EXPERIMENT 4

Electrophoresis

Theory

Basic Principles

Electrophoresis is the process of migration of charged molecules through solutions in an applied electric field. Electrophoresis is often classified according to the presence or absence of a solid supporting medium or matrix through which the charged molecules move in the electrophoretic system. Solution electrophoresis systems employ aqueous buffers in the absence of a solid support medium. Such systems can suffer from sample mixing due to diffusion of the charged molecules, with resultant loss of resolution during sample application, separation, and removal steps. Thus, solution electrophoresis systems must employ some means of stabilizing the aqueous solutions in the electrophoresis cell. For example, soluble-gradient electrophoresis systems use varying densities of a non-ionic solute (e.g., sucrose or glycerol) to minimize diffusional mixing of the materials being separated during electrophoresis (Fig. 4-1). Even with these refinements, solution electrophoresis systems have only limited application, usually when preparative scale electrophoretic separation is required.

Most practical applications of electrophoresis in biochemistry employ some form of zonal electrophoresis, in which the aqueous ionic solution is carried in a solid support and samples are applied as spots or bands of material. Paper electrophoresis, cellulose acetate strip and cellulose nitrate strip, and gel electrophoresis are all examples of zonal

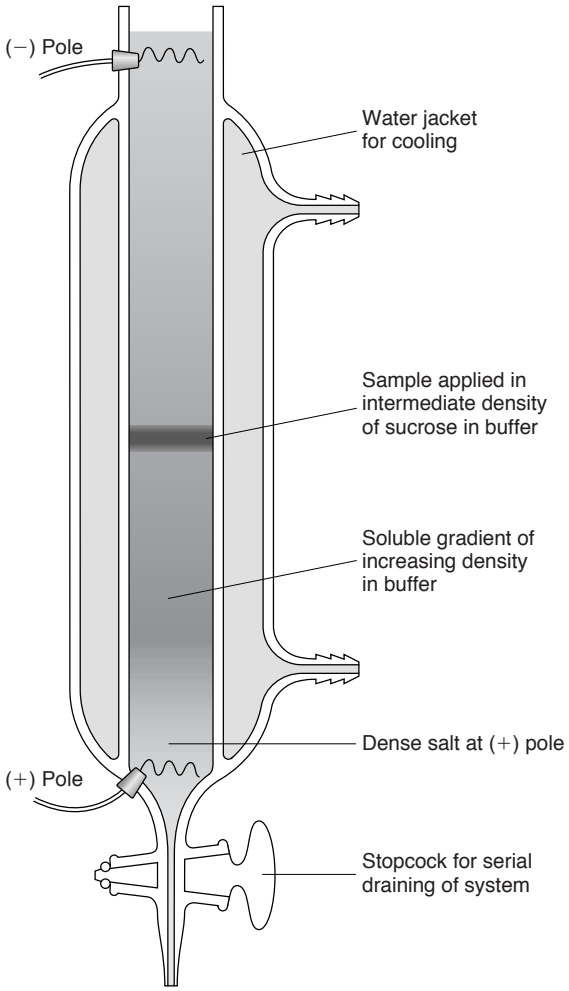

Figure 4-1 A solution electrophoresis system.

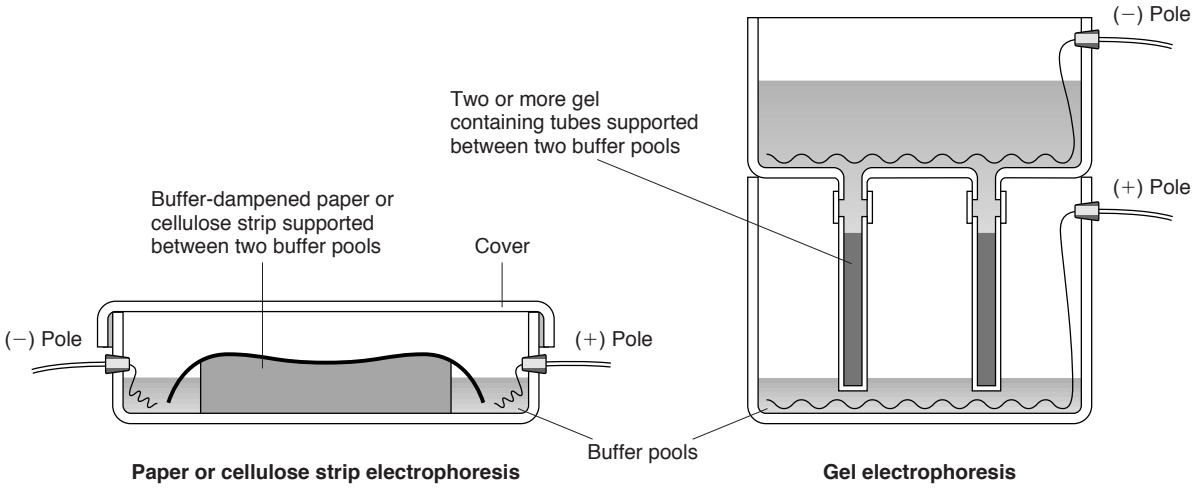

Figure 4-2 Two zonal electrophoresis systems.

electrophoresis systems (Fig. 4-2). Such systems are typically employed for analytical, rather than preparative scale, separations.

All types of electrophoresis are governed by the single set of general principles illustrated by Equation 4-1:

(4-1)

$$\text{Mobility of a molecule} = \frac{(\text{applied voltage})(\text{net charge on the molecule})}{(\text{friction of the molecule})}$$

The mobility, or rate of migration, of a molecule increases with increased applied voltage and increased net charge on the molecule. Conversely, the mobility of a molecule decreases with increased molecular friction, or resistance to flow through the viscous medium, caused by molecular size and shape. Total actual movement of the molecules increases with increased time, since mobility is defined as the rate of migration.

An understanding of the relationships described in Equation 4-1 is essential for many practical aspects of experimental biochemistry. Most electrophoretic systems employ an equal and constant voltage on all of the cross-sectional areas of the paper strips, gels, or solutions employed in the electrophoretic separation. These electric fields are best defined in terms of volts per linear centimeter. However, Ohm's law ($V = IR$) dictates that voltage (V) is a function of current (I) and resistance (R). The nature of the electrophoresis apparatus and buffer composition dictates the resistance in the system. Therefore, current (e.g., mA) is often used to define the voltage requirements of an electrophoretic separation. The resistance of the system is important because it will determine the amount of heat generated during electrophoresis. Since electrophoretic mobility is also a function of temperature, heating of the separation matrix must be controlled. If significant heating occurs during electrophoresis, it will be necessary to provide some means of cooling the apparatus so as to maintain a constant temperature. The "smiling" pattern often seen on slab gel electrophoresis (see below) is the result of nonuniform heating of the gel.

If the voltage or current applied to an electrophoresis system is constant throughout an electrophoretic separation, the mobilities of the molecules being resolved will reflect the other terms of Equation 4-1, namely, the net charge and frictional characteristics of the molecules in the sample. Consider the paper electrophoretic separation of glutamic acid, glutamine, asparagine methyl ester, and glycinamide at pH 6.0. The charges and molecular weights of these compounds at pH 6.0 are indicated in Figure 4-3. As seen, the equal-sized amino acids and the asparagine methyl ester separate strictly as a function of their net charges at pH 6.0, whereas

