10 Electrophoretic techniques

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10.1 GENERAL PRINCIPLES

The term electrophoresis describes the migration of a charged particle under the influence of an electric field. Many important biological molecules, such as amino acids, peptides, proteins, nucleotides and nucleic acids, possess ionisable groups and, therefore, at any given pH, exist in solution as electrically charged species either as cations (+) or anions (−). Under the influence of an electric field these charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge.

The equipment required for electrophoresis consists basically of two items, a power pack and an electrophoresis unit. Electrophoresis units are available for running either vertical or horizontal gel systems. Vertical slab gel units are commercially available and routinely used to separate proteins in acrylamide gels (Section 10.2). The gel is formed between two glass plates that are clamped together but held apart by plastic spacers. The most commonly used units are the so-called minigel apparatus (Fig. 10.1). Gel dimensions are typically 8.5 cm wide × 5 cm high, with a thickness of 0.5−1 mm. A plastic comb is placed in the gel solution and is removed after polymerisation to provide loading wells for up to 10 samples. When the apparatus is assembled, the lower electrophoresis tank buffer surrounds the gel plates and affords some cooling of the gel plates. A typical horizontal gel system is shown in Fig. 10.2. The gel is cast on a glass or plastic sheet and placed on a cooling plate (an insulated surface through which cooling water is passed to conduct away generated heat). Connection between the gel and electrode buffer is made using a thick wad of wetted filter paper (Fig. 10.2); note, however, that agarose gels for DNA electrophoresis are run submerged in the buffer (Section 10.4.1). The power pack supplies a direct current between the electrodes in the

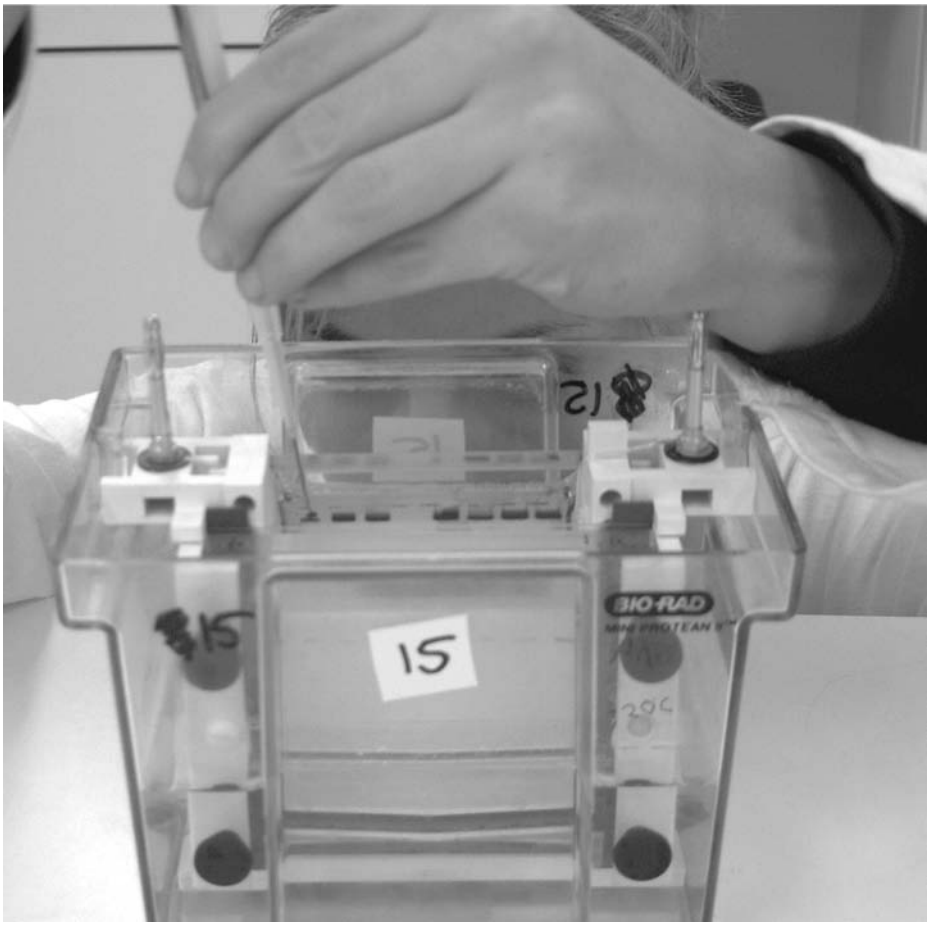


Fig. 10.1 Photograph showing samples being loaded into the wells of an SDS–PAGE minigel. Six wells that have been loaded can be identified by the blue dye (bromophenol blue) that is incorporated into the loading buffer.

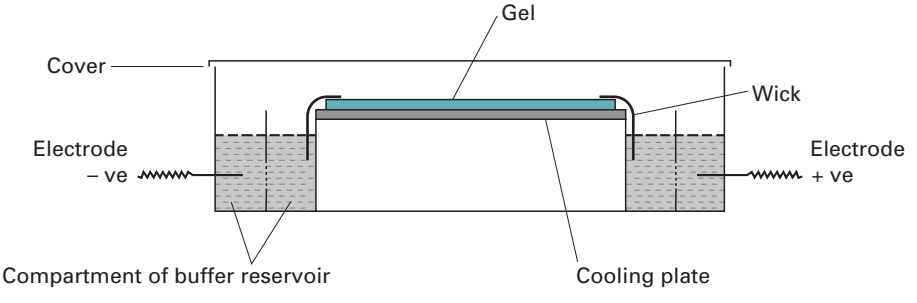


Fig. 10.2 A typical horizontal apparatus, such as that used for immunoelectrophoresis, isoelectric focussing and the electrophoresis of DNA and RNA in agarose gels.

electrophoresis unit. All electrophoresis is carried out in an appropriate buffer, which is essential to maintain a constant state of ionisation of the molecules being separated. Any variation in pH would alter the overall charge and hence the mobilities (rate of migration in the applied field) of the molecules being separated.

In order to understand fully how charged species separate it is necessary to look at some simple equations relating to electrophoresis. When a potential difference (voltage) is applied across the electrodes, it generates a potential gradient, E, which is the applied voltage, V, divided by the distance, d, between the electrodes. When this potential gradient E is applied, the force on a molecule bearing a charge of q coulombs is Eq newtons. It is this force that drives a charged molecule towards an electrode. However, there is also a frictional resistance that retards the movement of this charged molecule. This frictional force is a measure of the hydrodynamic size of the molecule, the shape of the molecule, the pore size of the medium in which electrophoresis is taking place and the viscosity of the buffer. The velocity, ν, of a charged molecule in an electric field is therefore given by the equation:

$$\nu = \frac{Eq}{f} \tag{10.1}$$

where f is the frictional coefficient.

More commonly the term electrophoretic mobility (μ) of an ion is used, which is the ratio of the velocity of the ion to field strength (ν/E). When a potential difference is applied, therefore, molecules with different overall charges will begin to separate owing to their different electrophoretic mobilities. Even molecules with similar charges will begin to separate if they have different molecular sizes, since they will experience different frictional forces. As will be seen below, some forms of electrophoresis rely almost totally on the different charges on molecules to effect separation, whilst other methods exploit differences in molecular size and therefore encourage frictional effects to bring about separation.

Provided the electric field is removed before the molecules in the sample reach the electrodes, the components will have been separated according to their electrophoretic mobility. Electrophoresis is thus an incomplete form of electrolysis. The separated samples are then located by staining with an appropriate dye or by autoradiography (Section 14.3.3) if the sample is radiolabelled.

The current in the solution between the electrodes is conducted mainly by the buffer ions, a small proportion being conducted by the sample ions. Ohm's law expresses the relationship between current (I), voltage (V) and resistance (R):

$$\frac{V}{I} = R \tag{10.2}$$

It therefore appears that it is possible to accelerate an electrophoretic separation by increasing the applied voltage, which would result in a corresponding increase in the current flowing. The distance migrated by the ions will be proportional to both current and time. However, this would ignore one of the major problems for most forms of electrophoresis, namely the generation of heat.

During electrophoresis the power (W, watts) generated in the supporting medium is given by

$$W = I^2R \tag{10.3}$$

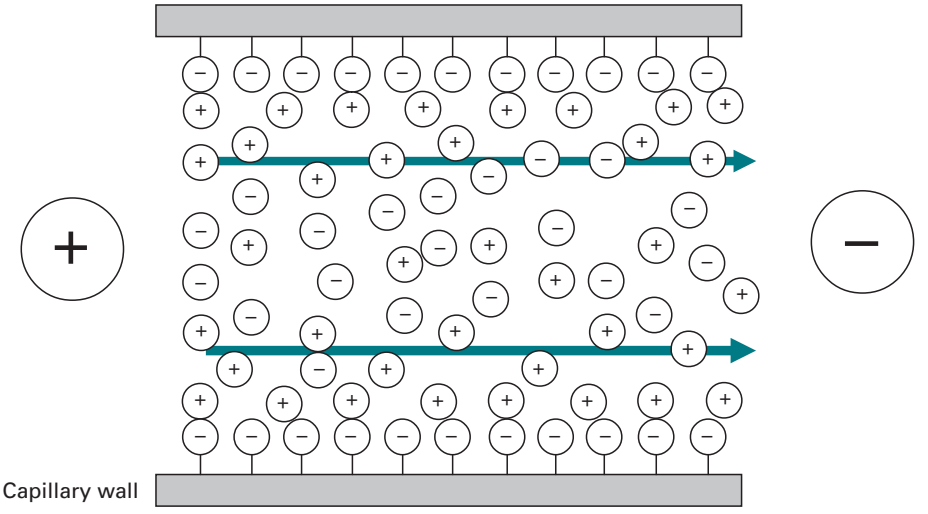
Most of this power generated is dissipated as heat. Heating of the electrophoretic medium has the following effects:

- An increased rate of diffusion of sample and buffer ions leading to broadening of the separated samples.
- The formation of convection currents, which leads to mixing of separated samples.
- Thermal instability of samples that are rather sensitive to heat. This may include denaturation of proteins (and thus the loss of enzyme activity).
- A decrease of buffer viscosity, and hence a reduction in the resistance of the medium.

If a constant voltage is applied, the current increases during electrophoresis owing to the decrease in resistance (see Ohm's law, equation 10.2) and the rise in current increases the heat output still further. For this reason, workers often use a stabilised power supply, which provides constant power and thus eliminates fluctuations in heating.

Constant heat generation is, however, a problem. The answer might appear to be to run the electrophoresis at very low power (low current) to overcome any heating problem, but this can lead to poor separations as a result of the increased amount of diffusion resulting from long separation times. Compromise conditions, therefore, have to be found with reasonable power settings, to give acceptable separation times, and an appropriate cooling system, to remove liberated heat. While such systems work fairly well, the effects of heating are not always totally eliminated. For example, for electrophoresis carried out in cylindrical tubes or in slab gels, although heat is generated uniformly through the medium, heat is removed only from the edges, resulting in a temperature gradient within the gel, the temperature at the centre of the gel being higher than that at the edges. Since the warmer fluid at the centre is less viscous, electrophoretic mobilities are therefore greater in the central region (electrophoretic mobilities increase by about 2% for each 1 °C rise in temperature), and electrophoretic zones develop a bowed shape, with the zone centre migrating faster than the edges.

A final factor that can effect electrophoretic separation is the phenomenon of electroendosmosis (also known as electroosmotic flow), which is due to the presence of charged groups on the surface of the support medium. For example, paper has some carboxyl groups present, agarose (depending on the purity grade) contains sulphate groups and the surface of glass walls used in capillary electrophoresis (Section 10.5) contains silanol (Si—OH) groups. Figure 10.3 demonstrates how electroendosmosis occurs in a capillary tube, although the principle is the same for any support medium that has charged groups on it. In a fused-silica capillary tube, above a pH value of about 3, silanol groups on the silica capillary wall will ionise, generating negatively charged sites. It is these charges that generate electroendosmosis. The ionised silanol groups create an electrical double layer, or region of charge separation, at the capillary wall/electrolyte interface. When a voltage is applied, cations in the electrolyte near the capillary wall migrate towards the cathode, pulling electrolyte solution with them. This creates a net electroosmotic flow towards the cathode.



- Acidic silanol groups impart negative charge on wall
- Counter ions migrate toward cathode, dragging solvent along

Fig. 10.3 Electroosmotic flow through a glass capillary. Electrolyte cations are attracted to the capillary walls, forming an electrical double layer. When a voltage is applied, the net movement of electrolyte solution towards the cathode is known as electroendosmotic flow.

