

# Electrophoresis in Practice

A Guide to Methods and Applications of DNA and Protein Separations

Bearbeitet von  
Reiner Westermeier

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# 1

## Electrophoresis

### 1.1

#### General

##### 1.1.1

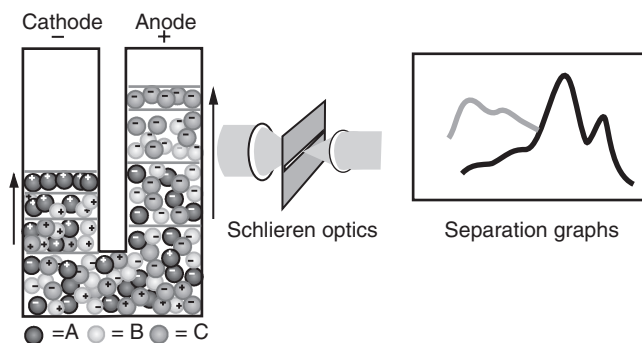
#### Electrophoresis in Free Solution

*Moving boundary electrophoresis:* Tiselius (1937) developed the moving boundary technique for the electrophoretic separation of substances, for which, besides his work on adsorption analysis, he received the Nobel Prize in 1948. The sample, a mixture of proteins, is applied into a U-shaped glass tube filled with a buffer solution and containing electrodes at each end. Under the influence of the applied electric field, the charged compounds migrate at different velocities toward the anode or the cathode, respectively. The resolution is quite poor: only the first zone in each direction consists of the same compound, the following zones contain mixtures of two different ones, the following mixtures of three different ones, and so on. The change in the refractive index at the boundary between the zones can be detected with Schlieren optics. With this approach, Tiselius could resolve human serum proteins into four zones. Nowadays, moving boundary electrophoresis in free solution is mainly used in fundamental research to determine exact electrophoretic mobilities (Figure 1.1).

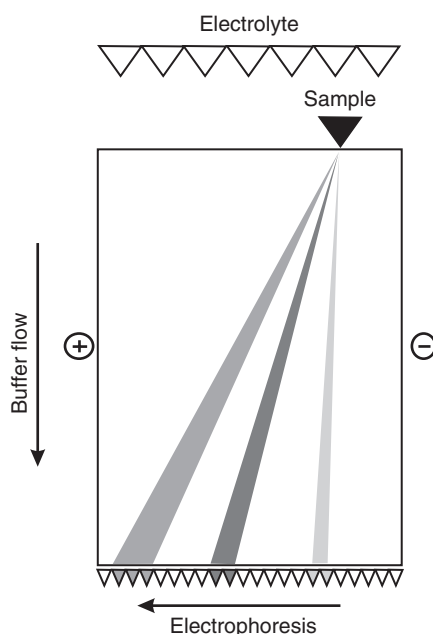
*Free-flow electrophoresis:* In this technique, developed by Hannig (1982), a thin buffer film between two parallel plates flows – under laminar conditions – perpendicular to the electrical field. At one end, the sample is injected at a defined spot, and at the other end the fractions are collected through an array of tubings, which leads to a 96-well microtiter plate. This is the only continuous electrophoretic separation method.

The sample components are deflected perpendicular to the flow depending on their charges; they reach the end of the separation chamber at different though stable positions (Figure 1.2).

Besides the separation of soluble substances, this technique is also used for the identification, purification, and isolation of cell organelles and membranes or whole cells such as erythrocytes, leukocytes, tissue cells, the causal agent



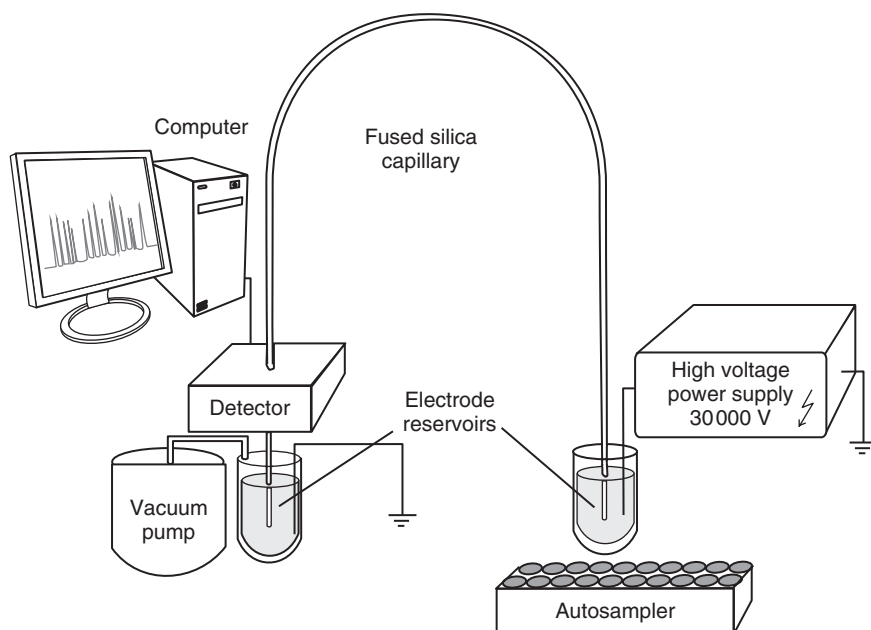
**Figure 1.1** Moving boundary electrophoresis in a U-shaped tube according to Tiselius. Measurements of the electrophoretic mobilities are done with Schlieren optics on both ends.



**Figure 1.2** Schematic drawing of a continuous free-flow electrophoresis system.

of malaria and other parasites. This method is very effective since even minimal differences in the surface charge of particles and cells can be used for separation.

Lately, a number of further developments have helped to make this method increasingly attractive for a wide range of applications: Besides overall simplified operation, the gap size between the plates has been reduced to 0.175 mm, offering new dimensions of speed and resolution; and applying more than  $800 \text{ V cm}^{-1}$  field strength allows separations in less than 1 min.



**Figure 1.3** Schematic representation of the set-up of capillary electrophoresis.

Unfortunately, electrophoresis in free solution cannot yet be applied at an industrial scale. The upscaling of the instrumentation is limited by thermal convection, which results from insufficient dissipation of Joule heat from the flowing electrolyte. Loading cannot be freely increased because the highly concentrated samples begin to sediment. Both these limiting factors occur only under gravity. Since 1971, since the launching of Apollo 14, experiments have been conducted in space to try and develop production in an orbital station.

*Capillary electrophoresis (CE):* This technique is being used increasingly for analytical and micropreparative electrophoresis (Jorgenson and Lukacs, 1981; Hjertén, 1983): as for HPLC (high-performance liquid chromatography), the abbreviation HPCE for high-performance capillary electrophoresis is often used. Separation is carried out in a fused silica capillary tube 20–30 cm long and with an internal diameter of 50–100  $\mu\text{m}$ . Both ends of the capillary are immersed in a buffer container into which the electrodes are built (see Figure 1.3).

Fused silica capillaries are otherwise used in gas chromatography.

The amount of chemicals and sample needed is very low. The volume of the injected material is usually not more than 2–4 nl, so nanograms of sample material only is required.

Field strengths of up to  $1 \text{ kV cm}^{-1}$  and currents of 10–20 mA are used; for this reason, a power supply that can yield voltages up to 30 kV is needed. Joule heat can be dissipated very effectively from these thin capillaries with a fan.

CE separation typically takes 10–20 min. There are many detection methods possible: UV–vis, fluorescence, conductivity, electrochemistry, and so on. In most applications, the fractions are detected by UV measurement at 280, 260 or, in some cases, even 185 nm directly in the capillary. For some substances and applications, the limit of detection can go as low as the attomole level. In general, the results are then further processed by HPLC interpretation software on personal computers.

To prevent adsorption of components on the surface of the capillary and electro-osmotic effects, the inside of the capillary can be coated with linear polyacrylamide or methyl cellulose. CE instruments can be used for all three separation methods: that is, electrophoresis, isotachopheresis (ITP), and isoelectric focusing (IEF). Even an additional new method – a hybrid of electrophoresis and chromatography – has been developed: The buffer used depends on the nature of the separation: for example, 20–30 mmol l<sup>-1</sup> sodium phosphate buffer, pH 2.6, for the electrophoresis of peptides.

*Micellar electrokinetic chromatography* (MEKC) was introduced by Terabe *et al.* (1984). It is the only electrophoretic method that can separate neutral and charged compounds. Surfactants are used at concentrations above the critical micelle concentration. The charged micelles migrate in a direction opposite to the electro-osmotic flow created by the capillary wall. The electro-osmotic counterflow is faster than the migration of the micelles. During migration, the micelles interact with the sample compounds in a chromatographic manner, through both hydrophobic and electrostatic interactions.

It has become one of the most widely used CE methods.

One great advantage of CE lies in its automation. Every step can be controlled by semiautomatic or fully automatic instrumentation. An autosampler is a standard part of this equipment. However, the investment for such an instrument is much higher compared to that in a gel electrophoresis equipment.

Another advantage is the possibility of linking with other analytical instruments either before electrophoresis (HPLC/HPCE) or after (HPCE/MS).

For preparative separations, a fraction collector is attached to the UV detector. The identification of the individual substances is done by the relative mobility or the molecular weight; or else, the collected fractions are analyzed. In contrast to reversed-phase chromatography (RPC), proteins are not damaged during HPCE and, in addition, the resolution is better.

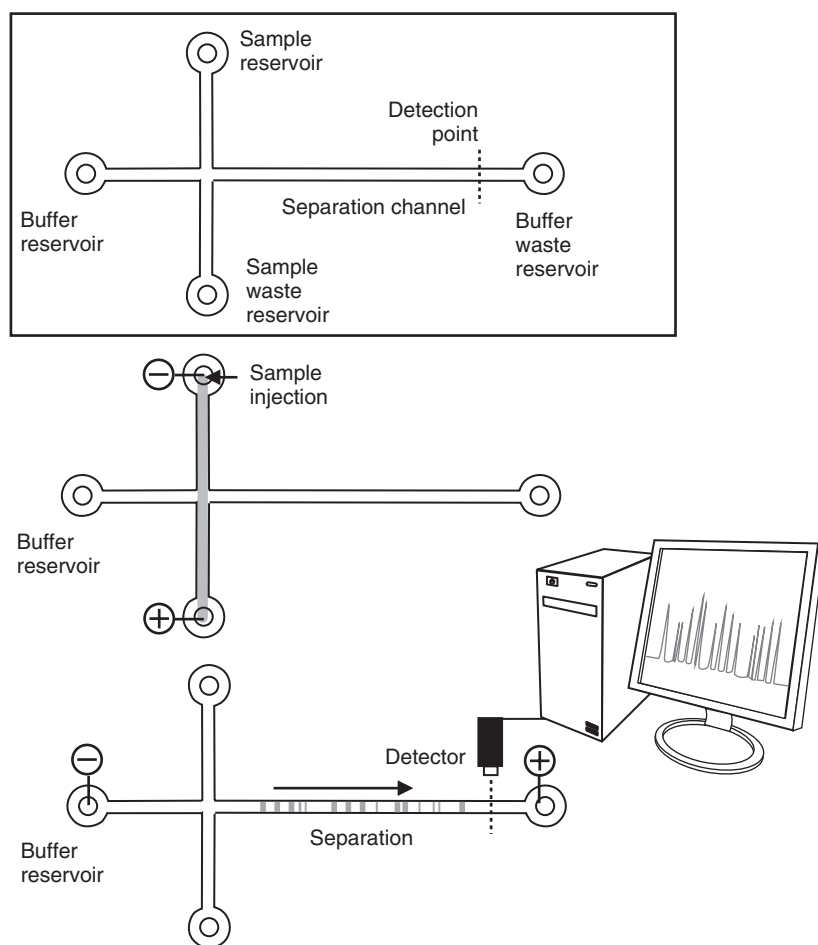
For the separation of proteins, peptides, and nucleic acids according to molecular weights, capillaries filled with linear (noncross-linked) polyacrylamide are used (Cohen and Karger, 1987).

Very successful applications of CE are separations of DNA fragments. Because of the possibility of automation and the repeated use of a large number of capillaries in parallel, this technique is ideal for high-throughput (HTP) DNA sequencing. It had been predicted that the complete knowledge of the human genome would be available in the year 2005. But the introduction of the new multicapillary sequencers has speeded up the Human Genome Project considerably. Since “Next Generation Genome Sequencing” tools – which are no longer based on electrophoresis – have conquered the labs, HTP multicapillary sequencers are

available for other applications such as HTP glycoprofiling (Schwarzer, Rapp and Reichl, 2008).

Practical information on CE can be obtained from the book edited by Weinberger (2000).

*Microchip electrophoresis (MCE)* is basically a miniaturized form of CE in a planar device, which allows a higher degree of automation and quicker analyses. Sample application and separation is done via and in narrow channels, which have been introduced into the surface of the glass, silicone or polymer material by using photolithographic processes. Typically, the channels are arranged in the shape of a cross (Figure 1.4) of size 1–10 cm and have diameters of about 50  $\mu\text{m}$ . All the reservoirs are equipped with electrodes for sample injection and separation steps. The sample volume required for MCE is about 1/10th of that of CE, that is,



**Figure 1.4** Microchip electrophoresis. For details see text.

~0.1–0.5 nL, mostly injected electrokinetically. The separation voltage is 1–4 kV, and separation time is about four times shorter than in CE, that is, 50–200 s. The most commonly used detection method is laser-induced fluorescence (LIF) as, for instance, described by Schulze *et al.* (2010), and the others are variations of electrochemical detection. The latter methods are optimal for the direct detection of small-molecule analytes.

### 1.1.2

#### Electrophoresis in Supporting Media

Compact materials such as paper, films or gels are used. In order to monitor the progress of the separation and to recognize the end of the run, dyes with a high electrophoretic mobility are applied together with the sample as front markers. The instructions in the second part are limited to electrophoresis in supporting media since these techniques require only minimal equipment.

For separation of proteins in the anodal direction, bromophenol blue, xylene cyanol or orange G is used, whereas in the cathodal direction bromocresol green, pyronine, or methylene blue is employed.

*Detection* of the separated zones can be done directly in the medium by positive staining with Coomassie Brilliant Blue or silver, negative staining with zinc imidazole, spraying with specific reagents, enzyme–substrate coupling reactions, immune precipitation, autoradiography or fluorography, or indirectly by immunoprinting or blotting methods. Comprehensive surveys on enzyme staining methods have been published by Rothe (1994) and Manchenko (2002). Blotting is the transfer of the separated molecules to immobilizing membranes followed by staining or specific ligand binding.

*Paper and thin-layer electrophoresis:* These methods have mostly been abandoned in favor of gel electrophoresis because of the latter's improved separation and the higher loading capacity of agarose and polyacrylamide gels. Electrophoretic separations on thin-layer silica gel plates linked to buffer tanks are carried out only for the analysis of polysaccharides of high molecular weight and lipopolysaccharides, which would obstruct the pores of the gels (Scherz, 1990).

*Cellulose acetate membrane electrophoresis:* Cellulose acetate membranes have large pores and therefore hardly exert any sieving effect on proteins (Kohn, 1957). This means that these electrophoretic separations are entirely based on charge.

