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Slab Gel Electrophoresis for Protein Analysis

David E. Garfin

INTRODUCTION

Gel electrophoresis holds a special place among the methods for protein analysis. It is fair to state that it is the most widely used procedure in protein studies. At some point in their work on proteins, researchers are likely to use gel electrophoresis or at least contemplate using it. The technique is familiar to everyone working with biomolecules and to the general public as well through accounts of its use in genomics, proteomics, diagnostics, and forensics. The widespread utilization of gel electrophoresis is undoubtedly because of its high resolution and its ease of use.

Of all the forms of electrophoresis, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) is by far the most commonly practiced method. It has become an everyday procedure in protein laboratories. Many people do not realize that there is any kind of gel electrophoresis other than SDS-PAGE. It provides an easy way to assess the complexity of a sample or the purity of a preparation. SDS-PAGE is particularly useful for monitoring the fractions obtained during protein purification by other techniques such as chromatography. It also allows samples from different sources to be compared for protein content. One of the more important features of SDS-PAGE is that it is a simple, reliable method with which to estimate the molecular weights of proteins.

SDS-PAGE is a denaturing technique in which proteins are broken down to their constituent polypeptide chains. Nondenaturing procedures are also available

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for use when it is desirable to maintain biological activity or antigenicity. However, it is more difficult to extract easily interpretable information from nondenaturing gels than from SDS-PAGE.

Although nondenaturing systems can give information about the charge isomers of proteins, this information is best obtained by isoelectric focusing (IEF). An IEF run can show charge heterogeneity in proteins not apparent with other types of electrophoresis. Proteins thought to be a single species by SDS-PAGE analysis are sometimes found by IEF to consist of multiple species. A more thorough determination of the composition of a protein preparation is obtained upon combining IEF with SDS-PAGE in two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). 2-D PAGE is the primary separation tool in the field of proteomics research because it is capable of separating thousands of proteins in a single gel. When desired, protein identifications are obtainable by immunoblotting, which combines antibody specificity with the separation power of 1- or 2-D gel electrophoresis.

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GEL ELECTROPHORESIS

Despite some refinements in the methods, the basic principles and protocols of gel electrophoresis have not changed appreciably since their introduction. Proteins are introduced into a gel matrix and separated by the combined effects of an electrical field, buffer ions, and the gel itself, which acts as a protein sieve. At the completion of the electrophoresis run, separated proteins in the gel are stained to make them visible, then analyzed qualitatively or quantitatively. The topic has been covered in numerous texts, methods articles, and reviews.[1–11] In addition, apparatus and reagents for analytical and preparative gel electrophoresis are available from several suppliers.

Proteins are charged molecules and migrate under the influence of electric fields. For the purposes of gel electrophoresis, the two most important physical properties of proteins are their electrophoretic mobilities and their isoelectric points (pIs). The surrounding medium influences both the electrophoretic mobilities and pIs of proteins, and this is exploited in various ways. In gel electrophoresis, the largest influence on protein migration comes from the sieving properties of the gels. Factors such as pH and the amounts and kinds of ions and denaturants in the system also influence migration.

The rate of migration of a protein per unit of field strength [velocity ÷ (magnitude of electric field)] is called its *electrophoretic mobility*. It is relatively easy to show[1,6,12] that the electrophoretic mobility of a particle is given by the ratio of its charge to its friction coefficient [charge ÷ (friction coefficient)]. Attempts to derive physical properties from the electrophoretic mobilities of proteins have been generally unsuccessful because of the complexity and size of protein molecules. Thus, electrophoretic mobility is used as a descriptive concept rather than as an analytical tool. It is relatively unimportant except in discussions

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of how the properties of gels and the compositions of buffer systems influence migration rates during electrophoresis.

The pH of the electrophoresis buffer determines the charges on the proteins in the sample being run. This means that the directions of motion of proteins in electrical fields depend on the pH of the electrophoresis buffer. Proteins are amphoteric molecules with net charges that vary with the pH of their local environment. For every protein there is a specific pH at which its net charge is zero. This pH is the so-called *isoelectric point* or pI of the protein. A protein is positively charged in solutions at pH values below its pI and negatively charged when the pH is above its pI. Thus, proteins are cationic when the pH is below their pIs and anionic when pH is above their pIs. On the other hand, when ionic detergents are employed in electrophoresis, the charges on the detergents determine the directions of migration and the pH of the buffer becomes relatively unimportant.

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Apparatus for Gel Electrophoresis

The equipment and reagents for gel electrophoresis are readily available and familiar to laboratory workers. Particularly noteworthy is the steady increase in the popularity of precast polyacrylamide gels since their introduction in the early 1990s (see Section 8.2.4). Precast gels provide researchers with off-the-shelf convenience and reproducibility and help to make gel electrophoresis and IEF commonplace laboratory practices.

Cells for gel electrophoresis are relatively simple. Most electrophoresis cells are variants of a standard design (Figure 8.1). Gels are formed in glass or plastic cassettes that are suspended vertically between anode and cathode buffer compartments. Electrodes of platinum wire and jacks for making electrical contact with the electrodes connect the gels and buffers to the power source. Samples are introduced into wells formed in the gels at the time of casting. The rectangular slab format allows multiple samples to be run and compared in the same gel. Manufacturers provide thorough instructions for using their electrophoresis cells.

Conventional gels are of the order of 20 cm wide $\times$ 20 cm long. The so-called mini-cells and midi-cells allow rapid analysis and are adequate for relatively uncomplicated samples. The design of the small cells allows runs to be completed in as little as 35 to 45 min as compared to 6 to 7 h for the larger gels. Mini-gels are about 7 cm long $\times$ 8 cm wide while midi-gels are of the order of 15×10 cm. Gel thickness is varied by means of spacers inserted into the cassettes. Standard gel thickness is 0.75 or 1 mm, which allows for adequate loads, high sensitivity staining, and good heat dissipation. Some gels can hold as many as 26 samples. All three size categories of gels can be used for nearly all purposes. However, the separation between bands is greater with longer gels. Closely spaced bands are easier to distinguish from one another, their bandwidths are easier to measure, and they are more easily cut out from a large gel than from a small one.

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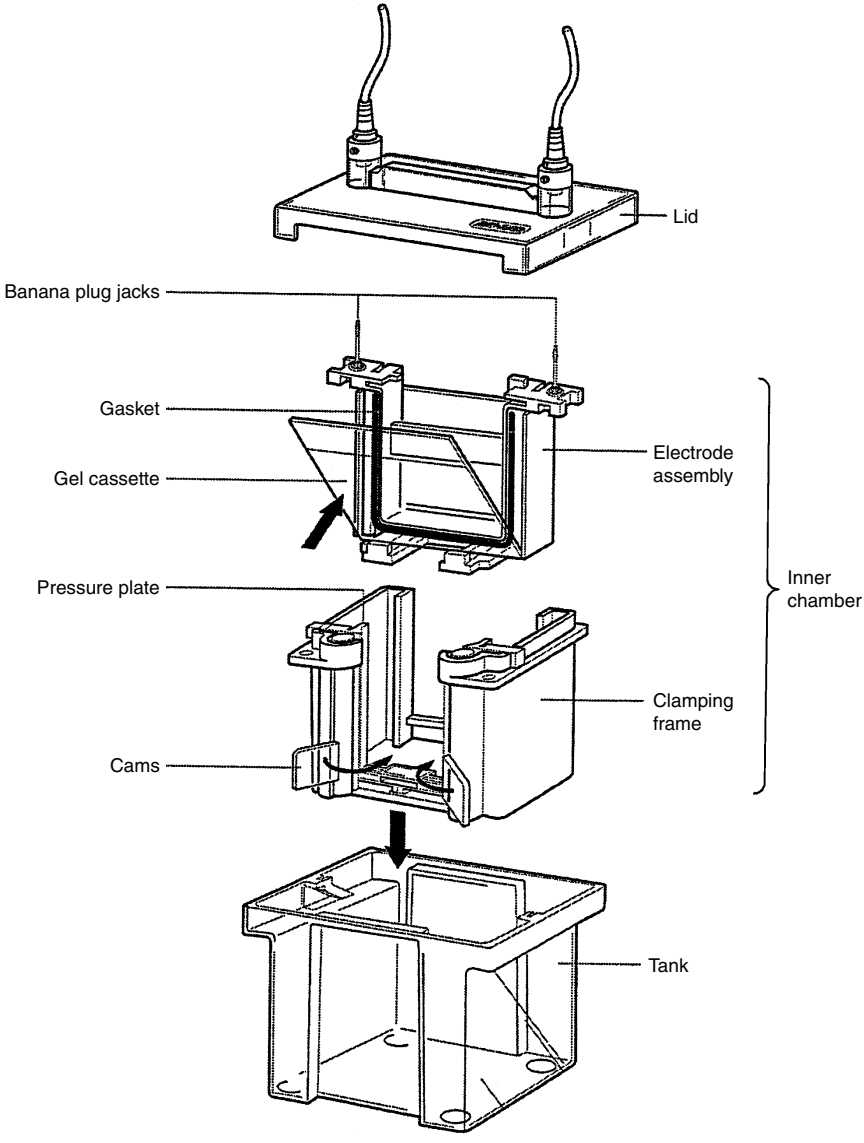

Figure 8.1 Exploded view of an electrophoresis cell. The components of the Bio-Rad Mini-PROTEAN 3 are shown. The inner chamber can hold one or two gels. It contains an electrode assembly and a clamping frame. The interior of the inner assembly constitutes the upper buffer compartment (usually the cathode compartment). The chamber is placed in the tank to which buffer is added. This constitutes the lower (anode) buffer compartment. Electrical contact is made through the lid.

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It is good practice to use small gels while the conditions for sample preparation are being optimized or when the optimum nondenaturing system is being determined. This is because small gels provide rapid results to encourage thorough optimization studies. Large gels should be used for comprehensive analyses.

Regulated direct current (DC) power supplies designed for electrophoresis allow control of every electrophoretic mode. Constant voltage, constant current, or constant power conditions can be selected. Many power supplies have timers and some have integrators allowing runs to be automatically terminated after a set time or number of volt-hours (important in IEF). All modes of operation can produce satisfactory results, but for best results and good reproducibility some form of electrical control is important. The choice of which electrical parameter to control is almost a matter of preference. The major limitation is the ability of the chamber to dissipate the heat generated by the electrical current.

Gels

No other separation medium can resolve the components of complex mixtures of proteins as well and as easily as a gel electrophoresis system. Gels and the associated buffer systems establish the migration rates of proteins as appropriate for the intended separation. Gels also hold proteins in place at the ends of runs until they can be stained for visualization. Polyacrylamide gels are the principal media for protein electrophoresis. Agarose gels are used in some applications such as for the separation of proteins larger than about 500 kDa and for immunoelectrophoresis,[1,10] but they are not as useful or versatile as polyacrylamide gels.

Polyacrylamide gels are particularly well suited for protein electrophoresis for several reasons. (1) The pores of polyacrylamide gels are roughly the same size as proteins and function as three-dimensional sieves. (2) Pore size is determined by the conditions of polymerization and can be easily altered. (3) Gel formation is easy and reproducible and gels can be cast in different sizes and shapes. (4) Polyacrylamide gels are hydrophilic and electrically neutral. (5) Polyacrylamide does not bind proteins. (6) Polyacrylamide gels are transparent to light at wavelengths above nearly 250 nm (important for visualization of stained gels).

The standard gel-forming reaction is shown in Figure 8.2. Acrylamide and the cross-linker *N,N*-methylenebisacrylamide (*bis*) are mixed in aqueous solution and then copolymerized by means of a vinyl addition reaction initiated by free radicals.[13–17] Gel formation occurs as acrylamide monomer polymerizes into long chains cross-linked by bis molecules. The resultant interconnected meshwork of fiberlike structures has both solid and liquid components. It can be thought of as a mass of relatively rigid fibers that create a network of open spaces (the pores) all immersed in liquid (the buffer). The liquid in a gel maintains the gel's three-dimensional shape. Without the liquid, the gel would dry to a thin film. At the same time, the gel fibers retain the liquid and prevent it from flowing away.

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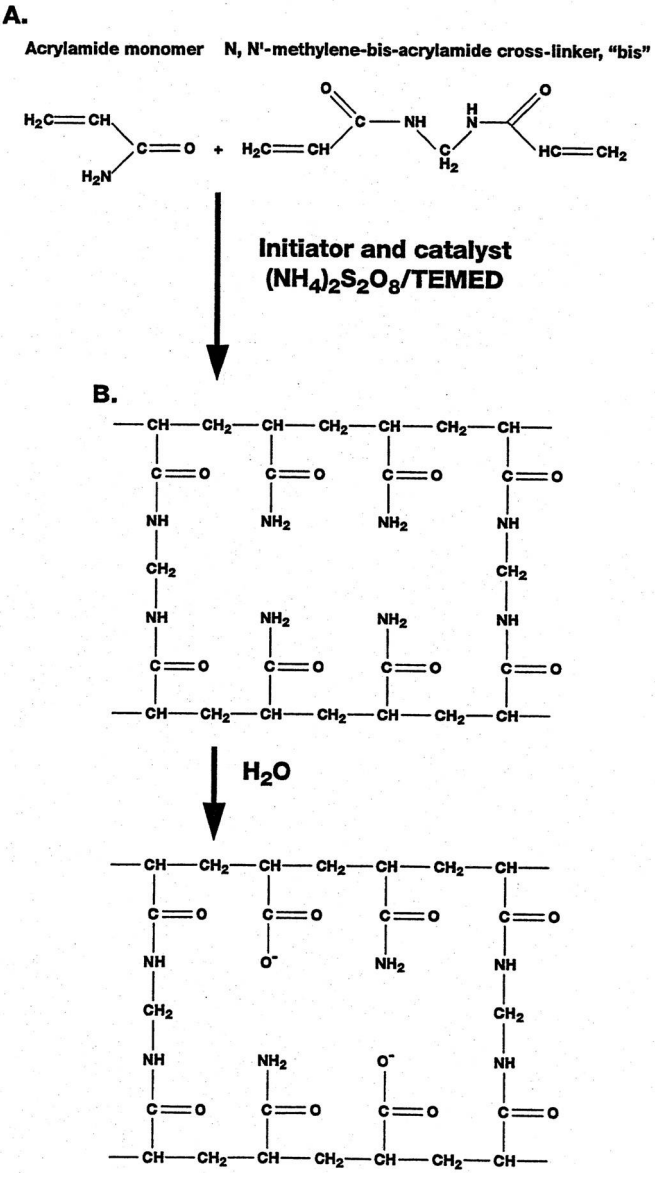

Figure 8.2 Polyacrylamide gel formation and hydrolysis of acrylamide to acrylate. (A) Acrylamide and *N,N*-methylenebisacrylamide (bis) are copolymerized in a reaction catalyzed by ammonium persulfate [$(NH_4)_2S_2O_8$] and TEMED. (B) A very short stretch of cross-linked polyacrylamide is represented. Cross-linking between similar structures leads to the formation of ropelike bundles of polyacrylamide that are themselves cross-linked together forming the gel matrix. In the lower portion of (B) is shown how pendant, neutral carboxamide groups can become hydrolyzed to charged carboxyls.

