,

which is certainly capable of giving fast, accurate and precise data. Reversed-phase HPLC, in particular, is proving to be an extremely versatile technique. The application of HPLC techniques to protein separations, via FPLC, is also proving to be a quick, robust technique, particularly in cases where protein denaturation is not a problem. The simplicity of TLC and its facility to separate multiple samples simultaneously makes it attractive for routine, mainly quantitative separations.

11.12 SUGGESTIONS FOR FURTHER READING

MONDELLO, L., LEWIS, A. C. and BARTLE, K. D. (2002). *Multidimensional Chromatography*. Wiley, Chichester. (Discussion of such developments as the coupling of HPLC to GC and SCFC to GC; 2D GC and the applications of these techniques.)

SHERMA, J. and FRIED, B. (eds.) (2003). *Handbook of Thin-Layer Chromatography*, 3rd edn. Marcel Dekker, New York. (Detailed discussion of the principles, practice and applications of TLC to a wide range of biological applications.)

SIMPSON, N. J. K. (ed.) (2000). *Solid Phase Extraction – Principles, Techniques and Applications*. Marcel Dekker, New York. (Comprehensive coverage of this important analytical technique.)

VARIOUS AUTHORS. *Trends in Analytical Chemistry* (2002), **21**. (Whole volume of the journal devoted to a review of recent developments and applications of GC.)

Chapter 12

Spectroscopic techniques: I Atomic and molecular electronic spectroscopy

12.1 INTRODUCTION

12.1.1 Properties of electromagnetic radiation

The interaction of electromagnetic radiation with matter is essentially a quantum phenomenon and is dependent upon both the properties of the radiation and the appropriate structural parts of the material involved. This is not surprising, as the origin of the radiation is due to energy changes within the matter itself. An understanding of the properties of electromagnetic radiation and its interaction with matter leads to a recognition of the variety of types of spectra and consequently spectroscopic techniques and their application to the solution of biological problems. Also the transitions which occur within matter (see e.g. Section 12.1.2) are quantum phenomena and the spectra which arise from such transitions are, at least in principle, predictable. Table 12.1 shows the various interactions, with parts of matter, of the electromagnetic spectrum and corresponding wavelengths. The various parts of matter both give rise to and are affected by the radiation in the corresponding region of the spectrum.

Electromagnetic radiation (Fig. 12.1) is composed of both an electric vector and magnetic vector (which gives rise to the name), which oscillate in planes at right angles (normal) to each other and mutually at right angles to the direction of propagation.

12.1.2 Interaction with matter

Electromagnetic phenomena exhibit energy, frequency, wavelength and intensity. All these are interrelated and can be explained either in terms of waveforms or particles termed photons or quanta. These phenomena are best exemplified by considering electronic spectra. Electrons in either atoms or molecules may be distributed between several energy levels but principally reside in the lowest levels or ground state. In order for an electron to be promoted to a higher level (or excited state), energy must be put into the system and this gives rise to an absorption spectrum if the energy is derived from electromagnetic radiation. Only the exact amount of energy equivalent to the difference in energy level, in accordance with the rules of quantum mechanics, will be absorbed. This is termed one quantum of

Table 12.1 Interaction of electromagnetic radiation and the various parts or 'structures' of matter

Phenomenon	Region of spectrum	Wavelength
Nuclear	Gamma	0.1 nm
Inner electrons	X-rays	0.1–1.0 nm
Ionisation	Ultraviolet	0–200 nm
Valency electrons	Near ultraviolet and visible	200–800 nm
Molecular vibrations	Near infrared and infrared	0.8–25 μm
Rotation and electron spin orientation in magnetic fields	Microwaves	400 μm–30 cm
Nuclear spin orientation in magnetic fields	Radiowaves	100 cm and above

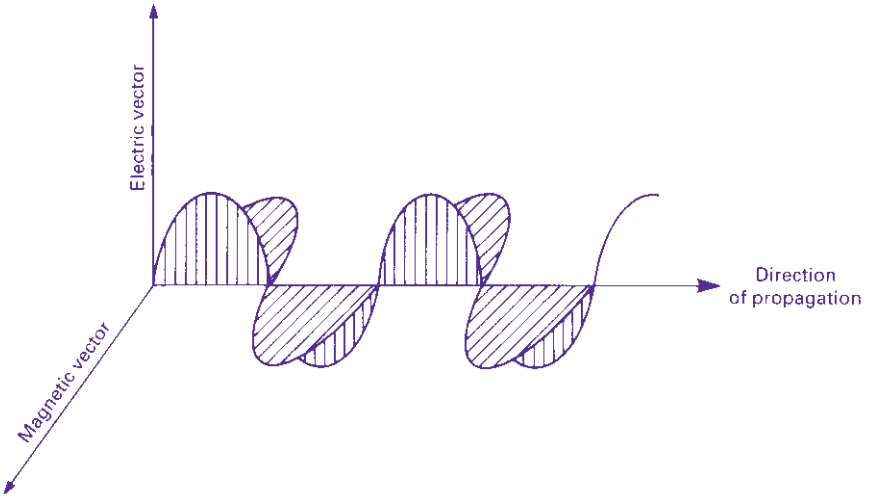

Fig. 12.1. The electric and magnetic vectors or 'oscillations' of electromagnetic radiation and the direction of propagation.

energy for a single-electron transition, and the absolute magnitude of each quantum will differ according to the difference in energy levels involved. When an electron falls from a higher to lower level, then exactly one quantum of energy is emitted from the system, giving rise to an emission spectrum. Energy in other forms may be put into the system; for example, the heating of metals achieves the promotion of electrons to higher energy levels and, if sufficient energy has been input, when they return to lower levels visible light is emitted. This gives rise to the effect of the glowing of heated metals.

Fig. 12.2a is a diagrammatic representation of electron transitions in the sodium atom. These transitions in most atoms give rise to relatively simple line spectra. The situation in molecules is somewhat more complicated, although the

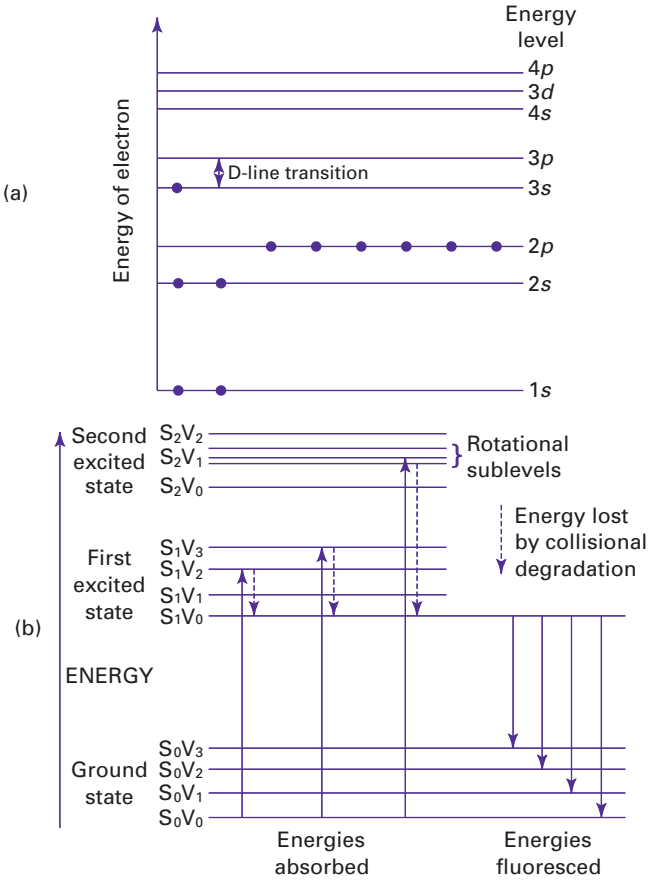

Fig. 12.2. Energy levels and transitions of electrons: (a) in the sodium atom and (b) in a fluorescent organic molecule. Note: for clarity, rotational sublevels have been indicated only for vibrational sublevel S_2V_1.

same basic principles apply, because more different kinds of energy level exist. Moreover the atoms in molecules may vibrate and rotate about a bond axis, which gives rise to vibrational and rotational sublevels. This situation is shown diagrammatically in Fig. 12.2b but, owing to the subdivision of energy levels in molecules, molecular spectra are usually observed as band spectra.

The energy change for an electron transition is defined in quantum terms by the following simple relation;

$$\Delta E = E_1 - E_2 = h\nu \tag{12.1}$$

where ΔE is the change in energy state of the electron or the energy of electromagnetic radiation absorbed or emitted by an atom or molecule, E_1 is the energy of the electron in its original state, E_2 is the energy of the electron in the final state, h is the Planck constant ($= 6.63 \times 10^{-34}$ J s), ν is the frequency of the electromagnetic radiation and is equal to the number of oscillations made by the wave in 1 s. It

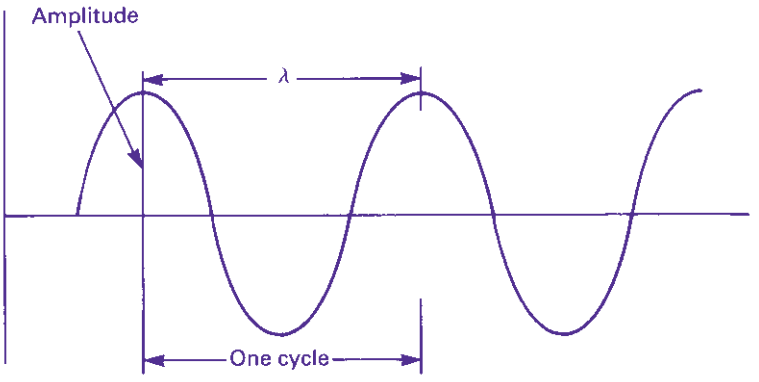

Fig. 12.3. Representation of terms in a single sinusoidal waveform. (The number of cycles occurring in unit time (second) is the frequency measured in hertz.)

therefore has units of reciprocal seconds (s^{-1}). One oscillation per second is called 1 Hz. Frequency is related to wavelength (λ) by the relationship $\nu = c/\lambda$ where c is the speed of light ($3 \times 10^8\,ms^{-1}$ in a vacum). The wavelength of electromagnetic radiation is equal to $c\bar{\nu}$, where $\bar{\nu}$ is the wave number of electromagnetic radiation in waves cm^{-1} (kaysers). Fig. 12.3 shows some of the interrelationships. It should be noted that wavelength should be expressed in submultiples of a metre, i.e. nanometre (nm), micrometre (μm), centimetre (cm) etc. (not ångströms (Å) or mμ or μ) and frequency expressed in hertz (not cycles s^{-1}).

In Fig. 12.2a,b, electron transitions in atoms or molecules give rise to the electronic spectra generally observed as absorption, emission or fluorescence phenomena (Section 12.5) in the ultraviolet and visible regions of the electromagnetic spectrum. The basic quantum relationships hold for other regions also. Of course, different energy transitions occur in these other regions and these will be indicated as each appropriate part of the system is dealt with.

In the following subsections, each region of the electromagnetic spectrum is treated in terms of the interaction involved, instrumentation used and application to appropriate biological problems. The treatment is unequal, however, and some sections are presented in considerably more detail to reflect usage.

12.2 γ-RAY SPECTROSCOPY AND γ-RAY RESONANCE SPECTROSCOPY

12.2.1 Principles

γ-Rays are of nuclear origin, but they are also part of the electromagnetic spectrum and so, in principle, it is possible to develop spectroscopic methods involving them. Owing to their considerable penetrating power, the main applications in a biological context are in imaging but also in radiotherapy. The rays arise from energy transitions occurring within the nucleus, the mechanisms of which are not described here. For further details, see the literature cited in Section 12.11.

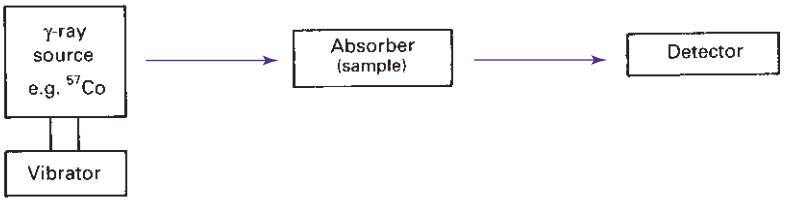

Fig. 12.4. Layout of a simple Mössbauer spectrometer.

An important application of γ-ray emission spectroscopy is the use of the element technicium, Tc, which does not occur naturally but is a product of the nuclear industry. This element may be used for medical studies because, if it is complexed to a compound that is preferentially concentrated in specific biological tissues, particularly bone, liver or brain, its location can be determined by its emission spectrum. The emitted radiation is detected using a device known as a γ-camera, enabling the shape and structure of the tissue under study to be investigated. Despite the name given to the instrument, in this type of application the technique is essentially spectroscopic.

Nuclear γ-resonance, the so-called Mössbauer effect, was discovered in 1957. Many isotopes exhibit the effect but the main emphasis appears to have centred around the ^{57}Fe isotope. Although the applications have been somewhat limited, there is considerable potential for the study of biologically important metal-containing complexes.

12.2.2 Mössbauer spectroscopy

Principles

The γ-ray energy from a radioactive nucleus may be modulated by giving a Doppler velocity to the source. The Doppler effect (observed in all waveforms, sound and electromagnetic) is recognised as the apparent change in frequency that occurs when the source is moving relative to the detector (observer). The change in frequency is proportional to the source velocity and any velocity may be chosen to give the required frequency. γ-Rays of discrete energy can be absorbed resonantly by appropriate nuclei. The source used is usually ^{57}Co; this emits a range of γ-rays with different energies, an appropriate one of which may be selected. The selected ray is then modulated by the imposed Doppler phenomenon.