The matrix has little effect on diffusion, so that the separated zones are relatively wide while the resolution and limit of detection are low. On the other hand, they are easy to handle, and the separation and staining are rapid. The cellulose acetate strips are suspended in the tank of a horizontal apparatus, so that both ends dip in the buffer; no cooling is necessary during separation. This technique is widely used for routine clinical analysis and related applications for the analysis of serum or isoenzymes. Because the resolution and reproducibility of separations in agarose and polyacrylamide gels are better, cellulose acetate membranes are more often replaced by gel electrophoresis.

## 1.1.3

**Gel Electrophoresis**

## 1.1.3.1

**Gel Types**

The gel matrix should have adjustable and regular pore sizes, be chemically inert and not exhibit electroendosmosis.

*Starch gels* were introduced by Smithies (1955) and are prepared from hydrolyzed potato starch, which is dissolved by heating and poured to a thickness of 5–10 mm. The pore size can be adjusted by the starch concentration in the solution. Because of the low reproducibility and the impractical handling, these gels have been largely replaced by polyacrylamide gels. Starch is a natural product whose properties can vary.

*Dextran gels* have been solely used for preparative methods without sieving effect, like IEF (Radola, 1975) and ITP (Battersby and Holloway, 1982).

*Agarose gels* are mostly used when large pores for the analysis of molecules >10 nm in diameter are needed. Agarose is a polysaccharide obtained from red seaweed.

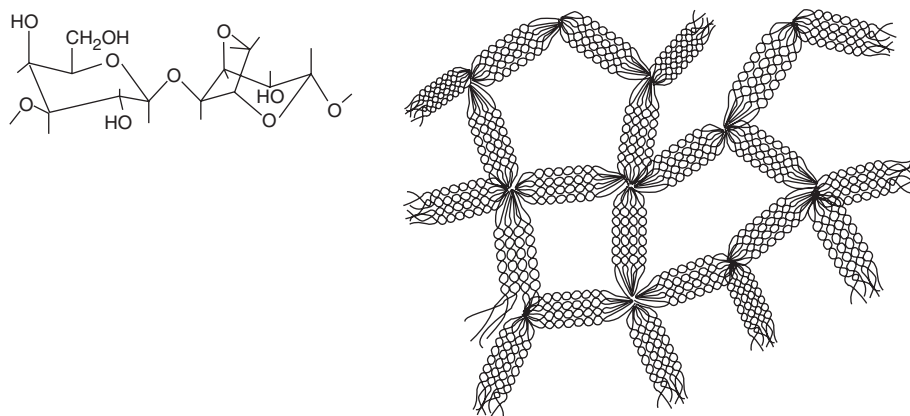
By the removal of the agarpectin, gels of varying electroendosmosis and degrees of purity can be obtained. They are characterized by their melting point (35–95 °C) and the degree of electroendosmosis ( $m_r$ ). The  $m_r$  value is dependent on the number of polar groups left and determined as explained above (see Part I “electroendosmosis”). The definition is the same as for relative electrophoretic mobility.

The pore size depends on the concentration of agarose: one usually refers to the weight of agarose and the volume of water. The unavoidable losses of water that occur during heating can vary from batch to batch, so in practice this value cannot be exact. Usually, gels with a pore size from 150 nm at 1% (w/v) to 500 nm at 0.16% are used. Serwer (1980) employed also very soft gels with pore diameters up to 800 nm (0.075% agarose) for the separation very large DNA molecules.

Agarose is dissolved in boiling water, which then forms a gel upon cooling. During this process, double helices form, which are joined laterally to form relatively thick filaments (Figure 1.5).

For DNA separation, 1–10-mm-thick gels are cast on UV-transparent trays because the bands are usually stained with fluorescent dyes: ethidium bromide, SYBR green or DNA stain. The gels are run under buffer in order to prevent drying out due to electroendosmosis.

For protein electrophoresis, the gels are prepared by coating flat glass plates or support films with a solution of agarose. The thickness of the gel – usually 1–2 mm – is determined by the volume of the solution and the surface it covers. Very even gel thicknesses are obtained by pouring the solution in prewarmed molds. Vertical agarose gel electrophoresis in presence of sodium dodecyl sulfate (SDS) of von Willebrand factor multimers followed by subsequent immune blotting has been described in detail by Ott *et al.* (2010).



**Figure 1.5** Chemical structure of agarose and structure of the polysaccharide polymers after gel formation.

The separated protein bands are mostly detected by Amido Black or Coomassie Brilliant Blue staining of the gels after drying them. In order to improve the protein detection limit, the first silver staining technique had been developed for agarose gels to detect oligoclonal IgGs in the cerebrospinal fluid (Kerenyi and Gallyas, 1972).

*Polyacrylamide gels* were first used for electrophoresis by Raymond and Weintraub (1959). They are chemically inert, fully transparent, and mechanically stable. By chemical copolymerization of acrylamide monomers with a cross-linking reagent – usually *N,N'*-methylenebisacrylamide (Figure 1.6) – a clear transparent gel with very little electroendosmosis is obtained. The reaction is started with ammonium persulfate as catalyst, and *N,N,N',N'*-tetramethylethylenediamine (TEMED) provides the tertiary amino groups to release the radicals. The pore size can be exactly and reproducibly controlled by the total acrylamide concentration  $T$  and the degree of cross-linking  $C$  (Hjertén, 1962):

$$T = \frac{a+b}{V} \times 100 \text{ [\%]}; \quad C = \frac{b}{a+b} \times 100 \text{ [\%]},$$

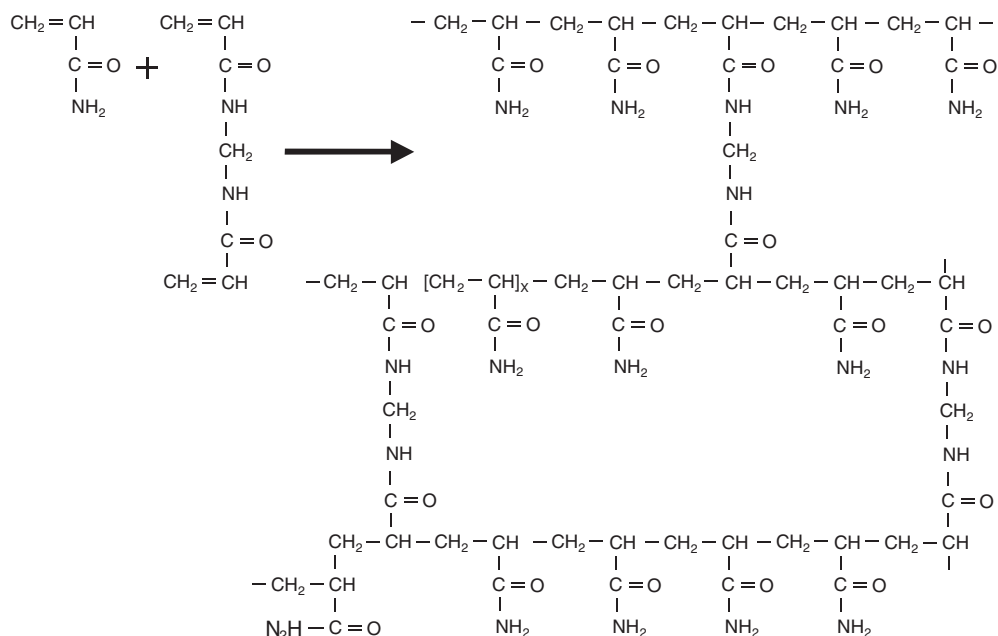
where

$a$  is the mass of acrylamide (g)

$b$  is the mass of methylenebisacrylamide (g)

$V$  the volume (ml)

When  $C$  remains constant and  $T$  increases, the pore size decreases. When  $T$  remains constant and  $C$  increases, the pore size follows a parabolic function: at high and low values of  $C$  the pores are large. For a gel with 10%  $T$ , the minimum pore size is at  $C = 5$ ; gels with 10%  $T$  and  $C > 5$  are brittle and relatively hydrophobic. They are only used in special cases, such as for the separation of mega protein complexes (Strecker *et al.*, 2010).



**Figure 1.6** Polymerization reaction of acrylamide and methylenebisacrylamide.

For separation of proteins and peptides, it is not recommended to use  $T$  values higher than 16% because proteins and peptides can become modified during the migration. The gels optimized by Schägger and von Jagow (1987) for the separation of peptides contain 16%  $T$  and 6%  $C$  (which produces the smallest pores at a 16%  $T$  concentration).

Besides methylenebisacrylamide (Bis), a number of alternative cross-linking reagents exist, which have been listed and compared by Righetti (1983). As an example,  $N,N'$ -bisacryloylcystamine is mentioned here, which contains a disulfide bond that can be cleaved by thiol reagents. Thus it is possible to solubilize the gel matrix after electrophoresis. However, alternative cross-linkers are used very rarely, because the sieving properties get compromised. The cross-linker piperidino propionamide (PPA), which produces slightly more hydrophilic gels, is, however, considerably more expensive than Bis.

Polymerization should take place under an inert atmosphere because oxygen would act as a free-radical trap and lead to interruption of the chain formation. The polymerization is temperature-dependent: to prevent incomplete polymerization, the temperature should be maintained above 20 °C. When the gel has apparently formed, there is still silent polymerization going on, which can take a few hours. It is therefore not advisable to leave the gel at room temperature for a few hours; rather, it is better to place it in a refrigerator shortly after polymerization.

To minimize oxygen absorption, the gels are usually polymerized in vertical casting cuvettes: cylindrical gels in glass tubes and flat gels in molds formed by two glass plates sealed together around the edges. The surfaces of the monomer

solution are usually overlaid with water-saturated butanol, but also 70% v/v isopropanol in water can be used. Alternatively, the monomer solution can be weighed with a portion of glycerol and overlaid with water. In horizontal gel casting, according to Radola (1980), the oxygen intake is considerably increased. This must be compensated by a higher amount of catalyst, often leading to problems during separation.

A few more parameters influence the gel properties:

- The pH value of the monomer solution has a great influence on the polymerization effectiveness: the optimum is around pH 7.
- If gels with an acidic buffer have to be polymerized, a combination of ascorbic acid,  $\text{FeSO}_4$ , and  $\text{H}_2\text{O}_2$  has been employed as catalysts (Jordan and Raymond, 1969).
- The quality of the monomers and all other reagents play an important role. If acrylamide is partly degraded to acrylic acid, the resulting gel will contain fixed carboxylic groups, which cause electroendosmosis.
- Some buffer components can inhibit polymerization; for example, the buffer must not contain any thiols.

#### **Note**

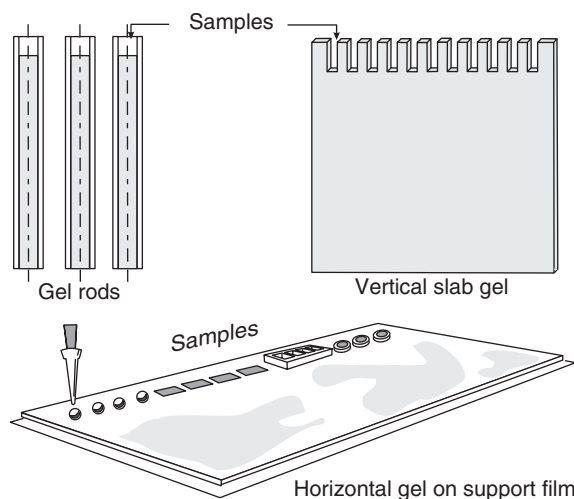
The monomers are toxic and should be handled with caution. The powder form is particularly dangerous. It is therefore recommended to use ready stock solutions rather than weighing acrylamide and Bis.

The addition of polyacrylamide gel strengtheners, such as rhinohide and duracryl, to the monomer solution improves handling of large-format gels. However, some interference with the separation performance and staining techniques has been observed.

Vertical cylindrical gel rods or plates as well as horizontal gel slabs are employed, the latter being usually cast on a stable film backing to facilitate handling (Figure 1.7).

The use of gel rods has become rare because of the complicated handling and difficult pattern comparison. Slab gels for vertical and flatbed systems can be easily polymerized in the laboratory, but prefabricated gels of many different types are available from various suppliers.

For electrophoresis in vertical systems, the gel in glass rods or cassettes are placed into the buffer tanks and in direct contact with the electrode buffers. If size stability of a gel is required, for example, for picking protein spots or bands from the gel for mass spectrometry (MS) analysis, gels can be fixed to one of the glass plates with bind-silane. Gels for flatbed systems are polymerized on a plastic support film, removed from the mold and run with open surfaces. For sample application, wells are formed at the upper edge of the gel during polymerization (Figure 1.7). These are made by insertion of a sample comb between the glass plates.



**Figure 1.7** Gel geometries for electrophoretic separations.

In horizontal gels, sample wells are not always necessary; the samples can be applied directly on the surface with strips of filter paper or silicone rubber. In homogeneous buffer systems, it is important to mold narrow sample wells on the surface of horizontal gels to obtain good results.

#### 1.1.3.2

##### **Instrumentation for Gel Electrophoresis**

The equipment for CE, MCE, and automated DNA sequencing has already been described.

The equipment for gel electrophoresis consists basically of three components:

- power supply
- cooling/heating thermostatic circulator
- separation chamber (partly with gel casting gear).

For electrophoresis, DC power supplies are needed, which yield high voltages and allow setting the maximum output of current, voltage, and power.

Many home-made systems are used without cooling or heating. Yet, it has been proved that better and more reproducible separations are obtained with temperature-controlled equipment.

The core of the electrophoretic equipment is the separation chamber. A number of different types exist because of the many different methods and modifications.

#### 1.1.3.3

##### **Current and Voltage Conditions**

To establish electrophoretic separation conditions, a few physical rules should be recalled.

This is also important when working under defined conditions.

The driving force behind electrophoresis is the product of the charge  $Q_{\pm}$  (net charge) of a substance and the electric field  $E$ , measured in volt per centimeter. For the speed of migration of a substance  $v$  in centimeter per second, this means (the net charge  $\Theta^{\pm}$  can be taken to be the sum of the elementary charges, measured in ampere second)

$$v = \frac{\Theta^{\pm} \times E}{R}$$

Thus adequate field strength is necessary for electrophoretic migration.

The frictional constant  $R$  is dependent on the molecular radius  $r$  (Stokes radius), in centimeter, and the viscosity  $\eta$  of the separation medium, measured in Newton second per square centimeter.

To reach the field strength, the voltage  $U$  must be applied, which is measured in volt [V], and the separation distance is  $d$  (cm).

$$\text{Voltage} = \text{field strength} \times \text{separation distance}$$

$$U = E \times d.$$

If an electric field is applied to a conducting medium (buffer), an electric current  $I$  will flow. It is measured in amperes [A] but is usually given in milliamperes for electrophoresis. The magnitude of the current depends on the ionic strength of the buffer. In electrophoresis, relatively high currents are used, whereas for IEF they are smaller because the pH gradient has a relatively low conductivity.