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During electrophoresis proteins move between the pores of the gels so that pore size has a great influence on the separation. However, pore size is difficult to measure directly. It is operationally defined by the size limit of proteins that can be forced through a gel. The collective experience of many years of gel electrophoresis has established gel compositions suitable for proteins of nearly every size range. From a macroscopic point of view, migrating proteins segregate into discrete regions or zones corresponding to their individual gel-mediated mobilities. When the electric field is turned off, the proteins stop moving. The gel matrix constrains the proteins at their final positions long enough for them to be stained to make them visible. An example of a one-dimensional separation of proteins is shown in Figure 8.3. In this configuration, the protein pattern is one of multiple bands with each band containing one protein or a limited number of proteins with similar molecular weights.

By convention, polyacrylamide gels are characterized by a pair of values, %T and %C. In this convention, %T is the weight percentage of total monomer (acrylamide + *bis*) in g/100 ml, and %C is the proportion of *bis* as a percentage of total monomer. The effective pore size of a polyacrylamide gel is an inverse function of the total monomer concentration (%T) and a biphasic function of %C. When %T is increased at a fixed %C, the number of polymer fiber chains increases and the pore size decreases. On the other hand, when %T is held constant and %C is increased from low values, pore size decreases to a minimum at about 5%C. With further increases in %C from the minimum, pore size increases, presumably because of the formation of shorter, thicker bundles of polymer fiber chains. For most protein separations, 2.6%C has been found appropriate (37.5 parts acrylamide and 1 part bis). Gels with low %T (e.g., 7.5%T) are used to separate large proteins, whereas gels with high %T (e.g., 15%T) are used for small proteins.

An example of the effect of pore size on the separation of a set of native proteins is shown in Figure 8.4. The 4%T, 2.67%C gel shown on the left is essentially nonsieving. Proteins in the artificial sample migrate in the gel more or less on the basis of their free mobility. The 8%T, 2.67%C gel on the right sieves the proteins shown and demonstrates the combined effects of charge and size on protein separation. The relative positions of some proteins are shifted in the sieving gel as compared to the nonsieving one.

Acrylamide gels can be cast so that they consist of gradients of pore size.[1,11,16] Pore-gradient gels are mostly used in SDS-PAGE of samples containing both large and small proteins. Acrylamide concentrations in gradient gels increase linearly from top to bottom so that the pores get smaller with the distance into the gels. As proteins move through gradient gels from regions of relatively large pores to regions of relatively small pores, they encounter greater and greater resistance to migration. Small proteins remain in gradient gels longer than in single percentage gels so that both large and small proteins can be retained in the same gel. Thus, gradient gels are popular for analyses of complex mixtures of proteins spanning wide molecular-mass ranges. However, gradient gels cannot match the resolution obtainable with properly chosen single-concentration gels.

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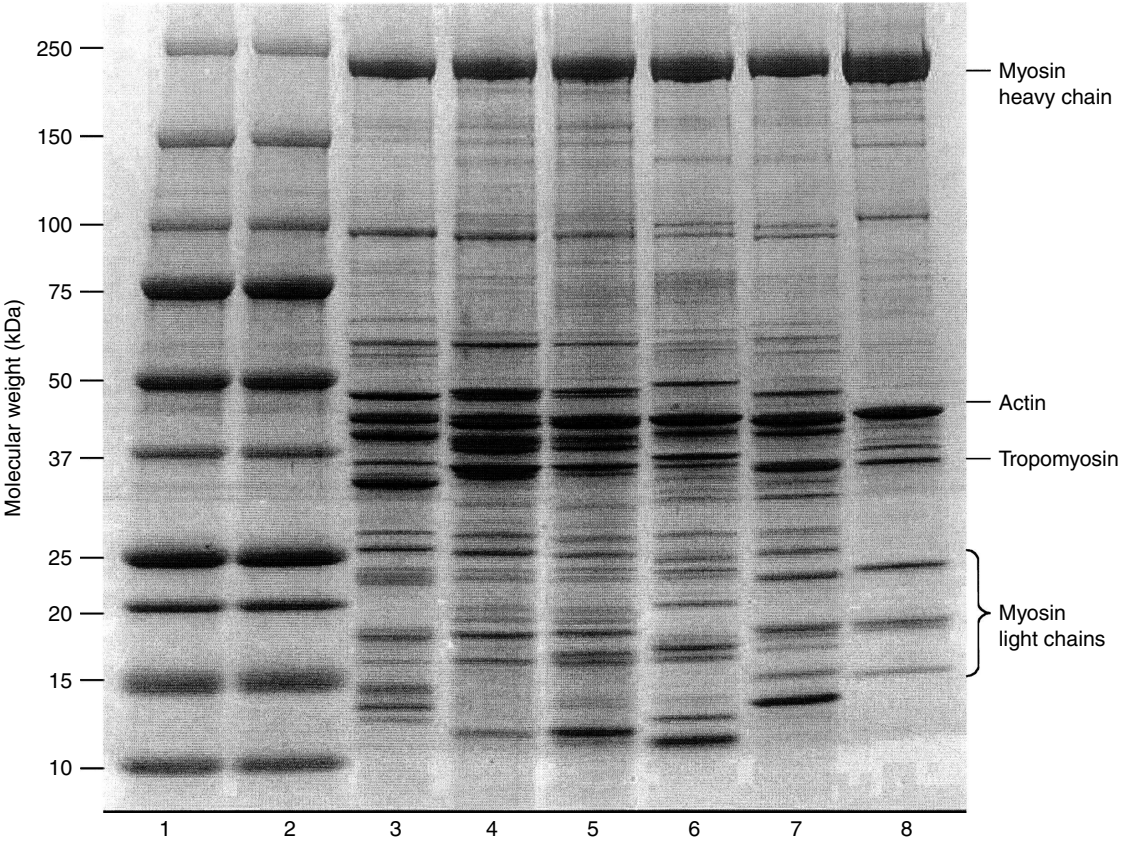

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Figure 8.3 A typical analytical SDS-PAGE gel. Extracts of muscle proteins from the meat of five different fish varieties were separated by SDS-PAGE in a precast mid-size gel (Bio-Rad Criterion Gel), consisting of a 4 to 20%T polyacrylamide gel gradient. Separated proteins were visualized with colloidal Coomassie Brilliant Blue G-250. The lanes contain proteins from the following sources: lanes 1 and 2, marker proteins; lane 3, shark; lane 4, salmon; lane 5, trout; lane 6, catfish; lane 7, sturgeon; lane 8, mixture of rabbit actin and myosin. The molecular weights of the marker proteins are shown at the left and serve to calibrate the gel. Rabbit muscle proteins are identified on the right. The salmon and trout patterns (lanes 4 and 5) are very similar as expected, given the close evolutionary relationship of the two species. All of the fish samples appear to contain muscle proteins similar to those of rabbit.

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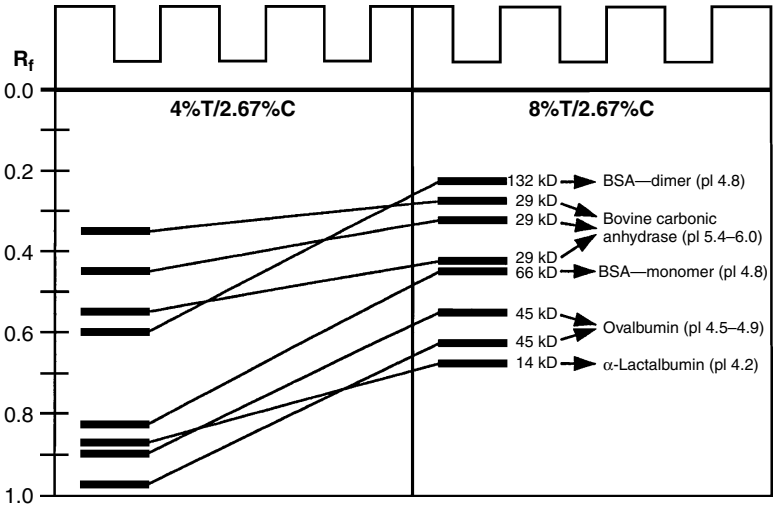

Figure 8.4 Effect of pore size on protein migration. The diagram shows the migration patterns of a set of proteins run in gels of differing %T with the Ornstein–Davis native, discontinuous buffer system. The diagram on the left was generated from 4%T, 2.67%C gels, whereas the pattern on the right was obtained from gels with 8%T, 2.67%C. The diagrams are drawn to scale. The slanted lines connect the bands representing the same proteins in the two diagrams. Note the large mobility shifts of BSA dimer and α-lactalbumin between the two gel types.

Buffer Systems

The electrical current in an electrophoresis cell is carried largely by the ions supplied by buffer compounds. Proteins constitute only a small proportion of the current-carrying ions in an electrophoresis cell. Buffer systems for electrophoresis are classified as either continuous or discontinuous, depending on whether one or more buffers are used. They are further classified as native or denaturing, depending on whether their compositions maintain or destroy protein structure and activity.

Continuous Buffer Systems

Continuous systems use the same buffer, at constant pH, in the gel, sample, and electrode reservoirs. With continuous systems, the sample is loaded directly on the gel in which separation will occur. The sample application buffer is the same as the gel and electrode buffer, but at about half the concentration. The localized voltage drop that results from decreased conductivity in the sample solution helps drive sample proteins into the gel and sharpens protein bands. Once inside a gel, proteins are separated on the basis of their individual (gel-mediated) mobility differences. Bandwidths are highly dependent on the height of the applied sample

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volume, which should be kept as small as possible, thus restricting continuous systems to high-concentration samples for best resolution.

Almost any buffer can be used for electrophoresis in a continuous system. Solutions of relatively low ionic strength are best suited for electrophoresis because these keep heat generation at a minimum. On the other hand, protein aggregation may occur if the ionic strength is too low. The choice of buffer will depend on the proteins being studied, but in general the concentrations of electrophoresis buffers are in the range of 0.01 to 0.1 *M*.

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Discontinuous Buffer Systems

Discontinuous buffer systems (often called multiphasic buffer systems) employ different ions in the gel and electrode solutions. These systems are designed to sharpen starting zones for high-resolution separations, even with dilute samples. The sharpening of sample starting zones is called *stacking*. It is an electrochemical phenomenon based on mobility differences between proteins and carefully chosen leading and trailing buffer ions.[2,6,13] Samples are applied in dilute gel buffer and sandwiched between the gel and the electrode buffer. When the electric field is applied, *leading ions* from the gel (e.g., Cl) move ahead of the sample proteins while *trailing ions* from the electrode buffer (e.g., glycinate) migrate behind the proteins. The proteins in the sample become aligned between the leading and trailing ion fronts in the order of decreasing mobility. Proteins are said to be *stacked* between the two buffer ion fronts. The analogy is that of a stack of coins. The width of the stack is no more than a few hundred micrometers with possible protein concentrations there approaching 100 mg/ml.[18] Electrophoretic stacking concentrates proteins into regions narrower than can be achieved by mechanical means. This has the effect of minimizing overall bandwidths. Dilute samples require discontinuous buffers for best results. With high-concentration samples above nearly 1 mg/ml, continuous systems provide adequate results.[19]

In order to allow the stack to develop, the gels used with discontinuous systems are divided into two distinct segments. The smaller, upper portion is called the *stacking gel*. It is cast with appreciably larger pores than the lower *resolving gel* (or separating gel) and serves mainly as an anticonvective medium during the stacking process. Separation takes place in the resolving gel, which has pores of roughly the same size as the proteins of interest. Once proteins enter the resolving gel, their migration rates are slowed by the sieving effect of the small pores. In the resolving gel, the trailing ions pass the proteins, and electrophoresis continues in the environment supplied by the electrode buffer. The proteins are said to become unstacked in the resolving gel. They separate there on the basis of size and charge.

The runs are monitored and timed by means of the buffer front. The migration of the buffer front as it moves through the gel can be followed by the change in the index of refraction between the regions containing the leading and trailing ions. It is usual to add tracking dye to the sample solution. Tracking dye moves with the buffer front and aids in visualization of its motion.

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Table 8.1 Continuous Buffers for Electrophoresis of Native Proteins

Buffer pH[a]	Basic Component	Amount for 5X Solution	Acidic Component	Amount for 5X Solution
3.8	β-Alanine 1X = 30 m*M*	13.36 g/l	Lactic acid 1X = 20 m*M*	7.45 ml/l[b]
4.4	β-Alanine 1X = 80 m*M*	35.64 g/l	Acetic acid 1X = 40 m*M*	11.5 ml/l
4.8	Gaba 1X = 80 m*M*	41.24 g/l	Acetic acid 1X = 20 m*M*	5.75 ml/l
6.1	Histidine 1X = 30 m*M*	23.28 g/l	Mes 1X = 30 m*M*	29.28 g/l
6.6	Histidine 1X = 25 m*M*	19.4 g/l	Mops 1X = 30 m*M*	31.40 g/l
7.4	Imidazole 1X = 43 m*M*	14.64 g/l	Hepes 1X = 35 m*M*	41.71 g/l
8.1	Tris 1X = 32 m*M*	19.38 g/l	Epps 1X = 30 m*M*	37.85 g/l
8.7	Tris 1X = 50 m*M*	30.29 g/l	Boric acid 1X = 25 m*M*	7.73 g/l
9.4	Tris 1X = 60 m*M*	36.34 g/l	Caps 1X = 40 m*M*	44.26 g/l
10.2	Ammonia 1X = 37 m*M*	12.5 ml/l	Caps 1X = 20 m*M*	22.13 g/l

[a] Listed buffer pH is ±0.1 unit. Do not adjust the pH with acid or base. Remake buffers outside the given range.