Instrumentation

Fig. 12.4 shows a very simplified diagram of the arrangement required to perform Mössbauer spectroscopy. Usually, because of the energies and wavelengths involved, the Doppler velocity can be imposed by rapidly vibrating the ^{57}Co source.

Applications

The major application of this technique is in the study of the coordination of metal atoms by ligands of an appropriate complexing agent. Model compounds have been investigated, enabling a better understanding of how certain metals of biological importance are affected by changes in the binding properties of the ligand either by chemical modification or because of local environment differences. An example is sickle cell anaemia, where, compared with normal haemoglobin, the iron atom is distorted out of the plane of the haem moiety.

12.3 X-RAY SPECTROSCOPY

12.3.1 Principles

Whereas γ-rays are of nuclear origin, X-rays arise from displacement of inner, extranuclear electrons. The electrons, with principle quantum numbers 1, 2 and 3, in an atom can be imagined to occupy shells – K, L and M, respectively. Should a bombarding electron from an external source have sufficient energy to displace a K shell (innermost) electron in a target atom, then this vacancy is filled within a time span of 10^{-4} s by an L shell electron and an X-ray of appropriate wavelength is emitted. The energy transition from L to K is, of course, governed by quantum rules and $E = h\upsilon$ must be satisfied; hence the frequency and wavelength of the emitted X-ray are determined.

X-rays can be absorbed by matter and this gives rise to X-ray absorption spectra. The rules applying to the relationship between an incident beam of monochromatic X-radiation (I_0) and the transmitted portion I, are similar to the Beer–Lambert case described in Section 12.4.1. If μ is the linear absorption coefficient of the absorbing material then

$$I = I_0 e^{-\mu x} \tag{12.2}$$

where x is the thickness of the absorber.

If X-rays have wavelength shorter than the so-called K absorption edge of an atom, then it is possible for the incident radiation to dislodge K electrons. This then results in the emission of X-rays (because of K electron displacement) of a frequency different from that of the incident ray. The phenomenon is called X-ray fluorescence and gives rise to X-ray fluorescence analysis (XRFA). The general principles of fluorescence are considered in Section 12.5.

12.3.2 Instrumentation and applications

A suitable X-ray source is required that can be focused into the specimen chamber where the substance under test is excited by the incident beam. A monochromator is required also to disperse the fluorescent (emitted) radiation and finally a suitable detector and data-processing facilities are needed. Fig. 12.5 is a simple representation of the required layout.

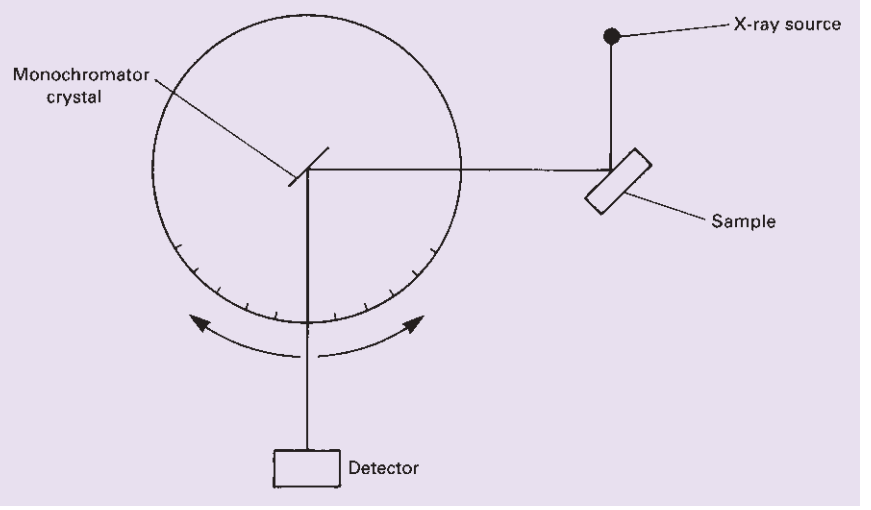

Fig. 12.5. X-ray fluorescence analysis. Dispersion of fluorescent X-rays may be detected at various angles.

The technique has wide applications in forensic science and environmental pollution studies because it enables many elements to be detected and concentrations measured. Of course the analysis is essentially concerned with elements but can be a useful adjunct to, for example, the detection and measurement of trace elements in fertilisers. Such elements may well find their way into the food chain, with possible toxic consequences if they potentially interfere with normal metabolism. An example of such an application would be the study of the uptake of lead in plants at various distances from, say, a heavily used thoroughfare.

Absorption and emission spectra are obtained in ways similar to those described below for the ultraviolet/visible region of the electromagnetic spectrum (Section 12.4). A clinical application for performing bone densitometry measurements involves either single-photon or dual energy X-ray absorptiometry (DEXA). These studies are useful for monitoring hormone replacement therapy (HRT) in female patients. X-ray spectrometers obviously require a more rigorous approach to the incorporation of safety features, but the essential requirements of source, monochromator and detector are the same.

12.4 ULTRAVIOLET AND VISIBLE LIGHT SPECTROSCOPY

These regions of the electromagnetic spectrum and their associated techniques are probably the most widely used, for both routine analytical work and research into biological problems. The energy transitions that occur here are exactly those described in Section 12.1.2. It is convenient, however, to deal here with the appropriate laws related to the absorption of 'light', that region of the electromagnetic spectrum for which these laws were developed.

12.4.1 Principles

The Beer–Lambert law is a combination of two laws, each dealing separately with the absorption of light, related to the concentration of the absorber (the substance responsible for absorbing the light) and the pathlength or thickness of the layer (related to the absolute amount of the absorber). Provided an absorbing substance is partially transparent it will transmit a portion of the incident radiation. The ratio of the intensities of transmitted and incident light gives the transmittance, T, expressed as:

$$T = I/I_0 \tag{12.3}$$

where I_0 is the intensity of incident radiation and I is the intensity of transmitted radiation. (Note: intensity = number of photons interacting in unit time (seconds).)

A 100% value of T represents a totally transparent substance, with no radiation being absorbed, whereas a zero value of T represents a totally opaque substance, which, in effect, represents complete absorption. For intermediate values we can define the absorbance (A) or extinction (E), which is given by the logarithm (base 10) of the reciprocal of the transmittance:

$$A = E = \log(1/T) = \log(I_0/I) \tag{12.4}$$

Absorbance used to be called optical density (OD) but continued use of this term should be discouraged. Also, as absorbance is a logarithm it is by definition unitless and has a range of values from 0 ($\equiv 100\%T$) to ∞ ($\equiv 0\%T$). However, the use of the term 'absorbance' is incorrect when the measurement is based on light scattering rather than absorption. This situation is commonly encountered in the estimation of bacterial cell numbers or measurements on isolated organelles. It is for this reason that microbiologists continue to use the term optical density for cell count studies. In such studies the more correct term is attenuance, symbol D. Attenuance reduces to absorbance when there is negligible light scattering.

It is now possible to define the Beer–Lambert law, which, as described above, states that the absorbance is proportional to both the concentration of absorber and thickness of the layer, as

$$A = \epsilon_\lambda cl \tag{12.5}$$

where ϵ_λ is the molar absorbance coefficient (or molar extinction coefficient) for the absorber at wavelength λ, c is the concentration of absorbing solution, and l is the pathlength through the solution (or thickness).

In the strictest use of SI units the concentration should be expressed as mol m^{-3} (which is not molar) and the pathlength in metres. As A is unitless this would give units for ϵ_λ as $mol^{-1}\ m^2$ (derived from 1/(mol m^{-3} m), if equation 12.5 is rearranged to give $\epsilon_\lambda = A/(cl)$). This is to be expected, as the value of the absorbance is also dependent upon the area of illumination by the incident radiation. As this area is identical for both sample and reference it can be ignored in any calculations. However, more practical units for ϵ_λ are $dm^3\ mol^{-1}\ cm^{-1}$, which conform to the

definition of molarity (despite being incoherent in SI terms) and the common use of 1 cm pathlength cuvettes (see Section 1.2.2). Sometimes molar absorbance coefficients are extremely large and in such cases a more convenient way of expressing values is to quote the absorbance of a 1 cm thick sample of a 1% solution of the absorbant. This is distinguished by writing the coefficient as $A^{1\%}_{1\text{cm}}$.

Example 1 CALCULATION OF MOLAR EXTINCTION COEFFICIENT

Question

An aliquot of a solution containing a light-absorbing substance at a concentration of 5 g dm^{-3}, was placed in a 2 cm light path cuvette. The cuvette was placed in a spectrophotometer and a beam of light of wavelength λ was passed through the cuvette containing the solution. A transmission value of 80% was recorded.

What is (i) the absorbance of the solution, and (ii) the molar extinction coefficient if the molecular mass of the substance is known to be 410?

Answer

(i) As the concentration is given in terms of absolute mass per unit volume, it is necessary to find a 'specific extinction coefficient', ϵ_s.

The transmission, T, is expressed as the percentage $100\times I_t$, of the incident light I_0;

$$T = 80\% = 100 \times \frac{I_t}{I_0}$$

If $I_0 \equiv 100\%$ of the light, then

$I_t \equiv 80\%$

$$\frac{I_t}{I_0} = \frac{80}{100} = 0.8$$

$$\text{Absorbance} = A = \log(1/T) = \log(1/0.8) = \log(1.25) = 0.0969$$

$$A = \epsilon_s \times c \times \ell = \epsilon_s \times 5 \times 2$$

Therefore,

$$\epsilon_s = \frac{0.0969}{10} = 9.69 \times 10^{-3}\,\text{g}^{-1}\,\text{dm}^3\,\text{cm}^{-1}$$

(ii) As the specific extinction coefficient relates to 'per gram', the molar extinction coefficient is obtained simply by multiplying by the relative molecular mass.

Hence,

$$\epsilon_s \times 410 = \epsilon_\lambda$$

$$9.69 \times 10^{-3} \times 410 = 3.973\,(\text{mol dm}^{-3})^{-1}\,\text{cm}^{-1}$$

12.4.2 Instrumentation

What material is used in the optical parts of the instrument depends on the wavelength used. In the ultraviolet region it is necessary to use prisms, gratings, reflectors and cuvettes made of silica. Above 350 nm wavelength, borosilicate glass may be used but also there are now some plastic materials (e.g. disposable cuvettes)

available that are transparent over virtually the whole of the visible region and into the near ultraviolet.

Wavelength selection is obviously of crucial importance. In the visible region where the analyte may not absorb, but can be readily modified chemically to produce a coloured product, coloured filters may be used that absorb all but a certain limited range of wavelengths. This limited range is known as the bandwidth of the filter. The methods that use filter selectors and depend on the production of a coloured compound are the basis of colorimetry; such methods give moderate accuracy, as even the best filters (interference types) do not have particularly narrow bandwidths. The usual procedure is to use two optically matched cuvettes, one containing a blank in which all the materials are mixed except the sample under test, an equivalent volume of solvent being added to this mixture, and the other containing the coloured material to be measured. It is necessary to standardise or zero the instrument using the blank, change cuvettes and read the absorbance. The best analytical procedure requires the zero to be reset between each measurement as colorimeters, and some filters, are influenced by temperature changes. It is also good practice to work from the most dilute (least colour) to the most concentrated because even if the cuvette is rinsed between each measurement the possibility of carryover should be minimised. Table 12.2 shows a number of commonly used colorimetric assays.

If the wavelength is selected using prisms or gratings, the technique is called spectrophotometry. In both colorimetry and spectrophotometry, the usual procedure is to prepare a set of standards and produce a concentration versus absorbance calibration curve, which is linear because it is a Beer–Lambert plot. Absorbances of unknowns are then measured and the concentration interpolated from the linear region of the plot. Interpolation is critical because:

- one should never extrapolate beyond the region for which any instrument has been calibrated,
- particularly in colorimetry, a phenomenon known as the Job effect (see below) occurs.

If we continue to take measurements beyond the colour reagent limit, it is observed that the linearity of the Beer–Lambert calibration does not continue indefinitely but forms a plateau, at a point which indicates that there is insufficient reagent to produce any more colour. This phenomenon is known as the Job effect. To extrapolate beyond the linear portion of the curve, therefore, would potentially introduce enormous errors. Furthermore, if a particular sample gives a very high absorbance reading, it is incorrect procedure merely to dilute that sample. This achieves nothing as all the materials in the sample are diluted to the same extent. The correct procedure is to return to the original material and dilute that appropriately and then perform all the steps required to produce colour.

