ELECTROPHORESIS

1. Introduction

Electrophoresis is a separation technique most often applied to the analysis of biological or other polymeric samples. It has frequent application to analysis of proteins (qv) and DNA fragment mixtures (see NUCLEIC ACIDS). The high resolution of electrophoresis has made it a key tool in the advancement of biotechnology (qv). Variations of this methodology are being used for DNA sequencing (see GENETIC ENGINEERING), isolating active biological factors associated with diseases such as cystic fibrosis, sickle-cell anemia, myelomas, and leukemia, and establishing immunological reactions between samples on the basis of individual compounds (see also CHEMOTHERAPEUTICS, ANTICANCER; IMMUNOASSAY). Electrophoresis is an extremely effective analytical tool because it does not necessarily affect a molecule's structure, and it is highly sensitive to small differences in molecular charge and mass.

The term electrophoresis refers to the movement of a solid particle through a stationary fluid under the influence of an electric field. The study of electrophoresis has included the movement of large molecules, colloids (qv), fibers (qv), clay particles (see CLAYS), latex spheres (see LATEX TECHNOLOGY), basically anything that can be said to be distinct from the fluid in which the substance is suspended. This wide range in particle size to which electrophoresis has been applied has required that the theory describing electrophoresis be very general.

The fundamental principle behind electrophoresis is the existence of charge separation between any surface and the fluid in contact with it. The surface carries an immobilized charge, and the electrolyte fluid in contact with the charged surface balances the electric charge with an increased density of ions of the opposite charge. An applied electric field can act on the resulting charge densities, causing the particle to move, the fluid around the particle to move, or both. An applied electric field also generates heat, through resistive heating, and gases, through electrolysis reactions. Each is important in understanding and designing working electrophoresis equipment.

There are three distinct modes of electrophoresis: zone electrophoresis, isoelectric focusing, and isotachophoresis. These three methods may be used alone or in combination to separate molecules on both an analytical (μL of a mixture separated) and preparative (mL of a mixture separated) scale. Separations in these three modes are based on different physical properties of the molecules in the mixture, making at least three different analyses possible on the same mixture.

Distinction is also made among electrophoretic techniques in terms of the type of matrix employed for analysis. Matrices include polymer gels such as agarose and polyacrylamide, paper, capillaries, and flowing buffers. Each matrix is used for different types of mixtures, and each has unique advantages.

There are a variety of techniques for detecting separated sample compounds using chemical stains, photographic media, and immunochemistry. Each detection technique also gives different information about the identity, quantity, and physical properties of the molecules in the mixture. Detection is

often the focus of electrophoresis, and usually yields basic information about the mixture being studied.

2. Principles

Electrophoresis uses the force of an applied electric field to move molecules or particles, often through a polymer matrix. The electric field acts on the intrinsic charge of a substance, and the force on each substance is proportional to the substance's charge or surface potential. The resulting force on the substance results in a distinct velocity for the substance that is proportional to the substance's surface potential. If two different substances have two different velocities, an electric field applied for a fixed amount of time results in different locations on the matrix for these substances.

The application of an electric field to a gel matrix or capillary tube results in heating in the media and gas generation at the electrodes. Thus special attention in the design and use of electrophoretic equipment is required.

2.1. Theory of Electrophoretic Motion. The study of the mechanics of electrophoresis focuses on the basis of electric potential on the surface of an object, and the relation of the electric potential to the velocity of the particle. Whereas research has been generally limited to nonmolecular particles of well-defined geometry and is not strictly applicable to molecules such as proteins and DNA fragments, this work is useful for understanding the physics of electrophoretic motion.