The product of the voltage and the current is the power  $P$  given in watt [W]:

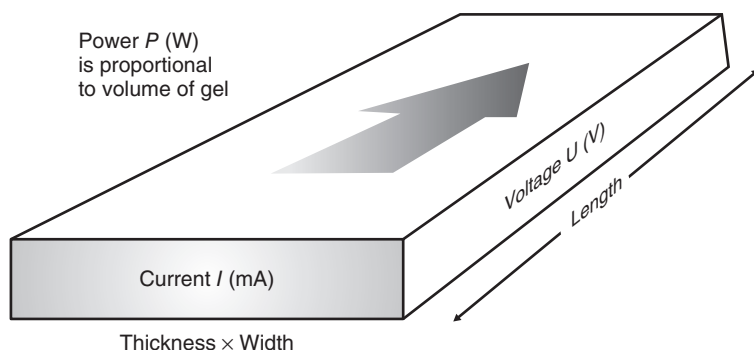
$$\text{Power} = \text{voltage} \times \text{current}$$

$$P = U \times I.$$

The product of (electrical) power and time is energy. During electrophoresis, a great portion of the electrical energy is transformed into Joule heat. For this reason, the temperature should be controlled during electrophoresis. Since the cooling efficiency, that is, the heat dissipation, cannot be increased indefinitely, there exists a certain maximum power that can be used.

Slab gels can develop a “*Smiling Effect*”: When in a gel more Joule heat is produced than can be dissipated, the temperature in the middle of the gel is higher than at the lateral edges. This causes higher mobility of the ions in the center, resulting in an uneven distribution of the zones. The zones bend up at the two lateral sides, causing “smiling.” When a slab gel is more efficiently cooled on one surface than on the other, a “*Venetian Blind Effect*” can occur: the zones migrate faster on one surface than on the other.

Figure 1.8 demonstrates the relationship between the voltage, current, power, and the dimension of the electrophoretic medium. The larger the separation distance, the higher the current necessary to reach the specific field strength. At a given ionic strength, the field strength is proportional to the cross-section: the thicker the gel, the greater the current. The power is proportional to the volume of the gel.



**Figure 1.8** Schematic diagram of the relationships between the separation medium and current, voltage, and power conditions during electrophoresis.

*Guidelines for cooled, 0.5-mm-thick horizontal gels:*

Electrophoresis: about  $2 \text{ W ml}^{-1}$  gel volume

IEF: about  $1 \text{ W ml}^{-1}$  gel volume.

This also means that the power and the current must be reduced if only part of a flatbed gel is used, but for the same separation distance, the setting on the power supply should be

*Half gel: half the current – half the power – same voltage.*

If two vertical gels are run instead of one, the current – and also the power – is doubled:

*Two gels: double current – double power – same voltage.*

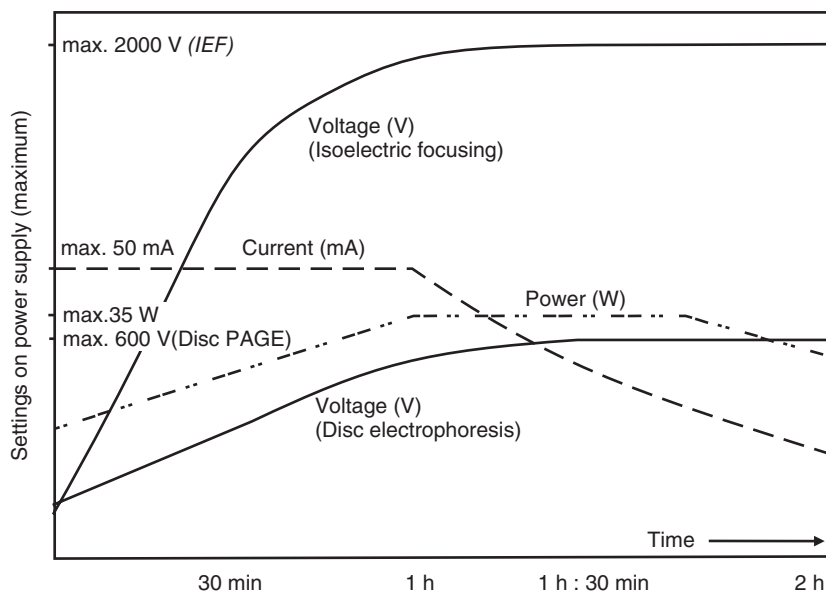
One should always be aware that the values set in the power supply are the maximum values; the real values change during the run, because they are controlled by the conductivity of the buffer and the gel. It is often forgotten that more concentrated gels have higher resistances than gels with low  $T$  values. The conductivity of the system changes during the run, particularly in disc electrophoresis and IEF experiments (Figure 1.9). To optimize separation conditions without overheating of the gel, it is very helpful to use a power supply that allows also the setting of the maximum power (some power supplies offer only voltage and current settings).

#### 1.1.4

##### Power Supply

Different models and degrees of specification exist:

- *Simple* power supplies can be regulated by the voltage. Maximum output can be up to 200 V.
- *Typical* electrophoresis power supplies can be run with constant current or constant voltage. Maximum output can be up to 1 kV and 200 mA.
- Power supplies for IEF need high voltages. They usually offer an automated cross-over (Figure 1.9) by setting also a maximum value for power. Most of these



**Figure 1.9** Course of electric parameters during disc electrophoresis and isoelectric focusing.

instruments are programmable with an in-built timer. Maximum output is up to 3 or 5 kV, 200 mA, 100 W. Additional control over the focusing conditions is provided by a volt per hour integrator.

### 1.1.5

#### Separation Chambers

An overview of the features, benefits, and drawbacks of vertical and horizontal slab gel systems is given in Table 1.1. The most practical instructions in the second part describe horizontal gels on support films since these can be used for all applications and with universally applicable equipment.

#### 1.1.5.1

##### Vertical Systems

Electrophoresis is mostly carried out in gel slabs, which are cast in glass cassettes. The samples are applied in gel pockets with a syringe or a micropipette. The current is conducted through platinum electrodes that are located in the buffer tanks.

A few examples of vertical system designs with and without temperature control are shown in Figure 1.10. To dissipate Joule heat in example B the “upper buffer” is cooled, in example C the lower buffer is cooled by a heat exchange system. Different ways to connect the gel to the upper buffer require different types of glass plates. Simple cassettes for home-made gels are first sealed at the

**Table 1.1** Comparison of flatbed and vertical gel systems.

| Flatbed systems                                                                                                                                  | Vertical systems                                                                                                                      |
|--------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------|
| Gel thickness is limited because cooling is possible only from one side                                                                          | Higher protein loading capacity because thicker gels (up to 3 mm) can be used, which are cooled from both sides                       |
| On flatbed systems, gels on film supports are used. Films can be removed for blotting                                                            | Blotting is easier because of higher gel thickness                                                                                    |
| Very versatile for different methods, ideal for isoelectric focusing                                                                             | Limited technical possibilities, not optimal for isoelectric focusing                                                                 |
| Direct cooling allows exact temperature control                                                                                                  | No cooling, or indirect cooling via buffer and glass or plastic plates                                                                |
| Thin layers can easily be employed, easy sample application; thin gels show higher sensitivity of detection, and are easier and quicker to stain | The thinner the gel, the more complicated is sample application and gel handling                                                      |
| Mostly gels on film backing are employed                                                                                                         | Gels without plastic backing are not easy to handle. But it is possible to cast and fix gels on glass plates treated with bind-silane |
| Versatile for gel sizes: different gel sizes can be applied. Gels can be cut into pieces if only a few samples are run                           | Multiple runs in one tank possible                                                                                                    |
| Electrode wicks soaked in concentrated buffer are used instead of large volumes of liquid buffers                                                | Large volume of buffer is required                                                                                                    |
| Easy to handle and to clean, no glass plates necessary; thus ideal for routine applications                                                      | Many pieces to set up and to clean                                                                                                    |
| Higher electric safety; the buffers cannot leak into each other                                                                                  | There are lower limits of voltage                                                                                                     |
| Readymade gels on film backing are environment friendly                                                                                          | Readymade gels in disposable plastic cassettes produce a lot of waste                                                                 |

bottom with agarose before filling it with the acrylamide monomer solution. Advanced systems seal the bottom with a soft rubber plate. Readymade gels are usually supplied in plastic cassettes (E).

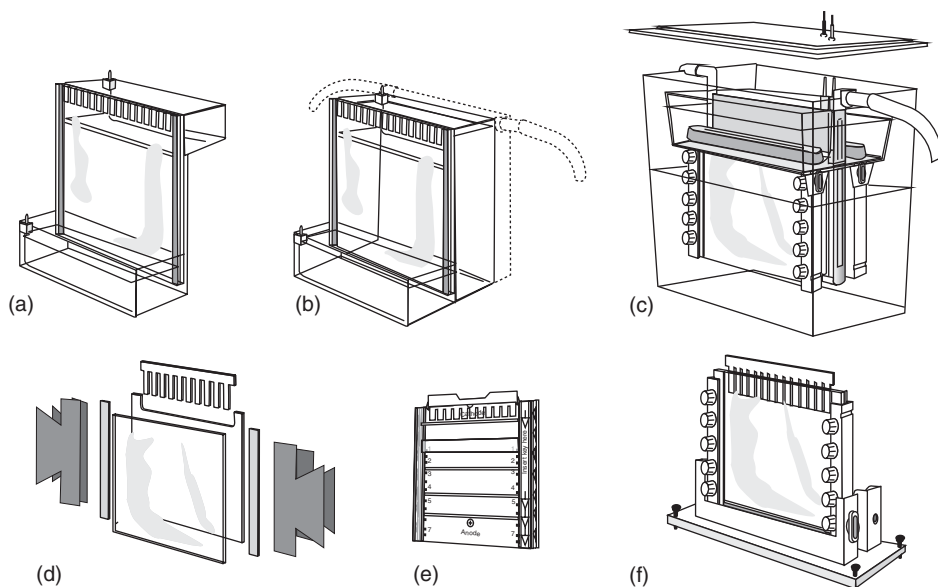
### 1.1.5.2

#### Horizontal Systems

##### 1.1.5.2.1

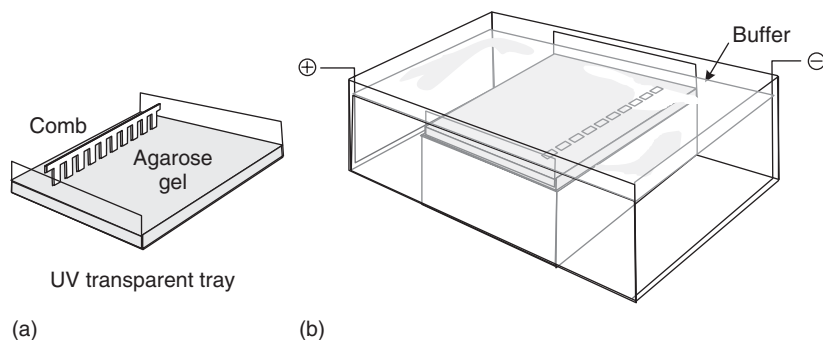
#### DNA Analysis in Agarose Gels

For analytical and preparative separation of DNA fragments and RNA restriction fragments, “submarine” chambers are used (Figure 1.11). The agarose separation gel is submerged under a thin layer of buffer between the lateral buffer tanks to



**Figure 1.10** Different designs of vertical chamber systems: (a) Simple noncooled setup with upper and lower buffer reservoir. (b) System with large “upper” buffer tank acting also as a heat sink, optionally with cooling via tubing. (c) System with large lower buffer tank acting also as a heat sink

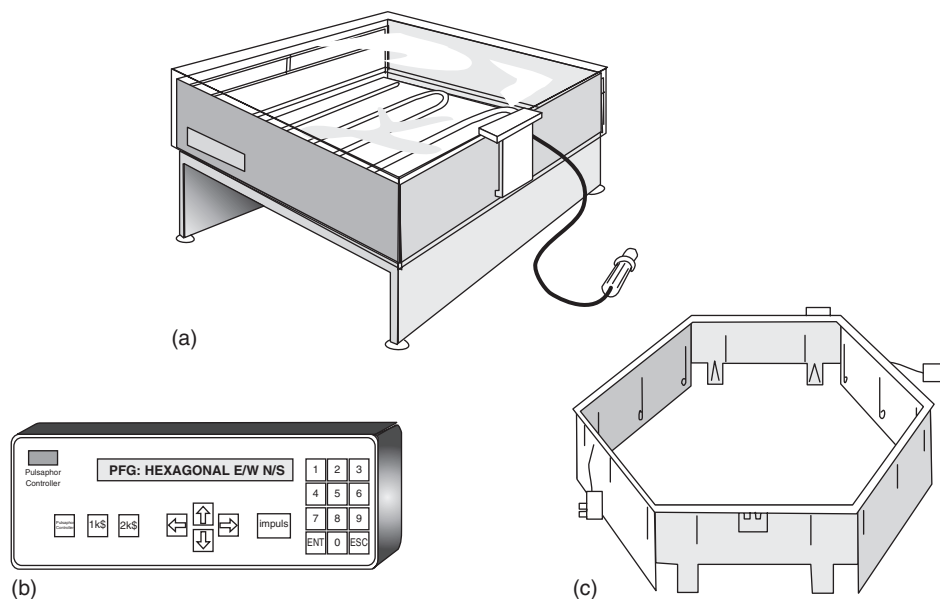
optionally with cooling via tubing. (d) Cassette design for set-ups (a) and (b) with a notched glass plate. (e) Plastic cassette containing readymade gel. (f) Casting stand with cassette for set-up (c) with two glass plates with even upper edges.



**Figure 1.11** Submarine chamber. (a) Casting tray. (b) Agarose chamber during electrophoresis.

prevent partial drying of the gel due to electroendosmosis. Such chambers are available in different sizes.

For electrophoresis in a pulsed field, a controlling device is connected to the power supply, which switches the electrodes – at predefined frequency – in the north/south and the east/west directions. Diodes are built in to the electrodes so



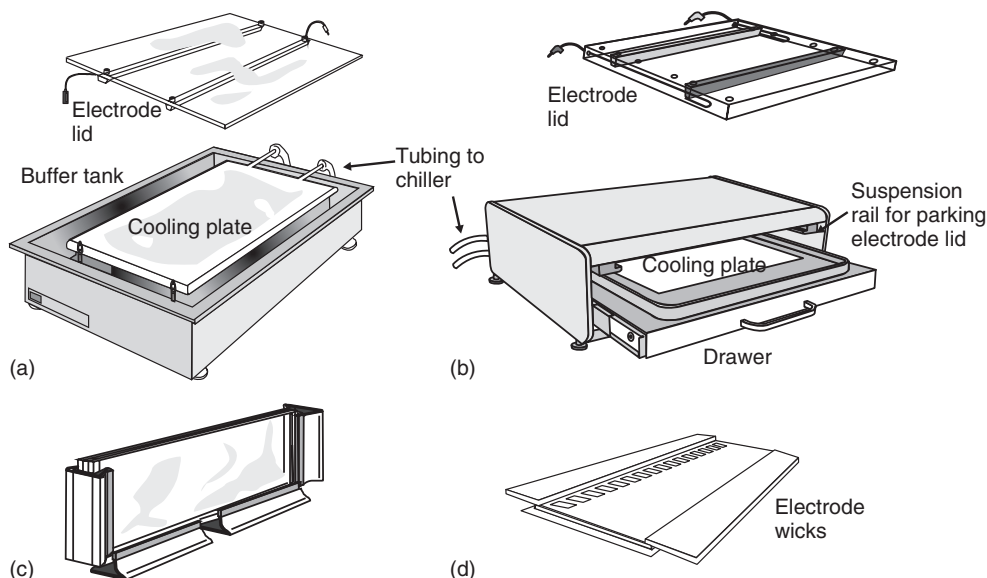
**Figure 1.12** System for pulsed-field DNA gel electrophoresis (PFG). (a) PFG submarine chamber with cooling coil and buffer circulation pump (not visible). (b) Programmable pulse controlling device. (c) Hexagonal electrode set-up for linear sample lanes.

that when they are switched off, they cannot influence the field. Since these separations can last for a long time – up to several days – the buffer must be cooled and circulated (Figure 1.12). For nonhomogeneous fields, point electrodes are placed in electrode grooves set at right angles.