If high precision is not required and the absorbances of the test and standard are close in value, the Beer–Lambert linear relation may be assumed for this experiment and an approximate concentration obtained from the simple relationship:

Table 12.2 Common colorimetric assays

Substance	Reagent	Wavelength (nm)
Inorganic phosphate	Ammonium molybdate; H_2SO_4; 1,2,4-amino-naphthol; $NaHSO_3$, Na_2SO_3	600
Amino acids	(a) Ninhydrin	570 (proline 420)
	(b) Cupric salts	620
Peptide bonds	Biuret (alkaline tartrate buffer, cupric salt)	540
Phenols, tyrosine	Folin (phosphomolybdate, phosphotungstate, cupric salt)	660 or 750 (750 more sensitive)
Protein	(a) Folin	660
	(b) Biuret	540
	(c) BCA reagent (bicinchoninic acid)	562
	(d) Coomassie Brilliant Blue	595
Carbohydrate	(a) Phenol, H_2SO_4	Varies, e.g. glucose 490, xylose 480
	(b) Anthrone (anthrone, H_2SO_4)	620 or 625
Reducing sugars	Dinitrosalicylate, alkaline tartrate buffer	540
Pentoses	(a) Bial (orcinol, ethanol, $FeCl_3$, HCl)	665
	(b) Cysteine, H_2SO_4	380–415
Hexoses	(a) Carbazole, ethanol, H_2SO_4	540 or 440
	(b) Cysteine, H_2SO_4	380–415
	(c) Arsenomolybdate	Usually 500–570
Glucose	Glucose oxidase, peroxidase, *o*-dianisidine, phosphate buffer	420
Ketohexose	(a) Resorcinol, thiourea, ethanoic acid, HCl	520
	(b) Carbazole, ethanol, cysteine, H_2SO_4	560
	(c) Diphenylamine, ethanol, ethanoic acid, HCl	635
Hexosamines	Ehrlich (dimethylaminobenzaldehyde, ethanol, HCl)	530
DNA	Diphenylamine	595
RNA	Bial (orcinol, ethanol, $FeCl_3$, HCl)	665
α-Oxo acids	Dinitrophenylhydrazine, Na_2CO_3, ethyl acetate	435
Sterols	Liebermann–Burchardt reagent (acetic anhydride, H_2SO_4, chloroform)	625
Steroid hormones	Liebermann–Burchardt reagent	425
Cholesterol	Cholesterol oxidase, peroxidase, 4-amino-antipyrine, phenol	500

$$\text{concentration} = \frac{\text{test absorbance}}{\text{standard absorbance}} \tag{12.6}$$

Of course, such an assumption for individual experiments is valid only if the Beer–Lambert relationship has been established for that particular reaction on a previous occasion.

It is important to note that, when plotting calibration curves, despite the fact that the Beer–Lambert relationship implies that there is zero absorbance at zero concentration, and that the instrument is physically zeroed, it is wrong to force the drawn line through zero. This would be to give greater credence to this point than any other and assume an unjustified level of precision. The best straight line should be drawn through the points by regression methods (Section 1.6.6).

A further point to note is that the accuracy of the instrument is not uniform throughout the transmission range. The final measurement is an electrical one involving a galvanometer. The maximum accuracy can be shown to occur at 36.8% transmission, and between 20% and 80% the relative error is about $\pm 2\%$. Owing to the nature of galvanometric measurements, the errors at low and high absorbance can be large. This indicates that the analysis should be designed to give absorbance readings in the middle of the range of the transmission scale.

In a colorimeter, the bandwidth of the wavelengths is determined by the filter. A filter that appears red to the human eye is transmitting red light and absorbing almost everything else. This kind of filter would be used to examine blue solutions as they would absorb red light. In general the filter should be of a colour complementary to that of the solution under test.

The arrangement in such an instrument can be very simple, consisting merely of a light source (lamp), filter, cuvette and photosensitive detector to collect the transmitted light. Another detector is required to measure the incident light, or a single detector is used to measure incident and transmitted light alternately. This latter design is both cheaper and analytically better as it eliminates variation between detectors.

The spectrophotometer is a much more sophisticated instrument. A photometer is a device for measuring 'light' and 'spectro' implies the whole range of continuous wavelengths that the light source is capable of producing. The detector in the photometer is generally a photocell in which a sensitive surface receives photons and a current is generated that is proportional to the intensity of the light beam reaching the surface. In instruments for measuring ultraviolet/visible light, two lamps are usually required: one, a tungsten filament lamp, produces wavelengths in the visible regions; the second, a hydrogen or deuterium lamp, is suitable for the ultraviolet. There is a switchover point, usually at 350 nm, although often both lamps are lit all the time that an instrument is in use if both ultraviolet and visible are to be used. The 'switch' in the latter case is then just a mechanical means of directing the appropriate beam along the optical axis, using mirrors or lenses. Mirrors are more frequently used, owing to cheapness and the fact that less light is lost, due to chromatic aberration, in a reflectance than a refraction system. The arrangement may be very simple, as in a colorimeter, but this really defeats the object of the instrument. Fig. 12.6a shows the optical arrangement in a single-beam instrument. Here, first the blank and then the sample must be moved into the beam, adjustments made and readings taken. Fig. 12.6b illustrates the double-beam device. In this arrangement the beam is split into two parts, one passing through the blank, or reference, at the same time as the other part passes through the sample. This approach obviates any problems of variation in light intensity, as both

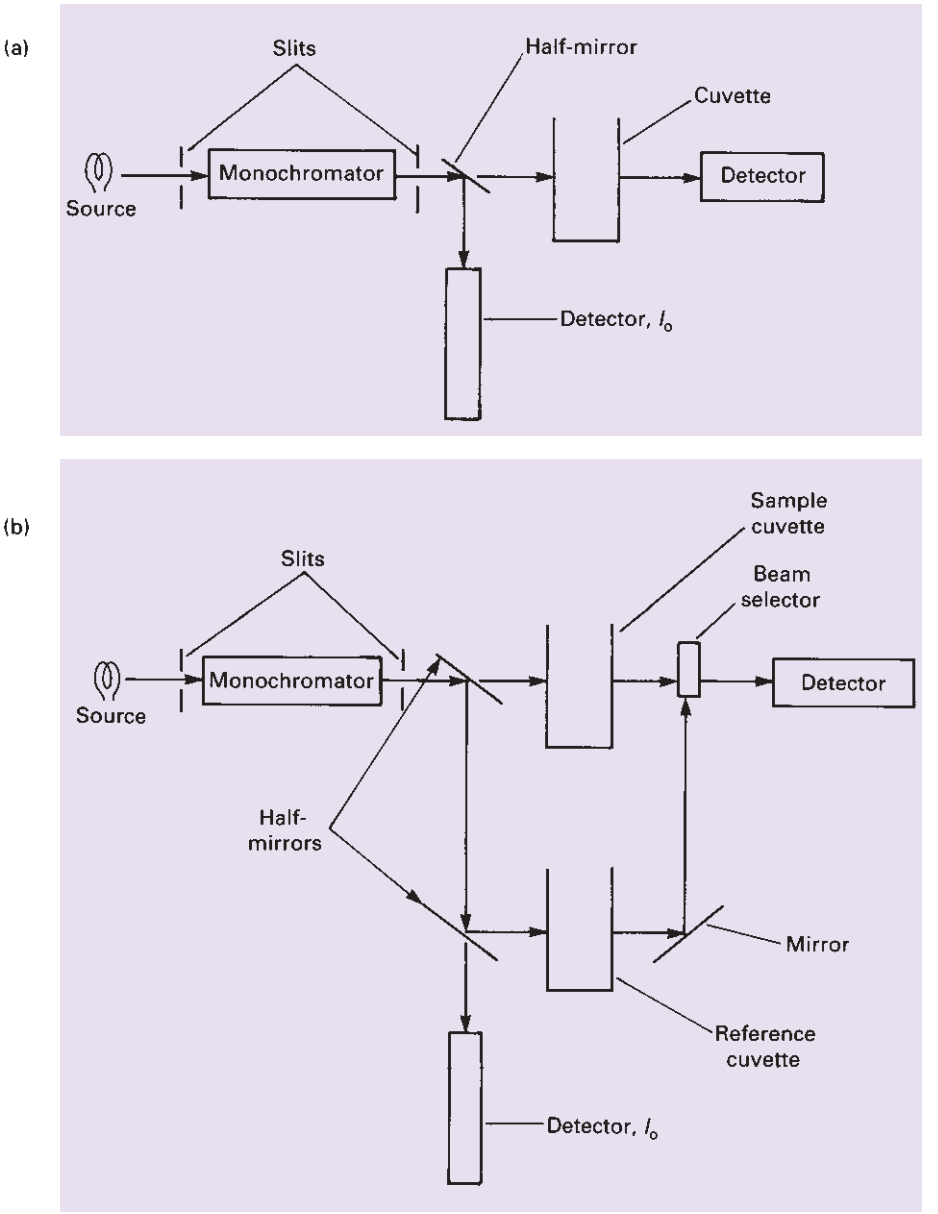

Fig. 12.6. Optical arrangements in (a) a simple single-beam spectrophotometer and (b) a double-beam spectrophotometer.

reference and sample would be affected equally. The resultant measured absorbance is the difference between the two transmitted beams of light recorded by the matched detectors. Multibeam instruments are available that allow the simultaneous recording of absorbance changes at two or more predetermined wavelengths.

The light or radiation emitted from the source lamps covers the whole range of wavelengths that the lamp is capable of producing. In colorimeters, as described above, the filter is used to obtain an appropriate range of wavelengths within the bandwidth it is capable of selecting. In spectrophotometers the bandwidth is selected by the monochromator, which is the optical system used in these devices. Theoretically, these systems select a single wavelength of monochromatic radiation, the emergent light being a parallel beam. The bandwidth here is defined as twice the half-intensity bandwidth, which is the range of wavelengths for which the transmitted intensity is greater than half the intensity of the chosen wavelength and it is a function of the slit width.

The optical systems used are usually either prisms, which split the multiwavelength source radiation into its component parts by the phenomenon known as refraction (an analogy is the natural water-droplet prisms that produce a rainbow), or gratings, which achieve the same thing by diffraction. Refraction occurs because radiation of different wavelengths travels along different paths in the denser medium of the prism material. In order that velocity conservation is maintained overall, a potentially slower-moving wavepacket must travel a shorter distance in a dense medium than does a faster one. Diffraction occurs by reflectance at a surface upon which is engraved a series of fine lines. The distance apart of the lines has to be of the same order of magnitude as the wavelength of the radiation being diffracted. The resolution of wavelengths is greater from gratings than from prisms and, originally, gratings were only available in the most expensive research instruments because they were hand engraved. With the advent of photoreproduction in the semiconductor industry, gratings of high quality can be reproduced in large numbers and hence are now relatively cheap.

The optical slit width affects the bandwidth, and the narrower the slit width the more reproducible are measured absorbance values. In contrast, sensitivity becomes less as the slit narrows, because less radiation travels through to the detector. In the most sophisticated instruments, a high level of control is available to the operator, usually via a computer.

The cuvettes used in either spectrophotometry or colorimetry are an integral part of the system. They should be optically matched for the most precise and accurate work, the optical faces parallel and the pathlengths identical. In flow cells, used in continuous flow systems, the parallelism of the optical faces and the pathlength are less critical because the reference (baseline) solution and the sample both occupy the same cell successively in time. Microcells are available for limited specimens and the extreme of this is the microscale spectophotometer, where a very narrow parallel beam of monochromatic radiation passes through the microcell and then enters a microscope optical system.

The major advantage of the spectrophotometer, however, is the facility to scan the wavelength range over both ultraviolet and visible and obtain absorption spectra. These are plots of absorbance versus wavelength and a typical example is shown in Fig. 12.7. This shows the extent of absorbance (absorption peaks) at various wavelengths for reduced cytochrome *c*. Absorption spectra in the ultraviolet (200–400) and visible (400–700) nm ranges arise owing to the kinds of

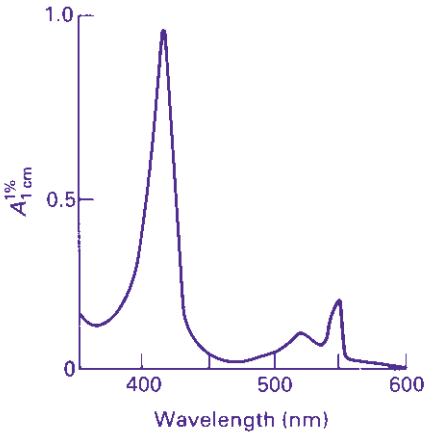

Fig. 12.7. Absolute absoption spectrum of reduced cytochrome *c*.

electron transitions described above (Section 12.1.2), usually the delocalised π-bonding electrons of carbon–carbon double bonds and the lone pairs of nitrogen and oxygen. The wavelengths of light absorbed are determined by the actual electronic transitions occurring and hence specific absorption peaks may be related to known molecular substructures. The term chromophore relates to a specific part of the molecule that independently gives rise to distinct parts of an absorption spectrum. Conjugation of double bonds lowers the energy (lower frequency) required for electronic transitions and hence causes an increase in the wavelength at which a chromophore absorbs. This phenomenon is termed a bathochromic shift. Conversely a decrease in conjugation (e.g. protonation of an aromatic ring nitrogen) causes a hypsochromic shift to lower wavelength. Changes in peak maxima (increase or decrease in absorbance) can also occur. A hyperchromic shift describes an increase and a hypsochromic shift a decrease in absorption maximum.

There are a number of specialised types of spectrophotometer available other than those already mentioned above. Recording spectrophotometers are usually capable of both scanning a predetermined spectrum (the prism or grating angle is changed by a motor-driven system, thereby emitting a continuously changing bandwidth along the optical axis) and monitoring changes at a predetermined wavelength. Although data are commonly recorded on a chart as hard copy, the more sophisticated devices capture and store data in computer systems and in some cases computer control is an option. Variable chart and scanning speeds and absorbance scale expansion are available. It is also possible to incorporate automatic cell changers and measurement at predetermined time intervals for time-dependent changes (e.g. kinetic studies, see Section 15.2.2). Measurement at the temperature of liquid nitrogen (−196 °C) increases the resolution, owing to the reduced thermal motion of the molecules. The absorbance generally increases also as the apparent pathlength is increased, because of internal reflections occurring in the frozen sample. Reflectance instruments measure the radiation absorbed when a light

beam is reflected by the sample, for example pastes and suspensions of microorganisms that are too opaque to transmit the radiation. In such cases, internal reflection and refraction is occurring and hence the true pathlength is unknown; the strict Beer–Lambert law is therefore inapplicable, making quantification difficult. A reference reflecting surface is required and magnesium oxide is frequently used.