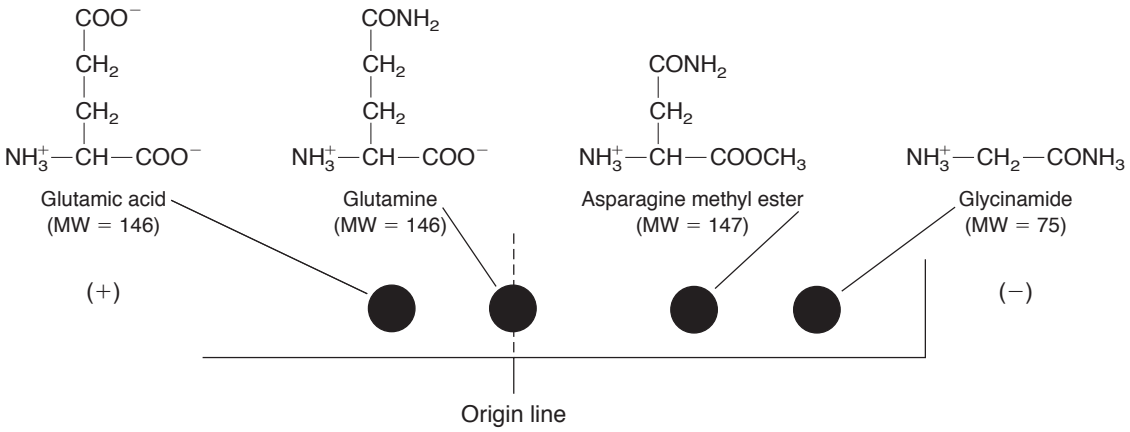

Figure 4-3 Paper electrophoretic separation of glutamic acid, glutamine, asparagine, methyl ester, and glycinamide at pH 6.0. The glycinamide, with a charge of +1 and a molecular weight of 75, may not necessarily migrate twice the distance migrated by asparagine methyl ester, with the same charge and ~2 times larger size. Specifically, the character of the buffer can influence the expression of the electrophoretic frictional contribution of small molecules. Higher ionic strength decreases the frictional contribution.

the glycinamide (which has the same net charge as asparagine methyl ester but has only half its size) migrates proportionally further toward the (−) pole of the system.

Generally, the principles illustrated in Figure 4-3 can be used to predict the relative mobility of small ionic molecules. These principles are particularly useful for predicting the potential for electrophoretic separation of small molecules containing weakly acidic or weakly basic groups that carry average partial charges in the pH ranges associated with their titration, as, for example, during the electrophoretic separation of nucleotides or amino acids.

The electrophoretic separation of larger macromolecules follows the general principles of Equation 4-1, but other factors influence the resolution of macromolecules. The friction experienced by molecules during electrophoretic migration reflects both molecular size and molecular shape. If the electrophoresis is carried out in a medium that offers significant barriers to the movement of macromolecules through it (as is the case with polyacrylamide and agarose, two very commonly used systems), *molecular size may prove to be the most important determinant of mobility*. If the charge to mass ratio on the macromolecules being separated is approximately equal (as in several of the cases discussed below), molecular size becomes the sole determinant of electrophoretic mobility. These conditions are exploited for the determination of the molecular weight of protein subunits by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE), and in the electrophoretic separation of oligonucleotide "ladders" during DNA sequencing. Molecular shape is not very significant in small molecules, in which bonds are free to rotate, so size alone defines their friction. However, macromolecules often have defined shapes with specific axial ratios (i.e., length to width ratios). As a result, both size and shape influence migration. Molecules with high axial ratios demonstrate lower electrophoretic mobility than more spherical molecules that have equal weight and equal charge. In addition, macromolecules may deviate from the electrophoretic principles of Equation 4-1 because of interaction with ions or because of charge-dependent intermolecular associations.

Specific Forms of Electrophoresis Commonly Used in Biochemistry

Paper Electrophoresis. Paper electrophoresis is a commonly used electrophoretic method for analysis and resolution of small molecules. This method

is not used to resolve macromolecules (e.g., proteins) because the adsorption and surface tension associated with paper electrophoresis usually alter or denature the macromolecules, causing poor resolution.

Two different methods are used routinely to apply samples to the electrophoretic paper. In the dry application procedure, a sample of solutes dissolved in distilled water, or a volatile buffer, is applied as a small spot or thin stripe on a penciled "origin line" on the paper. Appropriate standards of known compounds are applied at other locations on the origin line. If you anticipate electrophoretic migration toward both poles of the system, the origin line should be in the center of the paper. If you anticipate migration in only one direction, the origin line should be near one end of the paper. After the solvent containing the samples has evaporated, the paper is dampened with the electrophoresis buffer, either by uniform spraying or by dipping and blotting the ends of the paper so that wetting of the paper from both ends meets at the origin line simultaneously. In the wet application procedure, samples dissolved as concentrated solutions in distilled water are applied to paper predampened with electrophoresis buffer. The dry application procedure has the advantage of allowing small initial sample spots and better resolution of similarly mobile compounds. However, this method is awkward because the dipping or spraying requires considerable skill to avoid spreading the applied samples. The wet application procedure is simpler to perform, but usually yields larger spots and poorer resolution because of sample diffusion.

After the sample is applied to the dampened piece of paper, the paper is placed in the electrophoresis chamber so that both ends are in contact with reservoirs of the electrophoresis buffer at the electrodes (Fig. 4-4). If the origin line is not in the center of the paper, the paper must be positioned to allow maximum migration toward the correct electrode. After the chamber is covered or closed to protect against electric shock, an electric field is applied to the system.

Application of the electric field and the resultant resistance to current flow in the buffered paper generates heat. *This is the greatest source of difficulty with paper electrophoresis.* Heat dries the paper, which in turn leads to more resistance to current flow, which causes greater resistance, and so forth. Even if paper drying is prevented, heating will change the current flow and resistance properties of the system, which will distort the migration of molecules.

Because of these difficulties, modern paper electrophoresis systems have been designed to compensate for potential heating problems. Most low-voltage systems are portable and can be operated in cold rooms or refrigerated chambers. In contrast, most high-voltage systems either employ a cooled flat-bed system to dissipate the heat or operate in a cooled bath of inert and nonpolar solvent (e.g., Varsol, a petroleum distillate). This solvent absorbs the heat generated by the system without mixing with the water, buffers, or samples on the paper (Fig. 4-4). After electrophoretic resolution of the samples on the paper for the time and voltage required for optimal separation, the current is turned off, the paper is removed and dried, and the presence and location of the molecules of interest are determined.

Capillary Electrophoresis. A new method of analytical electrophoresis that is rapidly finding increasing application in biochemical research is capillary electrophoresis. As the name suggests, the material to be analyzed and the electrophoresis medium (a conducting liquid, usually aqueous) are placed in a long, fine-bore capillary tube, typically 50 to 100 cm long and 25 to 100 μm inside diameter. A very small sample (in the nanoliter range) is placed at one end of the capillary and subjected to electrophoresis under fields up to 20 to 30 kV. The analytes are separated by the principles illustrated in Equation 4-1 and detected as they emerge from the other end of the capillary by any of the methods commonly used in high-performance liquid chromatography (see Experiment 2). Capillary electrophoresis offers the advantages of extremely high resolution, speed, and high sensitivity for the analysis of extremely small samples, but is obviously not useful as a preparative method. It has proven especially useful in the separation of DNA molecules that differ in size by as little as only a single nucleotide. Because of its high resolution, capillary electrophoresis is the basis of separation of polynucleotides in some of the newer designs of DNA sequencers. Capillary electrophoresis can also be adapted to the separation of uncharged molecules by including charged micelles of a detergent (such