The Electric Double Layer. Any time an interface between two immiscible phases occurs, an electric potential can be developed at that interface. For example, the walls of a glass beaker have an electric potential when the beaker holds salt water. The potential is generated because some ions preferentially bind to or adsorb onto glass, and the glass naturally has silanol groups, SiO, on its surface that ionize in water causing the glass surface to be charged relative to the bulk salt water. Conceptually, there is a separation of charge that exists in a thin section of the glass and in a thin layer of the salt water adjacent to the glass. These two layers of charge are called the electric double layer. Detailed discussions of the properties of ions and the occurrence of electric double layers may be found in the literature (1–4). In electrokinetic phenomena, the primary concern is with the fluid half of the double layer (in the previous example, the layer is in the salt water), because the layer on the glass itself is immobilized. This charged layer is called the diffuse layer. The electric potential in the diffuse layer extends into the fluid phase, and drops off as a Poisson distribution:

$$\phi = \phi_o \exp(-\kappa x) \tag{1}$$

where ϕ_o = potential at interface, mV; ϕ = potential x cm from interface, mV; κ = characteristic length, cm^{-1}; and x = distance from the interface, cm. The characteristic length that the potential extends into the bulk fluid from the interface is called the double-layer thickness, $1/\kappa$. This length is a function of the concentration and charges of the salts in solution, the temperature, and the

permittivity of the solution:

$$\kappa^2 = \left(\frac{F^2 \Sigma c z^2}{\epsilon \epsilon_o RT}\right) \tag{2}$$

where ϵ = relative permittivity; ϵ_o = permittivity of free space = 8.854×10^{-14} C/(V · cm); z = valency of each ion; c = concentration of each ion; $R = 8.314$ J/(mol · K); T = temperature, K; and $F = 9.65 \times 10^4$C/mol. The relative permittivity is a measure of the conductance of the pure bulk material relative to a vacuum. In the salt water in the beaker example, the pure bulk material would be water, which has a relative permittivity of ~80.

Not all of the ions in the diffuse layer are necessarily mobile. Sometimes the distinction is made between the location of the true interface, an intermediate interface called the Stern layer (5) where there are immobilized diffuse layer ions, and a surface of shear where the bulk fluid begins to move freely. The potential at the surface of shear is called the zeta potential. The only methods available to measure the zeta potential involve moving the surface relative to the bulk. Because the zeta potential is defined as the potential at the surface where the bulk fluid may move under shear, this is by definition the potential that is measured by these techniques (3).

The physical separation of charge represented allows externally applied electric field forces to act on the solution in the diffuse layer. There are two phenomena associated with the electric double layer that are relevant: electrophoresis, when a particle is moved by an electric field relative to the bulk; and electroosmosis, sometimes called electroendosmosis, when bulk fluid migrates with respect to an immobilized charged surface.

Electrokinetics. The first mathematical description of electrophoresis balanced the electrical body force on the charge in the diffuse layer with the viscous forces in the diffuse layer that work against motion (6). By using this force balance, an equation for the velocity, V, of a particle in an electric field is

$$V = \mu E \tag{3}$$

where E = the electric field strength, V/cm; and $\mu = \epsilon\epsilon_o\zeta/\eta f(\kappa a)$ where ζ = zeta potential, mV; η = viscosity, g/(cm · s); and a = particle radius, cm. The function (κa) is 1 when the particle has a much larger diameter than its double layer (high ionic strengths) and is 1.5 for very low ionic strength solutions where the double layer is much greater than the particle diameter (7). The behavior of a particle is complex when the double layer and particle diameter are close to the same size, which is often the case (8,9).

Electroosmotic flow is also dependent on the zeta potential at the immobilized surface and the strength of the electric field. For electroosmosis, the flow rate generated is

$$F = \frac{\epsilon\epsilon_o\zeta}{\eta}\pi r^2 E \tag{4}$$

where F = flow rate in mL/s and r = radius of flow channel in centimeter. Electroosmotic flow is generally minimized in polymer networks such as gels, but is important in open channels such as capillaries.

2.2. Generation of Heat in Electric Fields. One of the practical problems encountered in electrophoresis is the generation of heat from resistive dissipation of energy in the electrophoretic medium. The generation of heat (joule heating) is given by

$$W = EI \tag{5}$$

where W = power in watts and I = current, A. The current and the electric field strength are related by the conductivity of the electrophoretic medium by Ohm's law

$$E = \frac{I}{C} \tag{6}$$

where C = medium conductivity in $(\Omega \cdot \mathrm{cm})^{-1}$. The higher the conductivity of the electrophoretic medium, the more difficult electrophoresis becomes because highly conductive solutions result in a lower field strength per current, and the heat load on the system increases as the current is squared. Electrophoresis is usually performed in highly resistive media, using just enough salt to solubilize the compounds of interest. The addition of polymer matrices, such as gels, also serves to increase the resistivity of the media by increasing the viscosity.