[b] Lactic acid from an 85% solution.

Source: Adapted from McLellan, T., *Anal. Biochem.*, 126: 94 (1982).

For detailed descriptions of the electrochemical processes that operate with discontinuous buffer systems, consult References 1, 2, 4–7, 13, and 20. Mathematically inclined readers might want to follow the development of multiphasic buffer theory as presented in References 21 to 23.

Native Buffer Systems

The choice of native electrophoresis system depends on the particular proteins of interest. There is no universal buffer system ideal for the electrophoresis of all native proteins. Both protein stability and resolution are important considerations in buffer selection. Recommended choices are the Ornstein–Davis discontinuous system[21,24] and McLellan's continuous buffers.[25]

The set of buffers compiled by McLellan provides the simplest way to carry out the electrophoresis of proteins in their native state.[25] McLellan's buffers range from pH 3.8 to pH 10.2, all with relatively low conductivity (Table 8.1). By using different buffers from the set it is possible to compare the effect of pH changes on protein mobility while maintaining similar electrical conditions. This is demonstrated

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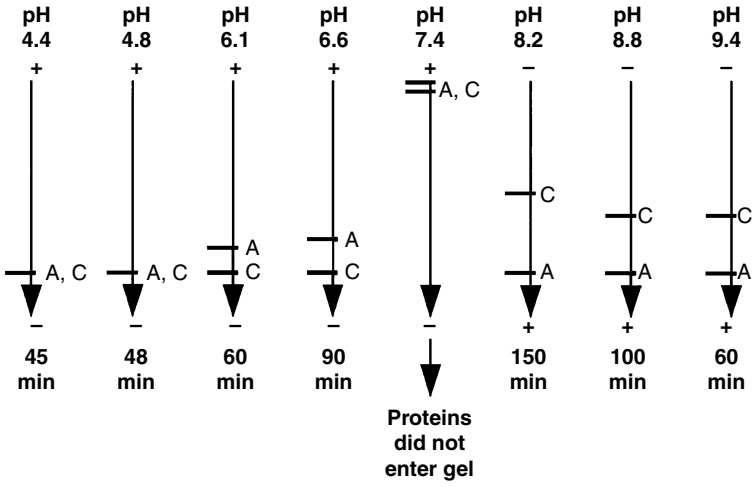

Figure 8.5 Effect of pH on protein mobility. Hemoglobin A (pI 7.1) and Hemoglobin C (pI 7.4) were electrophoresed in eight of the McLellan native, continuous buffer systems (Table 8.1). The diagram is drawn to scale. Migration is from top to bottom as shown by the vertical arrows. Bands marked A or C indicate the positions of the two hemoglobin variants in each gel representation. The polarities of the voltages applied to the electrophoresis cell are indicated by + and − signs above and below the vertical arrows. Run times are shown below the arrows. Note the polarity change between the gel at pH 7.4 and the one at pH 8.2. This reflects the pIs of the two proteins (and was accomplished by reversing the leads of the electrophoresis cell at the power supply).

in Figure 8.5. The illustration is a line-drawing representation, drawn to scale, of the relative positions of two hemoglobin variants, A and C, run under comparable electrical conditions in different McLellan buffers. HbA has a lower isoelectric point (pI 7.1) than HbC (pI 7.4). At pH 7.4, neither protein carries enough charge to move into the gel. At the acidic pHs tested, HbC is more highly charged and moves farther through the gel than HbA. The situation is reversed at the basic pHs tested. Note the differences in polarity and run times of the various runs.

Other buffers that have been used for continuous, native electrophoresis are Tris-glycine (pH range 8.3 to 9.5),[19] Tris-borate (pH range 8.3 to 9.3),[26] and Tris-acetate (pH range 7.2 to 8.5).[27] Borate ions[26] can form complexes with some sugars and can therefore influence resolution of some glycoproteins.

Ornstein[21] and Davis[24] developed the first high-resolution PAGE system for native proteins. Their popular system is still in widespread use. It was designed for the analysis of serum proteins, but works well for a broad range of protein types. The Ornstein–Davis buffers should be the first discontinuous system tried when working with a new, native sample.

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Gels for the Ornstein–Davis method are cast in two sections. A large-pore stacking gel (4%T, 2.7%C) is cast on top of a small-pore resolving gel (from 5 to 30%T depending on the proteins being studied). The two gel sections also contain different buffers. The stacking gel contains 0.125 *M* Tris-Cl, pH 6.8, and the resolving gel contains 0.375 *M* Tris-Cl, pH 8.8. The sample is diluted in 0.0625 *M* Tris-Cl, pH 6.8 (half-strength stacking gel buffer). The electrode (or running) buffer is 0.025 *M* Tris, 0.192 *M* glycine, pH 8.3. The pH discontinuity between the two sections of the gel is designed to regulate the effective mobility of glycinate ions from the cathode chamber. The concentrations and pHs of the buffers are derived from electrochemical considerations pertaining to the properties of serum proteins. The porosity of the resolving gel must be empirically determined to match the mobilities of the proteins in the sample. There is no reliable way to predict the correct gel concentration of an untested protein mixture without analyzing the proteins in gels. The choice is made such that the proteins of interest in the sample mixture are resolved in the gel. It is common to begin with a 7.5%T gel for the initial electrophoresis of a sample of unknown mobilities and then to try higher-concentration gels (and sometimes lower-concentration gels such as 5%T).

Tris-sulfate/Tris-borate, Tris-formate/Tris-borate, and Tris-citrate/Tris-borate have been advocated as electrophoresis buffers.[5] For basic proteins, a low-pH alanine–acetate system[28] is often used.

Denaturing Buffer Systems

Because it is not yet possible to calculate the physical properties of proteins from mobility data alone, researchers turn to SDS-PAGE denaturing systems in order to estimate protein molecular weights. Sample treatment for SDS-PAGE includes reduction of disulfide bonds and heating proteins in the presence of the surfactant. Breakage of inter- and intramolecular bonds in this process dissociates proteins into their polypeptide subunits and converts the subunits to forms that can be separated on the basis of their molecular weights. Moreover, SDS solubilizes most proteins, so SDS-PAGE is applicable to a wide range of sample types. The electrophoretic band patterns obtained by SDS-PAGE are appreciably easier to interpret than those from native PAGE.

The most popular electrophoresis system is the discontinuous buffer system devised by Laemmli.[29] Laemmli added SDS to the standard Ornstein–Davis buffers and developed a simple denaturing treatment. Sample preparation for SDS-PAGE is quite easy.[3,11,16] Nevertheless, it is as important to the system as the electrophoresis buffers. Proteins are simply brought to near-boiling in sample buffer (0.0625 *M* Tris-Cl, pH 6.8) containing 5% (v/v) 2-mercaptoethanol (a thiol reducing agent) and 2% (w/v) SDS. The treatment simultaneously breaks disulfide bonds and dissociates proteins into their constituent polypeptide subunits. SDS monomer binds to the polypeptides and causes a change in their conformations. For most proteins, 1.4 g of SDS binds per gram of polypeptide (approximately one SDS

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molecule per two amino acids).[30] The properties of the detergent overwhelm the properties of the polypeptides. In particular, the charge densities of SDS–polypeptides are independent of the pH in the range from 7 to 10.[5,31] Negatively charged micelles of SDS coat polypeptides in a more or less regular manner. SDS–polypeptides assume similar shapes. However, it is not entirely clear as to what exactly this shape is. It was for a long time thought that SDS–polypeptide complexes were rodlike particles.[30,32] There is some evidence, however, that most SDS–polypeptide complexes adopt a structure like beads on a string in which spherical SDS micelles are distributed along the unfolded polypeptide chain.[33] Regardless of their exact shapes, the collective experience of many years is that the electrophoretic mobilities of SDS–polypeptides are so nearly identical that they can be compared on the basis of size by means of gel electrophoresis and that the sizes of the complexes are proportional to the molecular weights of the polypeptides.

Laemmli's buffers as usually described are more elaborate than strictly necessary. Most presentations of this method utilize the two different gel buffers of the Ornstein–Davis system with SDS added to them. Because SDS so dominates the electrophoresis system, the buffer in the stacking gel can be the same as the buffer in the resolving gel. Results are the same whether the stacking gel is cast at pH 6.8 or at pH 8.8. Also, gels do not need to be cast with SDS in them. The SDS in the sample buffer is sufficient to saturate the proteins with the detergent. The SDS in the cathode buffer overtakes the proteins in the sample and at 0.1% is sufficient for maintaining saturation during electrophoresis. This distinction is important for the commercial manufacturing of gels for SDS-PAGE (Subsection 8.2.4). When following standard protocols for SDS-PAGE, it is acceptable to use resolving gel buffer in both the stacking gel and the resolving gel and to omit SDS from the polymerization mixture.[11]

Other systems for SDS-PAGE have been developed. Weber and Osborn's continuous, denaturing SDS-PAGE system uses pH 7 sodium phosphate.[34] This system helped establish the utility of SDS in electrophoresis as a means for estimating the molecular weights of proteins. The Weber–Osborn system is a popular one, but the lack of stacking limits its use to high-concentration samples for best resolution. A Tris-sulfate/Tris-borate buffer system has been shown to fractionate SDS-saturated proteins in the 2- to 300-kDa range with very sharp bands.[35] Replacement of Tris in the Laemmli SDS-PAGE system with its analog ammediol (2-amino-2-methyl-1,3-propanediol) resolves polypeptides in the 1- to 10-kDa size range, but the bands are less sharp than with either the Laemmli or sulfate/borate systems.[31] The sulfate/borate and ammediol systems receive attention in discussions of SDS-PAGE, but neither is very popular. Systems that substitute taurine for glycine in the running buffer[36,37] can give improved resolution of the smaller polypeptides in the sample. In addition, the buffer systems that do not employ glycine in the running buffer are preferred when proteins are to be extracted from the gels for amino acid analysis.

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An SDS-PAGE system based on the use of Tricine instead of glycine in the electrode buffer provides excellent separation of small polypeptides.[38] Peptides as small as 1 kDa are resolvable in Tricine-SDS gels. In particular, 16.5%T, 3%C separating gels are used for separations in the range from 1 to 70 kDa. Stacking gels in this system are 4%T, 3%C. Resolution is sometimes enhanced by inclusion of a 10%T, 3%C spacer gel between the resolving and stacking gels. Tricine-SDS resolving gels contain 1 *M* Tris-Cl, pH 8.45, and 13% glycerol. It is not necessary to include SDS in the gel buffer, but the glycerol is important to impart a viscosity that seems necessary for resolving small peptides. Electrode buffer is 0.1 *M* Tris, 0.1 *M* Tricine, 0.1% (w/v) SDS, pH 8.25. Sample buffer is 0.1 *M* Tris-Cl, pH 8.45, 1% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 20% (w/v) glycerol, and 0.04% Coomassie Brilliant Blue (CBB) G-250. Sample buffer should contain no more than 1% SDS for best resolution of small polypeptides (1 to 5 kDa). Proteins of very low molecular mass are not completely fixed and may diffuse from the gels during staining. This system is quite popular for polypeptide analysis.

The cationic detergent cetyltrimethylammonium bromide (CTAB) has been used as an alternative to SDS for gel electrophoresis of proteins. The most successful application of CTAB employs a discontinuous buffer system with sodium (from NaOH) as the leading ion and arginine as the trailing ion with Tricine as the counter ion and buffer.[39] This method uses no reducing agent in the sample buffer, and protein solutions are not boiled prior to electrophoresis. As a result, many enzymes retain their activities. Nonetheless, CTAB coats proteins thoroughly enough that it can be used for molecular weight determinations in analogy with SDS.

Very basic proteins such as histones and ribosomal proteins are separated in acetic acid–urea gels without SDS.[40,41]

Precast Gels

The biggest change in gel electrophoresis since the advent of polyacrylamide gels in the 1960s is the commercial availability of precast gels. Since the early 1990s, several companies have made a wide variety of precast polyacrylamide gels available to the research community. Most of the precast gels offered are Laemmli SDS-PAGE gels of differing %T and numbers of wells. Because the Laemmli SDS-PAGE gel is so overwhelmingly popular, alternative types of electrophoresis gels have tended to be ignored by researchers, and the companies have focused on this bias.

It took more than 20 years for manufacturers to devise production and distribution networks for delivering consistently high-quality gels to customers. The problems that the companies faced stem from the limited shelf life of polyacrylamide gels. Because polyacrylamide gels hydrolyze over time as shown in Figure 8.2, they are inherently unstable. At basic pH, the pendant, neutral carboxamide groups (-CO-NH_2) of the acrylamide monomers hydrolyze to ionized carboxyl

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groups (-COO) that can interact with some proteins. In addition, counter ions from the buffer neutralize the carboxy groups. The waters of hydration associated with the counter ions disrupt the integrity of the pores. Over extended periods of storage, band sharpness and resolution slowly deteriorate. The shelf life of a gel cast in the Laemmli gel buffer (pH 8.8) is about 3 to 4 months at 4°C. After this time, band patterns begin to deteriorate noticeably.

It is not possible to cast large volumes of Laemmli-type gels and to hold them in a warehouse for long periods of time. Consequently, a great deal of planning goes into the decisions of how many gels of each different type are to be cast at any particular time. Manufacturing and distribution issues have now been largely addressed, and customers can now be guaranteed that they will receive gels that can be stored for several weeks before they are used. A limited number of precast gel products are available that are cast with neutral pH buffers.[42] These gels have longer shelf lives than gels made according to the Laemmli formulation. The band patterns obtained with neutral-pH gels are different from those obtained with Laemmli gels. Nevertheless, they are becoming popular because of the convenience of extended shelf life.

People were initially drawn to precast gels because of difficulties in casting gradient gels. The convenience of being able to buy gradient gels that are already made rather than casting them is very appealing. The same holds true for single-percentage gel types as well. For all but the most demanding situations there is little reason to cast gels by hand. The gel types most in demand are 7.5%T, 10%T, 12%T, 4 to 15%T, and 4 to 20%T.