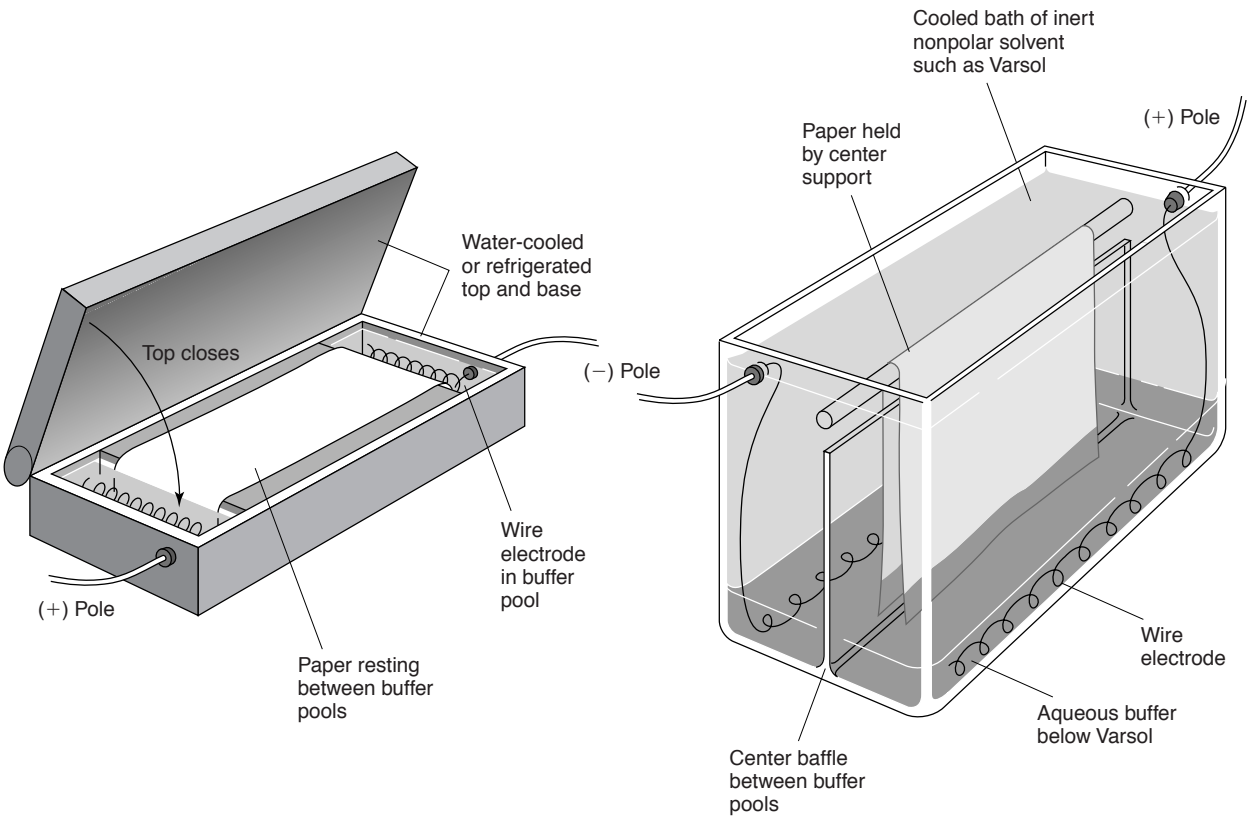

Figure 4-4 Two paper electrophoresis systems.

as SDS) in the aqueous electrophoresis medium. If a mixture of solute molecules that partitition between the aqueous medium and the hydrophobic interior of the micelles is introduced into such a system, they can be separated by electrophoresis. Capillary electrophoresis is a highly adaptable method, and the range of its applications and optimal methodology are still being explored.

Gel Electrophoresis. In gel electrophoresis, molecules are separated in aqueous buffers supported within a polymeric gel matrix. Gel electrophoresis systems have several distinct advantages. First, they can accommodate larger samples than most paper electrophoresis systems, and so can be used for preparative scale electrophoresis of macromolecules. Second, the character of the gel matrix can be altered at will to fit a particular application. This is possible because the gel enhances the friction that governs the electrophoretic mobility (see Equation 4-1). Low concentrations of matrix material or a low degree of cross-linking of the monomers in polymerized gel systems allow them to be used largely as a stabilizing or anticonvection device with relatively low frictional resistance to the migration of macromolecules. Alternatively, higher concentrations of matrix material or a higher degree of cross-linking of monomers are used to generate greater friction, which results in molecular sieving. Molecular sieving is a situation in which viscosity and pore size largely define electrophoretic mobility and migration of solutes. As a result, the migration of macromolecules in the system will be substantially determined by molecular weight.

Many gel-like agents are used in electrophoretic systems. Agarose (a polygalactose polymer) gels have proven quite successful, particularly when applied to very large macromolecules such as nucleic acids, lipoproteins, and others. Polyacrylamide gels are among the most useful and most versatile in gel electrophoretic separations because they readily resolve a wide array of proteins and nucleic acids (see Tables 4-1 and 4-2 for instructions on how to prepare SDS-PAGE gels and polyacrylamide gels for low molecular weight nucleic acid samples).

Polyacrylamide gels are formed as the result of polymerization of acrylamide (monomer) and *N*,*N*′-methylene-bis-acrylamide (cross-linker) (Fig. 4-5). The acrylamide monomer and cross-linker are stable by themselves or mixed in solution, but polymerize readily in the presence of a free-radical generating system. Biochemists use either chemical or photochemical free-radical sources to induce the polymerization process. In the chemical method (the most commonly used method), the free radical initiator, ammonium persulfate (APS), is added along with a *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) catalyst. These two components, in the presence of the monomer, cross-linker, and appropriate buffer, generate the free radicals needed to induce polymerization. In the photochemical method (less widely used), ammonium persulfate is replaced by a photosensitive compound (e.g., riboflavin) that will generate free radicals when irradiated with UV light. Many modifications can be made to produce a gel that will be useful for a particular application. If larger pores are required, you could decrease the amount of monomer and/or

Table 4-1 Recipe for Polyacrylamide Gels with Various Percent Acrylamide Monomer for Use with SDS-PAGE

	% Acrylamide in Resolving Gel				
Component (ml)	**7.5**	**10**	**12**	**15**	**20**
Distilled water	9.6	7.9	6.6	4.6	2.7
30% acrylamide solution	5.0	6.7	8.0	10.0	11.9
1.5 M Tris chloride (pH 8.8)	5.0	5.0	5.0	5.0	5.0
10% (wt/vol) SDS	0.2	0.2	0.2	0.2	0.2
TEMED	0.008	0.008	0.008	0.008	0.008
10% (wt/vol) ammonium persulfate	0.2	0.2	0.2	0.2	0.2

Prepare the ammonium persulfate fresh and add last to induce the polymerization process (polymerization of the resolving gel will take approximately 30 min).

4% Acrylamide Stacking Gel for SDS-PAGE

Component	**Volume (ml)**
Distilled water	2.7
30% acrylamide	0.67
1.0 M Tris chloride (pH 6.8)	0.5
10% (wt/vol) SDS	0.04
TEMED	0.004
10% (wt/vol) ammonium persulfate	0.04

Prepare the ammonium persulfate fresh and add last to induce the polymerization process (polymerization of the resolving gel will take approximately 30 min).

30% Acrylamide Solution

Dissolve 29.2 g of acrylamide and 0.8 g of *N*,*N*′-methylene-bis-acrylamide in 100 ml of distilled water. Filter through a 0.45-μM-pore-size membrane (to remove undissolved particulate matter) and store in a dark bottle at 4°C.

Table 4-2 Recipe for a 15% Polyacrylamide Gel for Use in Separating Nucleic Acids Smaller Than 500 Bases in Length

Component	Volume or Mass
Urea	15 g
40% acrylamide solution	11.3 ml
10× TBE buffer	3.0 ml
Distilled water	4.45 ml
TEMED	30 ml
10% (wt/vol) ammonium persulfate	200 ml

Prepare the ammonium persulfate fresh and add last to induce the polymerization process. This recipe will be enough to cast 3 to 4 gels, depending on the size of the plates that you use. The recipe for a 0.5× TBE solution is shown in Table 4-5. A 5× concentrated stock can be prepared and stored at room temperature for a short time by multiplying all of the masses and volumes of the Tris base, boric acid, and EDTA components by 5. After several days at room temperature, a precipitate may begin to form in the 5× TBE stock. When this occurs, a fresh solution should be made. This 5× working stock should be diluted 1:10 with distilled water to produce a 0.5× running buffer for use with separation of low-molecular-weight nucleic acids by polyacrylamide gel electrophoresis.

40% Acrylamide Solution

Dissolve 76.0 g of acrylamide and 4.0 g of *N,N′*-methylene-bis-acrylamide in 200 ml of distilled water. Filter through a 0.45-μM-pore-size membrane (to remove undissolved particulate matter) and store in a dark bottle at 4°C.

cross-linker in the polymerization solution. If smaller pores are required, one may increase the concentration of monomer and/or cross-linker.