10.2 SUPPORT MEDIA

The pioneering work on protein electrophoresis by Arne Tiselius (for which he received the Nobel Prize in Chemistry in 1948) was performed in free solution. However, it was soon realised that many of the problems associated with this approach, particularly the adverse effects of diffusion and convection currents, could be minimised by stabilising the medium. This was achieved by carrying out electrophoresis on a porous mechanical support, which was wetted in electrophoresis buffer and in which electrophoresis of buffer ions and samples could occur. The support medium cuts down convection currents and diffusion so that the separated components remain as sharp zones. The earliest supports used were filter paper or cellulose acetate strips, wetted in electrophoresis buffer. Nowadays these media are infrequently used, although cellulose acetate still has its uses (see Section 10.3.6). In particular, for many years small molecules such as amino acids, peptides and carbohydrates were routinely separated and analysed by electrophoresis on supports such as paper or thin-layer plates of cellulose, silica or alumina. Although occasionally still used nowadays, such molecules are now more likely to be analysed by more modern and sensitive techniques such as high-performance liquid chromatography (Section 11.3). While paper or thin-layer supports are fine for resolving small molecules, the separation of macromolecules such as proteins and nucleic acids on such supports is poor.

However, the introduction of the use of gels as a support medium led to a rapid improvement in methods for analysing macromolecules. The earliest gel system to be used was the starch gel and, although this still has some uses, the vast majority

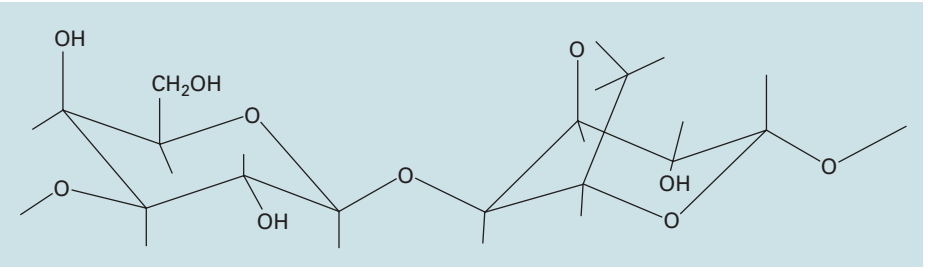


Fig. 10.4 Agarobiose, the repeating unit of agarose.

of electrophoretic techniques used nowadays involve either agarose gels or polyacrylamide gels.

10.2.1 Agarose gels

Agarose is a linear polysaccharide (average relative molecular mass about 12 000) made up of the basic repeat unit agarobiose, which comprises alternating units of galactose and 3,6-anhydrogalactose (Fig. 10.4). Agarose is one of the components of agar that is a mixture of polysaccharides isolated from certain seaweeds. Agarose is usually used at concentrations of between 1% and 3%. Agarose gels are formed by suspending dry agarose in aqueous buffer, then boiling the mixture until a clear solution forms. This is poured and allowed to cool to room temperature to form a rigid gel. The gelling properties are attributed to both inter- and intramolecular hydrogen bonding within and between the long agarose chains. This cross-linked structure gives the gel good anticonvectional properties. The pore size in the gel is controlled by the initial concentration of agarose; large pore sizes are formed from low concentrations and smaller pore sizes are formed from the higher concentrations. Although essentially free from charge, substitution of the alternating sugar residues with carboxyl, methyoxyl, pyruvate and especially sulphate groups occurs to varying degrees. This substitution can result in electro-endosmosis during electrophoresis and ionic interactions between the gel and sample in all uses, both unwanted effects. Agarose is therefore sold in different purity grades, based on the sulphate concentration – the lower the sulphate content, the higher the purity.

Agarose gels are used for the electrophoresis of both proteins and nucleic acids. For proteins, the pore sizes of a 1% agarose gel are large relative to the sizes of proteins. Agarose gels are therefore used in techniques such as flat-bed isoelectric focussing (Section 10.3.4), where the proteins are required to move unhindered in the gel matrix according to their native charge. Such large pore gels are also used to separate much larger molecules such as DNA or RNA, because the pore sizes in the gel are still large enough for DNA or RNA molecules to pass through the gel. Now, however, the pore size and molecule size are more comparable and fractional effects begin to play a role in the separation of these molecules (Section 10.4). A further advantage of using agarose is the availability of low melting temperature agarose (62–65 °C). As the name suggests, these gels can be reliquified by heating to 65 °C and

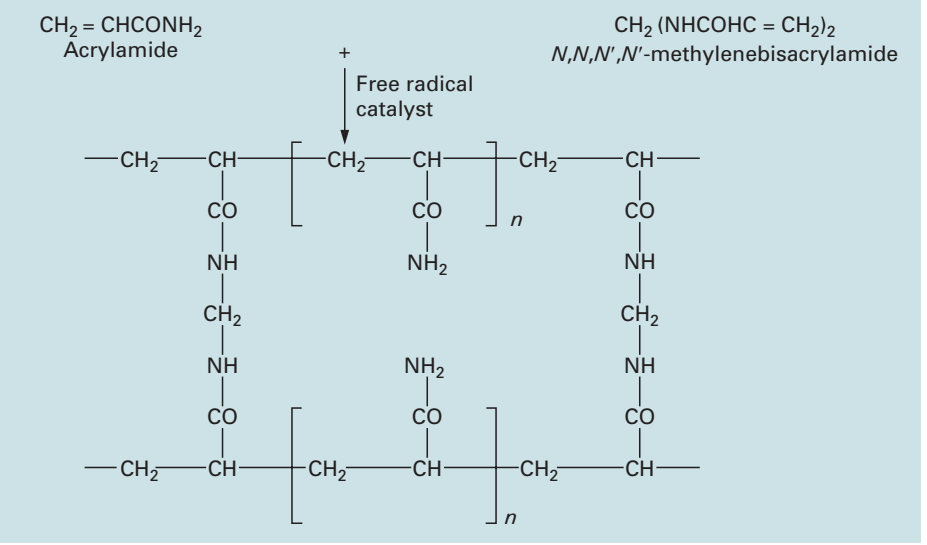


Fig. 10.5 The formation of a polyacrylamide gel from acrylamide and bis-acrylamide.

thus, for example, DNA samples separated in a gel can be cut out of the gel, returned to solution and recovered.

Owing to the poor elasticity of agarose gels and the consequent problems of removing them from small tubes, the gel rod system sometimes used for acrylamide gels is not used. Horizontal slab gels are invariably used for isoelectric focussing or immunoelectrophoresis in agarose. Horizontal gels are also used routinely for DNA and RNA gels (Section 10.4), although vertical systems have been used by some workers.

10.2.2 Polyacrylamide gels

Electrophoresis in acrylamide gels is frequently referred to as PAGE, being an abbreviation for *p*oly*a*crylamide *g*el *e*lectrophoresis.

Cross-linked polyacrylamide gels are formed from the polymerisation of acrylamide monomer in the presence of smaller amounts of *N,N′*-methylene-bisacrylamide (normally referred to as 'bis'-acrylamide) (Fig. 10.5). Note that bisacrylamide is essentially two acrylamide molecules linked by a methylene group, and is used as a cross-linking agent. Acrylamide monomer is polymerised in a head-to-tail fashion into long chains and occasionally a bis-acrylamide molecule is built into the growing chain, thus introducing a second site for chain extension. Proceeding in this way a cross-linked matrix of fairly well-defined structure is formed (Fig. 10.5). The polymerisation of acrylamide is an example of free-radical catalysis, and is initiated by the addition of ammonium persulphate and the base *N,N,N′,N′*-tetramethylenediamine (TEMED). TEMED catalyses the decomposition of the persulphate ion to give a free radical (i.e. a molecule with an unpaired electron):

$$S_2O_8^{2-} + e^- \rightarrow SO_4^{2-} + SO_4^{-\bullet}$$

If this free radical is represented as $R^{\bullet}$ (where the dot represents an unpaired electron) and M as an acrylamide monomer molecule, then the polymerisation can be represented as follows:

$$R^{\bullet} + M \rightarrow RM^{\bullet}$$

$$RM^{\bullet} + M \rightarrow RMM^{\bullet}$$

$$RMM^{\bullet} + M \rightarrow RMMM^{\bullet} \text{ etc.}$$

Free radicals are highly reactive species due to the presence of an unpaired electron that needs to be paired with another electron to stabilise the molecule. R● therefore reacts with M, forming a single bond by sharing its unpaired electron with one from the outer shell of the monomer molecule. This therefore produces a new free radical molecule $R - M^{\bullet}$, which is equally reactive and will attack a further monomer molecule. In this way long chains of acrylamide are built up, being cross-linked by the introduction of the occasional bis-acrylamide molecule into the growing chain. Oxygen mops up free radicals and therefore all gel solutions are normally degassed (the solutions are briefly placed under vacuum to remove loosely dissolved air) prior to use. The degassing of the gel solution also serves a second purpose. The polymerisation of acrylamide is an exothermic reaction (i.e. heat is liberated) and the warming up of the gel solution as it sets can liberate air bubbles that become trapped in the polymerised gel. The degassing step prevents this possibility.

Photopolymerisation is an alternative method that can be used to polymerise acrylamide gels. The ammonium persulphate and TEMED are replaced by riboflavin and when the gel is poured it is placed in front of a bright light for 2–3 h. Photodecomposition of riboflavin generates a free radical that initiates polymerisation.

Acrylamide gels are defined in terms of the total percentage of acrylamide present, and the pore size in the gel can be varied by changing the concentrations of both the acrylamide and bis-acrylamide. Acrylamide gels can be made with a content of between 3% and 30% acrylamide. Thus low percentage gels (e.g. 4%) have large pore sizes and are used, for example, in the electrophoresis of proteins, where free movement of the proteins by electrophoresis is required without any noticeable frictional effect, for example in flat-bed isoelectric focusing (Section 10.3.4) or the stacking gel system of an SDS–polyacrylamide gel (Section 10.3.1). Low percentage acrylamide gels are also used to separate DNA (Section 10.4). Gels of between 10% and 20% acrylamide are used in techniques such as SDS–gel electrophoresis, where the smaller pore size now introduces a sieving effect that contributes to the separation of proteins according to their size (Section 10.3.1).

Proteins were originally separated on polyacrylamide gels that were polymerised in glass tubes, approximately 7 mm in diameter and about 10 cm in length. The tubes were easy to load and run, with minimum apparatus requirements. However, only one sample could be run per tube and, because conditions of separation could vary from tube to tube, comparison between different samples was not always accurate. The later introduction of vertical gel slabs allowed running of up to 20 samples under identical conditions in a single run. Vertical slabs are now used routinely both for the analysis

of proteins (Section 10.3) and for the separation of DNA fragments during DNA sequence analysis (Section 10.4). Although some workers prepare their own acrylamide gels, others purchase commercially available ready-made gels for techniques such as SDS–PAGE, native gels and isoelectric focusing (IEF) (see below).

10.3 ELECTROPHORESIS OF PROTEINS

10.3.1 Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) is the most widely used method for analysing protein mixtures qualitatively. It is particularly useful for monitoring protein purification and, because the method is based on the separation of proteins according to size, it can also be used to determine the relative molecular mass of proteins. SDS($CH_3-(CH_2)_{10}-CH_2OSO_3^-Na^+$) is an anionic detergent. Samples to be run on SDS–PAGE are firstly boiled for 5 min in sample buffer containing β-mercaptoethanol and SDS. The mercaptoethanol reduces any disulphide bridges present that are holding together the protein tertiary structure, and the SDS binds strongly to, and denatures, the protein. Each protein in the mixture is therefore fully denatured by this treatment and opens up into a rod-shaped structure with a series of negatively charged SDS molecules along the polypeptide chain. On average, one SDS molecule binds for every two amino acid residues. The original native charge on the molecule is therefore completely swamped by the negatively charged SDS molecules. The rod-like structure remains, as any rotation that tends to fold up the protein chain would result in repulsion between negative charges on different parts of the protein chain, returning the conformation back to the rod shape. The sample buffer also contains an ionisable tracking dye, usually bromophenol blue, that allows the electrophoretic run to be monitored, and sucrose or glycerol, which gives the sample solution density thus allowing the sample to settle easily through the electrophoresis buffer to the bottom when injected into the loading well (see Fig. 10.1). Once the samples are all loaded, a current is passed through the gel. The samples to be separated are not in fact loaded directly into the main separating gel. When the main separating gel (normally about 5 cm long) has been poured between the glass plates and allowed to set, a shorter (approximately 0.8 cm) stacking gel is poured on top of the separating gel and it is into this gel that the wells are formed and the proteins loaded. The purpose of this stacking gel is to concentrate the protein sample into a sharp band before it enters the main separating gel. This is achieved by utilising differences in ionic strength and pH between the electrophoresis buffer and the stacking gel buffer and involves a phenomenon known as isotachophoresis. The stacking gel has a very large pore size (4% acrylamide), which allows the proteins to move freely and concentrate, or stack, under the effect of the electric field. The band-sharpening effect relies on the fact that negatively charged glycinate ions (in the electrophoresis buffer) have a lower electrophoretic mobility than do the protein–SDS complexes, which, in turn, have lower mobility than the chloride ions (Cl^-) of the loading buffer and the stacking gel