#### 1.1.5.2.2

##### Protein and DNA Analysis in Horizontal Polyacrylamide Gels

Horizontal chambers with thermostatic plates are very versatile (Figure 1.13): they are equipped for analytical and preparative IEF, for several variations of immune and affinity electrophoresis, all zone electrophoresis (ZE) techniques in restrictive and nonrestrictive gels and high-resolution 2D electrophoresis. High voltages can be applied because there are no problems with insulation of buffer tanks; and many techniques can be applied without using a buffer tank at all. Most of the instructions in Part II are designed for this kind of electrophoresis equipment because almost all methods can be performed on it. The design marked with “A” is on the market for more than 40 years without major modifications. The chamber type B is the latest development. It no longer contains buffer tanks because the alternative of using electrode wicks is much more practical. The separation unit is built into a cabinet. There are several benefits: it saves space on the lab bench; because there is a parking position for the electrode lid, the power supply is placed on top of it, and several instruments can be stacked on top of each other. Also the new electrode set-up is much sturdier than the platinum wires: they consist of platinated



**Figure 1.13** Different designs of horizontal flatbed electrophoresis systems. (a) Separation chamber with cooling plate and lateral buffer tanks. (b) Separation cabinet chamber without buffer tanks and a drawer-cooling

plate. (c) Gel casting cassette for thin, homogeneous and gradient gels. (d) Set-up of a horizontal gel on film backing with electrode wicks containing concentrated buffer.

titanium rods. The electrode distances can be adjusted for several different gel sizes. Figure 4.7c shows a modification of this design with four cooling plates for multiple runs. Furthermore, during the run the gel is not exposed to light. This is beneficial for separating proteins that have been prelabeled with light-sensitive fluorescent tags.

In order to provide even and efficient cooling, a thin layer of cooling contact fluid is pipetted between the supporting glass plate or film of the gel and the cooling plate. This fluid should be nonconductive, isosmotic, free of surface tension, and compatible with MS. In the past, kerosene was used. As a replacement, an aqueous solution containing glycerol, sorbitol, and the zwitterionic detergent CHAPS (3-(3-cholamidopropyl)dimethylammonio-1-propane sulfonate) is recommended; it is easier to get and handle, and is less messy.

It should be noted that, because of their good electric safety features, these chambers are particularly useful for IEF.

The automated mini-apparatus PhastSystem® has everything built in: Peltier cooling, power supply, separation compartment, and automated staining. The instrument has meanwhile been discontinued by the manufacturer, but the consumables are still available.

The various GE methods can be divided into those in restrictive and nonrestrictive media. Restrictive gels work against diffusion, so the zones are more distinctly separated and better resolved than in nonrestrictive gels. The limit of detection is

thus improved. In restrictive gels, the molecule's size has a major influence on the result of the separation.

## 1.2

### Electrophoresis in Nonrestrictive Gels

For these techniques, the frictional resistance of the gel is kept negligibly low so that the electrophoretic mobility depends only on the net charge of the sample molecule. Horizontal agarose gels are used for high molecular weight samples such as nucleic acids, proteins, or enzymes, and polyacrylamide gels for low molecular weight proteins, peptides, or polyphenols.

#### 1.2.1

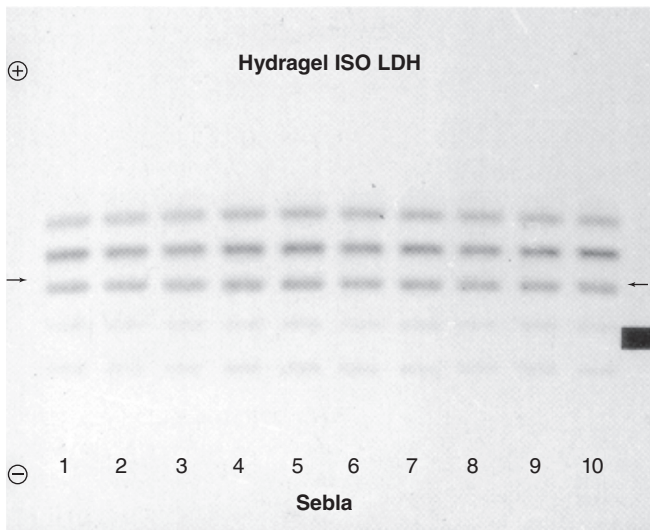
##### Agarose Gel Electrophoresis

##### 1.2.1.1

##### Zone Electrophoresis

Agarose gels with concentrations of 0.7–1% are often used in clinical laboratories for the analysis of serum proteins. The separation times are extremely low: ~30 min. Agarose gels are also used for the analysis of isoenzymes of diagnostic importance, such as lactate dehydrogenase (Figure 1.14) and creatine kinase.

Because of their large pore size, agarose gels are especially suited to specific protein detection by immunofixation: after electrophoresis, the specific antibody is allowed to diffuse through the gel. The insoluble immune complexes formed with



**Figure 1.14** Agarose gel electrophoresis of lactate dehydrogenase isoenzymes. Specific staining was performed with zymogram technique.

the respective antigen result in insoluble precipitates, and the nonprecipitated proteins can be washed out. In this way, only the desired fractions are detected during development.

*Immunoprinting* functions in a similar way: after the electrophoretic separation, an agarose gel containing antibodies or a cellulose acetate membrane impregnated with antibodies is placed on the gel. The antigens then diffuse toward the antibodies, and the identification of the zone is done in the antibody-containing medium. Immunoprinting is mainly used for gels with small pores. Immunoblotting is explained in Chapter 7.

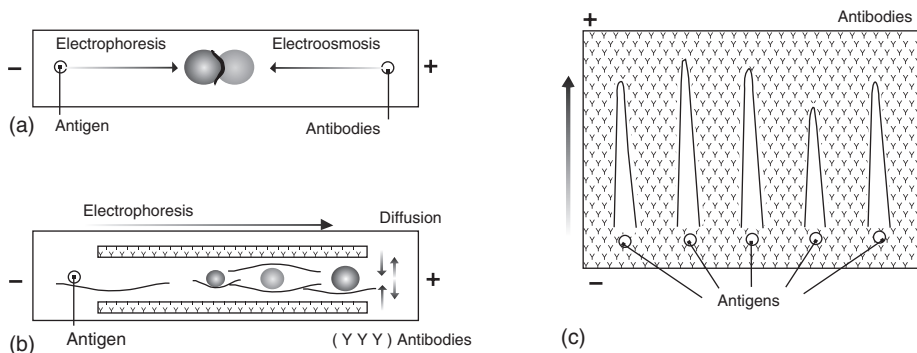
### 1.2.1.2

#### Immuno-electrophoresis

The principle of immuno-electrophoresis is the formation of precipitate lines at the equivalence point of the antigen and its corresponding antibody. In this method, it is important that the ratio between the quantities of antigen and antibody is correct (antibody titer).

When the antibody is in excess, statistically, at most one antigen binds to each antibody, whereas when the antigen is in excess, at most one antibody binds to each antigen. Yet at a specific antigen/antibody ratio (equivalence point), huge macromolecules are formed. They consist of an antigen–antibody–antigen–antibody ... sequence and are immobilized in the gel matrix as an immune precipitate. The white precipitate lines are visible in the gel and can be revealed with protein stains. The method is specific and the sensitivity is very high because distinct zones are formed. Immuno-electrophoresis can be divided into three based on their principles (Figure 1.15):

- a) *Counterimmuno-electrophoresis*. According to Bussard and Huer (1959), in an agarose gel exhibiting high electroendosmosis, the buffer is set at a pH about 8.6 so that the antibody does not carry any net charge. The sample and the antibody are placed in their respective wells and move toward each other: the charged antigens migrate electrophoretically and the antibodies are carried by the electro-osmotic flow.



**Figure 1.15** Three principles of immuno-electrophoresis, see text for details.

- b) *Zone electrophoresis/immunodiffusion*. According to Grabar and Williams (1953), first a ZE is run in an agarose gel, followed by the diffusion of the antigen fraction toward the antibody, which is pipetted into troughs cut in the side parallel to the electrophoretic run.
- c) The *rocket* technique (and related methods). According to Laurell (1966), the antigens migrate in an agarose gel that contains a definite concentration of antibody. As in method A, the antibodies are not charged because of the choice of the buffer pH. As the sample migrates, one antibody will bind to one antigen until the ratio of the concentrations corresponds to the equivalence point of the immune complex.

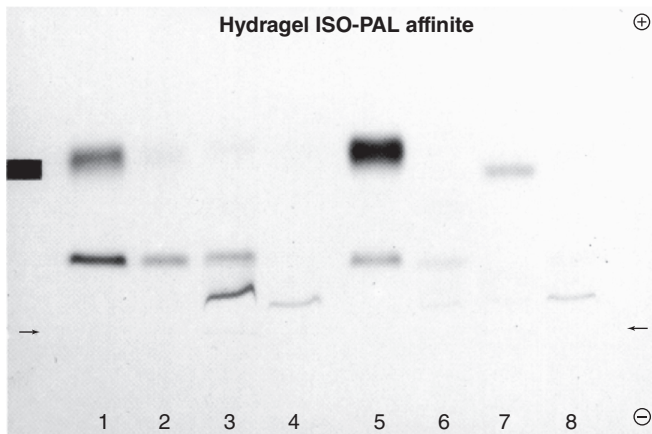
As a result, rocket shaped precipitation lines are formed, in which the enclosed areas are proportional to the concentration of antigen ions in the sample. A series of modifications to this technique exist, including two-dimensional ones.

### 1.2.1.3

#### Affinity Electrophoresis

This is a method related to immunoelectrophoresis and is based on the interactions between various macromolecules, for example, lectin–glycoprotein, enzyme–substrate, and enzyme–inhibitor complexes (Bøg-Hansen and Hau, 1981).

All the techniques known from immunoelectrophoresis can be employed. For example, specific binding lectins collected worldwide from plant seeds are examined with line-affinity electrophoresis. In this way, carbohydrate changes in glycoproteins during different biological processes can be identified. In Figure 1.16,



**Figure 1.16** Affinity electrophoresis of human alkaline phosphatase isoenzymes from liver and bones. The gel contains the lectin wheat germ agglutinin, which specifically interacts with the bone fraction.

The retarded zone can be recognized as a characteristic band close to the application point. Alkaline phosphatase zymogram detection.

an application of affinity electrophoresis is shown, which is regularly employed in clinical diagnosis in order to differentiate between human alkaline phosphatase of liver and bone.

### 1.2.2

#### Polyacrylamide Gel Electrophoresis of Low Molecular Weight Substances

Since low molecular weight fractions cannot be chemically fixed in the matrix, horizontal ultra-thin-layer polyacrylamide gels on film supports are used. Those are dried at 100 °C immediately after electrophoresis and then sprayed with specific reagents. With this method, for example, dyes with molecular weights of ~500 Da can be analyzed.

#### **Note**

According to the guidelines of the SI, the use of the term *Dalton (Da)* for  $1.6601 \times 10^{-27}$  kg is no longer recommended. However, it is still a current unit in biochemistry.

## 1.3

### Electrophoresis in Restrictive Gels

#### 1.3.1

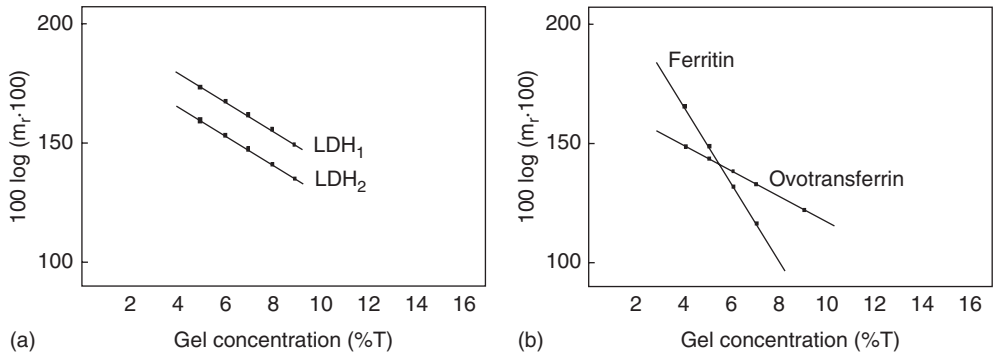
##### The Ferguson Plot

Although during electrophoresis in restrictive gels electrophoretic mobility depends on both net charge and molecular radius, this method can also be used for the physico-chemical determinations of proteins. The principle was formulated by Ferguson (1964). The samples are separated under identical buffer, time, and temperature conditions but with different gel concentrations (g/100 ml for agarose, %T for polyacrylamide). The distances traveled will vary:  $m_r$  is the relative mobility. A plot of  $\log_{10} m_r$  versus the gel concentration yields a straight line.

The slope (Figure 1.17) is a measure of the molecular size and is called the *retardation coefficient*  $K_R$ .

For globular proteins, there is a linear relationship between  $K_R$  and the molecular radius  $r$  (Stokes radius), so the molecular size can be calculated from the slope of the plot. Once the free mobility and the molecular radius are known, the net charge can also be calculated (Hedrick and Smith, 1968). For protein mixtures, the following deductions can be made according to the appearance of the plots:

- *The lines are parallel:* The proteins have the same size but different mobilities, for example, isoenzymes (Figure 1.17a).



**Figure 1.17** Ferguson plots: plots of the electrophoretic migrations of proteins versus gel concentrations. (a) Lactate dehydrogenase isoenzymes and (b) different proteins. See text for further details.

- *The slopes are different but the lines do not cross:* The protein corresponding to the upper curve is smaller and has a higher net charge.
- *The lines cross beyond  $T = 2\%$ :* The larger protein has a higher charge density and intercepts the  $y$ -axis at a higher value (Figure 1.17b).
- *Several lines cross at a point where  $T < 2\%$ :* These are obviously the various polymers of the same protein. They have the same net charge, but different molecular sizes.