12.4.3 Applications

Qualitative and quantitative analysis

Qualitative analysis may be performed in the ultraviolet/visible regions to identify certain classes of compound both in the pure state and in biological mixtures, for example proteins, nucleic acids, cytochromes and chlorophylls. The technique may also be used to indicate chemical structures and intermediates occurring in a system. The most precise analysis, however, is obtained by infrared methods.

Quantitative analysis may be performed by making use of the fact that certain chromophores, for example the aromatic amino acids in proteins and the heterocyclic bases in nucleic acids, absorb at specific wavelengths. Proteins may be measured at 280 nm and nucleic acids at 260 nm, although corrections are usually necessary to account for interfering substances. Such corrections commonly require the measurement of the absorbance, by the interfering substance, at a wavelength remote from that for the compound under test, plus a knowledge of the absorbance at the test wavelength. If the ratio of the absorbances of the interfering substance is known for the remote and test wavelengths then the correction is simple, for example (Section 8.3.2) the $A_{280/260}$ ratio for proteins in the presence of nucleic acid. More sophisticated algebraic techniques are available for the more complicated cases, for example R. A. Morton's and D. W. Stubbs' correction for the amount of vitamin A in saponified oils.

The amounts of substances with overlapping spectra, such as chlorophylls *a* and *b* in diethylether may be estimated if their extinction coefficients are known at two different wavelengths. For n components absorbance data are required at n wavelengths.

A phenomenon known as Rayleigh light scattering (Section 12.7) occurs with moderate concentrations of some biological macromolecules (e.g. large DNA fragments) measured at 260 nm. This introduces an interference leading to error but may be accounted for by measuring the scattering in a region of the spectrum where DNA does not absorb, for example at 330–430 nm.

Difference spectra

A difference spectrum is the difference between two absorption spectra. There are essentially two ways in which difference spectra may be obtained: first, indirectly, by subtraction of one absolute spectrum from another (Fig. 12.8a); secondly, directly, by placing one compound in the reference cell and the other in the test cuvette (Fig. 12.8b). Fig. 12.8a shows the two absolute spectra of ubiquinone and ubiquinol and differences in absorbance may be calculated at wavelength points

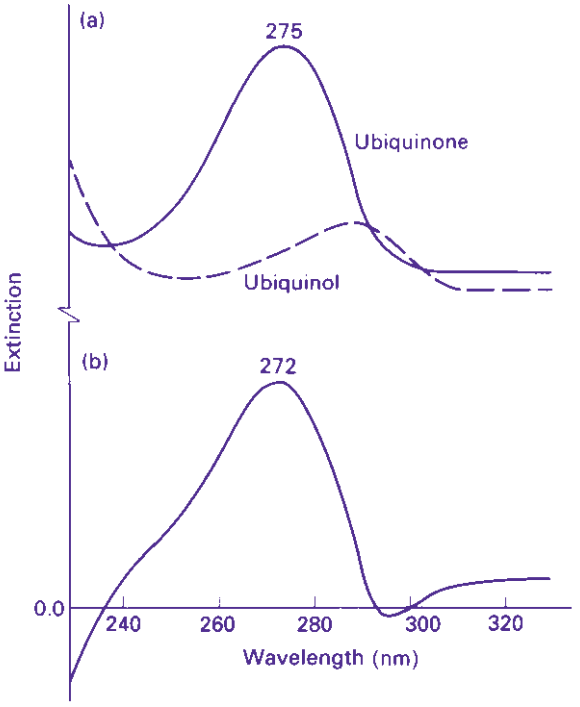

Fig. 12.8. (a) Absolute and (b) difference spectra of ubiquinone and ubiquinol.

with suitable regular intervals between them. The resultant absorbance values may then be plotted at the same wavelength points. Fig. 12.8b shows this difference spectrum, which is obviously the same, although obtained in a different manner. Difference spectrophotometry has the advantage of enabling the detection of small absorbance changes in a system with a high background absorbance. An example of this kind of investigation is the measurement of changes in the oxidation state of components of the respiratory chain in intact mitochondria and chloroplasts.

The following important observations should be made:

- Difference spectra may contain negative absorbance values.
- Both absorption maxima and minima may be displaced and extinction coefficients are different from those of absolute absorption peaks.
- There are points of zero absorbance in the difference spectrum, equivalent to those wavelengths where both the reduced and oxidised forms of the compound exhibit identical absorbances (isobestic points) and which may be used for checking for the presence of interfering substances.

A more complex example is that of the cytochrome a_3–CO complex minus cytochrome a_3, the difference spectrum being obtained by using anaerobic bacteria in the reference cuvette and the same system complexed with CO in the sample or

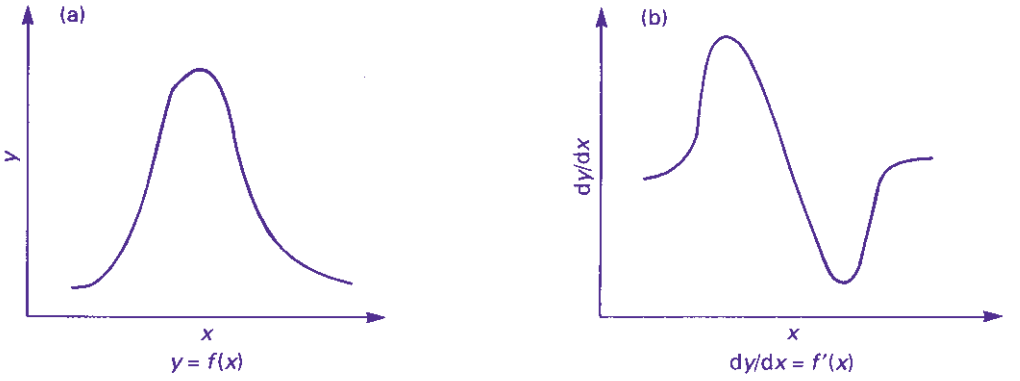

Fig. 12.9. First-differential spectra.

test cuvette. Cytochrome a_3 is the terminal electron carrier and is the only component in the system that reacts with carbon monoxide.

Frequently the term difference spectrum refers specifically to the absolute reduced spectrum minus the absolute oxidised spectrum, for example the difference spectrum of cytochrome *c* corresponds to the cytochrome c_{red} minus cytochrome c_{ox} difference spectrum. A similar difference spectrum may be obtained for a suspension of mitochondria using the so-called reversal technique. This involves measuring the change in absorbance at each wavelength when the preparation passes from the aerobic to the anaerobic state. The resultant spectrum obtained is a combined difference spectrum, for the cytochromes *a*, a_3, *b*, *c*, c_1 and NAD^+ and flavoprotein. Shoulders on peaks observed in difference spectra obtained at room temperature may be resolved into distinct peaks at −196 °C by measuring low temperature difference spectra.

An alternative to low temperature studies of unresolved absorption spectra (difference or absolute) is a purely mathematical one and is termed differential spectroscopy. If the algebraic relationship that governs the shape of a symmetrical peak is known then it may be differentiated and the differential plotted against the original variable. An ideal example is shown in Fig. 12.9a,b.

Almost always the algebraic relationship is unknown. However, the results may be readily obtained by digital computer techniques by sampling the curve at small intervals of the *x*-axis (wavelength). This process gives $\Delta y/\Delta x$ or $\Delta abs/\Delta\lambda$. If the $\Delta\lambda$ intervals were infinitesimally small, the limiting value would be dy/dx. Furthermore, higher order differential spectra may be obtained by feeding the data back to the processor chip as many times as are required. The value of higher-order calculations is in many cases dubious but second-order differential spectra (d^2y/dx^2) solve a number of otherwise intractable problems and instruments are commercially available, with the facility for making the calculations. The binding of a monoclonal antibody to its antigen may be monitored using second-order differential spectroscopy.

Binding spectra

Binding spectra or substrate binding spectra may be used to study the extent of interaction between an enzyme and its substrate. The binding of a substrate to a haem group containing a ferric ion in the high spin state perturbs the spectrum by displacing the ligand water from the sixth position of the ferric ion, causing it to change to the low spin state. The process may be followed spectrophotometrically. An example of this is the binding of a drug (substrate) to liver microsomal mono-oxygenase (mixed function oxidase), which causes a blue shift of the cytochrome P450 component of the enzyme from 420 nm to 390 nm (a hypsochromic shift).

Valuable structural studies may be performed on some particular biological macromolecules such as proteins and nucleic acids. In proteins, the spectrum of a chromophore depends largely on the polarity of the microenvironment. A change in the polarity of a solvent in which the protein is dissolved changes the spectrum of a particular amino acid chromophore without changing the conformation of the protein. This phenomenon is known as solvent perturbation and obviously, to be accessible to the solvent, the amino acid residue must be on the surface of the protein. Solvents or solutions miscible with water must be used and examples are dimethylsulphoxide, dioxane, glycerol, mannitol, sucrose and polyethylene glycol.

The aromatic amino acids are powerful chromophores in the ultraviolet. Processes such as denaturation (unfolding) of a polypeptide chain by pH, temperature and ionic strength can be monitored as more of these residues become exposed to the incident radiation.

Many other processes may be followed, particularly if the amino acid residue tyrosine is involved, for example protein–protein binding, protein–metal or protein–small molecule interactions. The range may be extended by the use of reporter group techniques in which an artificial chromophore is attached to the appropriate region of the protein.

In nucleic acid studies, solvent perturbation may be used to estimate the number of unpaired bases in RNA. If normal water is replaced by 50% ^2H_2O as solvent the ^2H_2O only changes the spectral components due to unpaired nucleotides. Also the denaturation of the helical structure of DNA in solution may be investigated when the double-stranded DNA is heated through its melting temperature (Section 5.2.3). The extinction at 260 nm increases (hyperchromic shift) on denaturation and decreases again (hypochromic shift) on renaturation, which occurs on cooling. Effects on the secondary structure of DNA by pH and ionic strength may be studied in a similar way.

Action spectra

In certain situations an action spectrum may be shown as a plot of a physiological (non-extinction) parameter against wavelength. In many complex biological systems such a spectrum often corresponds to the absorption spectrum of a single key compound. An example is the plotting of the rate of oxygen evolution by green plant tissue against the wavelength of light used to irradiate the system. This results in a graph similar to the spectrum of the chlorophylls.

Example 2 CALCULATION OF CONCENTRATION AND ABSORBANCE

Question

If a solution containing ATP is found to have an absorbance of 0.17 in a 1 cm cuvette and the molar extinction coefficient is 1.54×10^4 (mol dm^{-3})$^{-1}$ cm^{-1}, what is

(i) the concentration of ATP solution,
(ii) the transmission of the solution in a 1 cm cuvette and
(iii) the absorbance of a 2.5×10^{-2} mM solution of ATP in a 4 cm cuvette?

Answer

(i) The concentration is found by the direct application of the Beer–Lambert Law.

$$A = \log\left(\frac{I_0}{I_t}\right) = \epsilon_\lambda c\ell$$

$$0.17 = 1.54 \times 10^4 \times c \times 1$$

Therefore,

$$c = [\text{ATP}] = \frac{0.17}{1.54 \times 10^4 \times 1} = 1.104 \times 10^{-5}\ \text{M}$$

(ii) $$A = \log\left(\frac{I_0}{I_t}\right) = \log\left(\frac{1}{T}\right)$$

Therefore,

$$0.17 = \log\left(\frac{1}{T}\right)$$

$$\frac{1}{T} = \text{antilog}\,(0.17) = 1.4791$$

and $T = 0.676$ or 67.6%

(iii) As we have a value of the molar extinction coefficient we must convert the given concentration to mol dm^{-3} (M).

Hence
2.5×10^{-2} mmol dm^{-3} $= 2.5 \times 10^{-5}$ M

Then
absorbance $= A = \epsilon_m \times c \times \ell$

or
$A = 1.54 \times 10^4 \times 2.5 \times 10^{-5} \times 4 = 1.54$

As an aside, it is worth recalling that absorbance (because it is a logarithm) is unitless by definition. This is clearly demonstrated by performing 'quantity algebra' on the units of each term.

e.g. ϵ_λ (mol dm^{-3})$^{-1}$ cm^{-1}

c (mol dm^{-3})$^{-1}$ M

ℓ cm

Hence,
$\epsilon_\lambda \times c \times \ell \equiv (\text{mol dm}^{-3})^{-1}\,\text{cm}^{-1} \times (\text{mol dm}^{-3})^{-1} \times \text{cm}$
and all units cancel.

12.5 SPECTROFLUORIMETRY

12.5.1 Principles

Fluorescence is an emission phenomenon, the energy transition from a higher to lower state within the molecule concerned being measured by the detection of this emitted radiation rather than the absorption. In order for the transition from higher to lower states to occur, an earlier excitation event, for example caused by absorption of electromagnetic radiation, must have taken place. The wavelength(s) of absorbed radiation must be at lower values (higher energy) than the emitted (fluoresced) wavelength. The difference between these two wavelengths is known as the Stokes shift and in general the best results are obtained from compounds involving large shifts. It is possible for a compound to absorb (be excited) in the ultraviolet region and emit or fluoresce in the visible.