The pore size required for a particular electrophoretic separation will depend on the difference in size of the compounds that you wish to resolve. For instance, if you wish to resolve two small proteins of 8,000 Da and 6,000 Da, you will require a small-pore-size gel for this application (~15–20% acrylamide). This same percent acrylamide gel would not permit the resolution of two larger proteins of say 150,000 Da and 130,000 Da. A larger-pore-size gel would be required for this application (~7.5–10% acrylamide). A list of components required to produce polyacrylamide gels of varying percent acrylamide is shown in Table 4-1. A list of the effective separation range of proteins on acrylamide gels made with various percent acrylamide is shown in Table 4-3.

Most important, any methods used to produce polyacrylamide gels must be followed exactly each time they are cast, since reproducible electrophoretic separations require uniform gel-forming conditions. Current literature contains many electrophoresis procedures and applications for polyacrylamide gels. Most of these employ the polyacrylamide gel in some sort of "slab" format. One of the most commonly used of these procedures is discussed below.

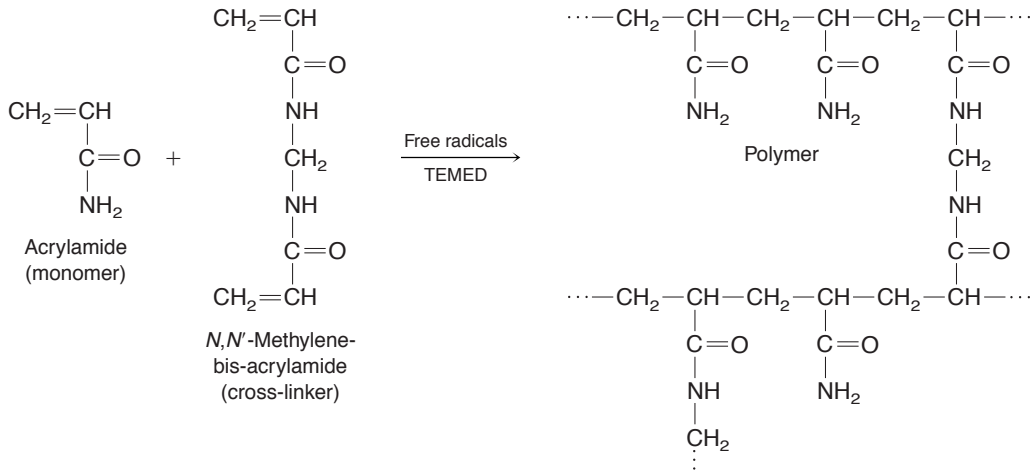

Figure 4-5 Formation of polyacrylamide gels.

Table 4-3 The Effective Separation Range of Polyacrylamide Gels of Various Percent Acrylamide Monomer for Use With SDS-PAGE

% Acrylamide in Resolving Gel	Effective Separation Range (Da)
7.5	45,000–200,000
10	20,000–200,000
12	14,000–70,000
15	5,000–70,000
20	5,000–45,000

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). Sodium dodecyl sulfate (SDS) gel electrophoresis systems are used to determine the number and size of protein chains or protein subunit chains in a protein preparation. Initially, the protein preparation is treated with an excess of soluble thiol (usually 2-mercaptoethanol) and SDS. Under these conditions, the thiol reduces all disulfide bonds (–S–S–) present within and/or between peptide units, while the SDS (an ionic or denaturing detergent) binds to all regions of the proteins and disrupts most noncovalent intermolecular and intramolecular protein interactions. These two components result in total denaturation of the proteins in the sample, yielding unfolded, highly anionic (negatively charged) polypeptide chains (Fig. 4-6).

The anionic polypeptide chains are then resolved electrophoretically within a polyacrylamide gel saturated with SDS and the appropriate current-carrying buffer. The excess SDS is included in the gel to maintain the denatured state of the proteins during the electrophoretic separation. The SDS-coated polypeptides (carrying approximately one SDS molecule per two amino acids) creates a situation in which the charge-to-mass ratio of all of the proteins in the sample is approximately the same. At this point, the intrinsic charge on the individual polypeptide chains (that is, in the absence of SDS) is insignificant as compared with the negative charge imposed on them by the presence of the SDS. The friction experienced by the population of molecules as they migrate through the polyacrylamide matrix is now the major factor influencing differences in their mobility. In addition, the fric-

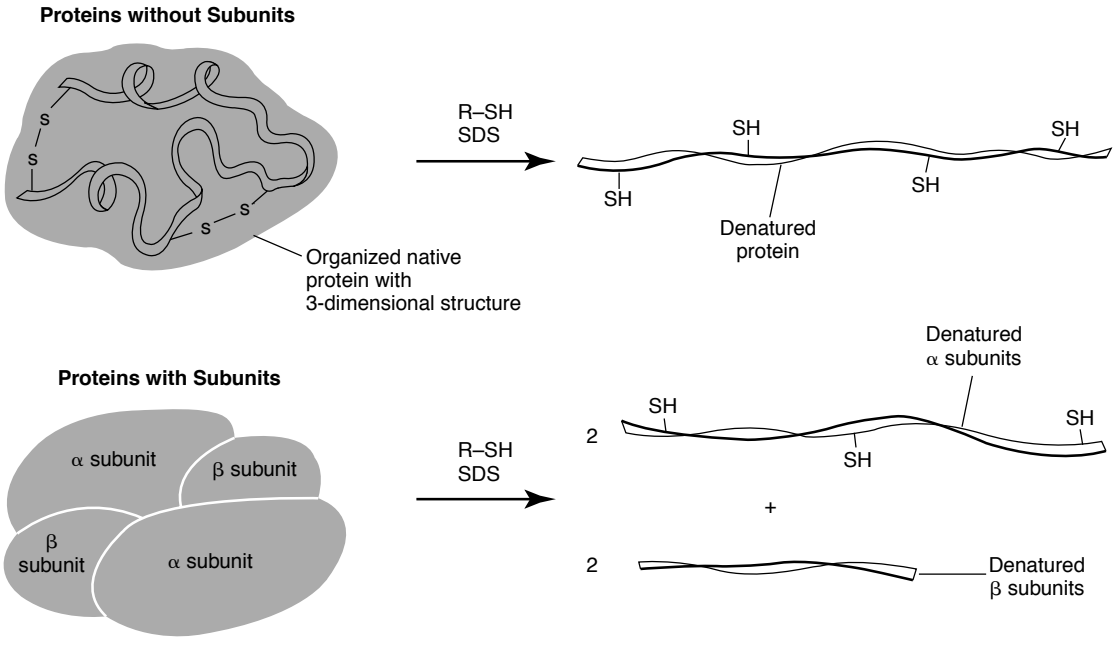

Figure 4-6 Disruption of proteins with excess thiol and SDS.

tion experienced by the molecules during the separation is governed by the pore size of the polyacrylamide matrix: *larger polypeptides will experience greater friction when passing through a gel of defined pore size (will migrate more slowly) than smaller polypeptides (which will migrate more rapidly)*. In summary, SDS-PAGE allows the separation of proteins on the basis of size.

The principles underlying the most commonly used form of SDS-PAGE are best illustrated by a description of the step-by-step progress of a protein migrating in the gel. As seen in Figure 4-7 and Table 4-1, SDS-PAGE employs two buffer/polyacrylamide gel compositions in a single slab. These are referred to as the "stacking gel" and the "running (or resolving) gel." Protein samples are first introduced into wells cast within the stacking gel after they are mixed with a viscous sample buffer (30% glycerol) containing SDS and thiols. After the samples have been loaded, voltage is applied to the system (current is carried through the gel, ~3–4 V/cm^2) between two separated pools of glycine buffer, pH 8.3 (Table 4-4).