### 1.3.2

#### Agarose Gel Electrophoresis

##### 1.3.2.1

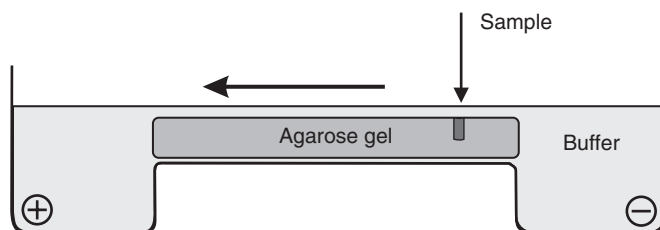
###### Proteins

Since highly concentrated agarose gels  $>1\%$  (1 g/100 ml agarose in water) are cloudy and the electro-osmotic flow is high, in research agarose gels are used only for the separation of very high molecular weight proteins or protein aggregates. However, they are widely used for clinical applications, such as for serum electrophoresis. Since agarose gels do not contain catalysts that can influence the buffer system, they have also been used to develop a series of multiphasic discontinuous buffer systems (Jovin, Dante and Chrambach, 1970).

##### 1.3.2.2

###### Nucleic Acids

Agarose electrophoresis is the standard method for separation, DNA restriction, fragment analysis and purification of DNA and RNA fragments (Green and Sambrook, 2012; Rickwood and Hames, 1982). The fragment sizes analyzed are in the range 1000–23 000 bp (base pairs). Horizontal “submarine” gels are used for these nucleic acid separations: the agarose gel lies directly in the buffer (Figure 1.18). This prevents the gel from drying out due to electroendosmosis.



**Figure 1.18** Set-up for the “submarine” technique for the separation of nucleic acids.

When a narrow-pore-size gel is required, agarose can be partially substituted by polysaccharides (Perlman, Chikarmane and Halvorson, 1987).

The gels are stained with fluorescent dyes such as ethidium bromide or SYBR Green; the bands are visible under UV light in a trans-illuminator. Ethidium bromide is a flat molecule, which has just the right size to get in between the stacked base pairs of double-stranded DNA (Waring, 1965). The sensitivity ranges between 100 pg and 1 ng per band. The gels can be cast with the dye already included, or they can be stained after electrophoresis. Because the dyes are intercalating in the helix, the sensitivity is dependent on the size of the DNA fragment and is lower for RNA detection. These dyes have to be handled with care because they are mutagens. New dyes, such as DNA Stain D, are nonmutagenic: instead of intercalating, they bind to the phosphate groups.

For a permanent record, mostly instant photos are taken from the gels in a darkroom. Video documentation systems take the images inside a box, print the results or feed them to a computer. For RFLP (restriction fragment length polymorphism) analysis, the separated DNA fragments are transferred onto an immobilizing membrane followed by hybridization with radiolabeled probes (see Chapter 7).

### 1.3.3

#### Pulsed-Field Gel Electrophoresis

For chromosome separation, pulsed-field electrophoresis (PFG), according to Schwartz and Cantor (1984), is used; it is a modified submarine technique.

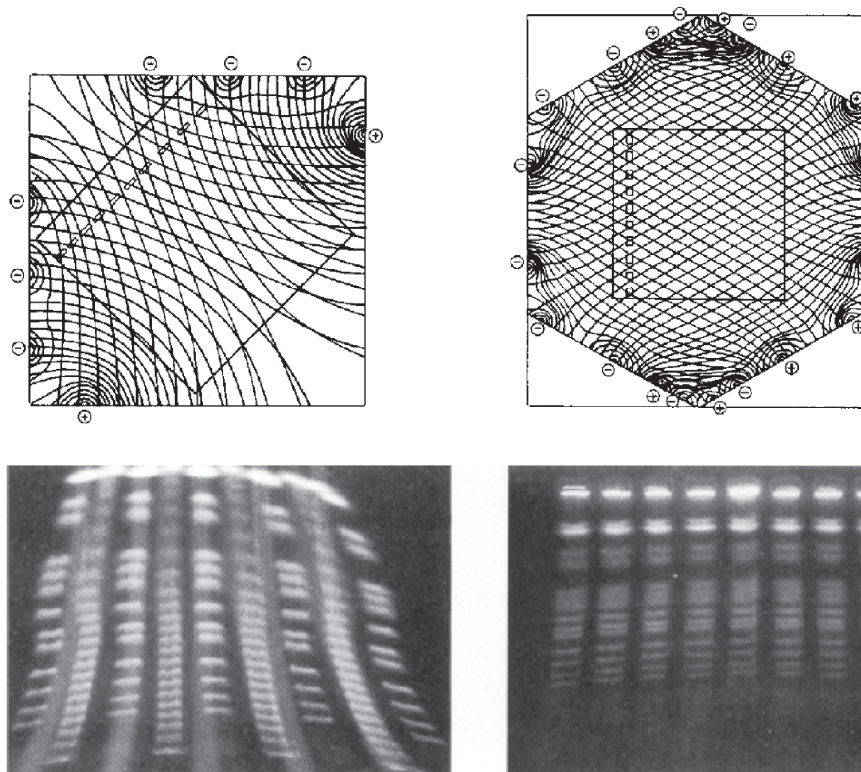
High molecular weight DNA molecules (larger than 20 kb) align themselves lengthwise during conventional electrophoresis and migrate with the same mobility so that no separation is achieved. In PFG, the molecules must change their orientation with changes in the electric field, and their helical structure is first stretched and then compressed. The “viscoelastic relaxation time” is dependent on the molecular weight. In addition, small molecules need less time to reorient themselves than large ones. This means that after renewed stretching and reorientation, larger molecules have – for a defined pulse – less time left for actual electrophoretic migration. The resulting electrophoretic mobility thus depends on the pulse time or on the duration of the electric field: a separation according to the molecular weight of up to the magnitude of 10 Mb is obtained. For shorter

DNA fragments, the resolution with PFG is also higher than with conventional submarine electrophoresis.

For the analysis of chromosomes, the sample preparation including cell disruption, is done in agarose blocks that are placed in the preformed sample pockets. These molecules would be broken by the shear forces; 1.0–1.5% agarose gels are used for the separation.

The electric fields should have an angle of at least  $110^\circ$  relative to the sample. This is obtained, for example, by an inhomogeneous field with point electrodes mounted on orthogonal rails or in a hexagonal configuration. The pulse time is  $\sim 1$  s to 90 min for these techniques, depending on the length of the DNA molecules to be separated. Large molecules are better separated when the pulse time is long, but separation of small molecules needs short pulse times. The separations can last for several days.

Figure 1.19 shows the field lines for an orthogonal configuration with an inhomogeneous field and for a hexagonal configuration with a homogeneous



**Figure 1.19** Field lines and separation results for two types of PFG electrophoresis: (left) orthogonal doubly inhomogeneous fields, and (right) homogeneous fields for hexagonally arranged point electrodes.

field, as well as the corresponding separations. There are, in addition, other field geometries:

*Field Inversion (FI) electrophoresis:* In this the electric field is pulsed back and forth in one direction.

*Transverse Alternating Field electrophoresis (TAFE):* The gel is mounted vertically in an aquarium-like tank and the field is pulsed back and forth between electrode pairs mounted on the top and the bottom of both sides of the gel.

Pulsed-field gel electrophoresis is mainly employed for basic research but has also found its place in routine analysis for bacterial taxonomy.

#### 1.3.4

#### Polyacrylamide Gel Electrophoresis of Nucleic Acids

##### 1.3.4.1

##### DNA Sequencing

In the DNA sequencing methods, according to Sanger and Coulson (1975) and Maxam and Gilbert (1977), the last step is electrophoresis in a polyacrylamide gel under denaturing conditions. The four reactions – containing variously long fragments of the DNA strand to be analyzed, each terminating with a specific base – are separated one beside the other. Determination of the order of the bands in these four lanes from the bottom to the top of the gel yields the base sequence, that is, the genetic information.

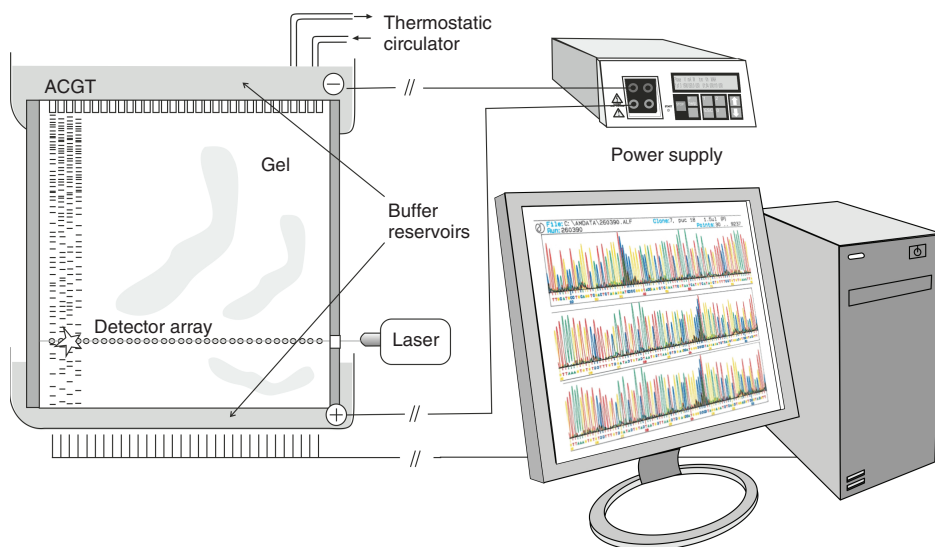
Tris-borate ethylenediaminetetraacetic acid (TBE) buffer is used. To completely denature the molecule, the process is usually carried out at a temperature  $>50^{\circ}\text{C}$  and in the presence of high molar urea. Irregular heat distribution results in the “smiling” effect (mentioned previously): the zones are bent up at the lateral sides. For this reason, it has proved effective to heat the gels with thermoplates independently of the electric field.

Manual sequencing: In the manual technique, the bands are mostly revealed by autoradiography. Nucleotides or primers labeled with  $^{33}\text{P}$  phosphate or  $^{35}\text{S}$  thiophosphate are separated. The gels are usually thinner than 0.4 mm since they must be dried for autoradiography. In practice, vertical gel slabs are used, which are – in most cases – heated by the electric field. An aluminum plate behind one of the glass plates distributes the heat evenly.

Alternative nonradioactive detection methods have been developed:

- Chromogenic or chemiluminescent detection on a membrane after the separated DNA fragments have been transferred from the gel. This requires biotinylated or fluorescent primers, nucleotides or probes.
- Silver staining of the gel. This requires cycle sequencing.

The use of wedge-shaped gels has proved useful: they generate a field strength gradient, which induces a compression of the band pattern in the low molecular weight area and enable the analysis of substantially more bases in one gel. The samples are introduced in sample wells (formed in the gel by a sample comb



**Figure 1.20** Instrumentation for automated DNA sequencing with a four-track system. On the computer screen is seen a typical trace after treatment of the crude data by a computer.

during polymerization) with microcapillaries or syringes with an extra-thin needle.

*Automated sequencing:* Samples with fluorescent tags are used. There are two principles:

- 1) *Single track system:* For the four necessary reactions – with the base endings A, C, G, and T – four different fluorescent markers are used. For separation, the four reagents are applied on the gel and the zones that migrate in one track are measured with selective photodetectors.
- 2) *Four-track system:* This principle is based on the traditional Sanger method (Sanger and Coulson, 1975). Only one dye is used, for example, fluorescein, which is used to mark the primer. The samples are separated in four tracks per clone (see Figure 1.20).

A fixed laser beam constantly scans the whole width of the gel in the lower fifth of the separation distance. At this height, a photovoltaic cell is fixed to the glass plate behind each band. When the migrating bands reach that spot, the fluorescent DNA fragments will be excited, and a light signal is emitted (Ansorge *et al.*, 1986).

Such “on-line electrophoresis” set-ups are nowadays mainly employed for DNA typing methods, since HTP multicapillary instruments have replaced the slab gel technique. Meanwhile, also this approach is no longer used; it has been replaced by several new – nonelectrophoretic – methods of “next-generation DNA sequencing.” Those are technologies that parallelize the sequencing process, producing thousands or millions of sequences concurrently, like 454 pyrosequencing, SOLiD technology (sequencing by ligation), ion torrent semiconductor sequencing, DNA

nanoball sequencing, single-molecule real time (SMRT) sequencing and single-cell DNA sequencing.

#### 1.3.4.2

##### **DNA Typing**

Many techniques and applications had been developed with DNA typing. Because these are almost exclusively based on polymerase chain reaction (PCR) technology, the size range of the DNA fragments lies between 50 and 1500 bp. In this range, the sensitivity and resolution of agarose electrophoresis with ethidium bromide staining are coming to its limits, because the gel pores are too large for proper sieving and the intercalating fluorescent dyes are less sensitive. Amplification of fragments larger than 1500 bp is possible; however, there are some issues with the reproducibility. Several DNA typing methods have been transferred to the capillary technique, such as VNTR (variable-number of tandem repeats) and STR (short tandem repeats) typing in forensic medicine.

##### 1.3.4.2.1

##### **PAGE and Silver Staining**

The use of polyacrylamide gels leads to much sharper bands and higher resolution; with subsequent silver staining, a sensitivity of 15 pg per band can be achieved (Bassam, Caetano-Annollés and Gresshoff, 1991). Whereas in agarose electrophoresis the mobilities of DNA fragments are solely proportional to their sizes, the band positions in polyacrylamide gels are partly influenced by the base sequence as well. A- and T-rich fragments migrate more slowly than others.

Silver-stained DNA bands can be directly reamplified after scratching them out of the gel without intermediate purification. About 20% of the DNA molecules of a band remain undestroyed by the silver staining procedure. As already mentioned above, DNA typing is mostly done in capillary systems, but reamplification of DNA fragments is possible only after slab gel electrophoresis.

##### 1.3.4.2.2

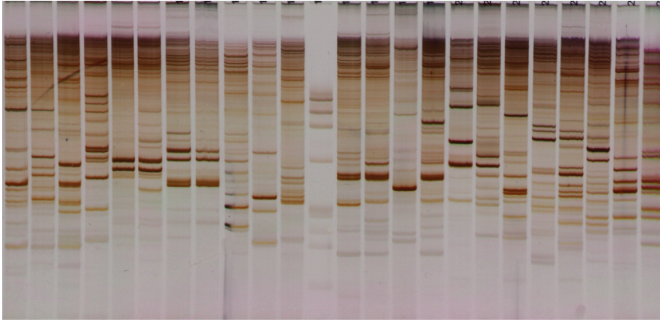
##### **Horizontal (Flatbed) Electrophoresis**

Flatbed polyacrylamide systems have a number of advantages over the vertical ones when ultrathin gels polymerized on support films are used (Görg *et al.*, 1980): simple handling; ease of use of readymade gels and buffer strips instead of large buffer volumes; good cooling efficiency and temperature control; possibility of washing, drying, and rehydrating the gels; and possibility of automation.