buffer. When the current is switched on, all the ionic species have to migrate at the same speed otherwise there would be a break in the electrical circuit. The glycinate ions can move at the same speed as Cl^- only if they are in a region of higher field strength. Field strength is inversely proportional to conductivity, which is proportional to concentration. The result is that the three species of interest adjust their concentrations so that [Cl^-] > [protein–SDS] > [glycinate]. There is only a small quantity of protein–SDS complexes, so they concentrate in a very tight band between glycinate and Cl^- boundaries. Once the glycinate reaches the separating gel it becomes more fully ionised in the higher pH environment and its mobility increases. (The pH of the stacking gel is 6.8, that of the separating gel is 8.8.) Thus, the interface between glycinate and Cl^- leaves behind the protein–SDS complexes, which are left to electrophorese at their own rates. The negatively charged protein–SDS complexes now continue to move towards the anode, and, because they have the same charge per unit length, they travel into the separating gel under the applied electric field with the same mobility. However, as they pass through the separating gel the proteins separate, owing to the molecular sieving properties of the gel. Quite simply, the smaller the protein the more easily it can pass through the pores of the gel, whereas large proteins are successively retarded by frictional resistance due to the sieving effect of the gels. Being a small molecule, the bromophenol blue dye is totally unretarded and therefore indicates the electrophoresis front. When the dye reaches the bottom of the gel, the current is turned off, and the gel is removed from between the glass plates and shaken in an appropriate stain solution (usually Coomassie Brilliant Blue, see Section 10.3.7) and then washed in destain solution. The destain solution removes unbound background dye from the gel, leaving stained proteins visible as blue bands on a clear background. A typical minigel would take about 1 h to prepare and set, 40 min to run at 200 V and have a 1 h staining time with Coomassie Brilliant Blue. Upon destaining, strong protein bands would be seen in the gel within 10–20 min, but overnight destaining is needed to completely remove all background stain. Vertical slab gels are invariably run, since this allows up to 10 different samples to be loaded onto a single gel. A typical SDS–polyacrylamide gel is shown in Fig. 10.6.

Typically, the separating gel used is a 15% polyacrylamide gel. This gives a gel of a certain pore size in which proteins of relative molecular mass (M_r) 10 000 move through the gel relatively unhindered, whereas proteins of M_r 100 000 can only just enter the pores of this gel. Gels of 15% polyacrylamide are therefore useful for separating proteins in the range M_r 100 000 to 10 000. However, a protein of M_r 150 000, for example, would be unable to enter a 15% gel. In this case a larger-pored gel (e.g. a 10% or even 7.5% gel) would be used so that the protein could now enter the gel and be stained and identified. It is obvious, therefore, that the choice of gel to be used depends on the size of the protein being studied. The fractionation range of different percentage acrylamide gels is shown in Table 10.1. This shows, for example, that in a 10% polyacrylamide gel proteins greater than 200 kDa in mass cannot enter the gel, whereas proteins with relative molecular mass (M_r) in the range 200 000 to 15 000 will separate. Proteins of M_r 15 000 or less are too small to experience the sieving effect of the gel matrix, and all run together as a single band at the electrophoresis front.

Table 10.1 **The relationship between acrylamide gel concentration and protein fractionation range**

Acrylamide concentration (%)	Protein fractionation range ($M_r \times 10^{-3}$)
5	60–350
10	15–200
15	10–100

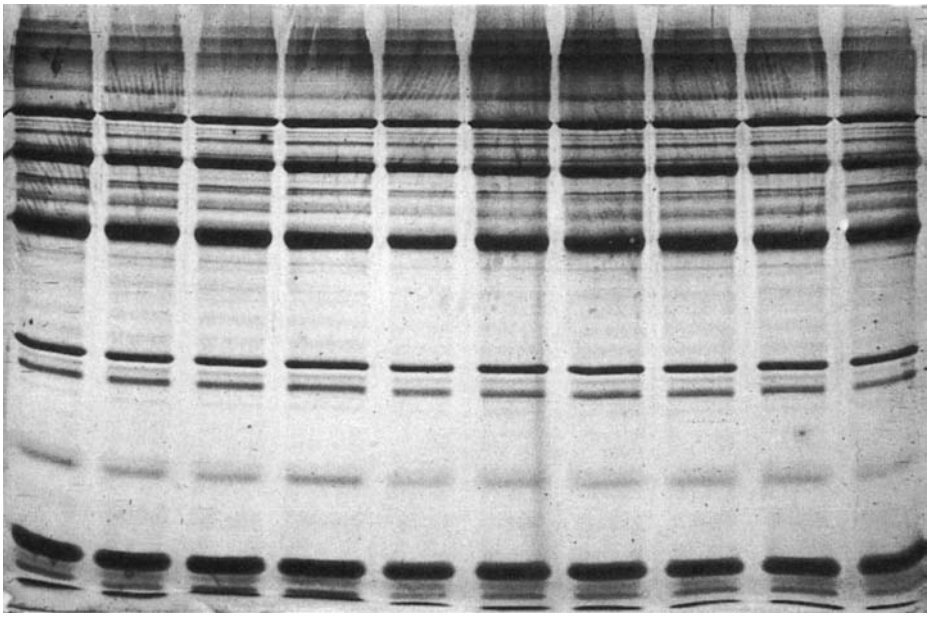


Fig. 10.6 A typical SDS–polyacrylamide gel. All 10 wells in the gel have been loaded with the same complex mixture of proteins. (Courtesy of Bio-Rad Laboratories.)

The M_r of a protein can be determined by comparing its mobility with those of a number of standard proteins of known M_r that are run on the same gel. By plotting a graph of distance moved against log M_r for each of the standard proteins, a calibration curve can be constructed. The distance moved by the protein of unknown M_r is then measured, and then its log M_r and hence M_r can be determined from the calibration curve.

SDS–gel electrophoresis is often used after each step of a purification protocol to assess the purity or otherwise of the sample. A pure protein should give a single band on an SDS–polyacrylamide gel, unless the molecule is made up of two unequal subunits. In the latter case two bands, corresponding to the two subunits, will be seen. Since only submicrogram amounts of protein are needed for the gel, very little material is used in this form of purity assessment and at the same time a value for the

Example 1 MOLECULAR MASS DETERMINATION BY ELECTROPHORESIS

Question The following table shows the distance moved in an SDS–polyacrylamide gel by a series of marker proteins of known relative molecular mass (M_r). A newly purified protein (X) run on the same gel showed a single band that had moved a distance of 45 mm. What was the M_r of protein X?

Protein	M_r	Distance moved (mm)
Transferrin	78 000	6.0
Bovine serum albumin	66 000	12.5
Ovalbumin (egg albumin)	45 000	32.0
Glyceraldehyde-3-phosphate dehydrogenase	36 000	38.0
Carbonic anhydrase	29 000	50.0
Trypsinogen	24 000	54.0
Soyabean trypsin inhibitor	20 100	61.0
β-Lactoglobulin	18 400[a]	69.0
Myoglobin	17 800	69.0
Lysozyme	14 300	79.0
Cytochrome *c*	12 400	86.5

Note: [a]β-lactoglobulin has a relative molecular mass of 36 800 but is a dimer of two identical subunits of 18 400 relative molecular mass. Under the reducing conditions of the sample buffer the disulphide bridges linking the subunits are reduced and thus the monomer chains are seen on the gel.

Answer Construct a calibration graph by plotting log M_r versus distance moved for each of the marker proteins. From a graph of log M_r versus the distance moved by each protein you can determine a relative molecular mass for protein X of approximately 31 000. Note that this method is accurate to ± 10%, so your answer is 31 000 ± 31 00.

relative molecular mass of the protein can be determined on the same gel run (as described above), with no more material being used.

10.3.2 Native (buffer) gels

While SDS–PAGE is the most frequently used gel system for studying proteins, the method is of no use if one is aiming to detect a particular protein (often an enzyme) on the basis of its biological activity, because the protein (enzyme) is denatured by the SDS–PAGE procedure. In this case it is necessary to use non-denaturing conditions. In native or buffer gels, polyacrylamide gels are again used (normally a 7.5% gel) but the SDS is absent and the proteins are *not* denatured prior to loading. Since all the proteins in the sample being analysed carry their native charge at the pH of the gel (normally pH 8.7), proteins separate according to their different electrophoretic mobilities *and* the sieving effects of the gel. It is therefore not possible to predict the behaviour of a given protein in a buffer gel but, because of the range of different

charges and sizes of proteins in a given protein mixture, good resolution is achieved. The enzyme of interest can be identified by incubating the gel in an appropriate substrate solution such that a coloured product is produced at the site of the enzyme. An alternative method for enzyme detection is to include the substrate in an agarose gel that is poured over the acrylamide gel and allowed to set. Diffusion and interaction of enzyme and substrate between the two gels results in colour formation at the site of the enzyme. Often, duplicate samples will be run on a gel, the gel cut in half and one half stained for activity, the other for total protein. In this way the total protein content of the sample can be analysed and the particular band corresponding to the enzyme identified by reference to the activity stain gel.

10.3.3 Gradient gels

This is again a polyacrylamide gel system, but instead of running a slab gel of uniform pore size throughout (e.g. a 15% gel) a gradient gel is formed, where the acrylamide concentration varies uniformly from, typically, 5% at the top of the gel to 25% acrylamide at the bottom of the gel. The gradient is formed via a gradient mixer and run down between the glass plates of a slab gel. The higher percentage acrylamide (e.g. 25%) is poured between the glass plates first and a continuous gradient of decreasing acrylamide concentration follows. Therefore at the top of the gel there is a large pore size (5% acrylamide) but as the sample moves down through the gel the acrylamide concentration slowly increases and the pore size correspondingly decreases. Gradient gels are normally run as SDS gels with a stacking gel. There are two advantages to running gradient gels. First, a much greater range of protein M_r values can be separated than on a fixed-percentage gel. In a complex mixture, very low molecular weight proteins travel freely through the gel to begin with, and start to resolve when they reach the smaller pore sizes towards the lower part of the gel. Much larger proteins, on the other hand, can still enter the gel but start to separate immediately due to the sieving effect of the gel. The second advantage of gradient gels is that proteins with very similar M_r values may be resolved, although they cannot otherwise be resolved in fixed percentage gels. As each protein moves through the gel the pore sizes become smaller until the protein reaches its pore size limit. The pore size in the gel is now too small to allow passage of the protein, and the protein sample stacks up at this point as a sharp band. A similar-sized protein but with slightly lower M_r will be able to travel a little further through the gel before reaching its pore size limit, at which point it will form a sharp band. These two proteins, of slightly different M_r values, therefore separate as two, close, sharp bands.

10.3.4 Isoelectric focussing gels

This method is ideal for the separation of amphoteric substances such as proteins because it is based on the separation of molecules according to their different isoelectric points (Section 8.1). The method has high resolution, being able to separate proteins that differ in their isoelectric points by as little as 0.01 of a pH unit. The most widely used system for IEF utilises horizontal gels on glass plates or plastic sheets.

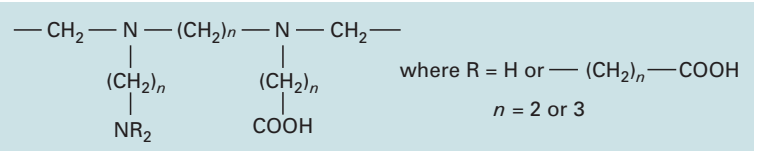


Fig. 10.7 The general formula for ampholytes.

Separation is achieved by applying a potential difference across a gel that contains a pH gradient. The pH gradient is formed by the introduction into the gel of compounds known as ampholytes, which are complex mixtures of synthetic polyaminopolycarboxylic acids (Fig. 10.7). Ampholytes can be purchased in different pH ranges covering either a wide band (e.g. pH 3–10) or various narrow bands (e.g. pH 7–8), and a pH range is chosen such that the samples being separated will have their isoelectric points (pI values) within this range. Commercially available ampholytes include Bio-Lyte and Pharmalyte.

Traditionally 1–2 mm thick IEF gels have been used by research workers, but the relatively high cost of ampholytes makes this a fairly expensive procedure if a number of gels are to be run. However, the introduction of thin-layer IEF gels, which are only 0.15 mm thick and which are prepared using a layer of electrical insulation tape as the spacer between the gel plates, has considerably reduced the cost of preparing IEF gels, and such gels are now commonly used. Since this method requires the proteins to move freely according to their charge under the electric field, IEF is carried out in low percentage gels to avoid any sieving effect within the gel. Polyacrylamide gels (4%) are commonly used, but agarose is also used, especially for the study of high M_r proteins that may undergo some sieving even in a low percentage acrylamide gel.