Precast gels differ from hand-cast gels in three ways: (1) they are cast with a single buffer throughout, (2) they are cast without SDS, and (3) they are cast without a sharp demarcation between the stacking and resolving gels. As pointed out previously, because SDS dominates the system, using different buffers in the stacking and resolving gels as in the original Laemmli formulation has no practical value. The two different buffers would mingle together on storage without elaborate means to keep them separate. In addition, during electrophoresis, SDS from the cathode buffer sweeps past the proteins in the resolving gel and keeps them saturated with SDS even when there is initially no SDS in the gel. Precast gels are thus made without SDS. This is beneficial to both the manufacturer and the user. SDS tends to form bubbles in the pumping systems used to deliver monomer solutions to gel cassettes, causing problems with monomer delivery. In addition, SDS micelles can trap acrylamide monomer and lead to heterogeneity of the gel structure.

When gels are cast by hand, it is customary to allow the resolving gel to harden before the stacking gel is placed on top of it. This practice is acceptable because hand-cast gels are usually used within a short period of time. On the other hand, when gels are cast this way and stored, the stacking gel eventually begins to pull away from the resolving gel. The gap that forms between the two gels leads to lateral spreading of the stacked proteins and destruction of the stack

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as it leaves the upper gel. For this reason, precast gels are cast in a continuous manner with the stacking-gel monomer solution added on top of the resolving-gel monomer solution before gelation. This means that the separation between the two gel segments is a gradual one rather than a sharp one. Even though the distance between the two gels is short, the transition between gel segments exists as a short gradient of %T. Proteins unstack gradually rather than abruptly. Because of this, the bands obtained with precast gels are not quite as sharp as those obtained with hand-cast gels.

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Choice of System

Different projects and protein samples have different requirements. The decision as to which gel electrophoresis system to use depends on the needs of each particular project.

Native Proteins

Continuous buffer systems are preferred for native work because of their simplicity. Furthermore, some native proteins have a tendency to aggregate and precipitate at the very high protein concentrations reached during the stacking process employed in discontinuous electrophoresis. Consequently, aggregated proteins might not enter the resolving gel or they might cause streaking as accumulated protein slowly dissolves during a run. If the proteins of interest behave in this manner, it is best to use some form of continuous buffer system.

The pH of the electrophoresis buffer must be in the range over which the proteins of interest are stable or where they retain their biological activity. The pH should also be properly chosen with respect to the pI. The pH of the gel buffer should be far enough away from the pIs of the proteins of interest that they carry enough net charge to migrate through the gel in a reasonable time (at least one-half pH unit). On the other hand, separation of two proteins is best near one of their pIs because the isoelectric protein will barely move in that pH range. (Figure 8.5 shows how the buffer choice determines migration rates.) The choice of pH is often a compromise between considerations of resolution and protein stability. For best results with continuous systems, the concentrations of the proteins of interest should be at least 1 mg/ml to keep sample volume at a minimum. The sample should be loaded in gel buffer diluted to one-fifth to one-half strength (some form of buffer exchange may be required). The decreased ionic strength of diluted buffer causes a voltage to develop across the sample, which assists in driving the proteins into the gel.

The choice of proper gel concentration (%T) is, of course, critical to the success of the separation because it heavily influences separation. Too high %T can lead to exclusion of proteins from the gel, and too low %T can decrease sieving (see Figure 8.4). One approach, useful with the McLellan continuous buffers (Table 8.1), is to use relatively large-pore gels (6%T or 7%T) and to alter mobilities with pH. An approach for discontinuous systems is to start with a

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7.5%T gel, and then, if that is not satisfactory, to try a number of gel concentrations between 5%T and 15%T.

Denatured Proteins

It is easier to choose suitable gel concentrations (%T) for SDS-PAGE than for native protein gels because the separation of SDS–polypeptides is dependent mainly on chain length. Laemmli gels with 7.5%T resolve proteins in the 40- to 200-kDa range, those with 10%T resolve 20- to 200-kDa proteins, 12%T gels separate proteins in the 15- to 100-kDa range, and 15% gels separate 6- to 90-kDa proteins (Figure 8.6).

Sample Preparation

Samples for SDS-PAGE by the Laemmli procedure are prepared in diluted gel buffer containing SDS, 2-mercaptoethanol, glycerol, and bromophenol blue tracking dye. It is most efficient to prepare a stock solution of sample buffer (0.0625 *M* Tris-Cl, pH 6.8, 2% SDS, 25% glycerol, and 0.01% bromophenol blue) containing everything but 2-mercaptoethanol and to add this reagent (to 5%) just before use. In some situations, it can be instructive to omit 2-mercaptoethanol and leave disulfide bonds intact.[3] The glycerol provides density for applying the sample on the stacking gel under the electrode buffer. The tracking dye allows both sample application and the electrophoretic run to be monitored (it migrates with the ion front). There is sufficient SDS present in the sample buffer to ensure saturation of most protein mixtures.[30] Except in the rare instances when the sample is in a very high-ionic-strength solution (>0.2 *M* salts), it can be dissolved 1:1 (v/v) in stock sample buffer. It is much better, though, to dilute the sample at least 1:4 (v/v) with the sample buffer stock. The amount of sample protein to load on a gel depends on the detection method to be used (Subsection 8.2.8). Enough of the protein of interest must be loaded on the gel for it to be subsequently located. Detection in gels requires on the order of 1 μg of total protein for easy visibility of bands stained with anionic dyes such as CBB R-250 or 0.1 μg of total protein with silver staining. Complete dissociation of most proteins is achieved by heating diluted samples to 95 to 100ºC for 2 to 5 min.

For native, discontinuous gels, upper gel buffer diluted twofold to fivefold for sample application is commonly used. Tracking dye and glycerol are added to these samples also, and protein concentrations should fall within the same limits as for SDS-PAGE. With discontinuous systems, the volume of sample is not very important as long as the height of the stacking gel is at least twice the height of the sample volume loaded on the gel. Continuous systems require minimal sample volumes for best resolution.

Careful sample handling is important when sensitive detection methods are employed. Silver-stained SDS-PAGE gels sometimes show artifact bands in the 50- to 70-kDa molecular mass region and irregular but distinctive vertical streaking parallel to the direction of migration. The appearance of these artifacts has been

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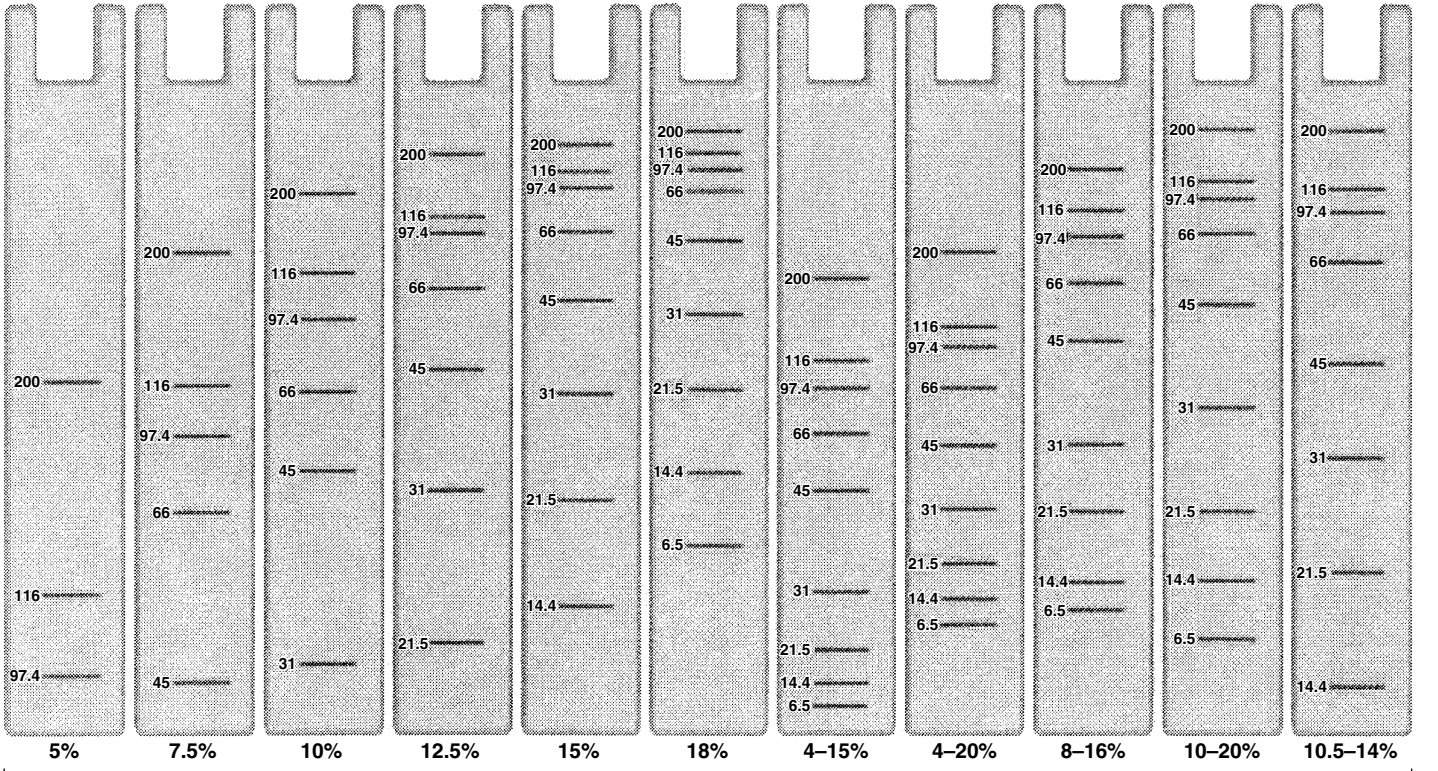

Figure 8.6 Protein migration charts. The relative positions of SDS-PAGE standards (Bio-Rad, broad range standards) are shown for several single percentage and gradient gels of the Laemmli type. The chart is useful as an aid in the selection of the appropriate gel types to match the molecular weight ranges of different protein samples.

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attributed to the reduction of contaminant skin keratin inadvertently introduced into the samples.[43] The best remedy for the keratin artifact is to avoid introducing it into the sample in the first place. Monomer solution, stock sample buffer, gel buffers, and upper electrode buffer should be filtered through nitrocellulose and stored in well-cleaned containers. It also helps to clean the gel apparatus thoroughly with detergent and to wear gloves while assembling the equipment.

Prepared samples are placed in sample wells of gels with microliter syringes or micropipettes. Both types of liquid-handling device provide good control of sample volume. Syringes must be thoroughly rinsed between applications to avoid cross-contamination of different samples. Standard pipette tips are too wide to fit into narrow sample wells, but several thin tips, specifically designed for sample application, are available. The choice of sample-loading device is one of personal preference.

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Electrical Considerations

During an electrophoresis run, electrical energy is converted into heat, called Joule heat. This heat can have many deleterious consequences, such as band distortion, increased diffusion, enzyme inactivation, and protein denaturation. All good electrophoresis chambers are designed to transfer the heat generated in the gel to the outside environment. In general, electrophoresis should be carried out at voltage and current settings at which the run proceeds as rapidly as allowed by the ability of the chamber to draw off heat. That is, the run should be as fast as possible without exceeding desired resolution and distortion limits, and these can only be determined empirically for any given system. Each experiment will impose its own criteria on cooling efficiency. Nearly all electrophoresis runs can be carried out on the laboratory bench, but some delicate proteins may require that the runs be conducted in the cold room or with circulated coolant.

It is important to bear in mind that an electrophoresis gel is an element in an electrical circuit and as such obeys the fundamental laws of electricity. Each gel has an intrinsic resistance, R, determined by the ionic strength of its buffer (R changes with time in discontinuous systems). When a voltage V is impressed across the gel, a current I flows through the gel and the external circuitry. Ohm's law relates these three quantities: $V = IR$, where V is expressed in volts, I in amperes, and R in ohms. In addition, power P, in watts, is given by $P = IV$. The generation of Joule heat, H, is related to power by the mechanical equivalent of heat, 4.18 J/cal, so that $H = (P/4.18)$ cal/sec.

With continuous buffer systems, the resistance of the gel is essentially constant, although it will decrease a bit during a run as the buffer warms. With the discontinuous Ornstein–Davis or Laemmli buffers, R increases during the course of a run as the chloride ions are exchanged by glycinate. For runs at constant current in Laemmli gels, the voltage (IR), power (I^2R), and consequently the heat generated in the gel chamber increase during the run. Under constant voltage conditions, current (V/R), power (V^2/R), and heat generation decrease

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during electrophoresis as R increases. Thus, runs carried out under constant current conditions are faster but hotter than runs done at constant voltage. Voltage and current should be set to keep H below the heat dissipation limit of the electrophoresis chamber. Follow the recommendations of the manufacturer for the proper electrical settings to use with any particular cell. Vertical cells are usually run at electric field strengths of 10 to 20 V/cm or currents in the range of 15 to 25 mA/mm of gel thickness.

The voltage applied to an electrophoresis cell is divided across three distinct resistance regions (Figure 8.7). The buffer paths from the open ends of the gel to the electrode wires form two of these regions. These two resistance regions are usually ignored, but they should be kept in mind for electrical analysis when experimenting with electrode buffers having very high or very low conductivity. The gel buffer is the third resistance region. It is the most important component in the system both electrically and electrochemically. With the Laemmli SDS system, the buffers create two different resistive sections. The low-resistance leading Cl ion forms a resistance segment that runs ahead of the higher-resistance trailing glycinate ion segment. Taken together, the two gel-segment resistors act as a voltage divider. The voltage across either one of the gel segments is proportional to the resistance of that segment (Figure 8.7B). The voltage across the chloride section provides the force that pulls the ion front through the gel, whereas the voltage across the glycinate section pulls the proteins through the gel. This proportioning of the applied voltage can cause two gels of the same %T to run differently if their gel buffers are different. For example, the final band pattern in a 12%T Laemmli gel with a gel buffer at pH 8.6 looks like the band pattern of a 10%T Laemmli gel with a gel buffer at pH 8.8. The gel at pH 8.6 also takes about 20% longer to run than the gel at pH 8.8. The differences in the properties of the two gel types are due to the increased conductivity of the pH 8.6 gel relative to the pH 8.8 gel. The extra chloride needed to drop the pH of 0.375 M Tris from 8.8 to 8.6 brings about the increased conductivity (0.12 M Cl vs. 0.19 M Cl). A subtle electrochemical process related to the ionization of glycine accentuates this effect, which can sometimes be used to advantage.[44] On the other hand, the electrode buffer is electrically less of a factor in the run than is the gel buffer. Its function is to provide a source of both glycinate and Tris ions to respectively replace the chloride ions that migrate from the gel at the anode and the Tris ions that migrate from the gel at the cathode. Concentration differences of as much as fivefold up or down from the prescribed formulation of electrode buffer are tolerated, but they do influence run time.