##### 1.3.4.2.3

##### **Amplified Ribosomal DNA Restriction Analysis (ARDRA)**

Fragments of ribosomal DNA with polymorphic restriction sites of an organism are amplified with a primer pair and subsequently digested with a restriction enzyme. After gel electrophoresis and silver staining, species-specific patterns are obtained. This method is derived from ribotyping and is mainly employed for the identification of bacterial species.



**Figure 1.21** RAPD electrophoresis of fungi varieties in a horizontal polyacrylamide gel. Silver staining. By kind permission of Birgit Jäger and Dr Hans-Volker Tichy, TÜV Südwest GmbH – Biological Safety Division, Freiburg im Breisgau.

#### 1.3.4.2.4

##### **Random Amplified Polymorphic DNA (RAPD)**

This method is applied for the rapid detection of DNA polymorphisms of a wide variety of organisms: bacteria, fungi, plants, and animals. One single, short oligonucleotide primer (10mer) of arbitrary sequence is used to amplify fragments of the genomic DNA (Welsh and McClelland, 1990; Williams *et al.*, 1990). The low-stringency annealing conditions lead to an amplification of a set of multiple DNA fragments of different sizes. A modification using 5mer primers is called DNA amplification fingerprinting (DAF) and has been introduced by Caetano-Annollés, Bassam and Gresshoff (1991). Figure 1.21 shows the RAPD patterns of different fungus varieties separated in a horizontal polyacrylamide gel and silver stained. The primers are based on those published by Berg and Akopyants (1984). Even one additional band detected can make a big difference in the evaluation. With optimized separation and detection, strain-specific patterns are achieved.

#### 1.3.4.3

##### **Mutation Detection Methods**

A comprehensive description of mutation detection methods can be found in the book *Laboratory Protocols for Mutation Detection*, edited by Landegren (1996).

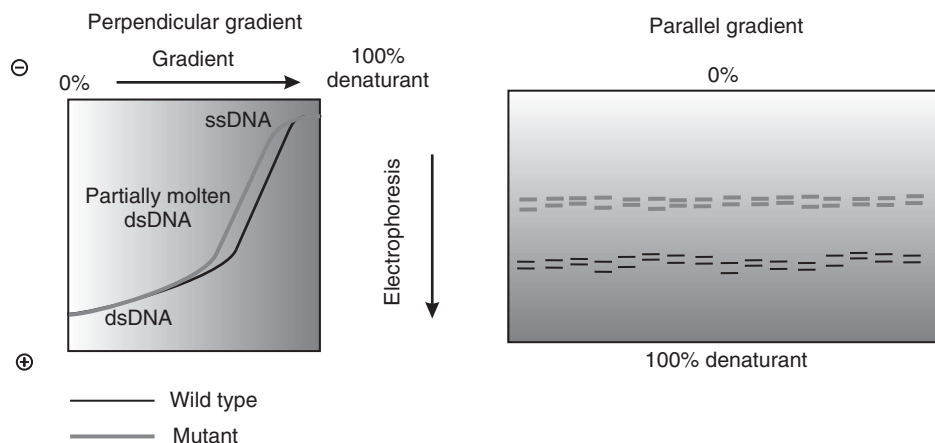
*Single-strand (ss) conformation polymorphism (SSCP):* Variations in the sequence as small as one base exchange alter the secondary structure of ssDNA, for example, by different intramolecular base pairing. The changes in the sequence cause differences in the electrophoretic mobility, which are observed as band shifts (Orita *et al.*, 1989). Differential transient interactions of the bent and curved molecules with the gel fibers during electrophoresis cause the various sequence isomers to migrate with different mobilities. Single strands migrate much slower than the corresponding double strands. A large number of samples can be screened with considerably lower effort than direct sequencing in a

relatively short time. However, the band shifts do not show up automatically for all mutations and under all conditions. The parameters influencing the result have been reviewed by Hayashi and Yandell (1993). For this SSCP analysis, an efficient temperature control system is very important.

**Heteroduplex:** Single base substitutions can also be detected by heating the mixtures of amplified wild-type and mutant DNA and running the resulting heteroduplexes on a native polyacrylamide gel electrophoresis (PAGE) (Keen *et al.*, 1991; White, 1992). The mobilities of heteroduplexes lie between the those of the corresponding homoduplexes and single strands. Different mutations cause different mobility shifts of the heteroduplexes.

**Denaturing gradient gel electrophoresis (DGGE):** With DGGE, single base exchanges in segments of DNA can be detected with almost 100% efficiency. The principle of DGGE is based on the different electrophoretic mobilities of partially denatured molecules caused by differences in DNA melting (Fischer and Lerman, 1983). With a denaturant gradient perpendicular to the electrophoresis direction, the region of a point mutation can be identified. Denaturant gradients parallel to the electrophoresis runs are better for screening applications. Typically, the 100% denaturant solution contains 6–7 mol l<sup>-1</sup> urea and 20–40% formamide. The gels are run at temperatures between 40 and 60 °C (Figure 1.22).

**Temperature gradient gel electrophoresis (TGGE):** TGGE resolves homo- and heteroduplexes according to their thermal stabilities (Riesner *et al.*, 1989). In this technique, denaturing gels are run on a plate with a cold (15 °C) side at the cathode and a hot side (60 °C) at the anode. The method is well suited for screening purposes. Suttorp *et al.* (1996) have described how to change a standard horizontal electrophoretic chamber into a TGGE device.



**Figure 1.22** Schematic representation of typical results of a perpendicular and a parallel DGGE.

## 1.3.4.4

**Denaturing PAGE of Microsatellites**

Denaturing gels provide a very high resolving power, and therefore are very useful for separating microsatellites with very short repeats down to 2 bp. Because the Taq-polymerase used in PCR adds an additional A to the 3'-end of a part of the single strands, double bands are frequently seen after silver staining of denaturing gels. It is not always necessary to apply completely denaturing conditions on the gel: 7 mol l<sup>-1</sup> urea in the gel and 25 °C separation temperature are often sufficient.

## 1.3.4.5

**Two-dimensional DNA Electrophoresis**

Complex DNA samples can also be displayed with a two-dimensional electrophoresis: First, the DNA is digested with a rare cutting restriction enzyme, and the fragments are separated in an agarose gel; then the agarose gel is soaked in a mixture of the selected restriction enzyme and applied on a polyacrylamide gel. Schickle, Lamb and Hanash (1999) have converted the time-consuming "hand-craft" procedure into a faster technique, which is based on readymade gels. The resulting tiny spots are visualized with autoradiography.

## 1.3.5

**Polyacrylamide Gel Electrophoresis of Proteins**

Because of their superior resolution, usually gel layers  $\leq 1$  mm are preferred. With the development of very sensitive staining methods such as silver and fluorescent staining, and fluorescent labeling, very small quantities of concentrated sample solutions can be applied for the detection of trace amounts of proteins.

The advantages of thinner gels are the following:

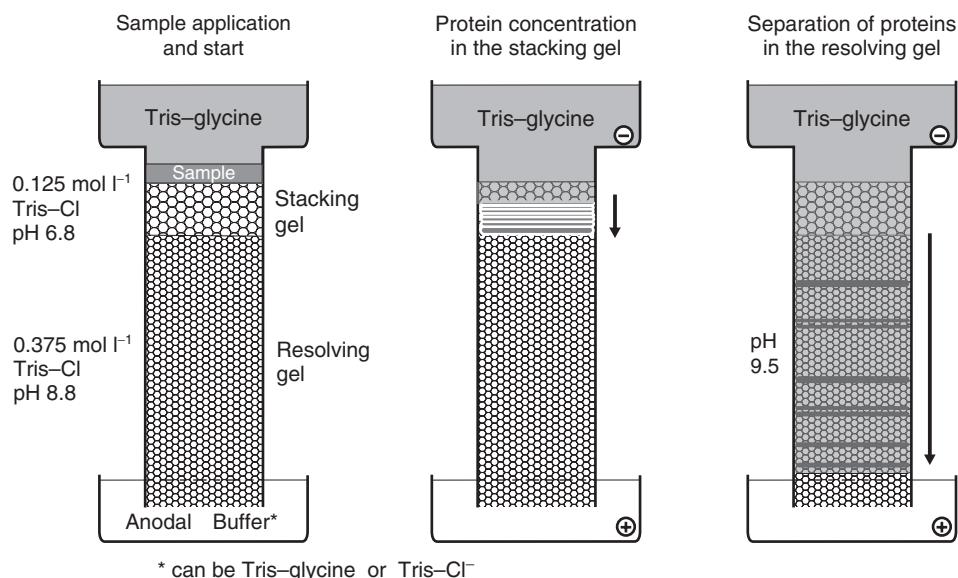
- faster separation
- better defined bands
- faster staining
- better staining efficiency and higher sensitivity

## 1.3.5.1

**Disc Electrophoresis**

Discontinuous (Disc) electrophoresis, according to Ornstein (1964) and Davis (1964), solves two issues of protein electrophoresis: it prevents aggregation and precipitation of proteins during the entry from liquid sample into the gel matrix, and it promotes the formation of well-defined bands. The discontinuity is based on four parameters (see also Chapter 2):

- the gel structure
- the pH value of the buffer
- the ionic strength of the buffer
- the nature of the ions in the gel and in the electrode buffer



**Figure 1.23** Schematic diagram of the principles of disc electrophoresis according to Ornstein (1964). The buffer system shown is also employed for discontinuous SDS electrophoresis.

Figure 1.23 shows the three important stages in disc electrophoresis. The gel is divided into two areas: the resolving gel and the stacking gel. The resolving gel with small pores (e.g., prepared with 12%*T*) contains  $0.375 \text{ mol l}^{-1}$  Tris-HCl buffer, pH 8.8, and the stacking gel with large pores (usually 4%*T*) contains  $0.125 \text{ mol l}^{-1}$  Tris-HCl, pH 6.8.

Besides the common counterion Tris, the electrode buffer contains solely glycine, and the gel contains exclusively  $\text{Cl}^-$  ions. Glycine has a pI of 6.7; therefore it has almost no net charge at pH 6.8, the pH of the stacking gel. This causes glycine to have a low mobility. Glycine is very hydrophilic and does not bind to proteins.

When the electric field is applied, the proteins migrate according to the *principle of isotachopheresis* and form stacks in the order of their mobilities ("stacking effect"). The individual zones become concentrated. Because of the large pores in the stacking gel, the mobilities are dependent only on the net charges and not on the size of the molecule. There is another valuable effect: because of the relatively slow migration velocity of glycine, the samples enter the gel slowly without suddenly increasing the concentration.

The protein stack migrates – slowly and at constant speed – toward the anode till it reaches the border to the resolving gel. The frictional resistance suddenly increases for the proteins, they migrate slower and the zones become higher concentrated. The low molecular weight glycine molecules are not affected, pass the proteins and become highly charged in the resolving zone because they are now in an environment of pH 8.8; the new  $\text{Cl}^-/\text{glycine}^-$  front moves ahead of the proteins.

Several events occur now simultaneously:

- The proteins suddenly find themselves in a homogeneous buffer medium, become de-stacked and start to separate according to the principles of ZE. The buffer discontinuity exists now at the front.
- Their mobility now depends on their charges as well as on their sizes. The ranking of the protein ions changes.
- Due to the pK value of the amino group of the glycine, the pH value raises to 9.5; therefore the net charges and hence the mobilities of the proteins increase. And the separation becomes faster.

Disc electrophoresis affords high resolution and high quality band definition. In the example cited above, proteins with isoelectric points (pIs) higher than pH 6.8 migrate in the direction of the cathode and are lost. Therefore, alternative buffer systems must be chosen to separate these proteins. A selection can be found in the works of Maurer (1978) and Jovin (1970). Alternatively, SDS can be added to the gel and running buffer to in order to have all proteins negatively charged (see Section 1.3.5.3).

The stacking gels is cast onto the resolving gel not longer than 1 h before electrophoresis because, when the complete gel is left standing for a long time, the ions diffuse toward each other.

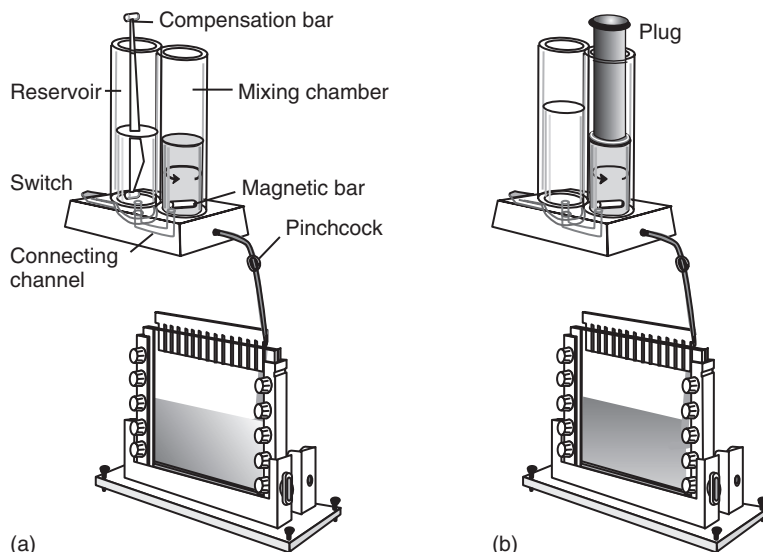
#### 1.3.5.2

##### **Gradient Gel Electrophoresis**

By continuously changing the acrylamide concentration in the polymerization solution, a pore gradient gel is obtained. Gradient gels exhibit an additional zone sharpening effect and can be used to determine the molecular diameter of proteins in their native state (Rothe and Purkhanbaba, 1982).

When the acrylamide concentration and cross-linking are high enough in the small pore area, the protein molecules can be driven to an end point where they are trapped in the tight gel matrix. Since the speed of migration of the individual protein molecules depends on their charge, the electrophoresis must be carried out long enough so that the molecule with the lowest net charge also reaches its end point. The determination of molecular weights in this manner can be problematic since different proteins have different tertiary structures. Structural proteins cannot be compared with globular proteins.

There are various ways of preparing gels with linear or exponential porosity gradients. All techniques are based on the same principle: two monomer solutions with different acrylamide concentrations are prepared. During casting, the concentrated solution is continuously mixed with the diluted solution so that the concentration in the casting mold decreases from bottom to top (Figure 1.24). For single gels, the solution is poured into the top of the cassette. When several gels are cast simultaneously, the solutions are injected from the bottom. Then the solutions in the mixing chamber and the reservoir have to be interchanged (see Method 8 in Part II).



**Figure 1.24** Casting of gradient gels with a gradient maker. The stirrer bar is rotated with a magnetic stirrer (not shown). (a) Linear gradient. (b) Exponential gradient.

The density of the highly concentrated solution is increased with glycerol or sucrose so that the layers in the molds do not mix. In principle, a concentration gradient is created. The mixing of the lighter solution with the high density solution is accomplished in the mixing chamber using a magnetic stirrer bar.