To prepare a thin-layer IEF gel, carrier ampholytes, covering a suitable pH range, and riboflavin are mixed with the acrylamide solution, and the mixture is then poured over a glass plate (typically 25 cm × 10 cm), which contains the spacer. The second glass plate is then placed on top of the first to form the gel cassette, and the gel polymerised by photopolymerisation by placing the gel in front of a bright light. The photodecomposition of the riboflavin generates a free radical, which initiates polymerisation (Section 10.2.2). This takes 2–3 h. Once the gel has set, the glass plates are prised apart to reveal the gel stuck to one of the glass sheets. Electrode wicks, which are thick (3 mm) strips of wetted filter paper (the anode is phosphoric acid, the cathode sodium hydroxide) are laid along the long length of each side of the gel and a potential difference applied. Under the effect of this potential difference, the ampholytes form a pH gradient between the anode and cathode. The power is then turned off and samples applied by laying on the gel small squares of filter paper soaked in the sample. A voltage is again applied for about 30 min to allow the sample to electrophorese off the paper and into the gel, at which time the paper squares can be removed from the gel. Depending on which point on the pH gradient the sample has been loaded, proteins that are initially at a pH region below their isoelectric point will be positively charged and will initially migrate towards the cathode. As they proceed, however, the surrounding pH will be steadily increasing, and therefore the positive

charge on the protein will decrease correspondingly until eventually the protein arrives at a point where the pH is equal to its isoelectric point. The protein will now be in the zwitterion form with no net charge, so further movement will cease. Likewise, substances that are initially at pH regions above their isoelectric points will be negatively charged and will migrate towards the anode until they reach their isoelectric points and become stationary. It can be seen that as the samples will always move towards their isoelectric points it is not critical where on the gel they are applied. To achieve rapid separations (2–3 h) relatively high voltages (up to 2500 V) are used. As considerable heat is produced, gels are run on cooling plates (10 °C) and power packs used to stabilise the power output and thus to minimise thermal fluctuations. Following electrophoresis, the gel must be stained to detect the proteins. However, this cannot be done directly, because the ampholytes will stain too, giving a totally blue gel. The gel is therefore first washed with fixing solution (e.g. 10% (v/v) trichloroacetic acid). This precipitates the proteins in the gel and allows the much smaller ampholytes to be washed out. The gel is stained with Coomassie Brilliant Blue and then destained (Section 10.3.7). A typical IEF gel is shown in Fig. 10.8.

The pI of a particular protein may be determined conveniently by running a mixture of proteins of known isoelectric point on the same gel. A number of mixtures of proteins with differing pI values are commercially available, covering the pH range 3.5–10. After staining, the distance of each band from one electrode is measured and a graph of distance for each protein against its pI (effectively the pH at that point) plotted. By means of this calibration line, the pI of an unknown protein can be determined from its position on the gel.

IEF is a highly sensitive analytical technique and is particularly useful for studying microheterogeneity in a protein. For example, a protein may show a single band on an SDS gel, but may show three bands on an IEF gel. This may occur, for example, when a protein exists in mono-, di- and tri-phosphorylated forms. The difference of a couple of phosphate groups has no significant effect on the overall relative molecular mass of the protein, hence a single band on SDS gels, but the small charge difference introduced on each molecule can be detected by IEF.

The method is particularly useful for separating isoenzymes (Section 8.2), which are different forms of the same enzyme often differing by only one or two amino acid residues. Since the proteins are in their native form, enzymes can be detected in the gel either by washing the unfixed and unstained gel in an appropriate substrate or by overlayering with agarose containing the substrate. The approach has found particular use in forensic science, where traces of blood or other biological fluids can be analysed and compared according to the composition of certain isoenzymes.

Although IEF is used mainly for analytical separations, it can also be used for preparative purposes. In vertical column IEF, a water-cooled vertical glass column is used, filled with a mixture of ampholytes dissolved in a sucrose solution containing a density gradient to prevent diffusion. When the separation is complete, the current is switched off and the sample components run out through a valve in the base of the column. Alternatively, preparative IEF can be carried out in beds of granulated gel, such as Sephadex G-75 (Section 11.7).

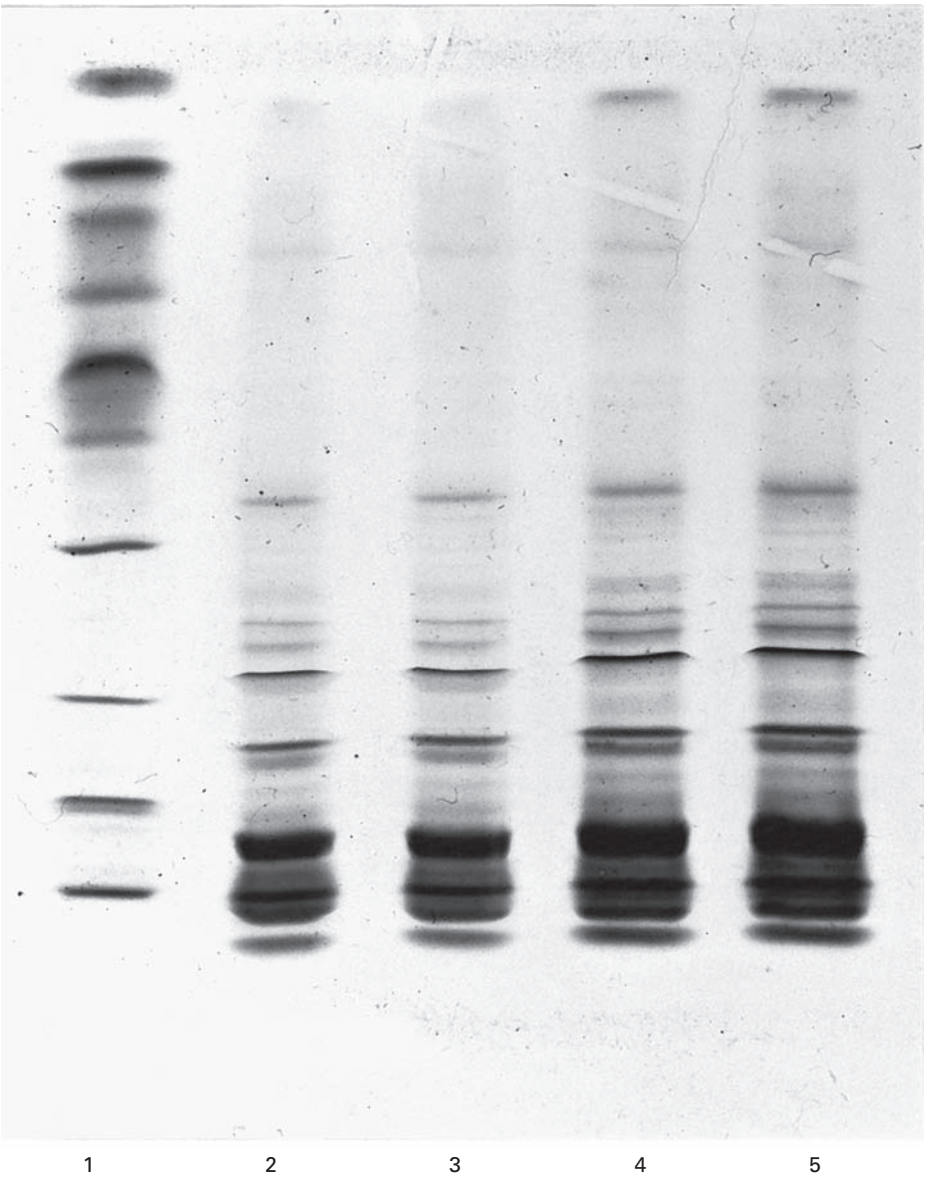


Fig. 10.8 A typical isoelectric focussing gel. Track 1 contains a mixture of standard proteins of known isoelectric points. Tracks 2–5 show increasing loadings of venom from the Japanese water moccasin snake. (Courtesy of Bio-Rad Laboratories Ltd.)

10.3.5 Two-dimensional polyacrylamide gel electrophoresis

This technique combines the technique of IEF (first dimension), which separates proteins in a mixture according to charge (pI), with the size separation technique of SDS–PAGE (second dimension). The combination of these two techniques to give two-dimensional (2-D) PAGE provides a highly sophisticated analytical method for analysing protein mixtures. To maximise separation, most workers use large format

2-D gels (20 cm × 20 cm), although the minigel system can be used to provide useful separation in some cases. For large-format gels, the first dimension (isoelectric focussing) is carried out in an acrylamide gel that has been cast on a plastic strip (18 cm × 3 mm wide). The gel contains ampholytes (for forming the pH gradient) together with 8 M urea and a non-ionic detergent, both of which denature and maintain the solubility of the proteins being analysed. The denatured proteins therefore separate in this gel according to their isoelectric points. The IEF strip is then incubated in a sample buffer containing SDS (thus binding SDS to the denatured proteins) and then placed between the glass plates of, and on top of, a previously prepared 10% SDS–PAGE gel. Electrophoresis is commenced and the SDS-bound proteins run into the gel and separate according to size, as described in Section 10.3.1. The IEF gels are provided as dried strips and need rehydrating overnight. The first dimension IEF run takes 6−8 h, the equilibration step with SDS sample buffer takes about 15 min, and then the SDS–PAGE step takes about 5 h. A typical 2-D gel is shown in Fig. 10.9. Using this method one can routinely resolve between 1000 and 3000 proteins from a cell or tissue extract and in some cases workers have reported the separation of between 5000 and 10 000 proteins. The applications of 2-D PAGE, and a description of the method's central role in proteomics is described in Section 8.5.1.

10.3.6 Cellulose acetate electrophoresis

Although one of the older methods, cellulose acetate electrophoresis still has a number of applications. In particular it has retained a use in the clinical analysis of serum samples. Cellulose acetate has the advantage over paper in that it is a much more homogeneous medium, with uniform pore size, and does not adsorb proteins in the way that paper does. There is therefore much less trailing of protein bands and resolution is better, although nothing like as good as that achieved with polyacrylamide gels. The method is, however, far simpler to set up and run. Single samples are normally run on cellulose acetate strips (2.5 cm × 12 cm), although multiple samples are frequently run on wider sheets. The cellulose acetate is first wetted in electrophoresis buffer (pH 8.6 for serum samples) and the sample (1−2 mm^3) loaded as a 1 cm wide strip about one-third of the way along the strip. The ends of the strip make contact with the electrophoresis buffer tanks via a filter paper wick that overlaps the end of the cellulose acetate strip, and electrophoresis is conducted at 6–8 V cm^{-1} for about 3 h. Following electrophoresis, the strip is stained for protein (see Section 10.3.7), destained, and the bands visualised. A typical serum protein separation shows about six major bands. However, in many disease states, this serum protein profile changes and a clinician can obtain information concerning the disease state of a patient from the altered pattern. Although still frequently used for serum analysis, electrophoresis on cellulose acetate is being replaced by the use of agarose gels, which give similar but somewhat better resolution. A typical example of the analysis of serum on an agarose gel is shown in Fig. 10.10. Similar patterns are obtained when cellulose acetate is used.