In Fig. 12.2b an example of the various permissible energy levels is shown. Most electrons will occupy the ground state S_0V_0 at room temperature. Elevation to a higher energy level, S_1, S_2, etc., may be achieved by absorption of electromagnetic energy (photons) in less than 10^{-15} s. Energy may be lost very rapidly (as heat) by collision degradation, resulting in minimal vibrational energy in the lowest excited state, S_1V_0. Electrons in this state return to the ground state in less than 10^{-8} s, the emitted energy being manifested as fluorescence. Many organic molecules absorb in the ultraviolet/visible regions but do not fluoresce. Fortunately, of those that do, many are of biological interest. Also, although a knowledge of the structure of an organic molecule may allow predictions about its absorption spectrum, this is not true with fluorescence. Aliphatic molecules, which are usually flexible, tend to photodissociate rather than fluoresce, whereas aromatic compounds with delocalised π-electrons sometimes fluoresce.

The emitted radiation appears as band spectra because there are many closely related values (for the wavelengths) dependent upon the final vibrational and rotational energy levels attained. These band spectra are usually independent of the wavelength of the exciting radiation and have a mirror image relationship with the absorption peak with the greatest wavelength.

An associated phenomenon is phosphorescence, but this emission has long decay times and usually persists when the exciting energy is no longer applied. Phosphorescence arises as a result of intersystem crossing to the lowest triplet state. This light emission usually occurs at longer wavelengths than does fluorescence.

Fluorescence spectra give information about events that occur in less than 10^{-8} s. The ratio:

$$Q = \frac{\text{quanta fluoresced}}{\text{quanta absorbed}} \tag{12.7}$$

gives Q as the quantum efficiency and is usually independent of the exciting wavelength. At low concentrations, the intensity of fluorescence (I_f) is related to the intensity of the incident radiation (I_0) by:

$$I_f = 2.3 I_0 \epsilon_\lambda \, cdQ, \qquad \text{i.e. } I_f \propto c \tag{12.8}$$

where c is the concentration of the fluorescing solution (molar), d is the light path in fluorescing solution (cm), and ϵ_λ is the molar extinction coefficient for the absorbing material at wavelength λ ($dm^3\,mol^{-1}\,cm^{-1}$).

The technique of spectrofluorimetry is most accurate at very low concentrations, whereas absorption spectrophotometry is least accurate at these concentrations. For example, 100 pg of catecholamines or NADH may be measured fluorimetrically, whereas absorption spectrophotometry requires 100 μg each of the catecholamines serotonin and adrenaline. This is due to increased sensitivity, which is easily adjustable over a large range by amplification of the detector signal. The technique allows great spectral selectivity because, owing to the Stokes shift, two monochromators may be used, one for the exciting wavelength and the other for the emitted fluorescence. Although no reference cuvette is required, a calibration curve must be obtained.

Susceptibility to pH, temperature, solvent polarity and the inability to predict whether a particular compound will fluoresce, are disadvantages but the major one is the phenomenon of quenching. This occurs because energy that might have been emitted as fluorescence is lost to other molecules by collisional interaction. This partly explains the increased sensitivity and accuracy in low concentrations because there are fewer molecules, and hence collisions, although the effects of solvent must not be neglected. Many materials such as detergents, stopcock grease, filter paper and some tissues may cause interference by the release of fluorescing agents.

12.5.2 Instrumentation

The direct relationship between fluorescence intensity and concentration allows relatively simple electronics and optics to be used. Two monochromators may be employed, the first (M_1) for selecting the excitation wavelength. Fluorescence emission occurs in all possible directions and one direction (90°) is chosen and the second monochromator (M_2) is used for determination of the fluorescence spectrum. The radiation source is generally either a mercury lamp or a xenon arc, excitation wavelengths frequently being selected in the ultraviolet region and the emission wavelengths in the visible region. The detector is usually a sensitive photocell, for example a red-sensitive photomultiplier for wavelengths greater than 500 nm. Temperature control is required for accurate work as the intensity of fluorescence may vary between 10% and 50% for a 10 deg.C change at approximately 25 °C.

Two approaches are possible for the illumination of the sample: the simplest is the basic 90° illumination (Fig. 12.10), the alternative approach being front-face

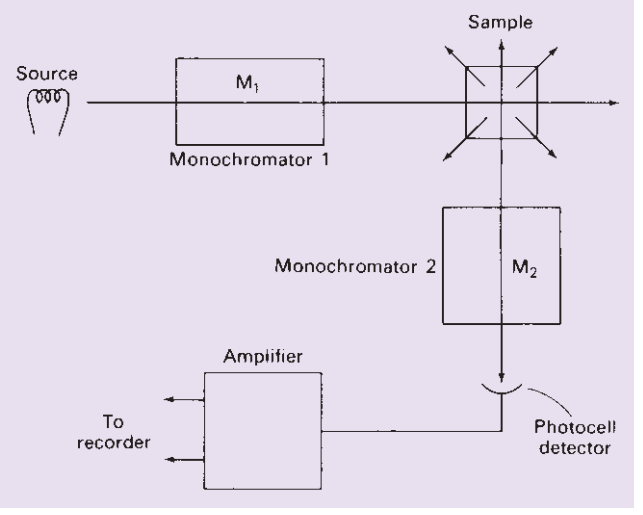

Fig. 12.10. The basic component of a spectrofluorimeter set up for 90° illumination.

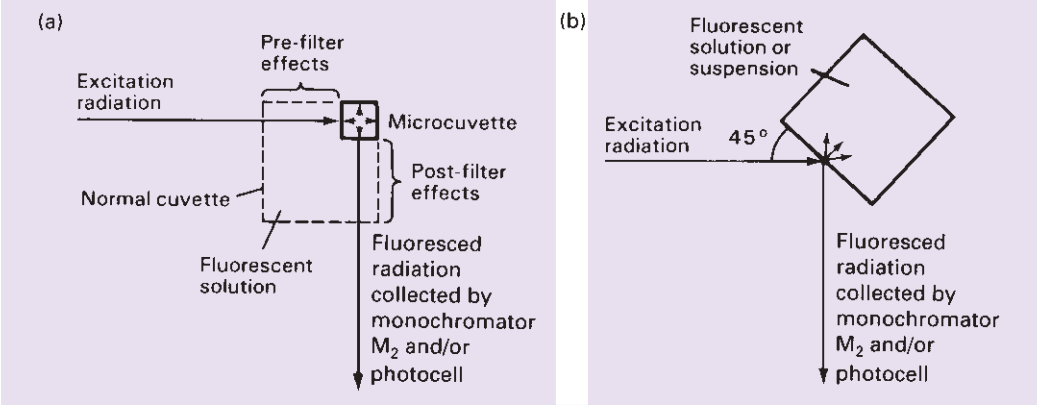

Fig. 12.11. Reduction of filter effects using (a) microcuvettes and (b) front-face illumination.

illumination (FFI; Fig. 12.11), which obviates pre- and postfilter effects. These latter effects arise owing to the absorption of radiation prior to it reaching the fluorescent molecules (prefilter absorption) and the reduction in the amount of emitted radiation escaping from the cuvette (postfilter effects). Such effects are more evident in concentrated solutions, and the use of microcuvettes (containing less material) can be of value (Fig. 12.11a). FFI is essential for examining suspensions, and cuvettes with only one optical face are required. Excitation and emission occur at the same face but generally the technique is somewhat less sensitive than 90° illumination.

12.5.3 Applications

Fluorescent probes

Applications of the technique are many and varied, despite the fact that relatively few compounds exhibit the phenomenon. A compound may have its fluorescence and absorption spectra compared as an aid to identification; the effects of pH, solvent composition and the polarisation of fluorescence may all contribute to structural elucidation. The measurement of phosphorescence and phosphorescence lifetimes can also be of value in compound identification.

The detection of non-fluorescent compounds may be achieved by coupling a fluorescent probe (or fluor) in a similar way to the use of reporter groups in absorption spectrophotometry (Section 12.4.3). This is termed extrinsic fluorescence as distinct from intrinsic fluorescence, where the native compound exhibits the property due to the presence of aromatic groups in amino acid side-chains in the case of proteins. The use of such probes is valuable in both qualitative and quantitative analysis. For instance, amino acids and peptides separated by chromatography or electrophoresis may be identified by coupling to their primary amino groups either dansyl chloride or *o*-phthalaldehyde (Section 8.4.2). The latter conjugates fluoresce intensely blue and the total oligopeptide fingerprint may be determined on only 10^{-5} g of protein. If the separation methods are used in column form, then quantification is possible by forming derivatives post-column. Acridine orange is an extrinsic fluor that can be used to determine the strandedness of polynucleotides as the Stokes shifts differ between conjugates of single- and double-stranded polynucleotides, which fluoresce red and green, respectively. The fluor should be tightly bound at a specific site, its fluorescence should be sensitive to environmental changes and it should not have adverse effects on the system being studied. Some structures of fluorescent probes are shown in Fig. 12.12.

The major use of fluorimetry in biochemistry is in quantitative determination of materials present in concentrations too low for absorption spectrophotometry. Assays of vitamin B_1 in foodstuffs, NADH, hormones, drugs, pesticides, carcinogens, chlorophyll, cholesterol, porphyrins and some metal ions indicate the range. Self- and contaminant quenching can be determined by adding a known quantity of a standard to an unknown quantity of a pure compound and measuring the fluorescence before and after the addition.

Ca^{2+} may be measured in the cytoplasm by the chelating agent Quin-2, which preferentially binds the metal. The fluorescence increases about five-fold on binding. More sensitive probes for this analysis are Fura-2 and Indo-1. Quin-1 is a chelating agent that may be used as a fluorescent probe to monitor intracellular pH changes in the range 5–9. Over this range, there is a 30-fold increase in fluorescence.

Enzyme assays and kinetic analysis

The general principles of enzyme assays, which are discussed in Section 15.2.2, often rely on the use of spectrofluorimetric assays. An example is the anion of

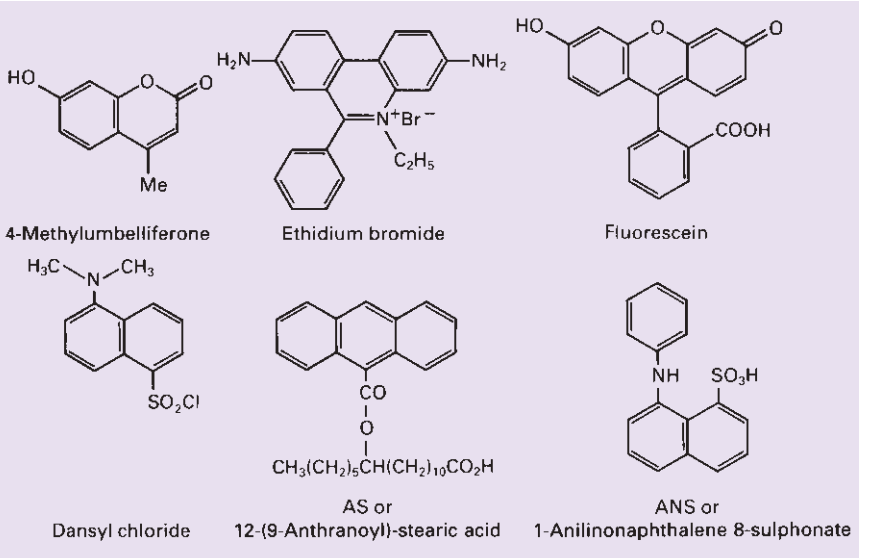

Fig. 12.12. Structure of some fluorescent probes.

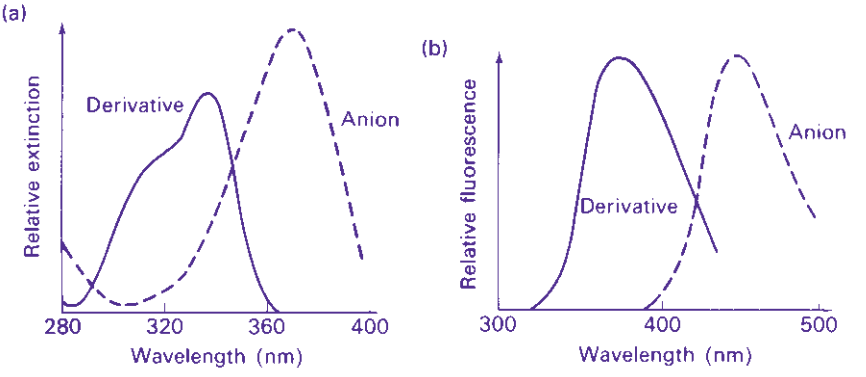

Fig. 12.13. Spectra of the methylumbelliferone anion and derivatives of 4-methylumbelliferone at pH 10: (a) absorption spectra; (b) fluorescence spectra.

4-methylumbelliferone, which fluoresces at 450 nm. Its rate of appearance may be monitored when it is produced as a result of enzymic action on an ether or ester derivative of the fluor. The enzymes used are group-specific hydrolases and their kinetics may be studied by fluorescence measurement; Fig. 12.13a,b shows typical absorption and fluorescence spectra. Irradiation is usually at 350–400 nm wavelength and virtually all the fluorescence measured between 450 nm and 500 nm is due to the anion product. It is claimed that one molecule of β-galactosidase may be detected when it acts on fluorescein bis(β-D-galactopyranoside) as substrate because the sensitivity of the method is so great. Hence actual numbers of molecules in a single bacterial cell may be determined, as may the synthesis of the enzyme in individual cells in a population.