Detection of Proteins in Gels

Gels are run for either analytical or preparative purposes. The intended use of the gel imposes restrictions on the amount of protein to be loaded and the means of detection. At one time it was popular to use radioactive labeling of proteins, with detection of proteins done by autoradiography. It is now more common to

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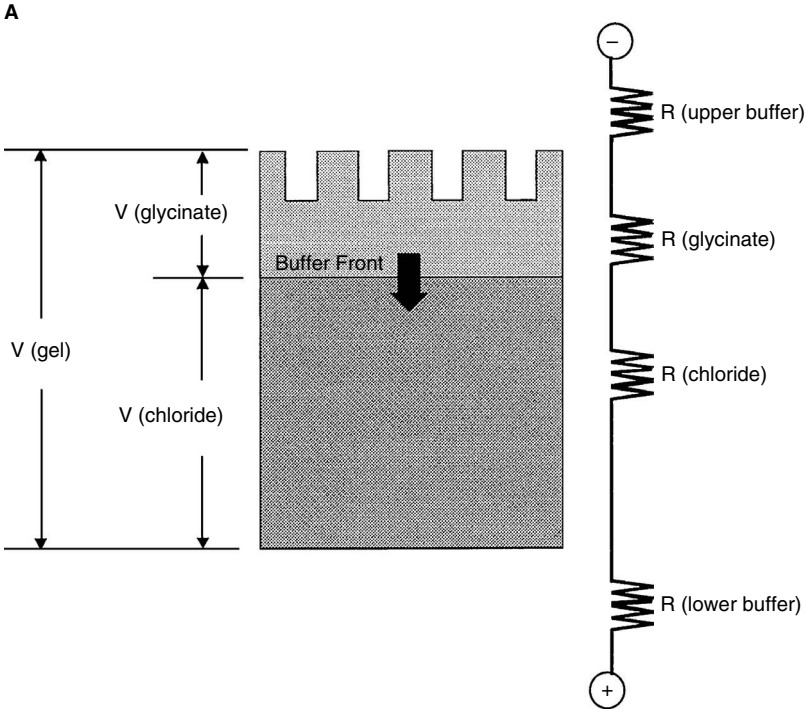

Figure 8.7 The electrophoresis gel as an element of an electrical circuit. With discontinuous buffer systems, the gel acts as a voltage divider as illustrated for the Ornstein–Davis or Laemmli system. (A) The voltage across the gel is divided between two resistors denoted by *R* (glycinate) and *R* (chloride). The resistors indicating the upper and lower buffer reservoirs are roughly equivalent and can be ignored. (B) The magnitudes of the voltages across the glycinate and chloride regions of a gel are shown in the two equations. They are proportional to the voltage across the gel and the relative resistances of the corresponding regions. The voltage across the lower portion of the gel, *V* (chloride), is responsible for pulling the buffer front through the gel, whereas the upper voltage, *V* (glycinate), acts to move the proteins in the samples. Migration rates and separations are influenced by the conductivities of the gel and running buffers.

make proteins in gels visible by staining them with dyes or metals.[45–48] Each type of protein stain has its own characteristics and limitations with regard to the sensitivity of detection and the types of proteins that best bind to the stain (Table 8.2).

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Table 8.2 Comparison of Stains for Proteins in Gels

Stain[a]	Sensitivity, in ng[b]	Steps[c]	Time[d]	Gel Types[e]
Coomassie stains				
CBB R-250	36–47	2	2.5 h	1-D and 2-D
Colloidal CBB G-250	8–28	3	2.5 h	1-D, 2-D, IPG, and Blots
Silver stains				
Silver Stain Plus[f]	0.6–1.2	3	90 min	1-D and 2-D
Silver stain[g]	0.6–1.2	7	2 h	1-D and 2-D
Negative stains				
Copper stain[h]	6–12	3	10 min	1-D
Zinc stain[i]	6–12	3	15 min	1-D
Fluorescent stains				
SYPRO[j] Ruby (Gel)	1–10	2	3 h	1-D and 2-D
SYPRO[j] Orange	4–8	1	45 min	1-D
IEF stains				
SYPRO[j] Ruby (IEF)	2–8	2	2 h[k]	IEF and IPG
IEF stain	40–50	2	3 h	IEF and IPG
Blotting stains				
SYPRO[j] Ruby (Blot)	2–8	3	50 min	N.C. and PVDF[l]
Colloidal Gold	1	3	2 h	N.C. and PVDF
Enhanced Colloidal Gold	0.01–0.1	4	3 h	N.C. and PVDF

[a] All stains listed are available from Bio-Rad in ready-to-use form.

[b] Sensitivities were determined with known masses of proteins run in 1-D gels or blots.

[c] Minimum number of hands-on steps.

[d] Estimated staining time. Actual manipulations require considerably less time.

[e] Recommended types of gels. The stains may work for other types of gels as well.

[f] Silver staining kit based on the method of Gottlieb, M. and Chavko, M., *Anal. Biochem.*, 165: 33 (1987).

[g] Silver staining kit based on the method of Merril, C.R., Goldman, D., Sedman, S.A., and Ebert, M.H., *Science*, 211: 1437 (1981).

[h] Lee, C., Levin, A., and Branton, D., *Anal. Biochem.*, 166: 308 (1987).

[i] Fernandez-Patron, C., Castellanos-Serra, L., and Rodriguez, P., *BioTechniques*, 12: 564 (1992).

[j] SYPRO is a trademark of Molecular Probes, Inc., Eugene, OR.

[k] SYPRO Ruby IEF stain requires an overnight incubation.

[l] N.C.: nitrocellulose membrane. PVDF: polyvinylidene fluoride membrane.

If the purpose of gel electrophoresis is to identify low-abundance proteins (e.g., low-copy-number proteins in a cell extract or contaminants in a purification scheme), then a high protein load (0.1 to 1 mg/ml) and a high-sensitivity stain such as silver or fluorescence should be used. When the intention is to obtain enough protein for use as an antigen or for sequence analysis, then a high protein load should be applied to the gel and the proteins visualized with a staining procedure that does not fix the proteins in the gel, e.g., colloidal CBB G-250 (Subsection 8.2.8.1). Furthermore, for purposes of quantitative comparisons, stains with broad linear ranges of detection response should be used.

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The sensitivity that is achievable in staining is determined by (1) the amount of stain that binds to the proteins, (2) the intensity of the coloration, and (3) the difference in coloration between stained proteins and the residual, background coloration in the body of the gel (signal-to-noise ratio). With all of the common stains, unbound stain molecules can be washed out of the bodies of the gels without removing much stain from the proteins.

Staining of all types of gels is done in the same way. Gels are removed from the cassettes, then washed, fixed, and incubated with staining solution in any convenient container such as a glass casserole or a photography tray. Staining is most commonly done at room temperature with gentle agitation (e.g., on an orbital shaker platform). Gloves should always be worn when staining gels because fingerprints (and fingers) will stain. Permanent records of stained gels can be obtained by photographing them, drying them with the appropriate apparatus on filter paper or between sheets of cellophane, or by capturing electronic images of them.

All stains interact differently with different proteins. No stain is universal in that it will stain all proteins in a gel proportionally to their quantities. The only observation that seems to hold for most of the positive stains is that they interact best with basic amino acids. For critical analyses, replicate gels should be stained with two or more different kinds of positive stain. Of all the stains available, colloidal CBB appears to stain the broadest spectrum of proteins. It is instructive, especially with 2-D PAGE gels, to follow a colloidal CBB-stained gel with silver staining[49–51] or to follow a fluorescence stain with colloidal CBB or silver. Very often, this double staining procedure will show a few differences in the two protein patterns. With CBB and silver stains, the order in which they are used often does not seem to be important.[52,53] With the fluorescent SYPRO stains (Subsection 8.2.8.4), CBB staining should always follow SYPRO staining rather than the reverse order. This is because the two dye molecules have such strong affinity for each other that proteins stained by the first dye are preferentially stained by the second dye.

Dye Staining

CBB R-250 is the standard stain for protein detection in polyacrylamide gels. It and the G-250 variety (CBB G-250) are wool dyes that have been adapted to the staining of proteins in gels. The R and G designations signify red and green hues, respectively. Easy visibility requires on the order of 0.1 to 1 μg of protein per band. The staining solution consists of 0.1% CBB R-250 (w/v) in 40% methanol (v/v), 10% acetic acid (v/v), which also fixes most proteins in gels. Absolute sensitivity and staining linearity depend on the proteins being stained.

CBB G-250 is less soluble than the R-250 variety. In acidic solutions it forms colloidal particles that are too large to penetrate surface gel pores and can be formulated into a staining solution that requires little or no destaining. It can also be formulated to be environmentally benign. This stain is somewhat more sensitive than CBB R-250, in part because of increased signal-to-noise ratios

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because the bulk of the gel matrix does not pick up excess stain. Staining with colloidal CBB G-250 can be linear over two orders of magnitude of protein concentration for some proteins. Gels containing low-molecular weight polypeptides (≈1000 Da) can be stained with CBB G-250 with minimum fixation time (and minimal potential loss of material).

Silver Staining

There are a number of different silver staining methods. Some are available in kit form from various manufacturers. Others do not lend themselves to commercial kits. For a discussion of the mechanisms of silver staining, see Reference 54. Silver staining can be as much as 100 times more sensitive than CBB dye staining. All silver staining procedures have many manual steps and the decision as to when to terminate color development is quite subjective.

Copper and Zinc Staining

Proteins in SDS-PAGE gels can be stained negatively with copper or zinc. For negative staining with copper, SDS-PAGE gels are incubated for a short time in a copper chloride solution, then washed with water.[55] Blue-green precipitates of copper hydroxide form in the bodies of the gels except where there are high concentrations of SDS, such as those bound to the proteins. Clear protein bands can be easily seen against the blue-green backgrounds and photographed with the gels on black surfaces. Proteins are not permanently fixed by this method and can be quantitatively eluted after chelating the copper.

A method using the combination of zinc and imidazole produces similar, negatively stained SDS-PAGE gels.[56] Zinc imidazolate forms precipitates in gels except at the sites where precipitation is inhibited by SDS–protein bands.[57] The resultant gels are opaque white with clear regions at the sites of the protein bands. They too are best viewed with the gel on a black surface.

With both the copper and zinc methods, the resultant negatively stained images of the electrophoresis patterns are intermediate in sensitivity between the CBB dyes and silver staining. Neither of the negative staining methods is recommended for 2-D PAGE gels because neither gives good quantification or discrimination of closely clustered spots.

Fluorescent Stains

The rare earth chelate stains have desirable features that make them popular in high-throughput laboratories.[58] They are end point stains with little background staining (high signal-to-noise characteristics), and they are sensitive and easy to use. The rare earth chelate compounds possess three distinct domains: (1) one domain binds a rare earth ion such as ruthenium, (2) a chromophoric domain is responsible for detection of the rare earth ion, and (3) a third domain reversibly binds to proteins. Because these compounds are fluorescent, they require an imaging device capable of providing high-intensity illumination at the excitation wavelength, band pass filters for excitation and emission wavelengths, and a

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detector such as a photographic or CCD camera (Section 8.6). Sensitivity varies from protein to protein, but can exceed that of silver stain. Linearity can extend to three orders of magnitude. The most popular types of fluorescent protein stains are the SYPRO™ class of compounds. SYPRO Orange is recommended for 1-D SDS-PAGE with SYPRO Ruby used for 1-D and 2-D SDS-PAGE and for native gels. (SYPRO is a trademark of Molecular Probes, Inc., Eugene, OR.)

Separation and Resolution

Stained proteins in gels appear visually as bands of variable width, each having a uniform cross section. This is because the eye tends to sharpen the boundaries between the colored bands and the clear background (ignoring the negative stains). In reality, proteins are distributed with Gaussian profiles with peaks at the centers of the bands and widths dependent on the amount of protein present. Image-acquisition instruments and analysis software (Section 8.6) display the true distributions and base quantifications on the true band shape. For much of the work in electrophoresis, measurements of bandwidths and interband spacing can be done with a ruler. Often mere qualitative comparisons of the band patterns of different protein samples are sufficient. For very accurate quantitative analyses of protein mixtures, digital image analysis is required.

Discussions of electrophoretic data handling usually include mention of *separation* and *resolution*. Although the two terms are not synonymous, they are often treated as such. In the terminology of separation science, separation refers to the distance between two adjacent band centers. Because bands are seen as being sharply defined with clearly evident blank spaces between adjacent bands, for practical purposes, separation is often taken to be the distance between the top of the faster running of two adjacent bands and the bottom of the slower one. It is the distance between the top of the bottom band and the bottom of the top band. This definition seems preferable to the rigorous one in electrophoresis.

Resolution, on the other hand, is a more technical term. It refers to the distance between adjacent bands relative to their bandwidths and acknowledges the fact that proteins are distributed in Gaussian profiles with overlapping distributions. The numerical expression for resolution is obtained by dividing the distance between the centers of adjacent bands by some measure of their average bandwidths. It expresses the distance between band centers in units of bandwidth and gives a measure of the overlap between two adjacent bands. For preparative applications, when maximal purity is desired, two proteins to be isolated should be separated by at least a bandwidth. In many applications it is sufficient to be able to simply discern that two bands are distinct. In this case bands can be less than a bandwidth apart.

Anomalous Migration in SDS-PAGE

Detergents disrupt protein–lipid and protein–protein interactions and play a large role in gel electrophoresis.[59,60] SDS is the most common detergent used in PAGE

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analysis. Most proteins are readily soluble in SDS, making SDS-PAGE a generally applicable method. In SDS-PAGE, the quality of the SDS is of prime importance. The effects of impurities in SDS are unpredictable. Of the contaminants, the worst offenders are probably the alkyl sulfates other than dodecyl sulfate (C_{12}), especially decyl sulfate (C_{10}), tetradecyl sulfate (C_{14}), and hexadecyl sulfate (C_{16}).[61,62] These bind to proteins with different affinities, thereby affecting mobilities. Lipophilic contaminants in SDS preparations, including dodecanol, can be trapped in SDS–protein complexes and SDS micelles leading to loss of resolution. Only purified SDS should be used for electrophoresis, but even with pure SDS, various glycoproteins, lipoproteins, and nucleoproteins can bind the detergent irregularly. The resultant SDS–polypeptides then migrate anomalously with respect to their molecular masses. Nevertheless, anomalous behavior in SDS-PAGE can serve as a diagnostic for certain posttranslational modifications.