Enzymes can easily be detected, in samples electrophoresed on cellulose acetate, by using the zymogram technique. The cellulose strip is laid on a strip of filter paper

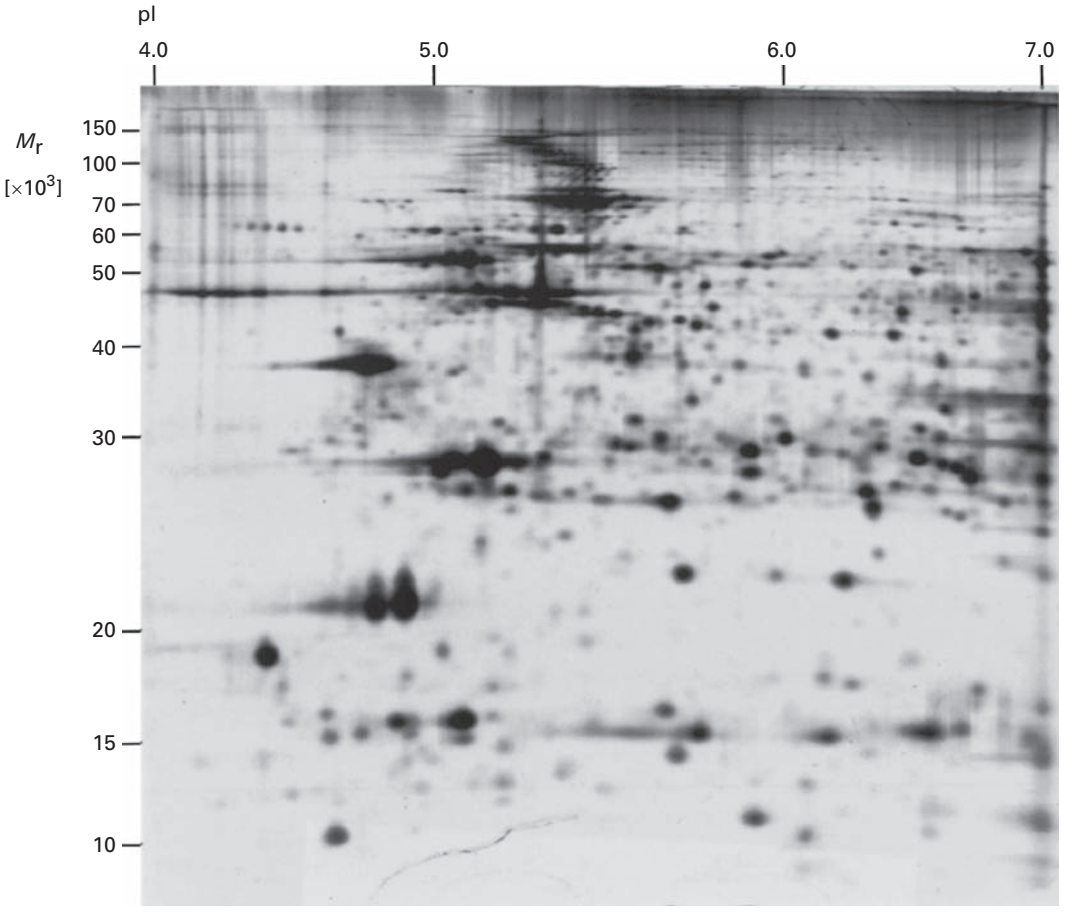


Fig. 10.9 A typical two-dimensional gel. The sample applied was 100 μg of total protein extracted from a normal dog heart ventricle. The first dimension was carried out using a pH 4–7 isoelectric focussing gel. The second dimension was a 12% SDS–PAGE vertical slab gel. The pattern was visualised by silver staining. (Courtesy of Monique Heinke and Dr Mike Dunn, Division of Cardiothoracic Surgery, Imperial College School of Medicine, Heart Science Centre, Harefield, UK.)

soaked in buffer and substrate. After an appropriate incubation period, the strips are peeled apart and the paper zymogram treated accordingly to detect enzyme product; hence, it is possible to identify the position of the enzyme activity on the original strip. An alternative approach to detecting and semiquantifying *any* particular protein on a strip is to treat the strip as the equivalent of a protein blot and to probe for the given protein using primary antibody and then enzyme-linked secondary antibody (Section 10.3.8). Substrate colour development indicates the presence of the particular protein and the amount of colour developed in a given time is a semiquantitative measure of the amount of protein. Thus, for example, large numbers of serum samples can be run on a wide sheet, the sheet probed using antibodies, and elevated levels of a particular protein identified in certain samples by increased levels of colour development in these samples.

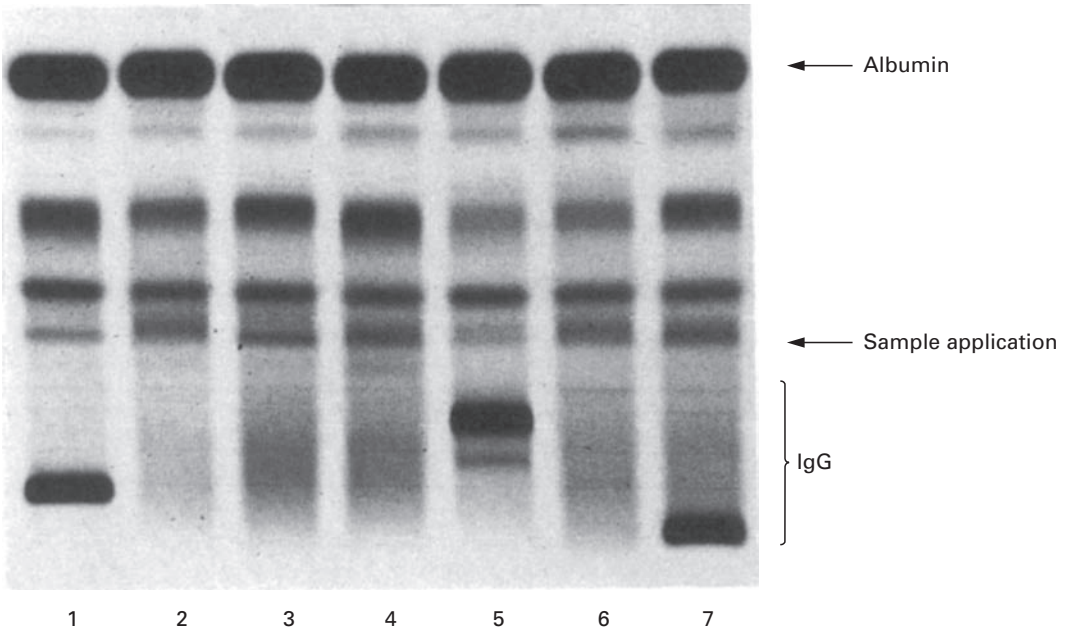


Fig. 10.10 Electrophoresis of human serum samples on an agarose gel. Tracks 2, 3, 4 and 6 show normal serum protein profiles. Tracks 1, 5 and 7 show myeloma patients, who are identified by the excessive production of a particular monoclonal antibody seen in the IgG fraction. (Courtesy of Charles Andrews and Nicholas Cundy, Edgware General Hospital, London.)

10.3.7 Detection, estimation and recovery of proteins in gels

The most commonly used general protein stain for detecting protein on gels is the sulphated trimethylamine dye Coomassie Brilliant Blue R-250 (CBB). Staining is usually carried out using 0.1% (w/v) CBB in methanol:water:glacial acetic acid (45:45:10, by vol.). This acid–methanol mixture acts as a denaturant to precipitate or fix the protein in the gel, which prevents the protein from being washed out whilst it is being stained. Staining of most gels is accomplished in about 2 h and destaining, usually overnight, is achieved by gentle agitation in the same acid–methanol solution but in the absence of the dye. The Coomassie stain is highly sensitive; a very weakly staining band on a polyacrylamide gel would correspond to about 0.1 μg (100 ng) of protein. The CBB stain is not used for staining cellulose acetate (or indeed protein blots) because it binds quite strongly to the paper. In this case, proteins are first denatured by brief immersion of the strip in 10% (v/v) trichloroacetic acid, and then immersed in a solution of a dye that does not stain the support material, for example Procion blue, Amido black or Procion S.

Although the Coomassie stain is highly sensitive, many workers require greater sensitivity such as that provided by silver staining. Silver stains are based either on techniques developed for histology or on methods based on the photographic process. In either case, silver ions (Ag^+) are reduced to metallic silver on the protein, where the silver is deposited to give a black or brown band. Silver stains can be used immediately after electrophoresis, or, alternatively, after staining with CBB. With the latter

approach, the major bands on the gel can be identified with CBB and then minor bands, not detected with CBB, resolved using the silver stain. The silver stain is at least 100 times more sensitive than CBB, detecting proteins down to 1 ng amounts. Other stains with similar sensitivity include the fluorescent stains Sypro Orange (30 ng) and Sypro Ruby (10 ng).

Glycoproteins have traditionally been detected on protein gels by use of the periodic acid–Schiff (PAS) stain. This allows components of a mixture of glycoproteins to be distinguished. However, the PAS stain is not very sensitive and often gives very weak, red-pink bands, difficult to observe on a gel. A far more sensitive method used nowadays is to blot the gel (Section 10.3.8) and use lectins to detect the glycoproteins. Lectins are protein molecules that bind carbohydrates, and different lectins have been found that have different specificities for different types of carbohydrate. For example, certain lectins recognise mannose, fucose, or terminal glucosamine of the carbohydrate side-chains of glycoproteins. The sample to be analysed is run on a number of tracks of an SDS–polyacrylamide gel. Coloured bands appear at the point where the lectins bind if each blotted track is incubated with a different lectin, washed, incubated with a horseradish peroxidase-linked antibody to the lectin, and then peroxidase substrate added. In this way, by testing a protein sample against a series of lectins, it is possible to determine not only that a protein is a *glyco*protein, but to obtain information about the type of glycosylation.

Quantitative analysis (i.e. measurements of the relative amounts of different proteins in a sample) can be achieved by scanning densitometry. A number of commercial scanning densitometers are available, and work by passing the stained gel track over a beam of light (laser) and measuring the transmitted light. A graphic presentation of protein zones (peaks of absorbance) against migration distance is produced, and peak areas can be calculated to obtain quantitative data. However, such data must be interpreted with caution because there is only a limited range of protein concentrations over which there is a linear relationship between absorbance and concentration. Also, equal amounts of different proteins do not always stain equally with a given stain, so any data comparing the relative amounts of protein can only be semiquantitative. An alternative and much cheaper way of obtaining such data is to cut out the stained bands of interest, elute the dye by shaking overnight in a known volume of 50% pyridine, and then to measure spectrophotometrically the amount of colour released. More recently gel documentation systems have been developed, which are replacing scanning densitometers. Such benchtop systems comprise a video imaging unit (computer linked) attached to a small ‘darkroom’ unit that is fitted with a choice of white or ultraviolet light (transilluminator). Gel images can be stored on the computer, enhanced accordingly and printed as required on a thermal printer, thus eliminating the need for wet developing in a purpose built darkroom, as is the case for traditional photography.

Although gel electrophoresis is used generally as an analytical tool, it can be utilised to separate proteins in a gel to achieve protein purification. Protein bands can be cut out of protein blots and sequence data obtained by placing the blot in a protein sequencer (see Section 8.4.3). Stained protein bands can be cut out of protein gels and the protein recovered by electrophoresis of the protein out of the gel piece

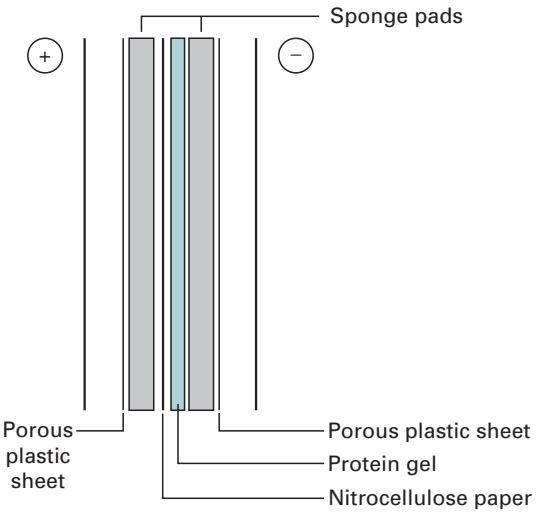


Fig. 10.11 Diagrammatic representation of electroblotting. The gel to be blotted is placed on top of a sponge pad saturated in buffer. The nitrocellulose sheet is then placed on top of the gel, followed by a second sponge pad. This sandwich is supported between two rigid porous plastic sheets and held together with two elastic bands. The sandwich is then placed between parallel electrodes in a buffer reservoir and an electric current passed. The sandwich must be placed such that the immobilising medium is between the gel and the anode for SDS–polyacrylamide gels, because all the proteins carry a negative charge.

(electroelution). A number of different designs of electroelution cells are commercially available, but perhaps the easiest method is to seal the gel piece in buffer in a dialysis sac and place the sac in buffer between two electrodes. Protein will electrophorese out of the gel piece towards the appropriate electrode but will be retained by the dialysis sac. After electroelution, the current is reversed for a few seconds to drive off any protein that has adsorbed to the wall of the dialysis sac and then the protein solution within the sac is recovered.