Remember that ion (current) flow must follow the principles dictated in Equation 4-1, namely, greater charge increases mobility, whereas greater size leads to greater friction and decreased mobility. Glycine carries an average charge of about −0.1 per molecule at pH 8.3 (running buffer) and almost no net negative charge at pH 6.8 (stacking gel pH). In contrast, the SDS-coated proteins in the system carry a high negative charge that is essentially independent of the pH of the system. At the low pH in the stacking gel, glycine anions lose negative charge and display decreased mobility in the system. In contrast, the chloride ions contained in the stacking gel migrate ahead of the proteins because of their small size (low friction) and full negative charge. Since the negatively charged proteins in the system have a larger frictional factor, they migrate into the stacking gel at a rate that is slower than that of the chloride population, but faster than that of the virtually uncharged glycine anion population. The resultant scale of anion mobilities in the low percentage acrylamide stacking gel ($Cl^- >$ proteins$^-$ > glycine$^-$) causes the proteins to accumulate ahead of the advancing glycine front (Fig. 4-7*b*) and eventually stack into concentrated, narrow bands at the interface between the stacking gel and the running gel (Fig. 4-7*c*). Meanwhile, the chloride

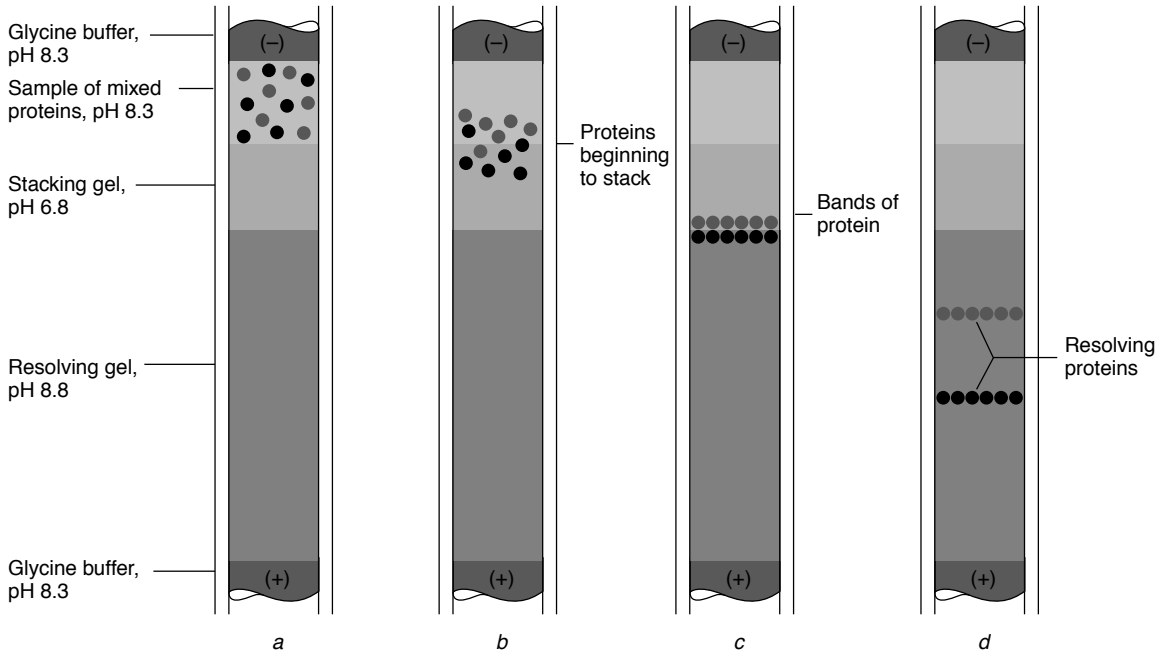

Figure 4-7 Migration of proteins through stacking and resolving gels during SDS-PAGE.

Table 4-4 Recipes for Buffers Used in Agarose Gel Electrophoresis of Nucleic Acids and SDS-PAGE

Tris/Borate/EDTA (TBE) Buffer (0.5×, working solution)

Dissolve 5.4 g of Tris base and 2.75 g of boric acid in 700 ml of distilled water. Add 2 ml of 0.5 M EDTA, pH 8.0. Bring the final volume of the solution to 1 liter with distilled water. *NOTE:* A 5× concentrated stock can be prepared and stored at room temperature for a short time by multiplying all of the masses and volumes of the Tris base, boric acid, and EDTA components by 10. This 5× concentrated stock will be diluted 1:10 with distilled water to give a 0.5× working solution.

Tris/Acetate/EDTA (TAE) Buffer (1×, working solution)

Dissolve 4.84 g of Tris base in 700 ml of distilled water. Add 1.14 ml of glacial acetic acid and 2 ml of 0.5 M EDTA, pH 8.0. Bring the final volume of the solution to 1 liter with distilled water. *NOTE:* A 50× concentrated stock can be prepared and stored at room temperature for long term by multiplying all of the masses and volumes of the Tris base, glacial acetic acid, and EDTA components by 50. This 50× concentrated stock will be diluted 1:50 with distilled water to give a 1× working solution.

Electrophoresis Buffer for SDS-PAGE (1X working solution)

Dissolve 3.02 g of Tris base and 18.8 g of glycine in 700 ml of water. Add 10 ml of 10% (wt/vol) SDS and adjust the pH of the solution to 8.3 with HCl. Bring the final volume of the solution to 1 liter with distilled water. *NOTE:* A 5× concentrated stock can be prepared by multiplying the masses and volumes of all of the components by five. This 5× concentrated stock will be diluted 1:5 with distilled water to give a 1× working solution described above.

ions in the stacking gel readily migrate into the running gel.

As the advancing front of anions enters the running gel, the high concentration of pH 8.8 buffer and the high concentration of acrylamide (decreased pore size) in the gel triggers two events. First, the high pH buffer imparts a greater negative charge to the glycine anions, whose migration was retarded in the pH 6.8 buffer in the stacking gel. Because of their small size and increased anionic character, the glycine ions quickly overtake the proteins as they migrate through the system. Second, the reduced pore size of the gel imparts a significant frictional component to the mobility of each individual protein present in the sample. The equal charge-to-mass ratio imparted on the proteins by the SDS present in the system (see above) now dictates that all of the proteins in the system will migrate through the gel on the basis of size.

As shown in Figure 4-8, the relative mobility of each of the anionic polypeptide chains is a function of the logarithm of its molecular weight. If a set of polypeptides of known molecular weight are included with the sample during the electrophoretic separation, their relative mobilities may be determined and plotted as log molecular weight versus relative mobility. The standard curve produced from this analysis of the polypeptides of known molecular weights can then be used, along with the relative mobilities of the unknown polypeptides in the sample, to estimate their molecular weight (see Fig. 4-8).

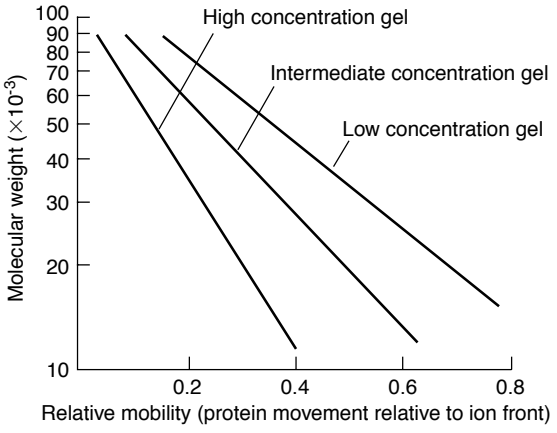

Figure 4-8 Relation between molecular weight and relative mobility of proteins on SDS gels.

How do you visualize the proteins that have been separated following SDS-PAGE? Two visualization methods are most often used. The choice between them depends largely on the sensitivity of detection that is required for your application. The first method involves saturating the gel with a solution of acetic acid, methanol, and water containing Coomassie Brilliant Blue R-250 dye. As the methanol and acetic acid in the solution work to "fix" the proteins within the gel matrix, Coomassie Brilliant Blue binds to the proteins in the gel. The interaction between the Coomassie dye and proteins has been shown to be primarily through arginine residues, although weak interactions with tryptophan, tyrosine, phenylalanine, histidine, and lysine are also involved. After the gel is "destained" with the same aqueous acetic acid/methanol solution without the dye (to remove the dye from portions of the gel that do not contain protein), the proteins on the gel are visible as dark blue "bands" on the polyacrylamide gel. When conducted properly, this method of detection is sufficiently sensitive to detect a protein band containing as little as 0.1 to 0.5 μg of a polypeptide. An alternative staining method, silver staining, is sensitive to about 10 ng of protein contained in a single band on the acrylamide gel. Silver staining begins by saturating the gel with a solution of silver nitrate. Next, a reducing agent is added to cause the reduction of Ag^+ ions to metallic silver (Ag), which precipitate on the proteins in the gel and cause the appearance of protein bands that are black in color. Silver staining is technically more difficult, and therefore is used only when extreme sensitivity is required.