Several types of proteins do not behave as expected during SDS-PAGE.[1,16] Incomplete reduction, which leaves some intra- or intermolecular disulfide bonds intact, makes some SDS-binding domains unavailable to the detergent so that the proteins are not saturated with SDS. Glycoproteins and lipoproteins also migrate abnormally in SDS-PAGE because their nonproteinaceous components do not bind the detergent uniformly. Proteins with unusual amino acid sequences, especially those with high lysine or proline content, very basic proteins, and very acidic proteins behave anomalously in SDS-PAGE, presumably because the charge-to-mass ratios of the SDS–polypeptide complexes are different from those that would be expected from size alone. Very large SDS–proteins, with molecular masses in the several hundred-kilodalton range, often do not migrate as expected. Polypeptides smaller than about 12,000 Da are not resolved well in most SDS-PAGE systems. In most cases, they do not separate from the band of SDS micelles that forms behind the leading ion front. The Tricine buffer system was devised to overcome this difficulty.

Molecular Weight Estimation

One of the reasons that SDS-PAGE became the most popular method of gel electrophoresis is that it can be used to estimate molecular masses.[1,4,10,16,63] To a first approximation, migration rates of SDS–polypeptides are inversely proportional to the logarithms of their molecular weights. The larger the polypeptide, the slower it migrates in a gel. Molecular weights are determined in SDS-PAGE by comparing the mobilities of test proteins to the mobilities of known protein markers. At one time, when samples for SDS-PAGE were run in individual tubes, it was necessary to normalize migration rates to a common parameter so that the different tube gels could be compared. This was because tube gels differ in length. The normalizing parameter that is still used is the relative mobility, R_f, defined as the mobility of a protein divided by the mobility of the ion front. In practice, when all gels are run for the same length of time, R_f is calculated as the quotient of the distance traveled by a protein from the top of the resolving gel divided by

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the distance migrated by the ion front. The distance to the ion front is usually taken as the distance to the tracking dye (measured or marked in some way before staining). With slab gels, this normalization is less important provided that a lane of standards is run in the same gel as the samples whose masses are to be determined. It is sufficient to compare migration distances of samples and standards. Plots of the logarithms of protein molecular weights (log M_r) vs. their migration distances fit reasonably straight lines.

In each gel, a lane of standard proteins of known molecular masses is run in parallel with the test proteins. After staining the gel to make the protein bands visible (Subsection 8.2.8), the migration distances are measured from the top of the resolving gel. The gel is calibrated with a plot of log M_r vs. migration distances for the standards. The migration distances of the test proteins are compared with those of the standards. Interpolation of the migration distances of test proteins into the standard curve gives the approximate molecular masses of the test proteins.

Pore-gradient SDS-PAGE gels can also be used to estimate molecular masses. In this case, log M_r is proportional to log (%T). With linear gradients,%T is proportional to distance migrated, so that the data can be plotted as log M_r vs. log (migration distance).

Standard curves are actually sigmoid in shape (Figure 8.8). The apparent linearity of a standard curve may not cover the full range of molecular weights for a given protein mixture in a particular gel. However, the mathematical function log M_r varies sufficiently slowly with changes in its argument (M_r) that fairly accurate molecular weight estimates can be made by interpolation, and even extrapolation, over relatively wide ranges. The approximate useful ranges of single-percentage SDS-PAGE gels for molecular-mass estimations is as follows: 40,000 to 200,000 Da, 7.5%T; 30,000 to 100,000 Da, 10%T; 15,000 to 90,000 Da, 12%T; 10,000 to 70,000 Da, 15%T. Mixtures of standard proteins with known molecular weights are available commercially for calibrating electrophoresis gels.

The semilogarithmic plots used for molecular weight determination are holdovers from the days when people had only graph paper and straight edges for curve fitting. (From a mathematical point of view, log M_r should be the independent variable [x-axis] and migration distance should be the dependent variable [y-axis], and not as usually drawn.) Several computer programs allow for standard curves to be fit with mathematical functions other than the semilogarithmic model. Some other types of curves actually fit the data better than the semilog function. Nevertheless, the semilogarithmic model for standard curves is the accepted form. It is important to bear in mind that the molecular weights obtained using Laemmli SDS-PAGE are those of the polypeptide subunits and not of native, oligomeric proteins. Moreover, proteins that are incompletely saturated with SDS, very small polypeptides, very large proteins, and proteins conjugated with sugars or lipids behave anomalously in SDS-PAGE, as mentioned in the preceding text. Nevertheless, SDS-PAGE provides reasonable molecular-mass estimates for most proteins.

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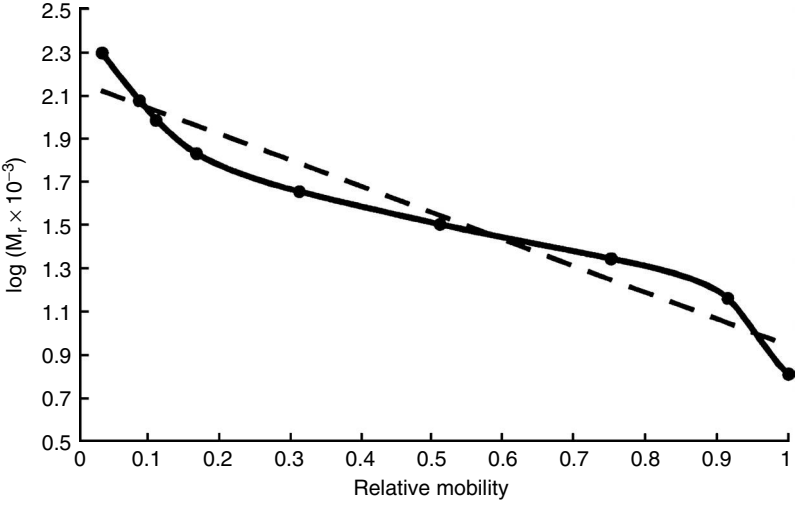

Figure 8.8 A representative calibration curve for molecular weight estimation. In the run that is plotted in this figure (solid line), Bio-Rad SDS-PAGE standards (broad range) with molecular weights of 200, 116.2, 97.4, 66.2, 45, 31, 21.5, 14.4, and 6.5 kDa (top to bottom, closed circles) were separated in a 15%T SDS-PAGE gel. The plot of $\log_{10}$ ($M_r \times 10^3$) vs. Relative mobility (R_f) shows the inherent nonlinearity of such curves. The straight-line segment in the middle of the plot is the most accurate range for molecular weight estimations. Larger polypeptides experience greater sieving than do those in the middle range of the plot, so that the upper portion of the curve has a different slope than does the middle. Small polypeptides experience less sieving than the others and also deviate from the straight line. It is customary to estimate molecular weights from a "best fit" straight line (dashed line). This is sufficient for many purposes and is acceptable because the mathematical logarithm function changes slowly with its argument.

ISOELECTRIC FOCUSING

IEF is an electrophoretic method in which proteins are separated on the basis of their pIs.[1,2,4–6,10,64–69] It makes use of the property of proteins that their net charges are determined by the pH of their local environments.

Proteins carry positive, negative, or zero net electrical charge, depending on the pH of their surroundings. The net charge of any particular protein is the (signed) sum of all of its positive and negative charges. The ionizable acidic and basic side chains of the constituent amino acids and prosthetic groups of the protein determine the net charge. If the number of acidic groups in a protein exceeds the number of basic groups, the pI of that protein will be at a low pH value and the protein is classified as being *acidic*. When the basic groups outnumber the acidic groups in a protein, the pI will be high and the protein is classified as being *basic*. Proteins show considerable variation in pIs, but pI values

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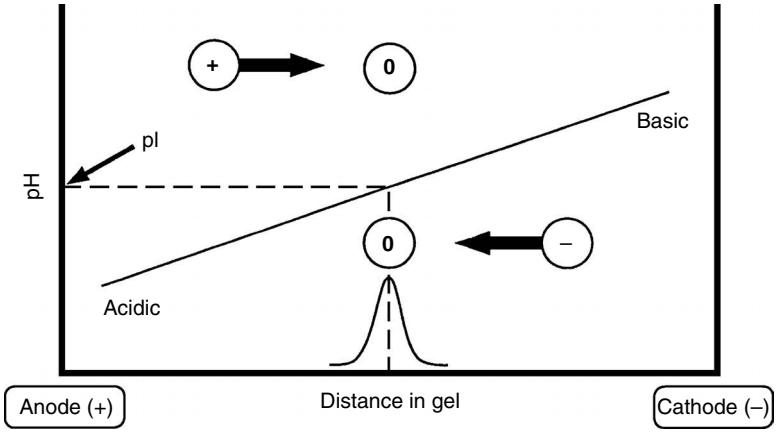

Figure 8.9 Isoelectric focusing. The motion of a protein undergoing isoelectric focusing is depicted (circles). The protein is shown near its pI in a pH gradient. Both the pH gradient and the motion of the protein are governed by an applied electric field. At pH values lower than the pI, the protein is positively charged (+) and it is driven toward the cathode as shown by the arrow. Above its pI, the protein is negatively charged (–) and it moves toward the anode. There is no net electrical force on the protein at its pI (0). The protein focuses in a Gaussian distribution centered at the pI.

usually fall in the range of pH 3 to 12 with a great many having pIs between pH 4 and pH 7.[70–72]

Proteins are positively charged in solutions at pH values below their pI and negatively charged above their pIs. Thus, at pH values below the pI of a particular protein, it will migrate toward the cathode during electrophoresis. At pH values above its pI, a protein will move toward the anode. A protein at its pI will not move in an electric field.

When a protein is placed in a medium with a linear pH gradient and subjected to an electric field, it will initially move toward the electrode with the opposite charge (Figure 8.9). During migration through the pH gradient, the protein will either pick up or lose protons. As it does so, its net charge and mobility will decrease and the protein will slow down. Eventually, the protein will arrive at the point in the pH gradient equaling its pI. There, being uncharged, it will stop migrating. If a protein at its pI should happen to diffuse to a region of lower pH, it will become protonated and be forced toward the cathode by the electric field. If, on the other hand, it diffuses into a pH higher than its pI, the protein will become negatively charged and will be driven toward the anode. In this way, proteins condense, or focus, into sharp bands in the pH gradient at their individual, characteristic pI values.

Focusing is a steady-state mechanism with regard to pH. Proteins approach their respective pI values at differing rates, but remain relatively fixed at those

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pH values for extended periods. This type of motion is in contrast to conventional electrophoresis in which proteins continue to move through the medium until the electric field is removed. Moreover, in IEF, proteins migrate to their steady-state positions from anywhere in the system. Thus, the sample application point is arbitrary. In fact, the sample can be initially distributed throughout the entire separation system.

Establishing pH Gradients

Stable, linear pH gradients are the keys to successful IEF. Establishment of such gradients is accomplished in one of two ways. The older, easier method makes use of mobile, oligomeric buffering compounds known as carrier ampholytes. In the other method, pH gradients are generated by means of buffering compounds grafted into the gel matrix. The latter method produces immobilized pH gradients.

Carrier ampholytes (amphoteric electrolytes) are mixtures of molecules containing multiple aliphatic amino and carboxylate groups. Some varieties contain sulfonic acid and phosphoric acid residues. Carrier ampholytes are small, multicharged organic buffer molecules about 300 to 1000 Da in size. They have closely spaced pI values and initially high conductivity. Ampholytes are included directly in IEF gel solutions at the time of casting. Under the influence of an electric field, carrier ampholytes partition into a smooth pH gradient that increases linearly from the anode (acidic) to the cathode (basic). The slope of a pH gradient is determined by the pH interval covered by the carrier ampholyte mixture and the distance between the electrodes. The use of carrier ampholytes is the most common and simplest means for forming pH gradients.

Immobilized pH gradients (IPGs) are formed by incorporating (acrylamido) buffers into polyacrylamide gels. Acrylamido buffers are derivatives of acrylamide. Each one contains both a reactive double bond and a buffering group. The general structure is CH_2 = CH-CO-NH-R, where R contains either a carboxyl [-COOH] or a tertiary amino group [e.g., -$N(CH_3)_2$]. Acrylamido buffers are covalently incorporated into polyacrylamide gels at the time of casting. In any given gradient, some of the acrylamido compounds act as buffers, whereas others serve as titrants. Published formulations and methods are available for casting the most common gradients.[66,68] Because the buffering compounds are fixed in place in the separation medium, gradients are stable over extended runs. This can prove important with many proteins that require long focusing times. IPGs are, however, more difficult and expensive to cast than carrier ampholyte gels. IPGs are commercially available in sheet form in a few pH ranges. A greater variety of pH ranges are available in IPGs that have been cut into strips for the IEF first dimension of 2-D PAGE.

IEF is a high-resolution technique that can routinely resolve proteins differing in pI by less than 0.05 pH unit. Under nondenaturing conditions, antibodies, antigens, and enzymes can retain their activities during IEF. The proper choice of ampholyte or IPG range is very important to the success of a fractionation.

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Ideally, the pH range covered by an IEF gel should be centered on the pI of the proteins of interest. This ensures that the proteins of interest focus in the linear part of the gradient with many extraneous proteins excluded from the separation zone.

With carrier ampholytes, concentrations of about 2% (w/v) are best. Ampholyte concentrations below 1% (w/v) often result in unstable pH gradients. At concentrations above 3% (w/v), ampholytes are difficult to remove from gels and can interfere with protein staining.

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Gels for Isoelectric Focusing

IEF is carried out in large-pore polyacrylamide gels that serve mainly as anticonvective matrices. A common composition of gels for IEF is 5%T, 3.3%C (29 parts acrylamide and 1 part bis). The best configuration for analytical IEF is the horizontal polyacrylamide slab gel. This is because the horizontal configuration allows use of ultrathin gels. Ultrathin gels (<0.5 mm) allow the use of very high field strengths and, therefore, very high resolution. Gels are cast with one exposed face on glass plates or specially treated plastic sheets. They are placed on cooling platforms and run with the exposed face upward or downward, depending on the particular setup. Electrolyte strips, saturated with 0.1 to 1 *M* phosphoric acid at the anode and 0.1 to 1 *M* sodium hydroxide at the cathode, are often placed directly on the exposed surface of the IEF gel. Electrodes of platinum wire maintain contact between the electrical power supply and the electrolyte strips. In another possible configuration, the gel and its backing plate are suspended between two carbon rod electrodes.