10.3.8 Protein (western) blotting

Although essentially an analytical technique, PAGE does of course achieve fractionation of a protein mixture during the electrophoresis process. It is possible to make use of this fractionation to examine further individual separated proteins. The first step is to transfer or blot the pattern of separated proteins from the gel onto a sheet of nitrocellulose paper. The method is known as protein blotting, or western blotting by analogy with Southern blotting (Section 5.9.2), the equivalent method used to recover DNA samples from an agarose gel. Transfer of the proteins from the gel to nitrocellulose is achieved by a technique known as electroblotting. In this method a sandwich of gel and nitrocellulose is compressed in a cassette and immersed, in buffer, between two parallel electrodes (Fig. 10.11). A current is passed at right angles to the gel, which causes the separated proteins to electrophorese out of the gel and into the nitrocellulose sheet. The nitrocellulose with its transferred protein is referred to as a blot. Once transferred onto nitrocellulose, the separated proteins can be examined further. This involves probing the blot, usually using an antibody to detect a specific

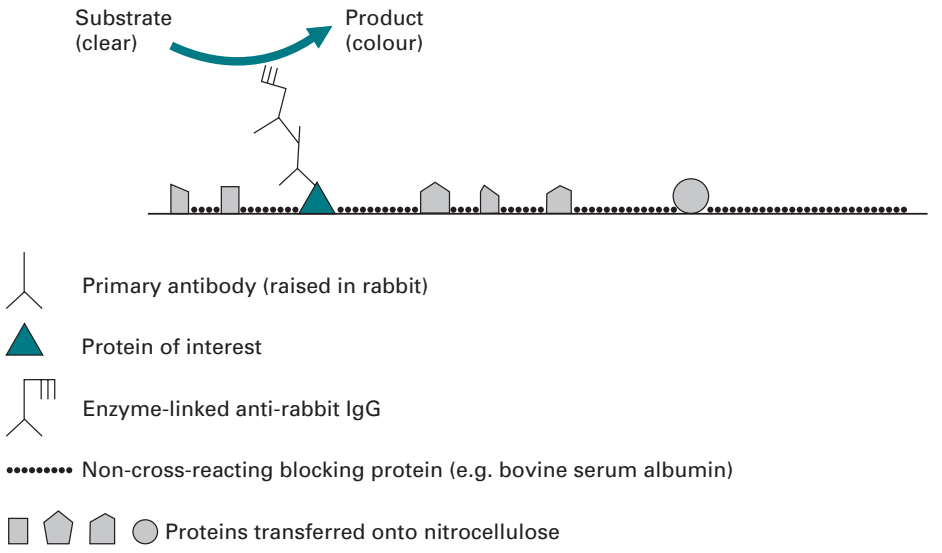


Fig. 10.12 The use of enzyme-linked second antibodies in immunodetection of protein blots. First, the primary antibody (e.g. raised in a rabbit) detects the protein of interest on the blot. Second, enzyme-linked anti-rabbit IgG detects the primary antibody. Third, addition of enzyme substrate results in coloured product deposited at the site of protein of interest on the blot.

protein. The blot is first incubated in a protein solution, for example 10% (w/v) bovine serum albumin, or 5% (w/v) non-fat dried milk (the so-called blotto technique), which will block all remaining hydrophobic binding sites on the nitrocellulose sheet. The blot is then incubated in a dilution of an antiserum (primary antibody) directed against the protein of interest. This IgG molecule will bind to the blot if it detects its antigen, thus identifying the protein of interest. In order to visualise this interaction the blot is incubated further in a solution of a secondary antibody, which is directed against the IgG of the species that provided the primary antibody. For example, if the primary antibody was raised in a rabbit then the secondary antibody would be anti-rabbit IgG. This secondary antibody is appropriately labelled so that the interaction of the secondary antibody with the primary antibody can be visualised on the blot. Anti-species IgG molecules are readily available commercially, with a choice of different labels attached. One of the most common detection methods is to use an enzyme-linked secondary antibody (Fig. 10.12). In this case, following treatment with enzyme-labelled secondary antibody, the blot is incubated in enzyme–substrate solution, when the enzyme converts the substrate into an insoluble coloured product that is precipitated onto the nitrocellulose. The presence of a coloured band therefore indicates the position of the protein of interest. By careful comparisons of the blot with a stained gel of the same sample, the protein of interest can be identified. The enzyme used in enzyme-linked antibodies is usually either alkaline phosphatase, which converts colourless 5-bromo-4-chloro-indolylphosphate (BCIP) substrate into a blue product, or horseradish peroxidase, which, with H_2O_2 as a substrate, oxidises either 3-amino-9-ethylcarbazole into an insoluble brown product, or 4-chloro-l-naphthol into an insoluble blue product. An alternative approach to the detection of

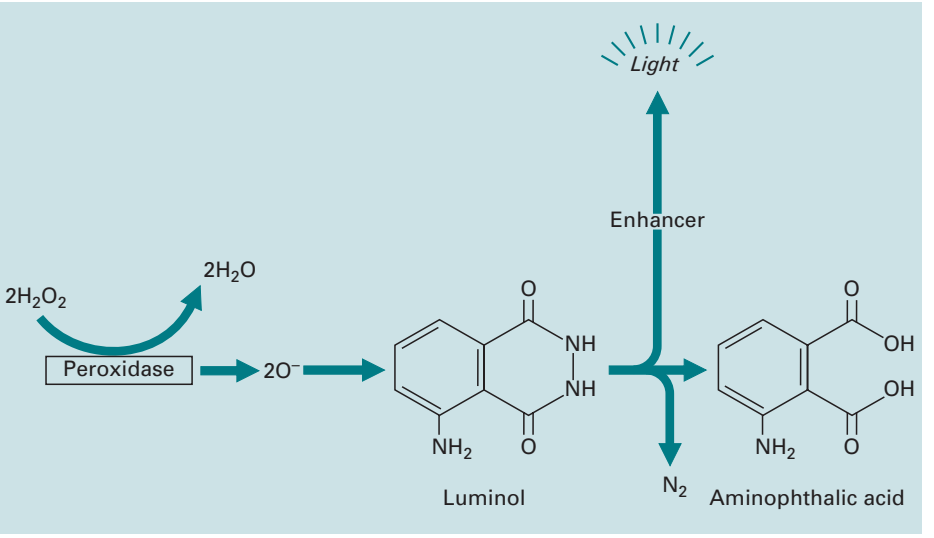


Fig. 10.13 The use of enhanced chemiluminescence to detect horseradish peroxidase.

horseradish peroxidase is to use the method of enhanced chemiluminescence (ECL). In the presence of hydrogen peroxide and the chemiluminescent substrate luminol (Fig. 10.13) horseradish peroxidase oxidises the luminol with concomitant production of light, the intensity of which is increased 1000-fold by the presence of a chemical enhancer. The light emission can be detected by exposing the blot to a photographic film. Corresponding ECL substrates are available for use with alkaline-phosphatase-labelled antibodies. The principle behind the use of enzyme-linked antibodies to detect antigens in blots is highly analogous to that used in enzyme-linked immunosorbent assays (Section 7.3.1).

Although enzymes are commonly used as markers for second antibodies, other markers can also be used. These include:

- *^{125}I-labelled secondary antibody:* Binding to the blot is detected by autoradiography (Section 14.3.3).
- *Fluorescein-labelled secondary antibody:* The fluorescent label is detected by exposing the blot to ultraviolet light.
- *^{125}I-labelled protein A:* Protein A is purified from *Staphylococcus aureus* and specifically binds to the Fc region of IgG molecules. ^{125}I-labelled protein A is therefore used instead of a second antibody, and binding to the blot is detected by autoradiography.
- *Biotinylated secondary antibodies:* Biotin is a small molecular weight vitamin that binds strongly to the egg protein avidin ($K_d = 10^{-15}$ M). The blot is incubated with biotinylated secondary antibody, then incubated further with enzyme-conjugated avidin. Since multiple biotin molecules can be linked to a single antibody molecule, many enzyme-linked avidin molecules can bind to a single biotinylated antibody molecule, thus providing an enhancement of the signal. The enzyme used is usually alkaline phosphatase or horseradish peroxidase.

- *Quantum dots*: These are engineered semiconductor nanoparticles, with diameters of the order of 2–10 nm, which fluoresce when exposed to UV light. Quantum dot nanocrystals comprise a semiconductor core of CdSe surrounded by a shell of ZnS. This crystal is then coated with an organic molecular layer that provides water solubility, and conjugation sites for biomolecules. Typically, therefore, second antibodies will be bound to a quantum dot, and the position of binding of the second antibody on the blot identified by exposing the blot to UV light.

In addition to the use of labelled antibodies or proteins, other probes are sometimes used. For example, radioactively labelled DNA can be used to detect DNA-binding proteins on a blot. The blot is first incubated in a solution of radiolabelled DNA, then washed, and an autoradiograph of the blot made. The presence of radioactive bands, detected on the autoradiograph, identifies the positions of the DNA-binding proteins on the blot.

10.4 ELECTROPHORESIS OF NUCLEIC ACIDS

10.4.1 Agarose gel electrophoresis of DNA

For the majority of DNA samples, electrophoretic separation is carried out in agarose gels. This is because most DNA molecules and their fragments that are analysed routinely are considerably larger than proteins and therefore, because most DNA fragments would be unable to enter a polyacrylamide gel, the larger pore size of an agarose gel is required. For example, the commonly used plasmid pBR322 has an M_r of 2.4×10^6. However, rather than use such large numbers it is more convenient to refer to DNA size in terms of the number of base-pairs. Although, originally, DNA size was referred to in terms of base-pairs (bp) or kilobase-pairs (kbp), it has now become the accepted nomenclature to abbreviate kbp to simply kb when referring to double-stranded DNA. pBR322 is therefore 4.36 kb. Even a small restriction fragment of 1 kb has an M_r of 620 000. When talking about single-stranded DNA it is common to refer to size in terms of nucleotides (nt). Since the charge per unit length (owing to the phosphate groups) in any given fragment of DNA is the same, all DNA samples should move towards the anode with the same mobility under an applied electrical field. However, separation in agarose gels is achieved because of resistance to their movement caused by the gel matrix. The largest molecules will have the most difficulty passing through the gel pores (very large molecules may even be blocked completely), whereas the smallest molecules will be relatively unhindered. Consequently the mobility of DNA molecules during gel electrophoresis will depend on size, the smallest molecules moving fastest. This is analogous to the separation of proteins in SDS–polyacrylamide gels (Section 10.3.1), although the analogy is not perfect, as double-stranded DNA molecules form relatively stiff rods and while it is not completely understood how they pass through the gel, it is probable that long DNA molecules pass through the gel pores end-on. While passing through the pores, a DNA molecule will experience drag; so the longer the molecule, the more it will be retarded by each pore. Sideways movement may become more important for very small double-stranded DNA

and for the more flexible single-stranded DNA. It will be obvious from the above that gel concentrations must be chosen to suit the size range of the molecules to be separated. Gels containing 0.3% agarose will separate double-stranded DNA molecules of between 5 and 60 kb size, whereas 2% gels are used for samples of between 0.1 and 3 kb. Many laboratories routinely use 0.8% gels, which are suitable for separating DNA molecules in the range 0.5–10 kb. Since agarose gels separate DNA according to size, the M_r of a DNA fragment may be determined from its electrophoretic mobility by running a number of standard DNA markers of known M_r on the same gel. This is most conveniently achieved by running a sample of bacteriophage λ DNA (49 kb) that has been cleaved with a restriction enzyme such as *Eco*RI. Since the base sequence of λ DNA is known, and the cleavage sites for *Eco*RI are known, this generates fragments of accurately known size (Fig. 10.14).

DNA gels are invariably run as horizontal, submarine or submerged gels; so named because such a gel is totally immersed in buffer. Agarose, dissolved in gel buffer by boiling, is poured onto a glass or plastic plate, surrounded by a wall of adhesive tape or a plastic frame to provide a gel about 3 mm in depth. Loading wells are formed by placing a plastic well-forming template or comb in the poured gel solution, and removing this comb once the gel has set. The gel is placed in the electrophoresis tank, covered with buffer, and samples loaded by directly injecting the sample into the wells. Samples are prepared by dissolving them in a buffer solution that contains sucrose, glycerol or Ficoll, which makes the solution dense and allows it to sink to the bottom of the well. A dye such as bromophenol blue is also included in the sample solvent; it makes it easier to see the sample that is being loaded and also acts as a marker of the electrophoresis front. No stacking gel (Section 10.3.1) is needed for the electrophoresis of DNA because the mobilities of DNA molecules are much greater in the well than in the gel, and therefore all the molecules in the well pile up against the gel within a few minutes of the current being turned on, forming a tight band at the start of the run. General purpose gels are approximately 25 cm long and 12 cm wide, and are run at a voltage gradient of about 1.5 V cm^{-1} overnight. A higher voltage would cause excessive heating. For rapid analyses that do not need extensive separation of DNA molecules, it is common to use mini-gels that are less than 10 cm long. In this way information can be obtained in 2–3 h.

Once the system has been run, the DNA in the gel needs to be stained and visualised. The reagent most widely used is the fluorescent dye ethidium bromide. The gel is rinsed gently in a solution of ethidium bromide (0.5 μg cm^{-3}) and then viewed under ultraviolet light (300 nm wavelength). Ethidium bromide is a cyclic planar molecule that binds between the stacked base-pairs of DNA (i.e. it intercalates) (Section 5.7.4). The ethidium bromide concentration therefore builds up at the site of the DNA bands and under ultraviolet light the DNA bands fluoresce orange-red. As little as 10 ng of DNA can be visualised as a 1 cm wide band. It should be noted that extensive viewing of the DNA with ultraviolet light can result in damage of the DNA by nicking and base-pair dimerisation. This is of no consequence if a gel is only to be viewed, but obviously viewing of the gel should be kept to a minimum if the DNA is to be recovered (see below). It is essential to protect one's eyes by wearing goggles when ultraviolet light is used. If viewing of gels under ultraviolet is carried out for long periods, a plastic mask that covers the whole face should be used to avoid 'sunburn'.