If the mixing chamber is left open at the top, the principle of communicating vases is valid: so the height of both fluids stays equal, half of the dilute solution flows in as the solution flowing out of the mixing chamber. A linear gradient is thus formed (see Figure 1.24a). A stick placed in the reservoir compensates for the volume of the stirrer bar and the difference in the densities of both solutions for porosity gradients, for pH gradients, and for additive gradients. Exponential gradients are formed when the mixing chamber is sealed (Figure 1.24b). The volume in the mixing chamber stays constant, and the same quantity of dilute solution flows in as the solution flows out of the mixing chamber. In order to prevent gel distortions caused by thermal convection, the light solution should receive about 20% more ammonium persulfate than the dense solution: this affects the polymerization starting at the top. An alternative technique – casting the gradient from the bottom – is shown in the section for blue native PAGE in Method 8.

### 1.3.5.3

#### SDS Electrophoresis

SDS electrophoresis was introduced by Shapiro, Viñuela and Maizel (1967), and separates exclusively according to molecular sizes. By loading the protein surfaces with the anionic detergent SDS, the charges of the proteins are masked so that

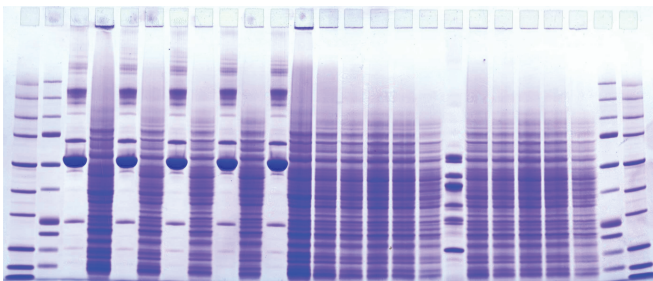
anionic micelles with a constant net charge per mass unit result: 1.4 g SDS per gram of protein.

In addition, the tertiary and secondary structures are canceled because of the disruption of the hydrogen bonds and unfolding of the molecules. Thus there is – in principle – no influence of the original protein shape on the running condition. However, disulfide bonds between cysteine residues can be cleaved only by a reducing reagent such as 2-mercaptoethanol, dithiothreitol, or Tris (2-carboxyethyl) phosphine. Nonreduced polypeptides are incompletely unfolded and migrate faster than reduced molecules, thus indicating a smaller size. Thiol treatment disrupts most quaternary structures. Therefore, SDS electrophoresis is mostly a separation method for protein subunits. In order to prevent back-folding and aggregation of the subunits, it is useful to protect the SH groups by a subsequent alkylation with iodoacetamide, iodoacetic acid or vinyl pyridine (Lane, 1978).

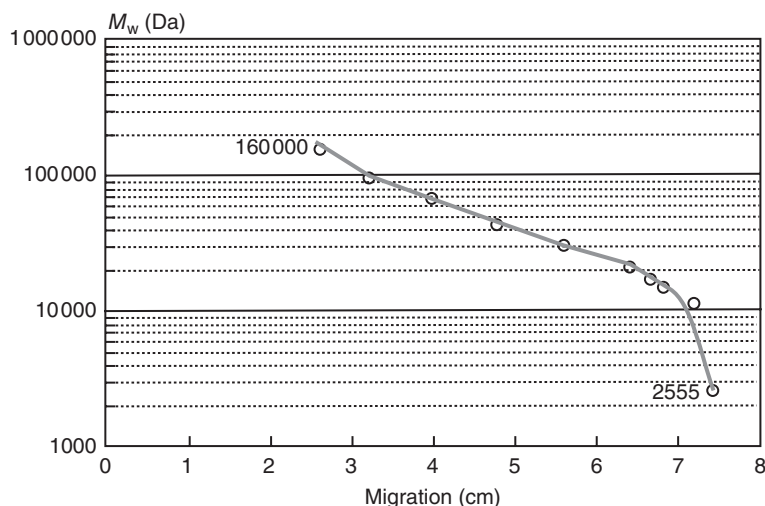
During electrophoresis in restrictive polyacrylamide gels in presence of SDS, there is a linear relationship between the logarithm of the molecular size and the relative distance of migration of the SDS–polypeptide micelle. This linear relationship is valid only for a certain interval, which is determined by the ratio of the molecular size to the pore diameter. Figure 1.25 shows an SDS polyacrylamide gel stained with Coomassie Blue R-250.

Gels with pore gradients show wider separation ranges and a larger linear relationship than gels with constant pore sizes. A few examples of the results obtained with SDS gradient gel electrophoresis can be seen in Figure 6.2. The molecular sizes of the proteins can be estimated with a calibration curve using marker proteins (Figure 1.26). Marker protein mixtures for various molecular weight intervals are available. Prestained marker proteins with different colors are very useful for monitoring the separation and estimation of the molecular sizes during the run. For fluorescence detection, fluorescent prelabeled markers are available.

For the separation of physiological fluids or the analysis of urine proteins, for example, the reduction step is left out to prevent the breakdown of the immunoglobulins into subunits. In these cases, the incomplete unfolding of certain proteins must be taken into account, and therefore the molecular sizes of



**Figure 1.25** Separation of proteins in a horizontal SDS polyacrylamide gel  $T = 12.5\%$  (cathode on top). Stained with Coomassie Brilliant Blue R 250. Samples: human serum, leguminosae seed extracts and different marker protein mixtures.



**Figure 1.26** Semilogarithmic representation of a molecular weight curve. The molecular weights of the marker proteins are represented as a function of their migration. (SDS linear pore gradient gel according to Figure 1.21.)

the proteins cannot be determined exactly. The migration behavior of albumin is a good example: when it is not reduced, it migrates with the velocity of a molecular weight of 54 kDa instead of 68 kDa because the polypeptide chain is only partially unfolded.

Several research groups, for example, Ibel *et al.* (1990), Samso *et al.* (1995), and Westerhuis, Sturgis and Niederman (2000), have described the structures of SDS–protein complexes as “necklace shapes.” This means that not all parts of the polypeptide chains are covered by SDS, and the molecular sizes interpolated with marker proteins will not always match with the molecular weights determined with MS. As an attempt to show such structures, drawings can be found in Section 5.3 describing protein sample preparation for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

There are a number of practical advantages to SDS electrophoresis:

- SDS solubilizes almost all proteins, even very hydrophobic and denatured proteins.
- Since SDS–protein complexes are highly charged, they possess high electrophoretic mobility. This ensures rapid separations.
- Since the fractions are uniformly negatively charged, they all migrate in one direction: toward the anode.
- The polypeptides are unfolded and stretched by the treatment with SDS, and the separation is carried out in strongly restrictive gels: this limits diffusion.
- This affords high resolution and sharp zones.
- The bands are easy to fix. No strong acids are necessary.
- The separation is based on one physico-chemical parameter, namely the molecular size. It is an easy method for the estimation of molecular weights.

- Charge microheterogeneities of isoenzymes are canceled out. There is *one* band for *one* enzyme.
- Proteins separated with SDS bind dyes better. The limit of detection decreases by 10-fold compared to native PAGE.
- After electrophoretic transfer on an immobilizing membrane, the SDS can be removed from the proteins without eluting the proteins themselves. See Chapter 7.

SDS electrophoresis can be carried out in a continuous phosphate buffer system (Weber and Osborn, 1969) or in a discontinuous system.

Lämmli (1970) has directly adopted the disc electrophoresis method according to Ornstein and Davis for proteins charged with SDS, though the discontinuities in pH value and ionic strength are in most cases unnecessary.

- Because the protein–SDS micelles have very high negative charges, the mobility of glycine is lower than that of the proteins in the stacking gel at the beginning of electrophoresis; even at pH 8.8 it does not bind SDS. However, the discontinuities of the anions and the different gel porosities are very important.
- During stacking, no field strength gradient results since there are no charge differences within the sample: therefore no lower ionic strength in the stacking gel area is necessary.

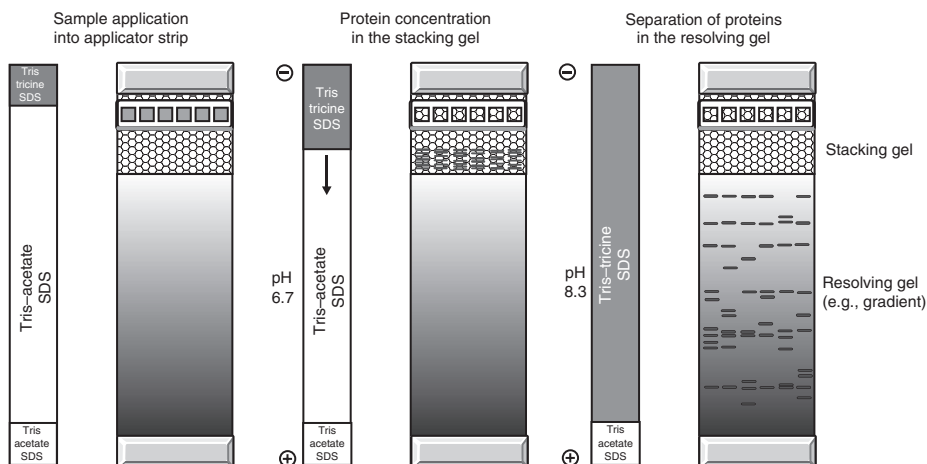
This means that SDS disc electrophoresis gels can be cast in one step: Glycerol is added to the resolving gel. The stacking gel, which does not contain glycerol, is directly cast on top of it. The stacking gel solution should contain the buffer with pH 6.8 because, due its low acrylamide concentration, it would be difficult to get adequate polymerization effectiveness at pH 8.8. The overlaying of the resolving gel with water-saturated butanol can thus be avoided, especially the laborious removal of the overlayer before pouring the stacking gel.

These gels can be stored longer than conventional disc gels because the diffusion of buffer does not matter. Yet, their shelf life is limited by the high pH value of the gel buffer since the polyacrylamide matrix will slowly become degraded by alkaline hydrolysis. For readymade gels with longer shelf lives, buffer systems with pH values below 7 should be employed.

#### 1.3.5.3.1

##### Long-Shelf-Life Gels

Tris–acetate buffer with a pH of 6.7 has proven to have very good storage stability and separation capacity. Tricine is used instead of glycine as the terminating ion. Figure 1.27 shows the principle of this buffer system, employing polyacrylamide electrode strips containing concentrated running buffers in a readymade SDS gel. Tricine is more expensive than glycine; therefore it is only used at the cathode. The anode contains Tris–acetate. These buffer strips simplify electrophoresis considerably and reduce chemical and radioactive liquid waste Kleine *et al.* (1992). Filter material wicks soaked in concentrated buffer solutions have the advantage that they are easier to store and do not break. Paper or cardboard can also be used.



**Figure 1.27** Principle of the buffer systems of readymade gels for discontinuous SDS electrophoresis. Horizontal gels with Tris–tricine buffer strips.

However, it has some limitation: it can interfere with sensitive detection methods, such as silver staining.

Alternative approaches to long-shelf-life gels either employ bis-Tris gel buffer, pH 7.0, or a combination of  $76 \text{ mmol l}^{-1}$  Tris–HCl and  $100 \text{ mmol l}^{-1}$  each of three amino acids serine, glycine, and aspartic acid (Ahn *et al.*, 2001). A new development made it possible to produce time-stable mini-gels for native and SDS electrophoresis containing Tris–glycine– $\text{Cl}^{-}$  with a pH value below 7. Results are shown in Figure 6.2.

#### 1.3.5.3.2

##### Low Molecular Weight Peptides

Because peptides with sizes smaller than 10 kDa are not resolved in Tris–glycine–HCl systems, several approaches have been made to establish alternative systems to separate or include also smaller molecules. Hashimoto *et al.* (1983) have proposed to add  $8 \text{ mol l}^{-1}$  urea to the gel buffer. Anderson, Berry and Telser (1983) added urea and increased the Tris concentration to  $1 \text{ mol l}^{-1}$ . The best resolution down to 1 kDa is achieved with the method according to Schagger and von Jagow (1987): In this method, a separation gel with 16%T and 6%C is employed, the molarity of Tris is increased to  $1 \text{ mol l}^{-1}$  at a pH of 8.4 and tricine is used as the terminating ion instead of glycine. This method yields linear resolution from 100 to 1 kDa.

Also, the “long-shelf-life gels” using tricine in the cathodal buffer show markedly better separation of small peptides. Patton *et al.* (1991) have discovered that Tris–tricine and Tris–borate buffer systems provide better estimates of molecular weights of human mesothelial cell intermediate filament proteins than the standard Tris–glycine system.

Wiltfang, Arold and Neuhoﬀ (1991) have introduced a multiphasic buffer system for the separation of proteins in the range between 1 and 100 kDa by using bicine as the trailing ion and sulfate as the leading ion, and BisTris and Tris as counterions during the stacking and separation phase, respectively.

### 1.3.5.3.3

#### SDS-PAGE in Low Sieving Media

A very wide separation range from 250 to 10 kDa can be achieved in low concentrated homogeneous gels (4.5%*T*, 2.6%*C*) with a taurine and Tris buffer using the method according to Maly and Nitsch (2007).

The gels contain a Tris–taurine buffer, pH 8.6, in the resolving zone and a Tris–imidazole buffer, pH 6.8, in the stacking zone. The cathodal buffer is prepared from 100 mmol l<sup>-1</sup> taurine and 0.1% SDS, which produces a pH 8.1 without titration; the anode buffer is made up with 100 mmol l<sup>-1</sup> Tris and 250 mmol l<sup>-1</sup> taurine, pH 8.8. For use in flatbed systems, the gels are cast in one piece with a buffer, pH 8.6; short before use the stacking gel area (up to 1 cm above the sample wells) is equilibrated in the stacking gel buffer pH 6.8, and then the narrow area, which is later overlapping with the cathodal wick is further equilibrated with cathodal buffer, using a vertical cuvette (see figure M07\_10 in Method 07). For vertical set-ups, the resolving gel and the stacking gel are cast independently. Because the low concentrated gel matrix is very soft, the gels should be bound to a film support or a glass plate. Remarkably, cathodal running buffer and separation gel buffer contain the same anion: taurine.

The prize for the most unorthodox technique for SDS-PAGE should be given to Zilberstein *et al.* (2007): they introduced SDS-PAGE under focusing conditions by grafting a concentration gradient of basic groups to a 4%*T* polyacrylamide gel with an acrylamide derivative bearing a tertiary amino group with a *pK* value of 10.3 (see also Section 3.5.2). The gel and the cathodal buffer contain 100 mmol l<sup>-1</sup> Tris–tricine buffer, pH 8.3, and 0.1% SDS, and the anodal buffer 100 mol l<sup>-1</sup> Tris–acetate, pH 6.4. Contrary to conventional SDS electrophoresis, the small proteins have the slowest migration velocity, and the larger molecules migrate much faster; thus, the plot of  $M_w$  versus  $m_r$  ( $M_w$ , molecular weight) is reversed, showing a positive slope. Furthermore, in contrast to standard SDS-PAGE where the plot of  $M_w$  versus  $m_r$  is semilogarithmic, with this method the plot is linear. Small polypeptides down to 2.5 kDa are well resolved. The separation mechanism is explained as follows: at the buffered pH 8.3, the gel contains an increasing concentration of positive charges. The negatively charged SDS–protein micelles migrate toward the anode in a gradient of increasing amounts of fixed positive charges. When such a charged micelle meets a density of charges, which is equal to its amount of negative charges, its charges will be neutralized and the molecule will not migrate further; they are “focused” at this position. This explains why the small polypeptides will migrate only for a short distance: they have less surface charges and will thus stop earlier than the large ones. There are a number of benefits discussed in the original paper mentioned above. However, it would seem that this method is not optimal for routine analyses because the pH in the gel is

alkaline with limited shelf-life, the casting procedure requires a gradient maker and immobilines of  $pK$  10.3 are required. Also these gels should be bound to a film support or a glass plate.