Spectrofluorimetry can be applied widely in metabolic studies where NAD^+ forms are involved as cofactors. This arises because NADH and NADPH fluoresce, whereas the oxidised equivalents do not. Therefore redox processses may be followed kinetically *in vitro* at concentrations similar to those encountered *in vivo*, and also followed in intact cells or organelles (e.g. mitochondria).

Protein structure

The presence of tryptophan and FAD as cofactors allows proteins to exhibit intrinsic fluorescence. The binding and release of cofactors, inhibitors, substrates etc., at sites close to the fluor, cause changes in the associated fluorescence spectra and as a consequence information about conformational changes, denaturation and aggregation may be gleaned. The absence of an intrinsic fluor can be overcome by coupling a suitable extrinsic fluor such as anilino-napthalene 8-sulphonate (ANS), dansyl chloride and derivatives of fluorescein or rhodamine. A recent development of the use of extrinsic fluors has been the use of the green fluorescent protein (GFP) of *Aequorea victoria* as the fluor. GFP has an intrinsic strong green fluorescence that requires no additional cofactors. By genetic engineering techniques, chimeras of GFP and the test protein can be produced without altering the normal functioning of the latter. Ligand-induced changes in the conformation of the test protein will be reflected in the fluorescence of the GFP. Similar studies can be carried out using the red fluorescent protein isolated from *Discosoma striata* (see Section 16.3.2).

Membrane structure

The fluorescent properties of a molecule are affected by its mobility and environment, particularly the polarity of the latter. These effects in the vicinity of a fluorescent probe may be monitored by measuring changes in fluorescence. Various probes having charged and hydrophobic regions (ANS and *N*-methyl-2-anilino-6-naphthalene sulphonate (MNS)) and hence able to orient themselves across lipid/aqueous interfaces may be used to study membrane structure and gain information about the properties of such interfaces. Incorporation of phospholipids containing 12-(9-anthroanoyl)-stearic acid and 2-(9-anthroanoyl)-palmitic acid into membranes yields information about the regions 0.5 nm and 1.5 nm, respectively, from the phosphate head groups of the lipid bilayer. The basic membrane structure and also the effects of temperature and certain biological phenomena may be studied. Changes in mitochondrial membranes during energy transduction have also been monitored using an ANS probe.

Fluorescence recovery after bleaching (FRAP)

If a fluor is exposed to a pulse of high intensity radiation it may be irreversibly bleached, i.e. permanently lose its ability to fluoresce. Fluorescently labelled phospholipids incorporated into a biological membrane may be subjected to this treatment and then the motion of such entities (in the membrane) can be studied by monitoring (with low intensity radiation) the re-emergence of fluorescence as the bleached and unbleached molecules interdiffuse. Applications include the lateral

motion of extrinsically labelled rhodopsin in the photoreceptor membrane, the study of polymerisation of proteins such as actin and the diffusion of fluorescently labelled proteins microinjected into cells (see also Section 16.3.3).

Fluorescence resonance energy transfer

In a number of cases energy may be transferred, by fluorescence resonance energy transfer (FRET), from a donor to an acceptor fluor, provided there is overlap between the donor fluorescence spectrum and the acceptor absorption spectrum. The fluors must also be closely situated and transfer efficiency is related to spatial separation. This efficiency may be measured either as quenching of the donor fluorescence by acceptor or as the intensities of fluorescence of acceptor when the latter is irradiated both in the presence and in the absence of the donor.

Intrinsic fluors such as tryptophan or extrinsic ones attached to amino acids, -SH groups, sugars or fluorescent analogues of substrates, inhibitors, cofactors or phospholipids may be employed in energy transfer experiments to deduce distances within protein molecules. Accuracy is limited to about ± 0.5 nm and determinations include the localisation of metals in metalloproteins, the measurement of the extent of conformational changes in enzymes and receptors when substrate or ligand binding occurs (Section 16.3.2), the distances between various pairs of proteins in the ribosome and the three-dimensional structure of transfer RNAs.

Fluorescence polarisation and depolarisation

The excitation wavelengths used may be polarised by introducing a suitable polariser between the first monochromator (M_1) and the sample. The emitted radiation may be totally unpolarised or partially polarised and may be detected by using a second polariser between the emission monochromator (M_2) and the detector.

Molecular rotations affect fluorescence depolarisation: for instance, the rotation of an absorber chromophore and energy transfer between chromophores increase the depolarisation effect. High concentrations of chromophore and high viscosity of the solvent result in the measurement of mainly energy transfer. At low concentrations and low viscosity the effects of molecular motion predominate.

The mobility of whole molecules, or parts thereof, may be investigated using this technique. The lifetimes of intrinsic fluors of proteins and nucleic acids are usually too short, as these biological macromolecules move relatively slowly. Hence extrinsic fluors are frequently used in these studies. Examples of such studies include the binding of fluorescent substrates, the binding of inhibitors and cofactors to enzymes and receptors (Section 16.3.2), reduction of mobility (increase of overall mass and hence inertia); and the antigen/antibody complexation reaction. The association and dissociation of multisubunit proteins, such as lactate dehydrogenase and chymotrypsin, and the viscosity of living cells may also be measured.

An interesting historical aside involves the use of highly viscous glycerol to slow down the rotation and translation of large molecules in depolarisation

experiments. It was knowledge of this totally unconnected fact that gave the clue to the use of glycerol as a matrix in fast atom bombardment mass spectrometry.

Microspectrofluorimetry

In this technique a microscope is combined with a spectrofluorimeter equipped with fibre optics to enable the examination of single bacterial cells binding fluorescent antibodies and also the fluorescent intensity of subcellular structures. The extra amount of nucleic acid that tends to be present in malignant cells will take up more of the fluorescent probe acridine orange than do normal cells. This observation may be used to detect malignant cells in biopsy tissue.

The fluorescence-activated cell sorter

This system, described in Section 7.8.5, makes use of the light emitted by cells carrying a fluorescently labelled antibody to trigger their physical separation from unlabelled cells as they flow through a fine capillary (Section 16.3.2).

Fluorescence immunoassay

These methods are dealt with extensively in Section 7.7.6 but are worthy of a brief mention here.

Several immunoassays have been developed using fluorescent probes to label either antigen or antibody. The binding of a labelled hapten by an antibody may alter the intensity of fluorescence, thus enabling the complex formation to be monitored. Changes in polarisation methods applied to immunoassay have been mentioned above. A major disadvantage of either of these approaches is the high background fluorescence that often accompanies the process and interferes with the measurement. The most promising development in this area is time-resolved fluorescence immunoassay. Two approaches have been combined to reduce the effects of background fluorescence and hence increase the sensitivity. First, europium chelates are usually used as the fluor, as they have large Stokes shifts and long-lived fluorescence. Secondly, a fluorimeter has been designed that delays the measurement of the emitted light by 400 μs, during which time the non-specific background fluorescence has almost completely decayed. Such an approach has led to the development of dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA).

Multicomponent analysis by synchronous luminescence spectrometry

Despite its name this is really a fluorescence technique and allows the simultaneous analysis of multicomponent mixtures without the need to resort to the use of rather complicated algorithms and sophisticated computer techniques. For this reason it is included in this section.

In conventional luminescence spectrometry an emission spectrum is monitored by scanning the emission wavelength λ_{em} whilst the luminescent (fluorescent) compound is excited at a fixed excitation wavelength λ_{exc}. Conversely an excitation spectrum is obtained by scanning λ_{exc} whilst the emission is monitored at a fixed λ_{em}.

A combined method involves scanning both λ_{em} and λ_{exc} together, i.e. varying both simultaneously or synchronously. This is feasible despite the loss of constant excitation energy employed in the conventional method.

The luminescence intensity, I_s, is obtained from equation 12.9, the right-hand side of which is derived from the Beer–Lambert law:

$$I_s = KcdE_X(\lambda - \Delta\lambda)E_M(\lambda) \tag{12.9}$$

where K is an aggregate constant; c is concentration of analyte; d is the optical pathlength; λ is the emission wavelength; $E_M(\lambda)$ is the emission spectrum; $E_X(\lambda - \Delta\lambda)$ is related to the experimentally determined exitation spectrum, which involves excitation at a wavelength λ'. It is a specific requirement of this technique that the difference between excitation and emission wavelengths remains fixed, i.e. $\Delta\lambda = \lambda - \lambda'$. Hence $\lambda' = \lambda - \Delta\lambda$ in the expression above. It is often convenient in practice to choose $\Delta\lambda$ to be the same as the Stokes shift.

A number of advantages of the method are that complex spectra may be reduced to a single peak, spectral bands are generally narrowed, a spectral range is produced (this is of particular advantage to the analytical scientist as against the requirements of the purist spectroscopist); and spectra of multicomponent systems may be simplified. In addition, multiplicity of scan rates for both monochromators allows for considerable variability in the operation of the technique.

Amongst the applications of the technique is the measurement of the fluorescence associated with benzo(a)pyrene (BP) molecules covalently attached to nucleic acids, both DNA and RNA, isolated from the epidermis of BP-treated mice. The measurements were made at 77 K in frozen aqueous solutions by use of a photon-counting fluorimeter operating in synchronous scanning mode. A $\Delta\lambda$ of 28 nm was chosen, which corresponds to the Stokes shift for the fluorescence of bound BP. Other applications have involved the measurement of the carcinogenic polycyclic hydrocarbon dibenz(a,h)anthracene in extracts of cigarette smoke, resolution of tyrosine and tryptophan in proteins and polypeptides, and quantitative determination of hallucinogens (e.g. LSD). Derivative spectra may also be obtained, enabling the resolution of phenylalanine, tyrosine and tryptophan in admixture, protein mixtures, and catecholamines. The method offers potential for applications in clinical analysis.

12.6 CIRCULAR DICHROISM SPECTROSCOPY

12.6.1 Principles

It has been known for some time that optical isomers (isomers whose mirror images are non-superimposable) possess the property of allowing the rotation of plane-polarised light. Electromagnetic radiation oscillates in all possible directions and it is possible to select preferentially waves oscillating in a single plane. This is achieved using a polarising material such as Polaroid or a nicol prism. The technique of polarimetry essentially measures the angle through which the plane of polarisation is changed after such light is passed through a solution containing

Example 3 FLUORIMETRIC ASSAYS AND QUENCHING

Question

A metabolite M was isolated from cerebrospinal fluid (CSF). After excitation at $\lambda_1 = 280$ nm the material fluoresced at $\lambda_2 = 360$ nm.

Using a standard spectrofluorimeter:

(i) the instrument scale was set to zero with solvent (e.g. buffer) and the 100% mark using a pure sample of M as standard (conc. 100 ng/100 cm^{-3}),
(ii) a blank was measured on a solution containing all the components except M and gave a reading of 11.2%,
(iii) an extract including M gave a total fluorescence measurement of 67%,
(iv) an overall fluorescence reading of 92% was observed when the extract above had an amount of pure M added, as internal standard, to give an equivalent concentration of 1 $\mu g\ dm^{-3}$. (Note this concentration is equivalent to 100 ng 100 cm^{-3}.)

Calculate the concentration of M in the sample of CSF in $\mu g\ dm^{-3}$ and the proportion of quenching, if any. Also state the Stokes shift for the assay.

Answer

The best way to proceed is to define the individual intensities.

Let I_s be the 100% value due to the standard.
Let I_b be the 11.2% value due to the blank.
Let I_t be the 67% value due to the total fluorescence.
Let I_f be the 92% value due to the overall fluorescence when sample is 'spiked' with internal standard.
Let I_u be the 55.8% value due to the blank correction $I_t - I_b\,(67 - 11.2)$.
Let I_{as} be the 25% value due to the 'quenched' internal standard $I_f - I_t\,(92 - 67)$.

Then

$$\frac{\text{assay fluorescence of unknown}}{\text{assay fluorescence of int. std}} = \frac{\text{amount of unknown}}{\text{amount of int. std}}$$

$$\frac{I_u}{I_{as}} = \frac{\text{amount of unknown}}{1\ \mu g\ dm^{-3}}$$

$$\text{amount of unknown} = \frac{I_u \times 1\ \mu g\ dm^{-3}}{I_{as}} = \frac{55.8}{25} = 2.2\ \mu g\ dm^{-3}$$

The degree of quenching is found as follows:

pure standard gives $I_s = 100\%$
equivalent amount of standard in assay $I_{as} = 25\%$

Hence, quenching,

$$Q = 100 \times \left(\frac{100 - 25}{100}\right) = 75\%$$

The value of the Stokes shift is the difference between the emission and excitation wavelengths,

$\Delta\lambda = \lambda_2 - \lambda_1 = 360 - 280 = 80$ nm

a chiral (optically active) substance. Optical rotary dispersion (ORD) spectroscopy is a technique for measuring this ability to rotate the plane of polarisation, as a function of the wavelength. However, such chiral substances may also absorb the plane-polarised radiation at certain wavelengths. In such cases the chromophore is termed an optically active chromophore or chiral centre, as it may only be part of a complex molecule. The technique of ORD has been largely supplanted by circular dichroism (CD) spectroscopy, which gives rather better information about the three-dimensional structure of macromolecules containing chiral centres. In CD, circularly polarised light is used and this is obtained by superimposing two plane-polarised light waves of the same wavelengths and amplitudes but differing in phase by one quarter of a wavelength and in their planes of polarisation by 90°. Just as plane-polarised light may be left (L) or right (R) handed, so can circularly polarised light. Whether R or L circularly polarised light is produced depends on the relative positions of the peaks of the two plane-polarised waves.