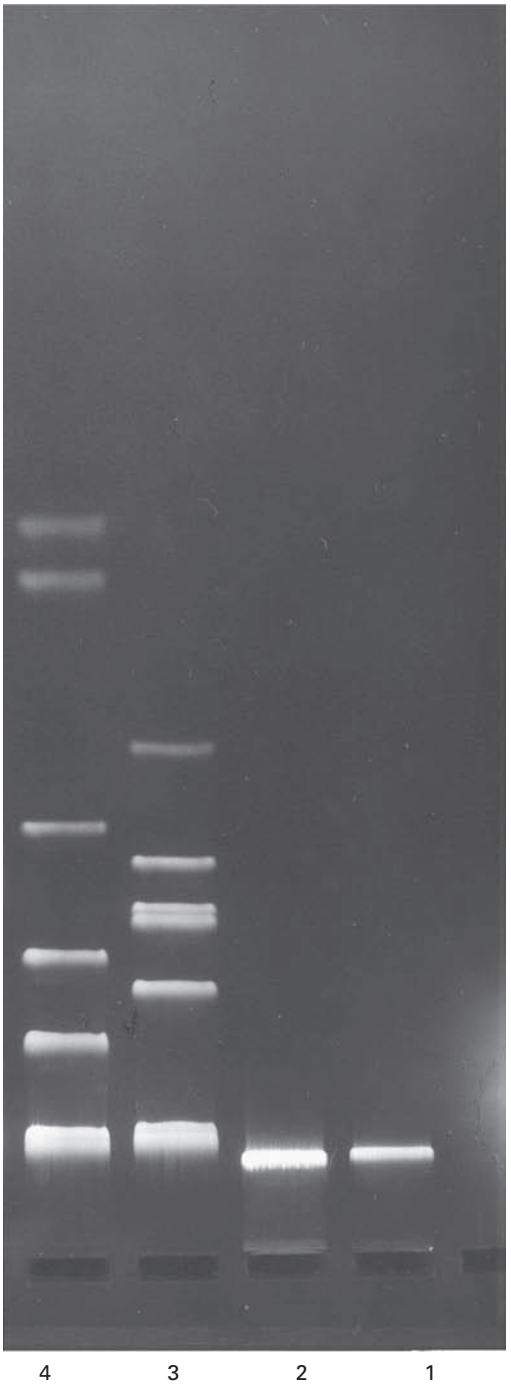


Fig. 10.14 Photograph showing four tracks from a 0.8% agarose submarine gel. The gel was run at 40 V in Tris/borate/EDTA buffer for 16 h, stained with ethidium bromide and viewed under ultraviolet light. Sample loadings were about 0.5 μg of DNA per track. Tracks 1 and 2, λ DNA (49 kb). Track 3, λ DNA cleaved with the enzyme *Eco*RI to generate fragments of the following size (in order from the origin): 21.80 kb, 7.52 kb, 5.93 kb, 5.54 kb, 4.80 kb, 3.41 kb. Track 4, λ DNA cleaved with the enzyme *Hin*dIII to generate fragments of the following size (in order from the origin): 23.70 kb, 9.46 kb, 6.75 kb, 4.26 kb, 2.26 kb, 1.98 kb. (Courtesy of Stephen Boffey, University of Hertfordshire.)

10.4.2 DNA sequencing gels

Although agarose gel electrophoresis of DNA is a 'workhorse' technique for the molecular biologist, a different form of electrophoresis has to be used when DNA sequences are to be determined. Whichever DNA sequencing method is used (Section 5.11), the final analysis usually involves separating single-stranded DNA molecules shorter than about 1000 nt and differing in size by only 1 nt. To achieve this it is necessary to have a small-pored gel and so acrylamide gels are used instead of agarose. For example, 3.5% polyacrylamide gels are used to separate DNA in the range 80–1000 nt and 12% gels to resolve fragments of between 20 and 100 nt. If a wide range of sizes is being analysed it is often convenient to run a gradient gel, for example from 3.5% to 7.5%. Sequencing gels are run in the presence of denaturing agents, urea and formamide. Since it is necessary to separate DNA molecules that are very similar in size, DNA sequencing gels tend to be very long (100 cm) to maximise the separation achieved. A typical DNA sequencing gel is shown in Fig. 5.38.

As mentioned above, electrophoresis in agarose can be used as a preparative method for DNA. The DNA bands of interest can be cut out of the gel and the DNA recovered by: (a) electroelution, (b) macerating the gel piece in buffer, centrifuging and collecting the supernatant; or (c), if low melting point agarose is used, melting the gel piece and diluting with buffer. In each case, the DNA is finally recovered by precipitation of the supernatant with ethanol.

10.4.3 Pulsed-field gel electrophoresis

The agarose gel methods for DNA described above can fractionate DNA of 60 kb or less. The introduction of pulsed-field gel electrophoresis (PFGE) and the further development of variations on the basic technique now means that DNA fragments up to 2×10^3 kb can be separated. This therefore allows the separation of whole chromosomes by electrophoresis. The method basically involves electrophoresis in agarose where two electric fields are applied alternately at different angles for defined time periods (e.g. 60 s). Activation of the first electric field causes the coiled molecules to be stretched in the horizontal plane and start to move through the gel. Interruption of this field and application of the second field force the molecule to move in the new direction. Since there is a length-dependent relaxation behaviour when a long-chain molecule undergoes conformational change in an electric field, the smaller a molecule, the quicker it realigns itself with the new field and is able to continue moving through the gel. Larger molecules take longer to realign. In this way, with continual reversing of the field, smaller molecules draw ahead of larger molecules and separate according to size. PFGE has proved particularly useful in identifying the course of outbreaks of bacterial foodborne illness (e.g. *Salmonella* infections). Having isolated the bacterial pathogen responsible for the illness from an individual, the DNA is isolated and cleaved into large fragments which are separated by PFGE. For example, DNA from *Salmonella* species, when digested with the restriction enzyme *Xba1*, gives around 15 fragments ranging from 25 kb to 680 kb. This pattern of fragments, or 'fingerprint', is unique to that strain. If the same fingerprint is found from bacteria

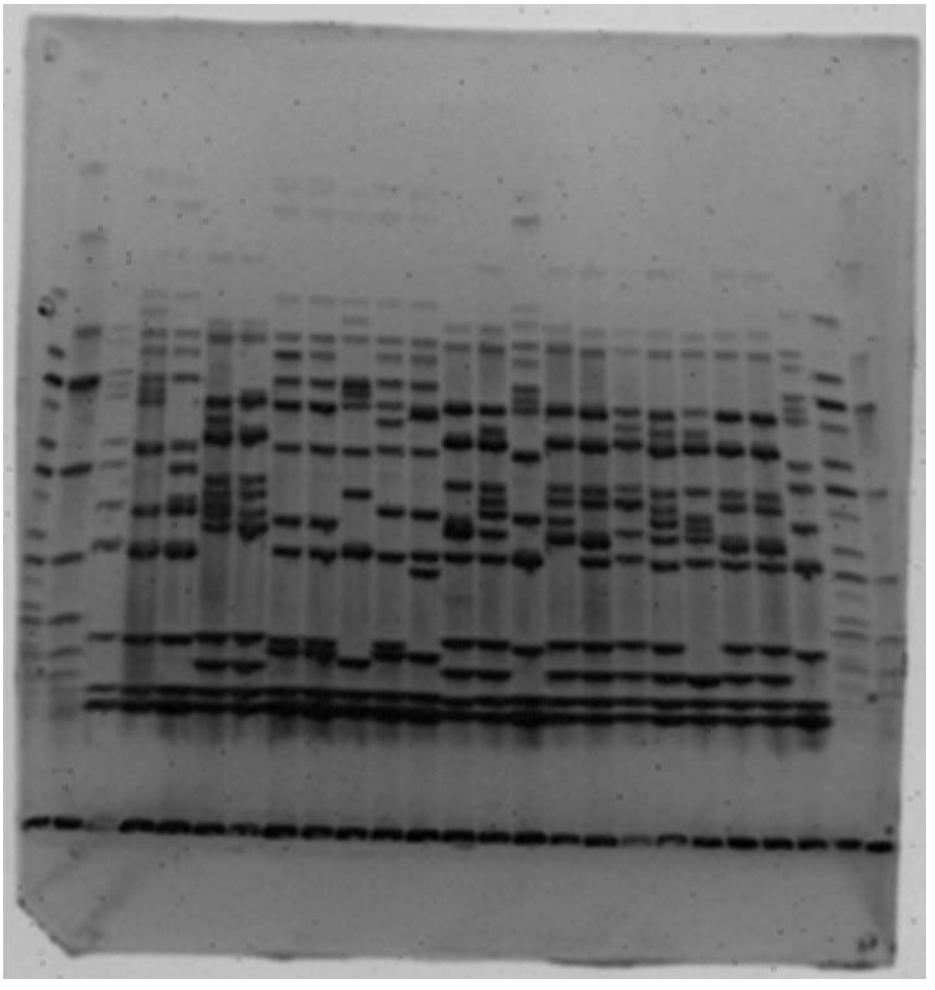


Fig. 10.15 PFGE separation of the digestion pattern produced with the restriction enzyme Nhe1, of 21 strains of *Neisseria meningitidis*. There are two molecular weight marker tracks at either end of the gel. (Courtesy of Dr Giovanna Morelli, Max-Planck Institute for Molecular Genetics, Berlin, Germany.)

from other infected people, then it can be assumed that they were all infected from a common source. Thus by comparing their eating habits, food sources, etc. the source of the infection can be traced to a restaurant, food item, etc. Figure 10.15 shows the restriction patterns from different strains of *Neisseria meningitidis*.

10.4.4 Electrophoresis of RNA

Like that of DNA, electrophoresis of RNA is usually carried out in agarose gels, and the principle of the separation, based on size, is the same. Often one requires a rapid method for checking the integrity of RNA immediately following extraction but before deciding whether to process it further. This can be achieved easily by electrophoresis in a 2% agarose gel in about 1 h. Ribosomal RNAs (18 S and 28 S) are clearly

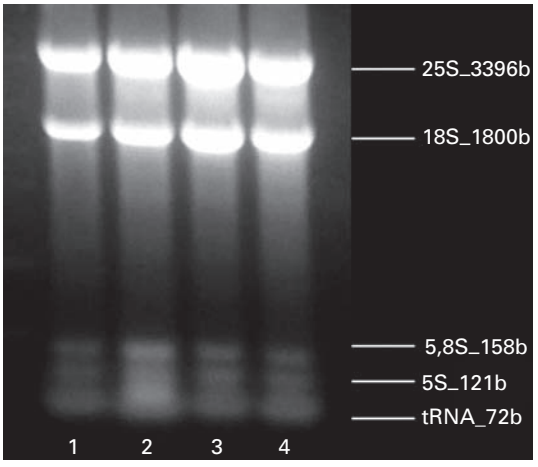


Fig. 10.16 Separation of yeast RNA on a 1.5% agarose gel. (Courtesy of Dr Tomas Masek, Department of Genetics and Microbiology, Charles University, Prague, Czech Republic.)

resolved and any degradation (seen as a smear) or DNA contamination is seen easily. This can be achieved on a 2.5–5% acrylamide gradient gel with an overnight run. Both these methods involve running native RNA. There will almost certainly be some secondary structure within the RNA molecule owing to intramolecular hydrogen bonding (see e.g. the clover leaf structure of tRNA, Fig. 5.6). For this reason native RNA run on gels can be stained and visualised with ethidium bromide. However, if the study objective is to determine RNA size by gel electrophoresis, then full denaturation of the RNA is needed to prevent hydrogen bond formation within or even between polynucleotides that will otherwise affect the electrophoretic mobility. There are three denaturing agents (formaldehyde, glyoxal and methylmercuric hydroxide) that are compatible with both RNA and agarose. Either one of these may be incorporated into the agarose gel and electrophoresis buffer, and the sample is heat denatured in the presence of the denaturant prior to electrophoresis. After heat denaturation, each of these agents forms adducts with the amino groups of guanine and uracil, thereby preventing hydrogen bond reformation at room temperature during electrophoresis. It is also necessary to run denaturing gels if the RNA is to be blotted (northern blots, Section 5.9.2) and probed, to ensure that the base sequence is available to the probe. Denatured RNA stains only very weakly with ethidium bromide, so acridine orange is commonly used to visualise RNA on denaturing gels. However, it should be noted that many workers will be using radiolabelled RNA and will therefore identify bands by autoradiography. An example of the electrophoresis of RNA is shown in Fig. 10.16.