#### 1.3.5.3.4

##### **Challenging Protein Types**

*High molecular weight proteins and protein multimers:* If the SDS–protein micelles are too big for polyacrylamide gels, SDS electrophoresis can also be performed in agarose gels, as described by Ott *et al.* (2010) for the example of the separation of the von Willebrand factor.

*Glycoproteins* migrate too slowly in SDS electrophoresis because the sugar moiety does not bind SDS. When a Tris–borate–EDTA buffer is used, the sugar moieties are also negatively charged, so the speed of migration increases (Poduslo, 1981). The use of gradient gels is also beneficial for better molecular size estimations.

*PEGylated proteins*, such as special forms of synthetic erythropoietin (EPO), cannot be detected with the same sensitivity in SDS-PAGE like epoetins without polyethylene glycol (PEG), because SDS binds to the protein as well to the PEG moiety. This creates an issue in doping control, where synthetic epoetins have to be differentiated from their biological counterparts in human urinary samples. The generally used anti-EPO antibody cannot interact with the fully SDS-solubilized PEGylated molecules. Reichel, Abzieher and Geisendorfer (2009) have replaced SDS by Sarcosyl, which binds only to the protein part and not to the PEG part, and have succeeded in detecting also these modified proteins.

#### 1.3.5.3.5

##### **Mobility Shift SDS-PAGE**

The mobility shift detection of phosphorylated proteins using phosphate affinity SDS-PAGE has been developed by Kinoshita *et al.* (2006). Manganese chloride/zinc chloride compounds are grafted into the resolving polyacrylamide gel matrix during polymerization. Phosphorylated proteins are bound by the Phos-tag as they migrate through the gel. This reduces the migration speed of the phosphorylated proteins, enabling the separation of phosphorylated and nonphosphorylated forms. The incorporated Phos-tag molecule contains two divalent metal ions and both manganese and zinc ions. Phos-tag SDS-PAGE can be followed by further downstream analysis such as Western blotting or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

#### 1.3.5.3.6

##### **Zymography SDS-PAGE**

For zymography detection, a substrate (e.g., gelatin, casein, albumin, hemoglobin, etc.) is copolymerized with the polyacrylamide matrix for SDS-PAGE (Lantz and Ciborowski, 1994). The concept will be further described in Chapter 6.

## 1.3.5.4

**Cationic Detergent Electrophoresis**

Strongly acidic proteins do not bind SDS, and very basic nucleoproteins behave abnormally in SDS gels. The alternative is to use cationic detergents, for instance, cetyltrimethyl ammonium bromide (CTAB), in an acidic medium at pH 3–5 (Eley *et al.*, 1979). This allows separation according to the molecular sizes in the direction of the cathode. However, the separation pattern is different from that obtained with SDS electrophoresis because the detergent–protein micelles are differently structured. This cationic detergent causes less damage to the protein than SDS, so CTAB electrophoresis can be used as a form of native electrophoresis (Atin, Shapira and Kinkade, 1985). Buxbaum (2003) has reported that membrane glycoproteins are separated much better in acidic CTAB-PAGE than in SDS-PAGE. Alternatively, benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC) can be used as a cationic detergent (MacFarlane, 1983). A discontinuous buffer system for 16-BAC electrophoresis with improved protein stacking has been developed by Kramer (2006). Note that, for the acidic gels, alternative catalysts according to Jordan and Raymond (1969) need to be employed for gel polymerization.

## 1.3.5.5

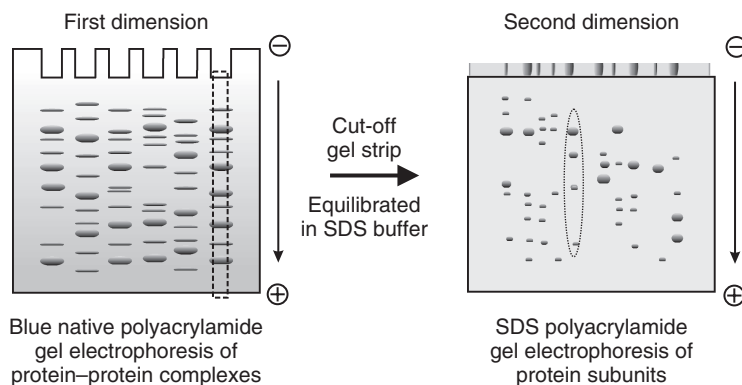
**Blue Native Electrophoresis**

## 1.3.5.5.1

**Membrane Proteins and Protein Complexes**

When membrane proteins are solubilized with nonionic detergents, these detergents would interfere with the SDS. Schägger and von Jagow (1991) have developed “blue native electrophoresis” of membrane protein complexes to solve this problem: After solubilization of the complexes with a mild nonionic detergent such as dodecyl-maltoside or digitonin, the anionic dye Coomassie Brilliant Blue G-250 is added to the sample prior to the run; it binds to the complexes without disturbing any protein–protein interactions. The separation occurs under physiological pH conditions, pH 7.5, at 4 °C. Coomassie Blue is also added to the cathodal buffer in a native PAGE. The membrane proteins and complexes can be isolated in enzymatically active form.

During the run, the dye competes with the nonionic detergent and binds to the membrane proteins and complexes and charges them negatively analogous to SDS. All these protein–dye complexes migrate toward the anode, so also the basic protein complexes. They are soluble in detergent-free solution, and – as the negatively charged protein surfaces repel each other – aggregation between proteins is minimized. Blue native PAGE gels contain a porosity gradient from 5%*T* to 16%*T*: this allows large supercomplexes to enter the gel, prevents small complexes and single proteins from migrating out of the gel and applies a band-sharpening effect. Detection and recovery of the proteins and complexes is easy, as the attached blue dye makes them visible. Particularly in functional proteomics, the technique has gained great interest because it allows the study of



**Figure 1.28** Principle of two-dimensional electrophoresis with blue native PAGE in the first and SDS-PAGE in the second dimension.

protein–protein interactions and the separation and analysis of very hydrophobic proteins, such as membrane proteins, their complexes, and even supercomplexes. The blue-stained native proteins can be recovered from the excised bands with electro-elution. In most cases, blue native PAGE is combined with a second dimension electrophoresis, which is either a second blue-native PAGE with an intermediate equilibration with a medium mild detergent or SDS-PAGE for mapping of the related subunits (Figure 1.28). After the second dimension run, the gels are stained with Coomassie Brilliant Blue, silver staining or with fluorescent staining. But also prelabeling with CyDye fluorophores or zymogram techniques can be employed for detection. More details and valuable troubleshooting can be found in the comprehensive review by Wittig, Braun and Schagger (2006). In the same year, Krause has described a number of applications of the technique in his review. Strecker *et al.* (2010) have increased the range for the separation of mega protein complexes larger than 10 MDa by employing polyacrylamide gels with a high cross-linking factor. Further insights into the internal architecture of protein complexes can be acquired by low SDS treatment of biological samples prior to native gel electrophoresis. The partially dissected protein complexes can be studied in a very defined and reproducible way, employing two-dimensional blue native PAGE/SDS-PAGE (Klodmann, Lewejohann and Braun, 2011).

If interference of Coomassie Blue with zymogram techniques or fluorescent detection is an issue, clear native (CN)-PAGE can be employed (Wittig and Schagger, 2005), but with a lower resolution than blue native PAGE.

#### 1.3.5.6

##### Rehydrated Polyacrylamide Gels

In washed gels, the SDS Tris–HCl/Tris–glycine buffer system exhibits poor results. However, good results are obtained with the Tris–acetate/Tris–tricine system. The performance of an SDS buffer system is obviously highly influenced by catalysts and/or monomers of acrylamide. In this method, the gel is rehydrated

in Tris–acetate, pH 8.0, using a horizontal tray. This procedure of washing, drying, rehydration and equilibration can be performed only with gels polymerized on film supports, which are used in horizontal systems.

#### 1.3.5.6.1

##### **Native Electrophoresis in Amphoteric Buffers**

The polymerization catalysts can be washed out of the polyacrylamide gels on support films used in horizontal systems with deionized water. By equilibration with amphoteric buffers such as *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 2-(*N*-morpholino)ethanesulfonic acid (MES) or 3-(*N*-morpholino)propanesulfonic acid (MOPS), for example, there is a wide spectrum for electrophoresis methods under native conditions.

This method proved to be particularly useful for acidic electrophoresis of basic hydrophobic barley hordeins (Hsam *et al.*, 1993) and basic fish sarcoplasmic proteins (Rehbein, 1995). The ionic catalysts ammonium persulfate (APS) and TEMED would destabilize these buffer systems, see Method 4.

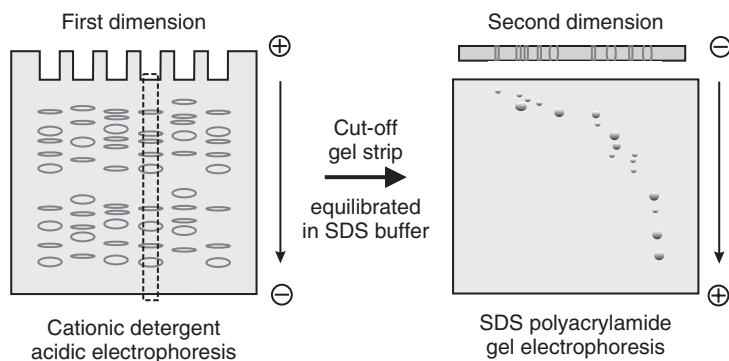
#### 1.3.5.7

##### **Two-Dimensional Electrophoresis Techniques**

Several aims are pursued by the combination of two different electrophoretic methods:

- Proteins separated by electrophoresis are identified by crossed immunoelectrophoresis.
- A complex protein mixture is first separated by ZE, and then further purified by IEF, or vice versa (Altland and Hackler, 1984).
- Hydrophobic proteins, such as membrane-bound proteins, are separated first in an acidic gel at pH 2.1 in presence of the cationic detergent 16-BAC, followed by SDS electrophoresis (MacFarlane, 1989; Hartinger *et al.*, 1996). As the separation patterns in 16-BAC and in SDS differ substantially, a decent resolution is obtained. The concept is shown in Figure 1.29.
- Membrane–protein complexes are first separated by blue native electrophoresis (see above) and then separated with SDS-PAGE for the display and identification of complex partners.
- Highly heterogeneous mixtures of proteins such as cell lysates or tissue extracts should be completely fractionated into individual proteins so as to obtain an overall picture of the protein composition and to enable location of individual proteins. The highest resolution is obtained by first separating according to the pIs, and the second dimension according to the molecular mass.

For these techniques, the first-dimensional runs are carried out in individual gel rods or strips and loaded onto the second-dimensional gels. The combination of IEF and SDS-PAGE – two completely orthogonal methods – has been introduced by Stegmann, Francksen and Macko (1973) and MacGillivray and Rickwood (1974). A flatbed IEF gel can also be cut into strips after the first separation and transferred onto the second gel.



**Figure 1.29** Principle of two-dimensional electrophoresis with a cationic detergent 16-BAC acidic PAGE in the first and SDS-PAGE in the second dimension.

### 1.3.5.7.1

#### High-Resolution 2-D Electrophoresis

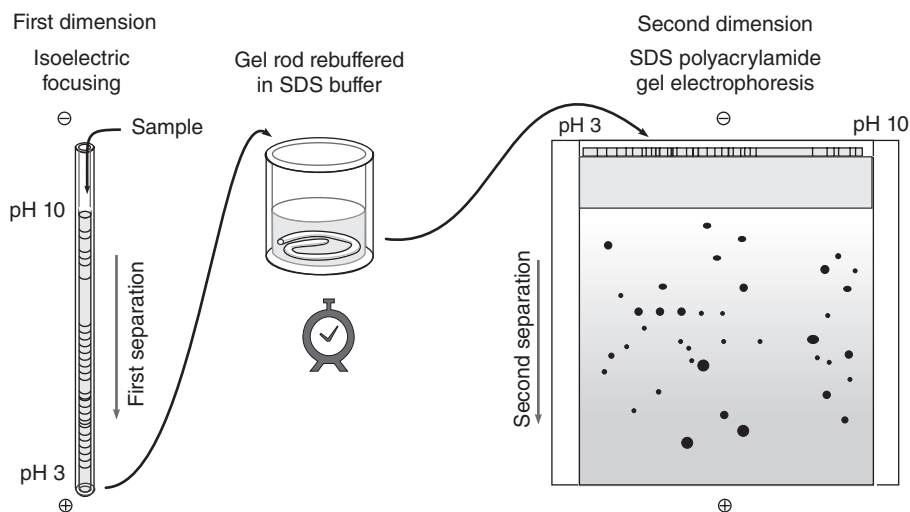
O'Farrell (1975) initiated a great step forward by employing completely denaturing conditions in both steps. First, the sample is denatured with a lysis buffer, which consists of saturated urea ( $9 \text{ mol l}^{-1}$ ), 2% (v/v) 2-mercaptoethanol for reduction and a nonionic detergent to increase the solubility. In the first dimension, IEF is performed in presence of 8 or 9 M urea and a nonionic detergent, and SDS electrophoresis is run as the second dimension. This measure disrupts protein complexes and aggregates, resulting in highly increased resolution. In the original work by O'Farrell, *Escherichia coli* extract proteins had been labeled with  $^{14}\text{C}$  or  $^{35}\text{S}$ , and detected in the gel by autoradiography. This method affords a very high dynamic range of up to five orders of magnitude, and thus 1100 different polypeptides could be detected. In Figure 1.30, the traditional way to perform high-resolution 2D electrophoresis is shown.

Since the protein spots obtained can be further analyzed with new methods of MS, this method has undergone a renaissance in the 1990s: the separated proteins can be identified and further characterized. This approach has been named "proteome analysis" (Wasinger *et al.*, 1995), and is described in more detail in Chapter 4 and in the book *Proteomics in Practice* by Westermeier, Naven and Höpker (2008).

### 1.3.5.8

#### GeLC-MS

The combination of SDS-PAGE with RPC of tryptic peptides and subsequent electrospray ionization (ESI) MS has become a widely used strategy for the identification and characterization of "proteomes." The whole tissue extract or cell lysate is first separated with SDS PAGE to reduce the complexity of the sample. The separation lanes are then divided into up to 48 gel slices with a grid cutter. The proteins are digested inside the gel slices with endoproteases, according to Hellman *et al.* (1995), mostly with trypsin. The peptides are eluted and loaded on an RPC column,



**Figure 1.30** Principle of the original high-resolution 2-D electrophoresis according to O'Farrell (1975).

which is on-line coupled with an ESI-MS instrument. In most cases, the peptides are further fragmented in the mass spectrometer for MS/MS analysis.

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