The asymmetry inherent in the structure of chiral molecules or centres interacts differently with polarised light. Not only are the R and L waves of plane-polarised light differentially absorbed and refracted, resulting in a beam in a different plane (the basis of polarimetry), but in the case of circularly polarised light a similar differential interaction occurs. In the latter case, the resultant beam, after having passed through the sample, is a recombination of the R and L components to give an emergent beam of elliptically polarised light. In polarimetry the specific rotation $[\alpha]_\lambda$ would be measured, whereas in CD spectroscopy it is the ellipticity, θ, which is measured:

$$\begin{aligned}\theta &= 2.303\,\Delta A \\ &= 33\,\Delta A \text{ degrees}\end{aligned} \qquad (12.10)$$

where ΔA is the difference in absorption between R and L components.

A CD spectrum is usually a plot of ellipticity versus wavelength and information regarding the structure of certain entities may be gleaned from it.

12.6.2 Instrumentation

The basic layout of a CD spectrometer is shown in Fig. 12.14. Both L and R circularly polarised light may be produced alternately, from a single monochromator, by the passage of plane-polarised light through an electrooptic modulator. This modulator is a crystal that, when subjected to alternating currents, transmits either the R or the L component, depending on the polarity of the electric field to which it is exposed. The photomultiplier detector produces a voltage proportional to the ellipticity of the resultant beam emerging from the sample container.

12.6.3 Applications

The major application of CD is the study of conformation of biological macromolecules and complements data generated from nuclear magnetic resonance experiments (Chapter 13).

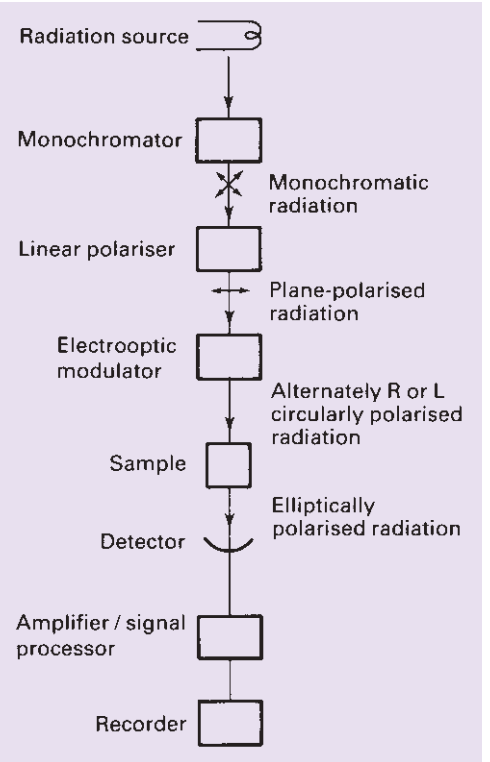

Fig. 12.14. The main components of a CD spectrometer.

Proteins

Information can be gained about the relative proportions of secondary structure, α-helical, β-sheet and random coil, in solution. The application of CD to tertiary structure is limited, owing to inadequate theoretical understanding of the influences of different parts of these molecules at this level of structure. The CD spectra of poly-L-amino acids have been obtained and are used as standards for calculating the percentage of each form of secondary structure in proteins. Curve-fitting procedures using computer processing have been used to apply the method to unknown proteins.

One of the most important benefits to be gained from CD spectroscopy is the study of conformational changes during, or because of, interactions with other entities. Examples are the determination of binding constants of substrates, cofactors, inhibitors or activators of virtually any enzyme. The binding of the inhibitor 3-cytidilic acid to the active site of pancreatic ribonuclease changes the CD spectrum of a remotely situated tyrosine residue. The binding of this inhibitor must therefore cause a conformational change in a distant part of the enzyme. CD spectroscopy is very sensitive and may be used to monitor the conversion of α- and β-structures to random coil (a major event of the denaturation process).

Nucleic acids

It is possible to calculate the CD spectrum of a single strand of DNA from the known nearest-neighbour frequency. Experimentally determined deviations from this calculated spectrum are indicative of a variation in structure, for example double strandedness. The CD spectrum of double-stranded DNA appears to be independent of the base composition in the range of wavelengths usually used.

A large increase in the CD spectrum of mononucleotides is observed when they link to form even short oligonucleotide chains. This observation provides evidence that hydrophobic interactions between stacked bases are important in stabilising the double-stranded structure of DNA.

All nucleotides exhibit chiral properties, and their CD is greatly increased on the adoption of a helical conformation. Hence the technique may be used to study structural changes in nucleic acids, for example loss of helicity of single-stranded DNA as a function of temperature and pH, structural changes on the binding of cations and proteins, transfer RNA–amino acid binding, transitions between single- and double-stranded DNA, DNA histone interactions in chromatin, the structure of ribosomal RNA in the ribosomes, and the interaction of double-stranded DNA and intercalating drugs.

12.7 TURBIDIMETRY AND NEPHELOMETRY

The two similar techniques of turbidimetry and nephelometry are both associated with the estimation of the concentrations of dilute suspensions. In turbidimetry, the apparent absorption of radiation by the suspension is measured. The apparent absorption should be measured at a wavelength where *true* absorption is not occurring; hence the Beer–Lambert law does not apply in turbidimetry. When radiation is passed through a transparent medium, for example a solution in a cuvette, one or both of two distinct physical phenomena might occur. In the case of extinction, true absorption of energy occurs and allows changes in the energy states of electrons, magnetic conditions, molecular vibrations, etc. The medium through which the radiation is passing, and in which the absorption is occurring, is termed optically empty, when this is the only phenomenon occurring. However, in the case of a suspension, a quite distinct radiative phenomenon may occur in which the light is scattered by the suspended particles. This scattering is due to reflection and refraction and gives rise to the Tyndall effect; it occurs in all directions and is an example of the more general Rayleigh scattering.

In turbidimetry the incident and transmitted radiation may be measured in an ordinary colorimeter or spectrophotometer, but the contribution of true absorption, if any, is small and the Beer–Lambert law is not strictly applicable as it holds only for very thin layers or very dilute suspensions.

The scattered light or Tyndall light may also be measured, usually at right angles (normal) to the incident radiation. This gives the Tyndall ratio, which is the ratio of the Tyndall intensity to that of the incident radiation. If this ratio

is measured directly, a Tyndall meter would be used. If, however, the Tyndall intensity is compared with that of a standard suspension of known concentration, then the instrument is known as a nephelometer (measures cloudiness). The concentrations of suspensions of microorganisms may be obtained using nephelometry and those of proteins and some other biological macromolecules by turbidimetry (Section 12.4.1).

These techniques are difficult to use but in experienced hands can be of value. The relationship between energy input (incident radiation) and measured output (transmitted or scattered), however, is complicated and non-linear. It should be noted that these techniques are not strictly spectroscopic but are included here for completeness.

12.8 LUMINOMETRY

12.8.1 Principles

The emission and radiative techniques discussed above all depend on some physical phenomenon within the molecules concerned. The phenomenon also depends on the prior input of energy, frequently obtained from electromagnetic radiation. The radiative phenomenon luminescence arises in a different way. Although it is essentially the emission of electromagnetic radiation in the visible region (i.e. light), it arises as the result of a chemical reaction. Luminometry is the technique used to measure this luminescence, and, although not a spectrophotometric technique, it is included for completeness as it is an important method in biological science.

Chemiluminescence occurs as a result of excited electrons relaxing to the ground state (see Fig. 12.2b). The prior excitation arises as a result of a chemical reaction that yields a fluorescent product, and the chemiluminescent spectrum of a reaction such as luminol with oxygen to produce 3-aminophthalate is the same as the fluorescent spectrum of the product. A similar phenomenon is bioluminescence, so-called because the light emission arises from an enzyme-catalysed reaction (Section 15.2.2) usually involving luciferase. The colour of the light emitted in the latter case depends on the source of the enzyme and varies between 560 nm (greenish yellow) and 620 nm (red) wavelengths. This method has the distinct advantage of high sensitivity, as a result of the reaction having a high quantum yield – 100% under favourable conditions.

12.8.2 Instrumentation

It is not electromagnetic radiation that is the source of the excitation energy, hence no monochromator is required. Luminometry can therefore be performed with relatively simple photometers. Two minor complications are the need to amplify the output signal prior to recording and the need to maintain fairly strict temperature control. This control is necessary owing to the sensitivity of reactions to temperature, particularly in the case of enzyme-catalysed reactions.

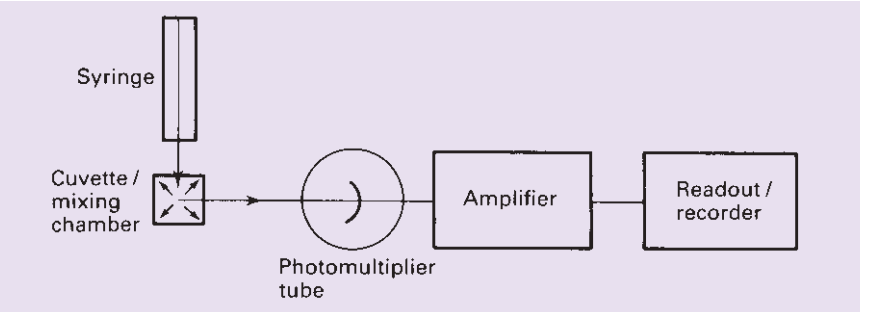

Fig. 12.15. Diagram of the main components of a simple luminometer.

Fig. 12.15 shows the layout of the main components. The reactants are introduced into a suitable light-protected reaction vessel in which adequate mixing takes place. The emitted light is collected by a photomultiplier tube, which is connected to a direct current amplifier with a wide range of sensitivity and linear response.

12.8.3 Applications

The firefly luciferase system

Details for the firefly luciferase system are given in Section 15.2.2. ATP concentration may be measured in an assay that is rapid to carry out and whose accuracy is comparable to spectrophotometric and fluorimetric assays. The sensitivity is, however, vastly increased, having a limit of detection of 10^{-15} M and a linear range of 10^{-12} to 10^{-6} M ATP. The concentrations of ADP, AMP and cyclic AMP may also be determined using appropriate enzymes, for example pyruvate kinase for ADP $\rightarrow$ ATP, adenylate kinase for AMP $\rightarrow$ ADP, and phosphodiesterase for cyclic AMP $\rightarrow$ AMP. In principle, all the enzymes and metabolites involved in ATP interconversion reactions may be assayed by this method. Examples are the enzymes creatine kinase, hexokinase and ATP sulphurase, and the substrates creatine phosphate, glucose, GTP, phosphoenolpyruvate and 1,3-diphosphoglycerate.

The bacterial luciferase system

Details of the bacterial luciferase system also are given in Section 15.2.2. The determination of nicotinamide adenine dinucleotides (and phosphates) and flavin mononucleotides, in their reduced states (i.e. NADH, NADPH and $FMNH_2$) may be made in assays which use this system. A concentration range of 10^{-9} to 10^{-12} M is achievable, which is much more sensitive than the corresponding spectrophotometric and fluorimetric assays, although the NADPH assay is less sensitive than the NADH assay by a factor of about 20. The method can be applied to a whole range of coupled enzyme reaction systems of the redox type that involve these nucleotides as coenzymes.

The aequorin system

Despite the development of calcium-specific electrodes, the calcium ion concentration may be determined with high sensitivity, intracellularly, using the phosphoprotein aequorin. The protein is isolated from luminescent medusae (jellyfish) and is practically non-fluorescent. In the presence of Ca^{2+}, however, it is converted from its natural yellow reflective colour to the blue fluorescent protein (BFP). The bioluminescent spectrum of the reaction is identical with the fluorescent spectrum of BFP:2Ca^{2+} but different from BFP:Ca^{2+}.

Ease of use, high sensitivity to, and relative specificity for, calcium and the non-toxicity of aequorin to living cells are advantages. The disadvantages are the scarcity of the protein, its large molecular size, consumption during the reaction and the non-linearity of the light emission relative to calcium concentration. Also the reaction is sensitive to its chemical environment and the limited speed in which it can respond to rapid changes in calcium concentration, for example influx and efflux in certain cell types.

Chemiluminescence

Luminol and its derivatives can undergo chemiluminescent reactions with high efficiency. For instance, enzymically generated H_2O_2 may be detected by the emission of light at 430 nm wavelength in the presence of luminol and microperoxidase (Section 15.2.2).

Competitive binding assays may be used to determine low concentrations of hormones, drugs and metabolites in biological fluids. Such assays depend on the ability of proteins such as antibodies and cell receptors to bind specific ligands with high affinity. Competition between labelled and unlabelled ligand for appropriate sites on the binding compound occurs. If the concentration of the binding compound is known, i.e. the number of available sites is known and a limited but known concentration of labelled ligand is introduced, then under saturation conditions all sites are occupied and the concentration of unlabelled ligand can be determined. Use of labelled ligand allows the concentration of only binding compound (the number of sites) to be determined. A variety of labels, including radioisotopes, is in common use, enabling the fractions in the bound and free states to be distinguished. Labelling with a luminol derivative, completing the binding reaction, separating bound and free fractions allows the protein to be assayed by its chemiluminescence. The system must be calibrated using standards and, under the most favourable conditions, 10^{-12} M of a compound may be determined.

Whilst polymorphonuclear leukocytes are phagocytosing, singlet molecular oxygen is produced that exhibits chemiluminescence. The effects of pharmacological and toxicological agents on these and other phagocytic cells can be studied by monitoring this luminescence.