10.5 CAPILLARY ELECTROPHORESIS

The technique has variously been referred to as high performance capillary electrophoresis (HPCE), capillary zone electrophoresis (CZE), free solution capillary electrophoresis (FSCE) and capillary electrophoresis (CE), but the term CE is the one most

common nowadays. The microscale nature of the capillary used, where only microlitres of reagent are consumed by analysis and only nanolitres of sample needed for analysis, together with the ability for on-line detection down to femtomole (10^{-15} moles) sensitivity in some cases has for many years made capillary electrophoresis the method of choice for many biomedical and clinical analyses. Capillary electrophoresis can be used to separate a wide spectrum of biological molecules including amino acids, peptides, proteins, DNA fragments (e.g. synthetic oligonucleotides) and nucleic acids, as well as any number of small organic molecules such as drugs or even metal ions (see below). The method has also been applied successfully to the problem of chiral separations (Section 11.5.5).

As the name suggests, capillary electrophoresis involves electrophoresis of samples in very narrow-bore tubes (typically 50 μm internal diameter, 300 μm external diameter). One advantage of using capillaries is that they reduce problems resulting from heating effects. Because of the small diameter of the tubing there is a large surface-to-volume ratio, which gives enhanced heat dissipation. This helps to eliminate both convection currents and zone broadening owing to increased diffusion caused by heating. It is therefore not necessary to include a stabilising medium in the tube and allows free-flow electrophoresis.

Theoretical considerations of CE generate two important equations:

$$t = \frac{L^2}{\mu V} \tag{10.4}$$

where t is the migration time for a solute, L is the tube length, μ is the electrophoretic mobility of the solute, and V is the applied voltage.

The separation efficiency, in terms of the total number of theoretical plates, N, is given by

$$N = \frac{\mu V}{2D} \tag{10.5}$$

where D is the solute's diffusion coefficient.

From these equations it can be seen, first, that the column length plays no role in separation efficiency, but that it has an important influence on migration time and hence analysis time, and, secondly, high separation efficiencies are best achieved through the use of high voltages (μ and D are dictated by the solute and are not easily manipulated).

It therefore appears that the ideal situation is to apply as high a voltage as possible to as short a capillary as possible. However, there are practical limits to this approach. As the capillary length is reduced, the amount of heat that must be dissipated increases owing to the decreasing electrical resistance of the capillary. At the same time the surface area available for heat dissipation is decreasing. Therefore at some point a significant thermal effect will occur, placing a practical limit on how short a tube can be used. Also the higher the voltage that is applied, the greater the current, and therefore the heat generated. In practical terms a compromise between voltage used and capillary length is required. Voltages of 10–50 kV with capillaries of 50–100 cm are commonly used.

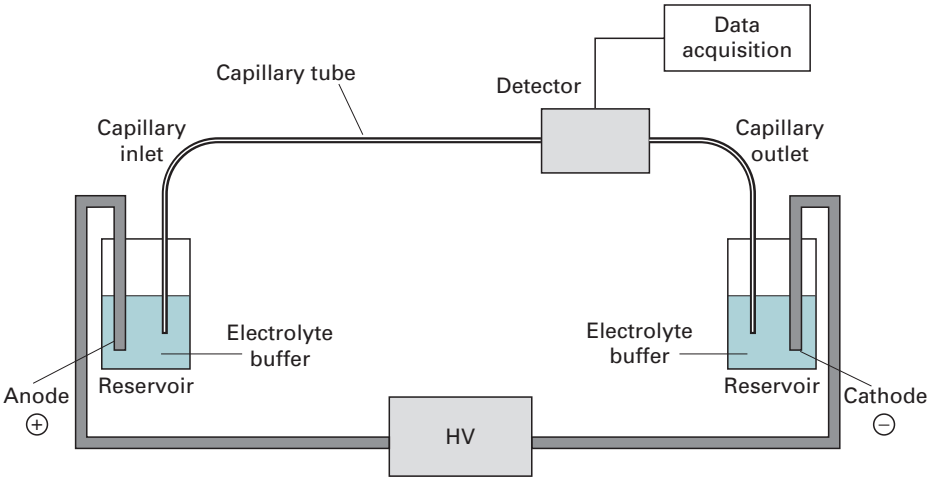


Fig. 10.17 Diagrammatic representation of a typical capillary electrophoresis apparatus.

The basic apparatus for CE is shown diagrammatically in Fig. 10.17. A small plug of sample solution (typically 5–30 μm^3) is introduced into the anode end of a fused silica capillary tube containing an appropriate buffer. Sample application is carried out in one of two ways: by high voltage injection or by pressure injection.

- *High voltage injection*. With the high voltage switched off, the buffer reservoir at the positive electrode is replaced by a reservoir containing the sample, and a plug of sample (e.g. 5–30 μm^3 of a 1 mg cm^{-3} solution) is introduced into the capillary by briefly applying high voltage. The sample reservoir is then removed, the buffer reservoir replaced, voltage again applied and the separation is then commenced.
- *Pressure injection*. The capillary is removed from the anodic buffer reservoir and inserted through an air-tight seal into the sample solution. A second tube provides pressure to the sample solution, which forces the sample into the capillary. The capillary is then removed, replaced in the anodic buffer and a voltage applied to initiate electrophoresis.

A high voltage (up to 50 kV) is then put across the capillary tube and component molecules in the injected sample migrate at different rates along the length of the capillary tube. Electrophoretic migration causes the movement of charged molecules in solution towards an electrode of opposite charge. Owing to this electrophoretic migration, positive and negative sample molecules migrate at different rates. However, although analytes are separated by electrophoretic migration, they are all drawn towards the cathode by electroendosmosis (Section 10.1). Since this flow is quite strong, the rate of electroendosmotic flow usually being much greater than the electrophoretic velocity of the analytes, all ions, regardless of charge sign, and neutral species are carried towards the cathode. Positively charged molecules reach the cathode first because the combination of electrophoretic migration and electroosmotic flow causes them to move fastest. As the separated molecules approach the cathode, they pass through a viewing window where they are detected by an ultraviolet monitor that

Peptide	
1	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
2	Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
3	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
4	Ser-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
5	Ile-Ser-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

Source: Courtesy of Patrick Camilleri and George Okafo, GSK Ltd.

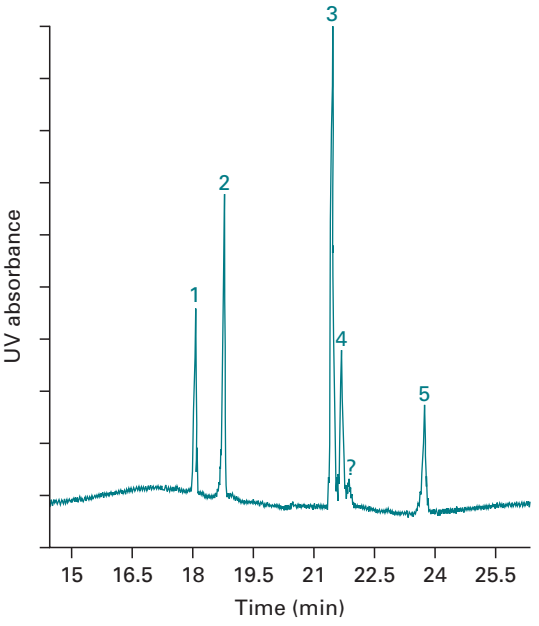


Fig. 10.18 Capillary electrophoresis of five structurally related peptides. Column length was 100 cm and the separation voltage 50 kV. Peptides were detected by their ultraviolet absorbance at 200 nm.

transmits a signal to a recorder, integrator or computer. Typical run times are between 10 and 30 min. A typical capillary electrophoretograph is shown in Fig. 10.18.

This free solution method is the simplest and most widely practised mode of capillary electrophoresis. However, while the generation of ionised groups on the capillary wall is advantageous via the introduction of electroendosmotic flow, it can also sometimes be a disadvantage. For example, protein adsorption to the capillary wall can occur with cationic groups on protein surfaces binding to the ionised silanols. This can lead to smearing of the protein as it passes through the capillary (recognised as peak broadening) or, worse, complete loss of protein due to total adsorption on the walls. Some workers therefore use coated tubes where a neutral coating group has been used to block the silanol groups. This of course eliminates electroendosmotic flow. Therefore, during electrophoresis in coated capillaries, neutral species are immobile while acid species migrate to the anode and basic species to the

cathode. Since detection normally takes place at only one end of the capillary, only one class of species can be detected at a time in an analysis using a coated capillary.

A range of variations on this basic technique also exist. For example, as seen above, in normal CE neutral molecules do not separate but rather travel as a single band. However, separation of neutral molecules can be achieved by including a surfactant such as SDS with the buffer. Above a certain concentration some surfactant molecules agglomerate and form micelles, which, under the influence of an applied electric field, will migrate towards the appropriate electrode. Solutes will interact and partition with the moving micelles. If a solute interacts strongly it will reach the detector later than one which partitions to a lesser degree. This method is known as micellular electrokinetic capillary electrophoresis (MECC). Since ionic solutes will also migrate under the applied field, separation by MECC is due to a combination of both electrophoresis and chromatography.

Original developments in CE concentrated on the separation of peptides and proteins, but in recent years CE has been successfully applied to the separation of a range of other biological molecules. The following provides a few examples.

- In the past, peptide analysis has been performed routinely using reversed-phase HPLC, achieving separation based on hydrophobicity differences between peptides. Peptide separation by CE is now also routinely carried out, and is particularly useful, for example as a means of quality (purity) control for peptides and proteins produced by preparative HPLC. Fig. 10.18 shows the impressive separation that can be achieved for peptides with very similar structures.
- High purity synthetic oligodeoxyribonucleotides are necessary for a range of applications including use as hybridisation probes in diagnostic and gene cloning experiments, use as primers for DNA sequencing and the polymerase chain reaction (PCR), use in site-directed mutagenesis and use as antisense therapeutics. CE can provide a rapid method for analysing the purity of such samples. For example, analysis of an 18-mer antisense oligonucleotide containing contaminant fragments (8-mer to 17-mer) can be achieved in only 5 min.
- Point mutations in DNA, such as occur in a range of human diseases, can be identified by CE.
- CE can be used to quantify DNA. For example, CE analysis of PCR products from HIV-I allowed the identification of between 200 000 and 500 000 viral particles per cubic centimetre of serum.
- Chiral compounds can be resolved using CE. Most work has been carried out in free solution using cyclodextrins as chiral selectors.
- A range of small molecules, drugs and metabolites can be measured in physiological solutions such as urine and serum. These include amino acids (over 50 are found in urine), nucleotides, nucleosides, bases, anions such as chloride and sulphate (NO_2^- and NO_3^- can be separated in human plasma) and cations such as Ca^{2+} and Fe^{3+}.

10.6 MICROCHIP ELECTROPHORESIS

The further miniaturisation of electrophoretic systems has led to the development of microchip electrophoresis, which has many advantages over conventional electrophoresis

methods, allowing very high speed analyses at very low sample sizes. For example, microchip analysis can often be completed in tens of seconds whereas capillary electrophoresis (CE) can take 20 min and conventional gel electrophoresis at least 2 h. Using new detection systems, such as laser-induced fluorescence, picomole to attomole (10^{-18} moles) sensitivity can be achieved, which is at least two orders of magnitude greater than for conventional CE. Detection systems for molecules that do not fluoresce include electrochemical detectors (Section 11.3.3), pulsed amperometric detection (PAD), and sinusoidal voltometry. All these detection techniques offer high sensitivity, are ideally suited to miniaturisation, are very low cost, and all are highly compatible with advanced micromachining and microfabrication (see below) technologies. Finally, the applied voltage required is only a few volts, which eliminates the need for the high voltages used by CE.

The manufacturing process that produces microchips is called microfabrication. The process etches precise and reproducible capillary-like channels (typically, 50 μm wide and 10 μm deep; slightly smaller than a strand of human hair) on the surface of sheets of quartz, glass or plastic. A second sheet is then fused on top of the first sheet, turning the etched channels into closed microfluidic channels. The end of each channel connects to a reservoir through which fluids are introduced/removed. Typically, the size of chips can be as small as 2 cm^2. Basically the microchip provides an electrophoretic system similar to CE but with more flexibility.

Current developments of this technology are based on integrating functions other than just separation into the chip. For example, sample extraction, pre-concentration of samples prior to separation, PCR amplification of DNA samples using infrared-mediated thermocycling for rapid on-chip amplification, and the extraction of separated molecules using microchamber-bound solid phases are all examples of where further functions have been built into a microchip electrophoresis system. An interface has also been developed for microchip electrophoresis–mass spectrometry (MCE–MS) where drugs have been separated by MCE and then identified by MS.

10.7 SUGGESTIONS FOR FURTHER READING

Walker, J. M. (2009). *The Protein Protocols Handbook*, 3rd edn. New York: Humana Press. (Detailed theory and laboratory protocols for a range of electrophoretic techniques and blotting procedures.)

Hames, B. D. and Rickwood, D. (2002). *Gel Electrophoresis of Proteins: A Practical Approach*, 3rd edn. Oxford: Oxford University Press. (Detailed theory and practical procedures for the electrophoresis of proteins.)