Isoelectric Focusing. Isoelectric focusing is an electrophoretic technique that separates macromolecules on the basis of their isoelectric points (pI, pH values at which they carry no net charge). As with SDS-PAGE, this process can be carried out in a "slab" format. A pH gradient is established in the polyacrylamide gel with the aid of ampholytes, which are small (~5000 Da) polymers containing random distributions of weakly acidic and weakly basic functional groups (e.g., carboxyls, imidazoles, amines, etc.). A polyacrylamide gel containing these ampholytes is connected to an electrophoresis apparatus that contains dilute acid solution (H^+) in the anode chamber and a dilute base (OH^-) solution in the cathode chamber (Fig. 4-9).

When voltage is applied to the system, the current flow will be due largely to migration of the charged ampholyte species present in the gel. The ampholytes migrate toward either pole in a manner consistent with their charge distributions; ampholytes with lower isoelectric points (e.g., those that contain more carboxyl groups and have a net negative charge) migrate toward the anode, while ampholytes with higher isoelectric points (e.g., those that contain more amine groups and have a net positive charge) migrate toward the cathode. Eventually, each ampholyte in the system will reach a position in the gel that has a pH equal to its isoelectric point. When this occurs, these ampholytes carry no net charge and will no longer migrate in the gel. The net effect is that, after a sufficient period of electrophoresis, the population of ampholytes will act as local "buffers" to establish a stable pH gradient in the polyacrylamide gel. This pH gradient forms the basis for the separation of macromolecules that are present in the system (or are subsequently introduced into the system).

Proteins present in the system act like ampholytes in that they migrate as a function of their net charge. As the ampholytes establish a pH gradient in the gel, the proteins in the system also migrate toward their respective poles until they reach a pH in the gel at which they too carry no net negative charge (the pI of the protein). At this point, the attractive forces applied to the protein by the anode and cathode are equal, and the protein no longer migrates in the system. In effect, the different proteins in the sample are "focused" to particular portions of the gel where the pH is equal to their pI values.

How do you determine when the electrophoretic separation is complete? As stated above, the separation is complete when the ampholytes and proteins reach a pH value in the gel equal to their pI and no longer migrate. Once the proteins and ampholytes no longer migrate, the current (I) in the system decreases dramatically. Because isoelectric focusing is performed in the presence of fairly low concentrations of ampholytes (2–4%), high voltage potential (150 V/cm^2) can be applied to the system without the generation of excess amounts of heat caused from high currents.

Since this method is designed to separate proteins on the basis of isoelectric point, one must take care not to impose a significant "friction factor" to

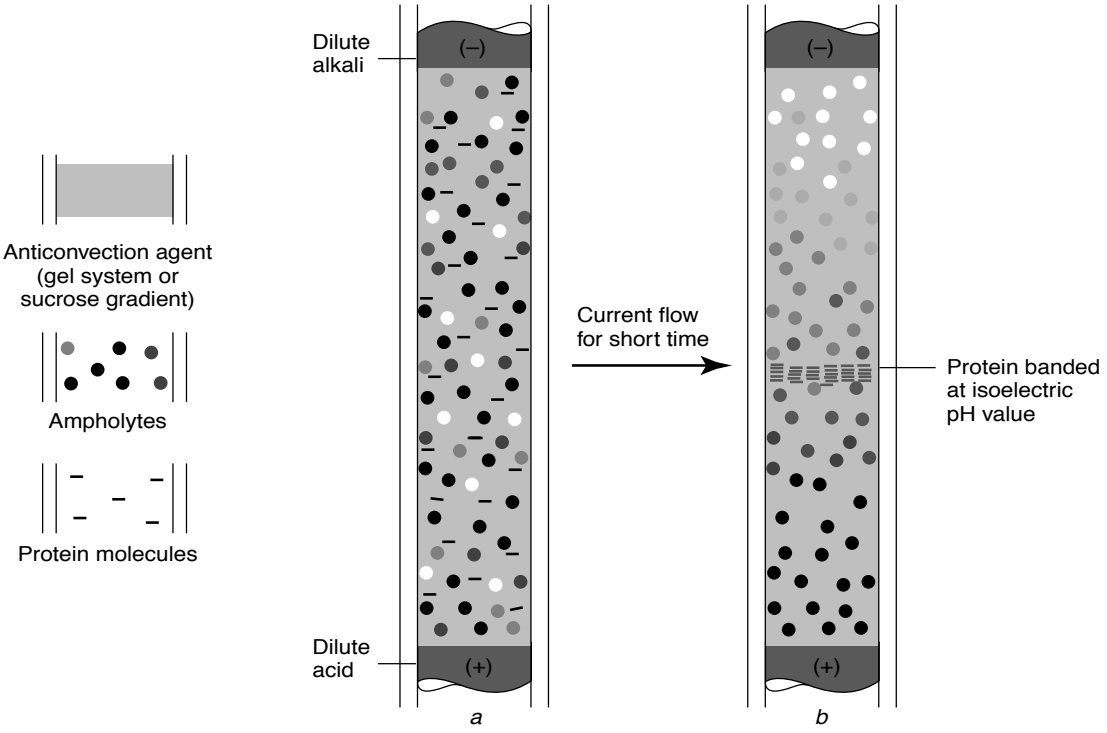

Figure 4-9 Steps of an isoelectric focusing procedure.

the macromolecules being separated. In other words, the pore size of the gel should be large enough that all of the macromolecules in the system can migrate freely to their appropriate isoelectric points. If the pore size of the matrix limits the rate of migration of the proteins with higher molecular weights, the separation may turn out to be influenced more, or as much as, by size as by isoelectric point. If polyacrylamide gels are used for this purpose, the concentration of acrylamide in the gel should not exceed 8%. If desired, you can perform isoelectric focusing using protocols that employ other "slab" matrix materials, such as agarose, or in a liquid matrix format that uses a sucrose density gradient as the anticonvection agent. You should also be aware that ampholytes are commercially available in a wide array of pH ranges that may be required for separation of a molecule of interest (e.g., pH 3–5, pH 2–10, pH 7–9, etc.). In the past, isoelectric focusing has been performed in the "slab" format and employed largely as an analytical technique. More recently, systems have been developed that will allow isoelectric focusing to be carried out in a preparative (large-scale, nondenaturing) format. Although the principles of the separation are the same as those described above, preparative isoelectric focusing is carried out using a liquid anticonvection agent rather than a gel slab.

Agarose Gel Electrophoresis. Agarose gel electrophoresis is the principal technique used to determine the size of high-molecular-weight nucleic acids (DNA and RNA). Agarose is a long polymer of galactose and 3,6-anhydrogalactose linked via α $(1 \rightarrow 4)$ glycosidic bonds. This material is readily isolated from seaweed. Agarose polymers may contain up to 100 monomeric units, with an average molecular weight of around 10,000 Da. Agarose gels are cast by dissolving the white agarose powder in an aqueous buffer containing EDTA and either Tris-acetate or Tris-borate as the buffering species (TAE or TBE buffer, respectively, see Table 4-4). When the sample is heated to just below boiling, the agarose powder dissolves in the buffer to form a clear solution. As the solution slowly cools

to room temperature, hydrogen bonding within and between the polygalactose units in the solution will cause the formation of a rigid gel with a relatively uniform pore size. The induction of this polymerization event involves no chemical reaction, unlike the polymerization process described above for polyacrylamide gels.

As with polyacrylamide gels, the pore size of the gel can be controlled by the percentage of the agarose dissolved in the solution. A high percent agarose gel (say, 3% wt/wt) will have a smaller pore size than a lower (0.8% wt/wt) agarose gel. The percent of agarose to be cast in the gel will be determined by the size of the various molecules to be resolved during electrophoresis; the smaller the molecular weight of the molecules to be resolved, the higher percent agarose (smaller pore size) the gel should contain. A list of the effective separation ranges with agarose gels of various percent agarose is shown in Table 4-5.