12.9 ATOMIC SPECTROSCOPY

All of the methods described above, with the exception of nuclear phenomena in the γ- and X-ray regions, have dealt essentially with molecular spectroscopy.

The general theory of electron transitions was discussed in Section 12.1.2 and for simplicity the phenomena were described mainly in atomic terms, although the extension to molecules is not too difficult. It was indicated above (Section 12.1.2) that, in general, molecules give rise to band spectra and atoms to clearly defined line spectra. These lines can be observed by eye either as light, associated with a particular wavelength, which are atomic emission spectra or black lines against a bright background, which are atomic absorption spectra. Some elements, particularly metals, have an important role to play in biological systems, whether as simple cofactors in enzymes, the central atom in biological macromolecules such as iron in haemoglobin or magnesium in chlorophyll, or as toxic substances that affect metabolism. Use of atomic spectroscopy will enable data to be obtained that are important in understanding the biological roles of these elements.

In a spectrum of an element, the wavelengths at which absorption or emission are observed are associated with transitions where the minimal energy change occurs. For example, in Fig. 12.2a is shown the $3s$–$3p$, or D-line transition in the sodium atom that gives rise to the emission of orange light. When electron transitions occur in an atom they are limited by the availability of an empty orbital or level. An orbital or level could not be overfilled without contravening the Pauli exclusion principle. In order for energy changes to be minimal, transitions tend to occur between levels close together in energy terms. These limitations mean that emission and absorption lines are absolutely characteristic of the element concerned. At least for simple atoms it is theoretically possible to deduce their electronic structure from their line spectra. The wavelengths emitted from excited atoms may be identified using a spectroscope, spectrograph or a direct reading spectrophotometer that uses as detectors the human eye, a photographic plate or a photoelectric cell, respectively.

In general, and in contrast to molecular spectroscopy, atom concentrations are not measured directly in solution. The atoms have to be volatilised either in a flame or electrothermally in an oven. In this state the elements will readily emit or absorb monochromatic radiation at the appropriate wavelength. Usually nebulisers (atomisers) will be used to spray the standard or test solution into the flame through which the light is passed. Alternatively the light beam is passed, in an oven, through a cavity containing the vaporised material.

12.9.1 Principles of atomic flame spectrometry

This technique takes advantage of the properties described above to determine the amounts of a specific element that may be present. The emission of light is measured by emission flame spectrophotometry and absorption by atomic absorption flame spectrophotometry.

The energy absorbed or emitted is proportional to the number of atoms in the optical path. In the case of emission it is strictly the number of excited atoms, but under reproducible standard conditions this will be the same as that for a calibrating standard. Flame instability, variation in temperature and composition of the flame make standard conditions difficult to achieve. Sodium gives high

backgrounds and hence should be measured first and then a similar amount added to all other standards. Excess hydrochloric is usually added as chloride compounds are often the most volatile salts. Calcium and magnesium emissions are enhanced by the addition of alkali metals and suppressed by addition of phosphate, silicate and aluminate (by the formation of non-dissociable salts). This suppression effect may be relieved by the addition of lanthanum and strontium salts. Cyclic analysis may be performed that involves the estimation of each interfering substance in a mixture and then the standards for each component in the mixture are 'doped' with each interfering substance. The process is repeated (usually only two to three cycles are necessary) with refined estimates of interfering substance, until self-consistent values are obtained for each component; this implies minimal interference effects resulting from the concentrations approaching those in the unknown sample.

Flame instability requires that assays are carried out in triplicate and it is advantageous to bracket a determination of an unknown with measurements of the same standard to achieve the greatest accuracy. The use of lithium as an internal standard improves the technique. Polythene bottles should be used for storage if possible, as metal ions are both absorbed and released by glass.

Biological samples are usually converted to ash prior to the determination of metals. This can be done dry if sublimation losses are prevented. Wet ashing (in solution) is often used; this employs an oxidative digestion similar to the Kjeldahl method (see Section 8.3.2).

12.9.2 Instrumentation

Atomic emission spectrophotometry

The nebulisers used are usually of the type that involves passing a stream of air over a capillary tube whose other end dips into the solution under test. Larger droplets tend not to remain in the hottest part of the flame long enough, in direct injection systems, for their constituents to be volatilised and hence are allowed to settle out in a cloud chamber. Combustion of air and natural gas gives a temperature of 1500 °C, which is adequate for sodium determination. Calcium is better assayed at 2000 to 2500 °C and magnesium and iron require 2500 °C, obtained from an air/acetylene gas mixture. Bandwidth selection using a filter device may be used for routine analyses of moderate accuracy. More accurate measurements require a monochromator. The best accuracy achieves a resolution of 0.1–0.2 nm over the range 200–1000 nm. Table 12.3 lists the wavelengths used for a number of metals, together with their detection limits. Detectors are often of the photocell type but flame instability limits their value as their potential accuracy is not realised. Multichannel polychromators allow the emission of up to six elements at one time to be measured. The basic layout of an atomic (flame) emission spectrophotometer is shown in Fig. 12.16.

Atomic absorption spectrophotometry

In these instruments either a double monochromator with a source of white light or a hollow cathode discharge lamp is used to produce radiation in a very narrow

Table 12.3 **The detection limits for various elements in emission and absorption flame spectrophotometry, flameless absorption spectrophotometry, and ion-selective electrodes**

	Emission		Absorption			
			Detection limit (p.p.m.)			
Element	Detection limit (p.p.m.)	Wavelength (nm)	Flame	Flameless	Wavelength (nm)	Ion-selective electrode: detection limit (p.p.m.)
Calcium	0.005	442.7	0.1	0.00007	442.7	0.02
Copper	0.1	324.8	0.1	0.0001	324.8	0.0006
Iron	0.5	372.0	0.2	0.0001	248.3	
Lead			0.5	0.0002	283.3	0.21
Lithium	0.001	670.7	0.03	0.0001	670.7	
Magnesium	0.1	285.2	0.01	0.00001	285.2	
Manganese	0.02	403.3	0.05	0.00004	279.5	
Mercury			10.0	0.018	253.8	
Potassium	0.001	766.5	0.03	0.00003	766.5	0.04
Sodium	0.0001	589.0	0.03	0.00001	589.0	0.02
Strontium	0.01	460.7	0.06	0.0001	460.9	

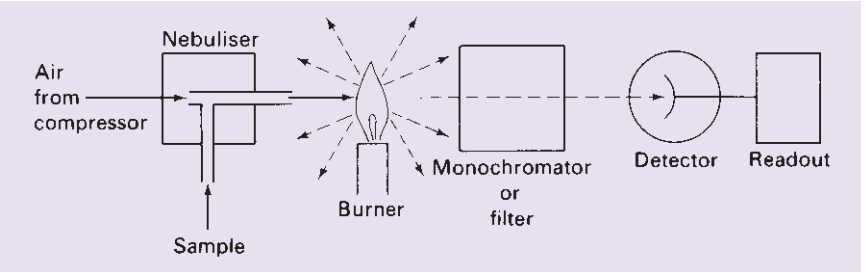

Fig. 12.16. The main components of an atomic emission (flame) spectrophotometer.

bandwidth. Discharge lamps emit radiation at a wavelength specific for the element being assayed. This specificity can be obtained only from a pure sample of the element that is excited electrically to produce an arc spectrum of that element, and electrodeless discharge lamps are now available. Nebulisers and burners are similar to the emission devices but 10 cm flames are often used to obtain an increased optical length. Both single and double beam instruments are available, the latter often incorporating a chopper to give intermittent pulses and prevent stray light from the flame reaching the detector. The most useful wavelength range is 190–850 nm.

Flameless instruments

A flameless atomic absorption spectrophotometer incorporates a graphite tube as an oven, which may be heated electrothermally to 3000 °C. Monochromatic light specific to the element being assayed is produced either by a hollow cathode discharge lamp or an electrodeless discharge lamp. The graphite tube forms an optical cavity, in which the sample resides and through which the monochromatic radiation is passed. Absorption is measured continuously as the temperature is raised and computer methods allow the superimposition of absorption and temperature profiles, with time, to be produced. This approach allows optimum conditions to be determined for future analyses. The flameless technique is 100 times more sensitive than flame methods and has the distinct advantage of being able to be automated as the inherent dangers of using combustible gases have been eliminated.

12.9.3 Applications

Sodium and potassium may be assayed at concentrations of a few parts per million (< 5) using simple filter photometers. The more sophisticated emission flame spectrophotometers may be used to assay some 20 elements in biological samples, the most common being calcium, magnesium and manganese. Absorption flame spectrophotometers are usually more sensitive than emission types and can usually detect < 1 p.p.m. of each of more than 20 elements. Exceptions to this are the alkali metals. Relative precision is about 1% in a working range of 20–200 times the detection limit (Table 12.3).

The techniques were widely used in clinical laboratories, for the determination of metals in body fluids. However, the technique has been largely superseded by the use of ion-selective electrodes (ISEs). These are amenable to automation and measurements can be carried out on very small samples (Sections 1.5.2 and 1.7.2). These determinations aid diagnosis and are valuable in the monitoring of many therapeutic regimes. In physiological and pharmacological research, sodium, potassium, calcium, magnesium, cadmium and zinc may be measured directly, but copper, lead, iron and mercury require prior extraction from the biological source. The methods are also widely used in element determination in soil and plant materials and, after suitable ashing procedures, may be used for metals in macromolecules, organelles, cells and tissues.

12.9.4 Atomic fluorescence spectrophotometry

Prior excitation of atoms by electromagnetic radiation rather than by thermal energy is required (cf. molecular fluorescence, Section 12.8.1). Atoms are again required to be in the vapour state: the phenomenon is not observed in solution as it is with molecules. The source beam must be intense but less spectrally pure than that required for atomic absorption spectrophotometry, as only the resonant wavelengths will be absorbed and lead to fluorescence. Direct emission from the flame being recorded by the detector must be avoided and this may be achieved by modulation of the detector amplifier to the same frequency as that of the primary source. Although limited to only a few metals, the extreme sensitivity achievable in appropriate cases makes it better than comparable methods. For example, zinc and cadmium may be detected at levels as low as 1 and 2 parts per 10^{10}, respectively.

12.10 LASERS

Laser is an acronym for *l*ight *a*mplification by *s*timulated *e*mission of *r*adiation. A detailed explanation of how laser light is generated is not possible here. A simple view is that electromagnetic radiation used as the excitation agent can be considered as the input of photons to an absorbing material. This results in elevation of an electron to a higher energy level as described above (Section 12.1.2). If, whilst the electron is in an excited state, another photon of precisely the correct energy arrives, then, instead of the electron being promoted to an even higher energy level, it returns to its original ground state. This return is accompanied by the emission of two coherent photons. These photons have associated wavelengths that are exactly in phase, hence the term coherent. A laser-producing material has to be pumped and this is often achieved by surrounding the material with a rapidly flashing high intensity flash tube that gives an ample supply of suitable photons.

The emitted, coherent light has considerable advantages, but in particular it can be produced with zero bandwidth, i.e. unique invariant wavelengths can be selected to excite molecules or atoms in a very precise way. It is also possible to

generate, from appropriate sources, groups of selected wavelengths should this be required. Various applications are under development in spectroscopic and spectrophotometric methods that take advantage of the spectral purity of laser light.

An important application is the laser reflectance method for determining complementary DNA (cDNA) in nucleic acid studies. The use of reverse transcriptase and DNA polymerase (see Section 6.2.5) allows the nucleotide sequence corresponding to the primary sequence of a peptide fragment or protein to be synthesised. Chain growth occurs at the 3′ end from a primer section, and chain termination occurs when a dideoxynucleotide is incorporated into the growing complementary strand (Chapter 5). Four 'channels' are required, each containing primer, all four deoxynucleoside triphosphates and one of each of the four dideoxy compounds. In each of the four channels, chain termination occurs at different points. Also, at the 5′ end of the primers a different fluorescent label is attached that has no influence on the subsequent reactions but can be used to identify uniquely components of the resulting mixtures in each channel. Mixtures are separated by gel electrophoresis (see Section 10.2.2) in which distance travelled in the gel is effectively inversely proportional to the mass of the fragment. The gel is illuminated with a narrow beam of laser light and fluorescent emission from each label is measured (a different wavelength is emitted from each label). The band on the gel can be identified by including, to interrupt the emitted beam, a rotating filter disc that contains four sectors, each of which allows only one fluorescent wavelength to pass. By design, which fluor relates to which dideoxy terminator is known and mobility, position and amount are determined. The system can be automated and avoids the use of radioisotopes. It is reliable and precise, and data interpretation can be done by computer.

12.11 SUGGESTIONS FOR FURTHER READING

BRAND, L. and JOHNSON, M. L. (eds.) (1997). *Fluorescence Spectroscopy*. Methods in Enzymology vol. 278. Academic Press, San Diego. (An authoritative coverage of the applications of the form of spectroscopy in biochemistry.)

HARWOOD, L. M. and CLARIDGE, D. W. (1997). *Introduction to Organic Spectroscopy*. Oxford Chemistry Primers no. 43. Oxford University Press, Oxford. (A good text for studying some of the principles of spectroscopy in greater depth.)

HOLLAS, J. M. (2002). *Basic Atomic and Molecular Spectroscopy*. Wiley/RSC, London. (Contains many worked examples that are ideal for self-directed study.)

PAVIA, D. L., LAMPMANN, N. G. M. and KRIZ, G. S. (2001). *Introduction to Spectroscopy*, 3rd edn. Harcourt, New York. (Good coverage of all the techniques with many worked examples.)