Precast IEF gels are available for carrying out carrier-ampholyte electrofocusing. A selection of IPG sheets is also available for horizontal IEF. Vertical IEF gels have the advantages that the electrophoresis equipment for running them is available in most laboratories and they can hold relatively large sample volumes. Because vertical electrophoresis cells cannot tolerate very high voltages, this orientation is not capable of the ultrahigh resolution of horizontal cells. To protect the proteins in the sample and the materials of the electrophoresis cells (mainly the gaskets) from caustic electrolytes, alternative catholyte and anolyte solutions are substituted in vertical IEF runs. As catholyte, 20 m*M* arginine, 20 m*M* lysine are recommended in vertical slab systems. The recommended anolyte is 70 m*M* H_3PO_4, but it can be substituted with 20 m*M* aspartic acid, 20 m*M* glutamic acid.

Electrofocusing can also be done in tubes, and this configuration once constituted the first dimension of 2-D PAGE.[73] Because of difficulties in handling and reproducibility with tube gels, IPG strips have largely replaced them.

Sample Preparation and Loading for IEF

A fundamental problem with IEF is that some proteins tend to precipitate at their pI values. Carrier ampholytes sometimes help overcome pI precipitation, and they

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are usually included in the sample solutions for IPG strips. In addition, nonionic detergents or urea are often included in IEF runs to minimize protein precipitation.

Urea is a common solubilizing agent in gel electrophoresis. It is particularly useful in IEF, especially for moderately soluble proteins. Urea disrupts hydrogen bonds and is used in situations in which hydrogen bonding can cause unwanted aggregation or formation of secondary structures that affect mobilities. Dissociation of hydrogen bonds requires high urea concentrations (7 to 8 *M*). If complete denaturation of proteins is sought, samples must be treated with a thiol-reducing agent to break disulfide bridges. Urea must be present in the gels during electrophoresis, but, unlike SDS, urea does not affect the intrinsic charge of the sample polypeptides. Urea solutions should be used soon after they are made, or they should be treated with a mixed-bed ion-exchange resin to avoid protein carbamylation by cyanate in old urea. Protein solutions in urea should never be left standing for extended periods of time or heated above 30°C.

Some proteins, especially membrane proteins, require detergent solubilization during isolation. Ionic detergents, such as SDS, are not compatible with IEF, although nonionic detergents, such as octylglucoside, and zwitterionic detergents, such as 2-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) and its hydroxyl analog CHAPSO, can be used. NP-40 and Triton X-100 often perform satisfactorily, but some preparations may contain charged contaminants.

Concentrations of CHAPS and CHAPSO, or octylglucoside of 1 to 2% in the gel, are recommended. Some proteins may require as high as 4% detergent for solubility. Even in the presence of detergents, some samples may have stringent salt requirements. Salt should be present in a sample only if it is an absolute requirement. Carrier ampholytes contribute to the ionic strength of the solution and can help to counteract a lack of salts in a sample. When urea or detergents are not needed, samples (1 to 10 µl) can be loaded in typical biochemical buffers, but better results can be obtained with solutions in deionized water, 1% glycerol, 2% ampholytes, or 1% glycine. Suitable samples can be prepared by dialysis or gel filtration.

Good visualization of individual bands generally requires a minimum of 0.5 µg of protein per band with dye staining or 50 ng of protein per band with silver staining (Subsection 8.2.8). One of the simplest methods for applying samples to thin polyacrylamide gels is to place filter paper strips impregnated with sample directly on the gel surface. Up to 25 µl of sample solution can be conveniently applied after absorption into 1-cm squares of filter paper. A convenient size for applicator papers is 0.2×1 cm, holding 5 µl of sample solution. Alternatively, 1- to 2-µl samples can be placed directly on the surface of the gel. In most cases, IPG strips for 2-D PAGE (which are provided in dehydrated form) are rehydrated in sample-containing solution prior to electrophoresis.[74] Rehydration loading allows higher protein loads to be applied to gels than do other methods. It is particularly popular because of its simplicity.

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There are few rules regarding the positioning of the sample on the IEF gel. In general, samples should not be applied to areas where they are expected to focus. To protect the proteins from exposure to extreme pH, the samples should not be applied closer than 1 cm from either electrode. Forming the pH gradients in carrier ampholyte IEF gels before sample application also limits the exposure of proteins to pH extremes. When acidic, narrow-range IPGs are used (e.g., pH 3 to 6), the sample is best applied at the cathode end of the strip. With basic, narrow-range IPGs (e.g., pH 7 to 10), the sample should be loaded at the anode side of the strip.

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Power Conditions and Resolution in Isoelectric Focusing

The pH gradient and the applied electric field determine the resolution of an IEF run. According to both theory and experiment,[1,64,69] the difference in pI between two resolved adjacent protein IEF bands (ΔpI) is directly proportional to the square root of the pH gradient and inversely proportional to the square root of the voltage gradient (field strength) at the position of the bands: $\Delta pI \propto [(\text{pH gradient})/(\text{voltage gradient})]^{1/2}$. Thus, to minimize ΔpI, a narrow pH range and high applied voltage give high resolution (small ΔpI) in IEF.

In addition to the effect on resolution, high electric fields also result in shortened run times. However, high voltages in electrophoresis are accompanied by large amounts of generated heat at the beginning of a run. Thus, there are limitations on the magnitudes of the electric fields that can be applied depending on the ionic strengths of the solutions used in IEF. Voltages are usually ramped up to the desired final values to give salts in the samples time to clear from the gel before high-resolution focusing is begun. Because of their higher surface-to-volume ratio, thin gels are better able to dissipate heat than thick ones and are therefore capable of higher resolution (high voltage). Electric fields used in IEF are generally of the order of 100 *V*/cm. At focusing, currents drop to nearly zero because the current carriers have stopped moving by then.

Detection of Proteins in Isoelectric Focusing Gels

IEF gels differ from those for gel electrophoresis in that they have relatively large pores and they contain relatively large amounts of carrier ampholytes. It is possible for proteins to diffuse in the large-pore gels during some staining procedures, and some stains will interact with carrier ampholytes. The standard staining solution for proteins in IEF gels uses a combination of CBB R-250 and Crocein Scarlet in an ethanol–acetic acid solution containing cupric sulfate. The Crocein Scarlet binds rapidly to proteins and helps fix them in the large-pore IEF gels.[75] The cupric sulfate enhances stain intensity.[76] The procedures of staining and destaining are similar to those for CBB R-250 used alone but yield better signal-to-noise ratios. IEF gels can also be silver stained for increased detection sensitivity. However, some silver stains will turn the plastic backing sheets of

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IPGs into mirrors. An easy way to stain IPGs is to immerse them for 1 h in colloidal CBB G-250 followed by two 10-min water washes. There is also a version of SYPRO Ruby stain specifically formulated for use with both carrier ampholyte and IPG-IEF gels.

TWO-DIMENSIONAL GEL ELECTROPHORESIS

2-D PAGE is the highest resolution method available for separating proteins.[73,77–82] The technique combines a first dimension of IEF, with a second separation by SDS-PAGE in a perpendicular direction. The technique is a true orthogonal procedure in that the two separation mechanisms are based on different physical principles (they are orthogonal in that sense) and the two separations are done at right angles to one another (they are geometrically orthogonal). The resolution obtained in the first dimension separation is retained when the IEF gel is mated to the second, SDS, gel.[82] It is this feature that gives 2-D PAGE exceptional resolution and distinguishes it from other separation methods. Thousands of polypeptides can be resolved in a single 2-D PAGE slab gel. The technique works best with soluble proteins such as those from serum or cytoplasm. It is relatively labor intensive for an electrophoresis technique, requiring a relatively high skill level for best results.

The best approach for 2-D PAGE is to run the IEF first dimension using IPG strips, and the best approach to obtaining IPG strips is to purchase them already made. Common practice is to denature proteins for 2-D PAGE to their constituent polypeptide chains so that polypeptide sequences can be matched to their corresponding gene sequences. This means that the IEF dimension is carried out in the presence of urea, CHAPS, carrier ampholytes, and a disulfide reducing agent such as dithiothreitol. Following IEF, an IPG strip is first treated with SDS and reducing and alkylating agents, then inserted into the gel cassette on top of the SDS-PAGE slab gel. The SDS-PAGE gel is run and stained as with one-dimensional electrophoresis. The difference between 1-D PAGE and 2-D PAGE gels is that the protein patterns in 2-D PAGE are spots rather than bands (Figure 8.10).

A large part of the success of a 2-D PAGE run is determined by careful sample preparation. This topic and several of the nuances of 2-D PAGE are outside the scope of this chapter. Those interested should consult References 77 to 85. See also www.expasy.ch and www.proteomeworkssystem.com and associated links.

IMMUNOBLOTTING

Proteins bound to the surfaces of synthetic membranes retain their antigenicity and are accessible to antibody probes. The most common membrane-based immunoassay technique is called *immunoblotting* or, more popularly, *Western blotting*. In Western blotting, proteins are transferred from an electrophoresis gel to a

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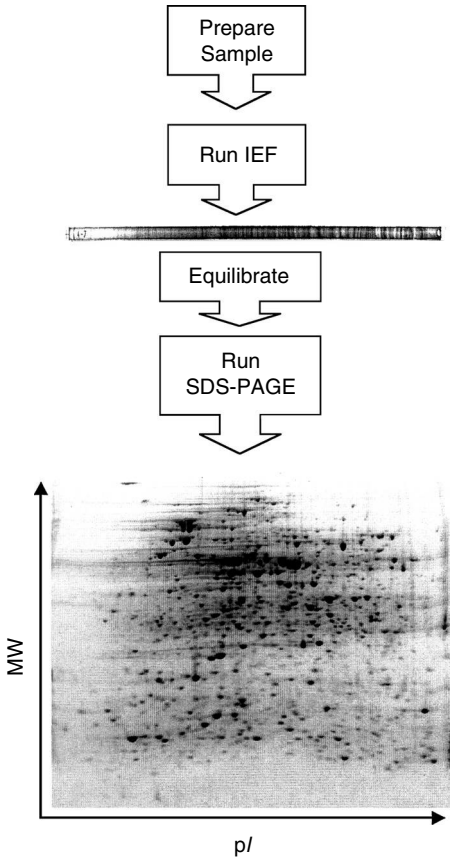

Figure 8.10 2-D polyacrylamide gel electrophoresis. Proteins from a lysate of *Escherichia coli* were subjected first to IEF in a 17-cm IPG strip spanning the pH range of 4 to 7. The strip containing focused proteins was prepared for SDS-PAGE, transferred to an 18 × 20 cm, 8 to 16%T gel, and electrophoresed in the Laemmli buffer system. At the end of the electrophoresis run, the separated proteins in the gel were stained with SYPRO Ruby Gel Stain, and the image shown was captured with a laser-based instrument. A second IPG strip was run in parallel and stained with colloidal CBB G-250. Its image is shown above the 1-D PAGE gel.

support membrane and then probed with antibodies. The method combines the resolution of PAGE (1-D or 2-D) with the specificity of immunoassays and enables the definitive identification of individual proteins in complex mixtures.[86–89]

Western blotting consists of the following steps (Figure 8.11): (1) Proteins are electrophoretically transferred from a gel to a membrane surface. The transferred proteins bind to the surface of the membrane and are immobilized in a

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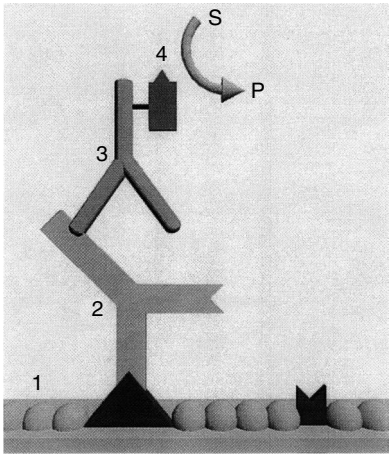

Figure 8.11 Specific enzymatic immunodetection of a blotted protein. Depicted are blocked binding sites on the membrane (1), a primary antibody (2) specifically bound to an antigenic protein, and a secondary antibody (3) bound to the primary antibody. The secondary antibody is conjugated to a reporter enzyme (4). Substrate (S) is converted to insoluble product (P) at the site of the antigen.

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pattern that is an exact replica of the gel. (2) Unoccupied protein-binding sites on the membrane are saturated with detergent and (or) some kind of inert protein that will not interact with the antibody probes. This is done to prevent nonspecific binding of antibodies to the membrane and is called either blocking or quenching. (3) The blot is probed with a specific primary antibody (or antibodies) in order to tag the proteins of interest with antibody molecules. (4) The blot is probed a second time with an antibody that recognizes the species of the primary antibody. Secondary antibodies are conjugated to some kind of reporter group. In Western blotting the reporter group is a detectable enzyme. The most common enzymes used in Western blotting are alkaline phosphatase and horseradish peroxidase. The site of the protein of interest is thus tagged with an enzyme through the intermediaries of the primary and secondary antibodies. (5) Enzyme substrates are incubated with the blot. They are converted into insoluble, detectable (visible) products leaving a colored trace at the site of the band or spot representing the protein of interest.

Apparatus for Blotting

Electrotransfer from a gel to a membrane is done by directing an electric field across the thickness of the gel to drive proteins out of the gel and on to the membrane. There are two types of apparatus for electrotransfer: (1) buffer-filled tanks and (2) "semidry" transfer devices (Figure 8.12).