Once the solution is heated to dissolve the agarose, the solution is cooled momentarily and poured into a slab mold fitted at one end with a Teflon or plastic comb. After the solution polymerizes, the comb is removed to create wells into which the desired DNA or RNA samples will be applied. The gel is then transferred to an electrophoresis chamber and is completely covered with the same TAE or TBE buffer that was used to cast the gel. Next, the nucleic acid sample is mixed with a viscous buffer (30% glycerol) containing one or more tracking dyes that will be used to monitor the progress of the electrophoresis. Bromophenol blue dye will migrate at the same rate as a DNA molecule of about 500 base pairs, while xylene cyanole dye will migrate at the same rate as a DNA molecule of about 4000 base pairs.

Table 4-5 The Effective Separation Range of Agarose Gels of Various Composition for Separation of Nucleic Acids

% Agarose (wt/vol)	Effective Separation Range (base pairs)
0.8	700–9000
1.0	500–7000
1.2	400–5000
1.5	200–3000
2.0	100–300

The nucleic acid samples are loaded into the wells of the gel, along with a sample of DNA fragments of known molecular weight (number of base pairs) in one of the wells. These standards will be used following the electrophoretic separation to aid in the determination of the size of the nucleic acid samples present in the unknown sample (see below). Remember that the DNA is negatively charged. Because of this, the cathode (negative electrode) should be connected to the side of the apparatus nearest the wells in the gel, while the anode (positive electrode) is connected to the opposite side of the apparatus. A voltage of about 4 to 6 V/cm is applied to the system (~50–70 mA of current), and the electrophoresis is continued until the bromophenol blue dye front reaches the end of the gel. You will notice that agarose gel electrophoresis, unlike SDS-PAGE, does not employ the use of a stacking gel. Since the nucleic acids in the sample have a much greater frictional component in the gel than they do in the buffer contained in the wells, the nucleic acids focus very quickly at the buffer–gel interface before entering the matrix.

How do you visualize the nucleic acids following the electrophoretic separation? Ethidium bromide is a fluorescent dye that has the ability to intercalate between the stacked bases of nucleic acid duplexes (i.e., the double helix of DNA). After electrophoresis, the gel is placed in a solution of TBE or TAE buffer containing ethidium bromide, which diffuses into the gel and associates with the nucleic acids. Following a short destaining period in buffer without ethidium bromide (to remove it from the areas on the gel that do not contain nucleic acids), the gel is briefly exposed to ultraviolet (UV) light (256–300 nm), revealing the nucleic acid fragments as orange or pink fluorescent bands in the gel. The pattern of fluorescent bands is recorded by photographing the gel in a dark chamber while the gel is exposed to UV light from below. A filter is included to screen out UV light, so that most of the light that exposes the film is from the fluorescence of the nucleic acid bands that contain tightly bound ethidium bromide.

The method of determining the molecular weight of an unknown nucleic acid sample is exactly the same as that described for the determination of protein molecular weight in SDS-PAGE.

The log molecular weight of the nucleic acid samples of known sizes are plotted against their relative mobilities to produce a standard curve. From the relative mobility of an unknown nucleic acid fragment on the same gel, the molecular weight and number of base pairs that the fragment contains can be readily determined. (A single base has an average molecular weight of approximately 320 Da, while a single base pair has an approximate average molecular weight of 640 Da).

In the experiment that follows, you will have an opportunity to gain experience with some of the foregoing principles by using SDS-PAGE to determine the number of polypeptides and its (their) molecular weight(s) in an unknown protein sample.

Supplies and Reagents

1.5 M Tris chloride, pH 8.8
1.0 M Tris chloride, pH 6.8
30% acrylamide (292 g/liter acrylamide, 8 g/liter *N*,*N*′-methylenebisacrylamide) [*CAUTION, ACRYLAMIDE IS TOXIC!*]
10% (wt/vol) SDS in water
TEMED (*N*,*N*,*N*′,*N*′-tetraethylethylenediamine)
10% (wt/vol) ammonium persulfate in water—freshly prepared
Running buffer (25 mM Tris, 250 mM glycine, 0.1% (wt/vol) SDS, pH to 8.3 with HCl)
4X Sample buffer:

- 0.25 M Tris chloride, pH 7.0
- 30% (vol/vol) glycerol
- 10% (vol/vol) 2-mercaptoethanol
- 8% (wt/vol) SDS
- 0.001% (wt/vol) bromophenol blue

Coomassie Blue staining solution (40% methanol, 10% glacial acetic acid, 0.25% (wt/vol) Coomassie Brilliant Blue R-250 in water)
Destaining solution (40% methanol, 10% glacial acetic acid in water)
Power supply and electrophoresis apparatus
Protein molecular weight standards (suggested standards: phosphorylase [97,400 Da], bovine serum albumin [66,200 Da], ovalbumin [45,000 Da], carbonic anhydrase [31,000 Da], soybean trypsin inhibitor [21,500 Da], and lysozyme [14,400 Da] at 1 mg/ml each in a single mixture)
Unknown protein solutions (approximately 1 mg/ml)

Protocol

NOTE: The quantities of materials to be used and details of preparation of the PAGE gels for electrophoresis will depend on the number of students and the characteristics of the electrophoresis apparatus to be used. A typical apparatus for SDS-PAGE in vertical slab gels is shown in Figure 4-10; the instructions that follow assume that you are using such a system. Your instructor will provide detailed instructions and will demonstrate the techniques to be used in preparing the gels.

1. Prepare the running gel. (The volume to be prepared will depend on the size of the electrophoresis apparatus you will use and the number of gels to be prepared; consult your instructor. The procedure described here is for 20 ml of a 10% monomer running gel.) Mix the following, in order, in a clean 100-ml beaker: 7.9 ml distilled H_2O, 6.7 ml 30% acrylamide solution, 5.0 ml 1.5 M TrisHCl, pH 8.8, 200 μl 10% SDS, 8 μl TEMED, and 200 μl freshly dissolved 10% ammonium persulfate. The ammonium persulfate should be added last, as it initiates polymerization.
2. Pour the freshly mixed running gel solution into the spaces between parallel glass or plastic gel-forming sheets of the electrophoresis apparatus, leaving suficient space at the top for the stacking gel to be added later. (This is usually about 1 cm from the top, but the exact distance depends on the apparatus being used; consult your instructor.) Create a flat surface above the running gel solution by gently adding a water layer on top of the more dense gel solution, and allow the gel to polymerize for at least 30 min.
3. Pour off the water from above the polymerized running gel. This should leave a very sharp flat surface at the top of the gel.
4. Prepare the stacking gel. (The procedure described here is for 4 ml of a 4% monomer stacking gel; again, consult your instructor to see if you should prepare a different amount.) Mix the following, in order, in a clean test tube: 2.7 ml distilled H_2O, 0.67 ml 30% acrylamide solution, 0.5 ml 1.0 M TrisHCl, pH 6.8, 40 μl 10% SDS, 4 μl TEMED, and, added last, 40 μl freshly dissolved 10% ammonium persulfate.