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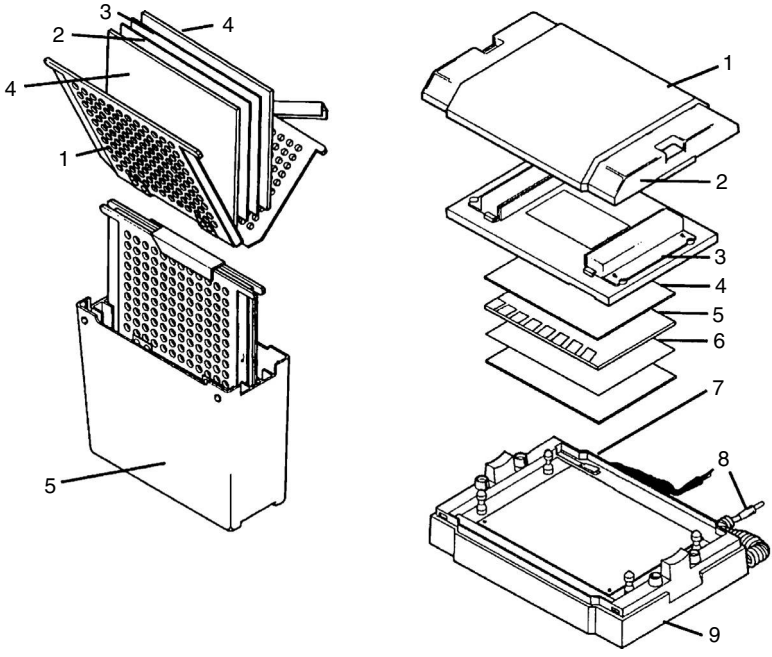

Figure 8.12 Two types of electrotransfer apparatus. At the left a tank transfer cell is shown in an exploded view. The cassette (1) holds the gel (2) and transfer membrane (3) between buffer-saturated filter paper pads (4). The cassette is inserted vertically into the buffer-filled tank (5) between positive and negative electrodes (not shown). A lid with connectors and leads for applying electrical power is not shown. On the right side of the figure is shown an exploded view of a semidry transfer unit. The gel (5) and membrane (6) are sandwiched between buffer-saturated stacks of filter paper (4) and placed between the cathode assembly (3) and anode plate (7). A safety lid (1) attaches to the base (9). Power is applied through cables (8).

Transfer tanks are made of plastic with two electrodes mounted near opposing tank walls. A nonconductive cassette holds the membrane in close contact with the gel. The cassette assembly is placed vertically into the tank parallel to the electrodes and submerged in electrophoresis buffer. A large volume of buffer in the tank dissipates the heat generated during the transfer.

In semidry blotting, the gel and membrane are sandwiched horizontally between two stacks of buffer-wetted filter papers in direct contact with two closely spaced solid-plate electrodes. The close spacing of the semidry apparatus provides for high field strengths. The term semidry refers to the limited amount of buffer that is used in the stacks of filter paper.

Tanks rather than semidry apparatus should be used for most routine work. With tanks, transfers are somewhat more efficient than with semidry devices.

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Under semidry electrotransfer conditions, some low-molecular weight proteins are driven through the membranes, and because low buffer capacity limits run times, some high-molecular weight proteins are poorly transferred.

Membranes and Buffers for Immunoblotting

The two membranes most used for protein work are nitrocellulose and polyvinylidene fluoride (PVDF). Both bind proteins at about 100 μg/cm^2. Nitrocellulose is the best membrane to use in the initial stages of an experiment. PVDF is used when proteins are to be sequenced or placed into a (matrix-assisted laser desorption ionization) mass spectrometer. PVDF can withstand the harsh chemicals of protein sequenators and the heat generated by mass spectrometer lasers, whereas nitrocellulose cannot.

Tank transfers from SDS-PAGE gels are done in modified electrophoresis buffer, 25 m*M* Tris, 192 m*M* glycine, 20% (v/v) methanol, and pH 8.3. With semidry transfers from SDS-PAGE gels, the buffer is 48 m*M* Tris, 39 m*M* glycine, 20% methanol, and pH 9. The methanol in the buffers helps remove SDS from protein–detergent complexes and increases the affinity between proteins and the membranes. Methanol is not used in transfers from nondenaturing gels. Nonfat dry milk and Tween 20 detergent are used to block unoccupied sites in membranes and are included as carriers for antibodies used to probe the membranes.

Immunodetection

Appropriate primary antibodies can be produced in any convenient animal, such as rabbits or mice. Antibodies to many important proteins can be purchased from a number of commercial vendors. Secondary antibodies (e.g., goat antirabbit immunoglobulin) conjugated to alkaline phosphatase or horseradish peroxidase are also commercially available. The preferred substrate for alkaline phosphatase is the mixture of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT). The substrate BCIP is dephosphorylated by the enzyme and then oxidized in a reaction coupled to reduction of NBT. The resultant highly visible product is a purple-colored formazan. Good substrates for horseradish peroxidase are 4-(chloro-1-naphthol) or diaminobenzidine (and hydrogen peroxide). Chemiluminescent substrates for horseradish peroxidase are based on oxidation of luminol. The luminol substrate provides the most sensitive signal of the blotting substrates, but requires photographic exposures or specially configured imaging devices. For procedures and more detail than can be provided here, consult References 86 to 89.

Total Protein Detection

For proper identification of the proteins of interest in a blot, immunodetected proteins must be compared to the total protein pattern of the gel. This requires the indiscriminate staining of all the proteins in the blot. Colloidal gold stain is

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a very sensitive reagent for total protein staining. It consists of a stabilized sol of colloidal gold particles. The gold particles bind to proteins on the surfaces of membranes. Detection limits are in the low hundreds of picogram range and can be enhanced by an order of magnitude by subsequent treatment with silver.

CBB G-250 is another popular total protein stain. Researchers blotting 2-D PAGE gels particularly favor it because it is compatible with mass spectrometry. Stained blots provide good media for archiving 2-D PAGE separations. A version of SYPRO Ruby, formulated for blots, is a very sensitive total protein stain.

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IMAGE ACQUISITION AND ANALYSIS

Several types of imaging systems and associated software are commercially available for analyzing gels stained with just about any kind of stain.[90–92] These instruments greatly simplify data acquisition and analysis and the archiving of gel patterns.

The three categories of image-acquisition devices used with electrophoresis gels are (1) document scanners, (2) charge-coupled device (CCD) cameras, and (3) laser-based detectors. Document scanners as configured for densitometry are for measurements on gels stained with one of the colored materials: CBB, silver, copper, or zinc. They operate in visible light illumination, 400 to 750 nm, with dynamic ranges extending to 3 O.D. The linear-array CCD detectors used with the better densitometers can distinguish adjacent features that are separated by 50 μm or greater (spatial resolution), which is more than adequate for most gel applications.

The better CCD camera instruments are cooled to increase their signal-to-noise ratios. They operate with illumination provided by either light boxes (UV or visible) for transmittance measurements or overhead lamps for epi-illumination. Filters are used to view fluorescent signals. CCD camera instruments are very versatile, and they can acquire images from gels stained with colored or fluorescent compounds. The epi-illumination feature allows CCD cameras to capture images of blots on opaque membranes. The spatial resolution obtainable with the cameras is entirely dependent on the properties of the lenses used and the area being imaged, but is generally in the 100- to 200-μm range. Their dynamic ranges for quantification often exceed four orders of magnitude.

Laser devices are the most sophisticated image-acquisition tools. They are particularly useful for gels labeled with fluorescent dyes because the lasers can be matched to the excitation wavelengths of the fluorophores. Detection is generally with photomultiplier tubes. Some instruments incorporate storage phosphor screens for detection of radiolabeled and chemiluminescent compounds (not discussed in this chapter). Resolution depends on the scanning speed of the illumination module and can be as low as 10 μm.

An imager is the most significant investment of all electrophoresis apparatus. As with all significant purchases, comparison shopping among the available products is highly recommended. In practice, researchers access the data in their

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gels through the analysis software, and the software should be a primary consideration in any imaging system. Good software will be able to use data from most imaging devices. However, dedicated software designed for use with particular instruments provides the desirable feature of controlling the imagers with the software.

Software for 1-D gel analysis (Figure 8.13) defines lanes and bands, quantifies bands, constructs standard curves, and determines molecular weights. Images can be adjusted for contrast, processed in various ways, annotated, and exported to other files for publication or document control. 2-D analysis software (Figure 8.14) defines and quantifies spots in 2-D PAGE gels. Those programs that use Gaussian spot modeling are better able to quantify proteins in overlapping spots than the programs that define spots by contours. Programs for 2-D analysis include statistical software designed for quantitative comparisons of large numbers of gels. The programs are also set up for analysis of spot patterns derived from differentially expressed proteins, and some can query databases to assist in protein identifications. They can also be used for image adjustments, annotation, and export in a variety of file formats.

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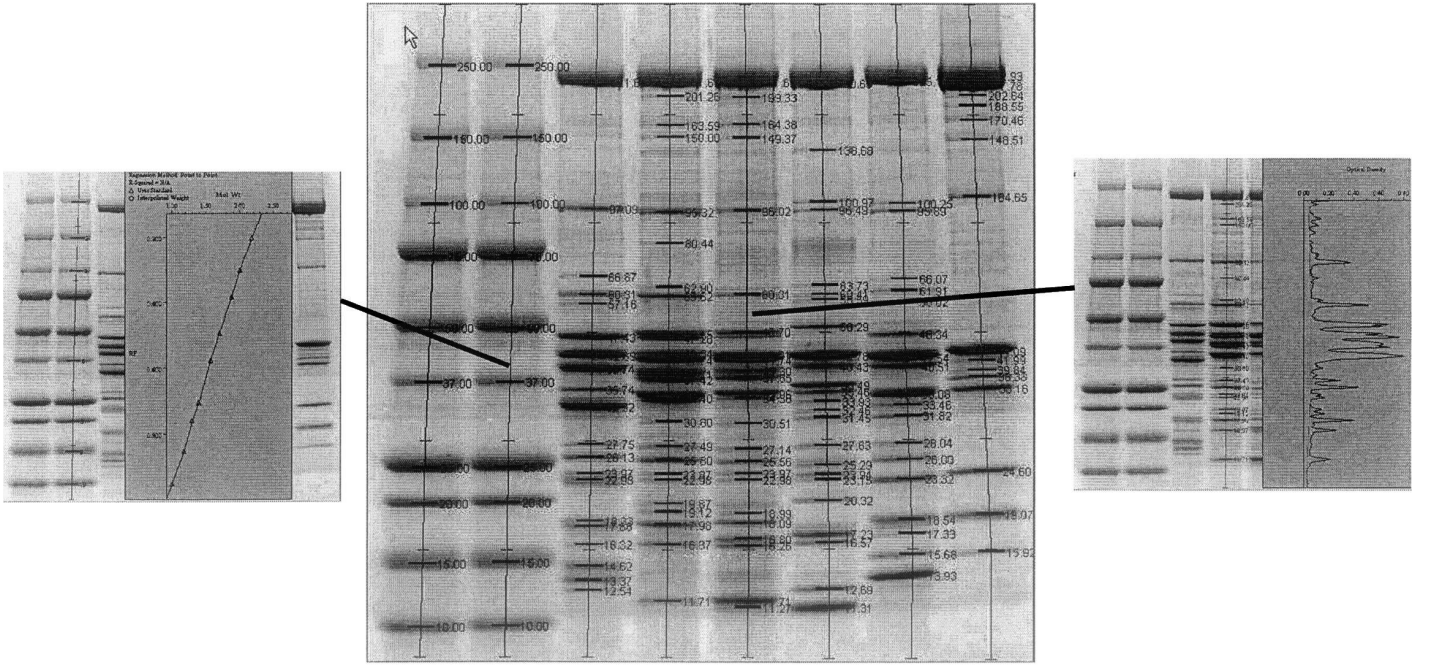

Figure 8.13 Image analysis software for 1-D gels. The molecular weights of the proteins in the gel of Figure 8.3 were digitally determined. The software first identified the individual lanes (vertical lines) and protein bands (horizontal bars). A standard curve was generated from the molecular weights of the marker proteins in lane 2 as shown in the insert at the left of the figure. The software automatically calculated the molecular weights of all the other protein bands in the gel and displayed them numerically on the image. The insert at the right of the gel image shows a densitometric scan of lane 5.

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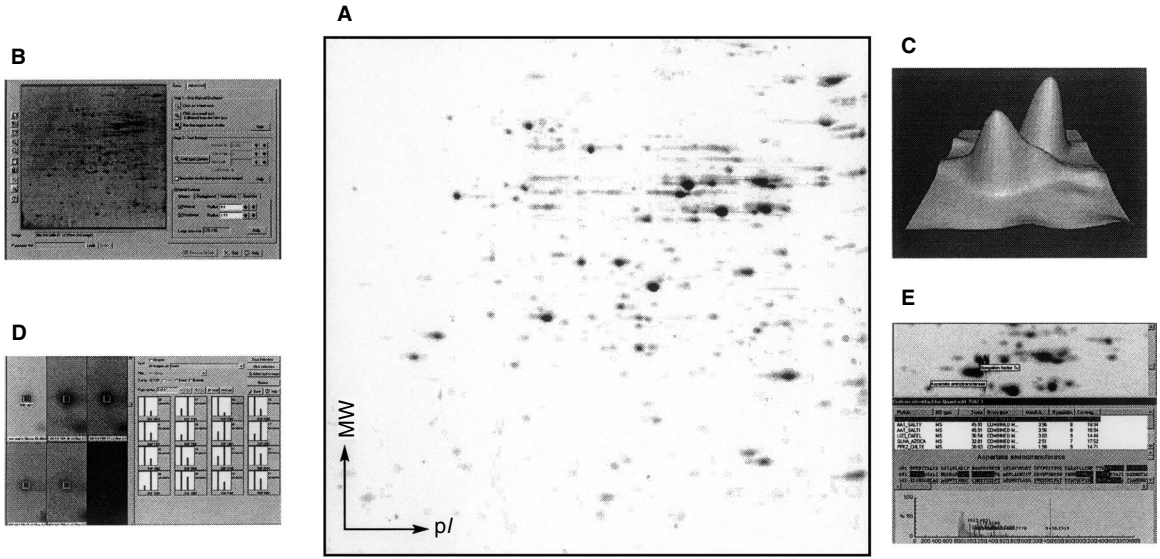

Figure 8.14 Image analysis software for 2-D gels. The image of a 2-D PAGE gel was filtered to remove streaks and other blemishes and background "noise" was subtracted. Protein spots on the filtered image were identified and converted to their Gaussian representations (A). Insert (B) shows the software "wizard" used to set the parameters for image filtering, background subtraction, and spot detection. A 3-D representation of two adjacent Gaussian-modeled spots is shown in (C). The heights of the cones represent the intensities of the two spots. Insert (D) shows one type of statistical analysis in which the relative intensities of identical spots in four different gels are compared. The gel image at the upper left corner of (D) is from a master image made from the four experimental gels. At the lower right (E) is a portion of the annotated gel showing two proteins identified by mass spectrometry. The bottom portion of insert (E) shows the mass spectrometer data used to identify one of the proteins. This type of mass spectrometer data is accessible from the spots on the image through use of the annotation function of the software.

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