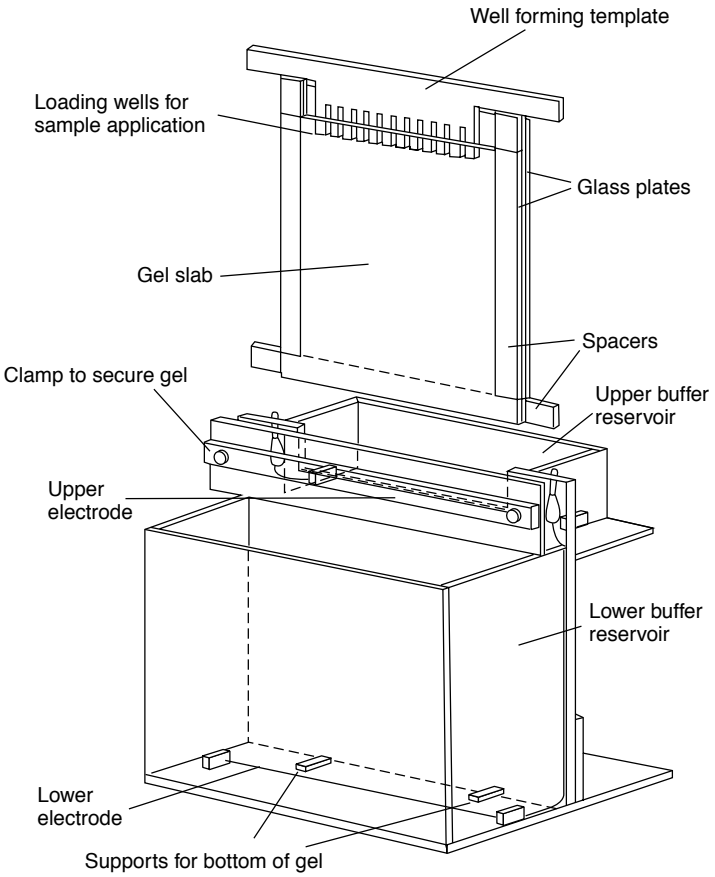

Figure 4-10 A typical apparatus for vertical slab SDS-PAGE. (From Wilson, K., and Walker, J. (1994) *Principles and Techniques of Practical Biochemistry,* 4th ed. Cambridge, UK. Cambridge University Press, Fig. 9-1. Reprinted with permission.)

Mix the solution gently and proceed immediately to Steps 5 and 6.

5. Pour the stacking gel into the space above the running gel.
6. Immediately insert the comb to create sample wells in the stacking gel before it polymerizes. Be sure that the comb is clean and free of foreign material and that no air bubbles form around the comb. If bubbles do form, tilt the gel slightly and tap the glass near the area of the bubble to dislodge it and allow it to rise to the top. Be sure that you fill the stacking gel slightly over the top of the small plate, as the stacking gel will shrink slightly during polymerization. Allow the gel to polymerize for at least 30 min at room temperature.
7. Remove the bottom spacer from between the plates, and place the polymerized gel in the plates in which it was formed into the electrophoresis apparatus.
8. Add running buffer to the chambers at the bottom and top of the gel. Gently remove the comb from between the plates. Be sure that no bubbles interfere with uniform contact of the liquid in the running buffer with the gel at the bottom or at the top. The wells in the stacking portion of the gel should be uniformly filled with buffer with no bubbles or gel debris in them. If bubbles need to be removed, use a syringe and buffer to gently force them out. Also, be sure that there is no leaking between the two buffer chambers. Remember that the current

will take the path of least resistance, and it will not pass through the gel unless the two buffer chambers are separated.

9. Prepare the standard and unknown protein samples for analysis by mixing 10 μl of each with 5 μl of 4X sample buffer in microcentrifuge tubes, mixing gently, and heating at 100°C for 5 min in a boiling water bath. This step is to ensure that all of the proteins in the sample are completely denatured. Remove the samples from the water bath and allow them to cool to room temperature.
10. Load the samples onto the gel with a syringe, taking care to place the needle so that the dense blue solution settles gently in a layer at the bottom of the sample wells, displacing the running buffer upward as it is added. Be careful not to tear or distort the soft stacking gel as you add the samples. Several students can share a gel; for example, if a 10-well gel is used, eight students can analyze their unknowns and two samples of standards can be analyzed, one in a center well and one in a well at the edge of the gel.
11. Connect the electrodes of the apparatus to the power source. The anode (+ pole) must be at the bottom of the apparatus. (Can you explain why?)
12. Apply a constant 200-V field to the apparatus, and continue the electrophoresis until the bromophenol blue tracking dye reaches the very bottom of the gel. Bromophenol blue is a small anionic dye, which will migrate faster than any protein during electrophoresis.
13. Turn off the power supply, and disconnect the electrodes from it.
14. Gently remove the gel from the apparatus. Using a spatula or razor blade, separate the two plates by gently prying up one corner of a single plate. The gel should adhere to one of the plates. Using the same spatula or razor blade, separate the soft stacking gel from the more rigid running gel. Discard the stacking gel in the specified container (polyacrylamide is toxic and should be properly disposed of).
15. Gently transfer the running gel to a pan filled with approximately 50 ml of Coomassie Blue staining solution and allow it to soak in the solution for 1 hour.
16. Pour off the staining solution (save it, because it can be reused many times), and replace it with destaining solution for 1 hour, replacing it with fresh destaining solution every 15 min. Destaining proceeds more rapidly if the gel is gently agitated in the solution continuously during destaining. Destaining is complete when the proteins appear as blue bands on a transparent gel background.
17. The gel can now be photographed or placed between two sheets of cellophane (using water to make the gel easier to slide around, then gently squeezing out the excess water, but leaving no bubbles) and dried under a vacuum on a commercial gel drier to keep a permanent record of the electropherogram.

Data Analysis

1. For each protein band in the lanes in which standards and the unknown sample were analyzed, measure the distance from the bottom of the well (in mm) to the center of the band. Also measure the distance from the bottom of the well to center of the dye front (in millimeters) in the same lane.
2. Calculate the R_f value for each protein band according to:

$$R_f = \frac{\text{distance migrated by the protein band (mm)}}{\text{distance migrated by the dye front (mm)}}$$

3. Use the R_f values for the protein standards to prepare a standard curve that plots the log of the molecular weight of each standard protein on the ordinate versus the R_f value for that protein on the abscissa. Do all of the values fall on a straight line? Can you predict what the curve would look like for proteins that are much larger and much smaller than the standards you used?
4. From the R_f value(s) of the protein band(s) in your unknown sample, determine the molecular weights of these proteins by interpolation on your standard curve. How many bands did you observe in your unknown? What was/were the molecular weight(s)? If there was more than one band in your unknown, what can you say from the intensity of Coomassie staining of the bands about the relative abundance of the proteins in the sample?

Exercises

1. You wish to resolve the compounds in Figure 4-11 by paper electrophoresis in a system that uses a pH 7.0 buffer. At pH 7.0, will all of these compounds migrate in one direction, so that you can place the origin line at one end of the paper? If so, would you place the origin line near the anode (+ pole) or cathode (− pole)? What is the predicted relative order of electrophoretic migration of these compounds at pH 7.0?
2. Will the three compounds in Figure 4-11 be clearly separated from one another by electrophoresis at pH 11? If so, describe the electrode toward which they will migrate and the order of migration. If not, why not?

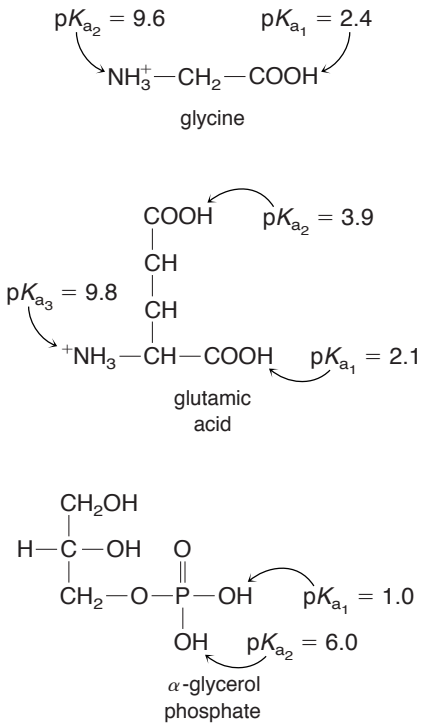

Figure 4-11 Compounds to be separated in the Exercises, questions 1 and 2.

REFERENCES

Dunbar, B. S. (1987). *Two Dimensional Electrophoresis and Immunological Techniques.* New York: Plenum Press.

Gersten, D. M. (1996). *Gel Electrophoresis* (Essential Techniques Series). New York: Wiley.

Kuhr, W. G. (1990). Capillary Electrophoresis. *Anal Chem* **62**:403R–414R.

Laemmli, U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **227**:680.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Tal, M., Silberstein, A., and Nusser, E. (1980). Why Does Coomassie Brilliant Blue Interact Differently with Different Proteins? *J Biol Chem* **260**:9976–9980.

Wilson, K., and Walker, J. M. (1994). *Principles and Techniques of Practical Biochemistry*, Chapter 9. Cambridge, UK: Cambridge University Press.