Heating in electrophoresis causes changes in the viscosity and density of the electrophoretic medium. High temperatures can also damage electrophoretic equipment by warping cooling blocks, melting plastic, or cracking glass plates. Poorly cooled electrophoretic systems have poor resolution. Usually bands are smeared or appear warped.

When fluids heat unevenly, the hot part of the fluid tends to rise with respect to the cooler part of the fluid because of differences in density. The flow is driven by gravity, and distorts resolution in electrophoretic separations.

The ability to remove heat from electrophoretic systems has severely limited the maximum capacity of these systems in terms of how large or thick the systems can be. Electrophoretic separations have been performed on space flights because the effect of gravity in outer space is small and mixing from heating is negligible. Whereas electrophoresis in outer space has been achieved (10), the economics for a scaleable process have not (see SPACE PROCESSING).

The heating effect is the limiting factor for all electrophoretic separations. When heat is dissipated rapidly, as in capillary electrophoresis, rapid, high resolution separations are possible. For electrophoretic separations the higher the separating driving force, ie, the electric field strength, the better the resolution. This means that if a way to separate faster can be found, the separation should be more effective.

2.3. Electrolyses Reactions. The electrodes in electrophoresis equipment are typically constructed from platinum wire, and sodium chloride generally carries the current in any electrophoretic medium. This results in the half-cell reactions

Cathode

$$2\,H_2O + 2\,e^- \longrightarrow 2\,OH^- + H_2$$

$$HA + OH^- \rightleftarrows A^- + H_2O$$

Anode

$$H_2O \longrightarrow 2\,H^+ + 0.5\,O_2 + 2\,e^-$$

$$H^+ + A^- \rightleftarrows HA$$

That is, water is electrolyzed. The hydrogen gas produced at the cathode can be hazardous, especially because it is in the vicinity of an electrode that is also producing heat. For this reason, electrode chambers are usually open to the atmosphere so that gases can vent.

The other reactions at the electrodes produce acid (anode) and base (cathode) so that there is a possibility of a pH gradient throughout the electrophoresis medium unless the system is well buffered (see HYDROGEN-ION ACTIVITY). Buffering must take the current load into account because the electrolysis reactions proceed at the rate of the current. Electrophoresis systems sometimes mix and recirculate the buffers from the individual electrode reservoirs to equalize the pH.

3. Modes of Electrophoretic Separations

Zone electrophoresis, isoelectric focusing, and isotachophoresis are all commonly practiced as analytical techniques. Zone electrophoresis is by far the most commonly practiced and there are several different zone electrophoresis methods and techniques available for different separation goals. Isoelectric focusing is also useful for separation, and possibly more useful for determining charge characteristics of sample proteins. Isotachophoresis takes advantage of the continuity of current across a medium to segregate a sample into contiguous zones of high purity. It is not useful as an analytical technique, but is applied as a potential preparative method.

3.1. Electrophoresis Equipment. Most electrophoresis equipment shares a basic design, a diagram of which is shown in Figure 1. Electrophoresis equipment usually consists of two buffer reservoirs, one for the anode and one for the cathode. Very often, electrophoretic buffers have a basic pH and sample compounds migrate to the anode. The equipment includes some sort of electrophoretic medium connecting the two reservoirs, such as a gel, paper, or capillary tubes, to which a sample is applied. A direct current power supply connects the two electrodes that are immersed in the buffer reservoirs. The power supply is usually interlocked to the electrophoresis equipment through a cover, isolating the medium and buffer reservoirs from human interaction because contact can be very dangerous. Small dc currents through the human heart can cause cardiac arrest or arrhythmia. An electric field is applied between the two electrode reservoirs, causing sample compounds to migrate through the medium and heat to be generated in the medium. The heat is usually removed by a chilled water bath or running tap water. At completion of the electrophoretic run the sample appears resolved into several different components along the length of the medium. In this respect, gel electrophoresis resembles thin-layer chromatography (see CHROMATOGRAPHY).

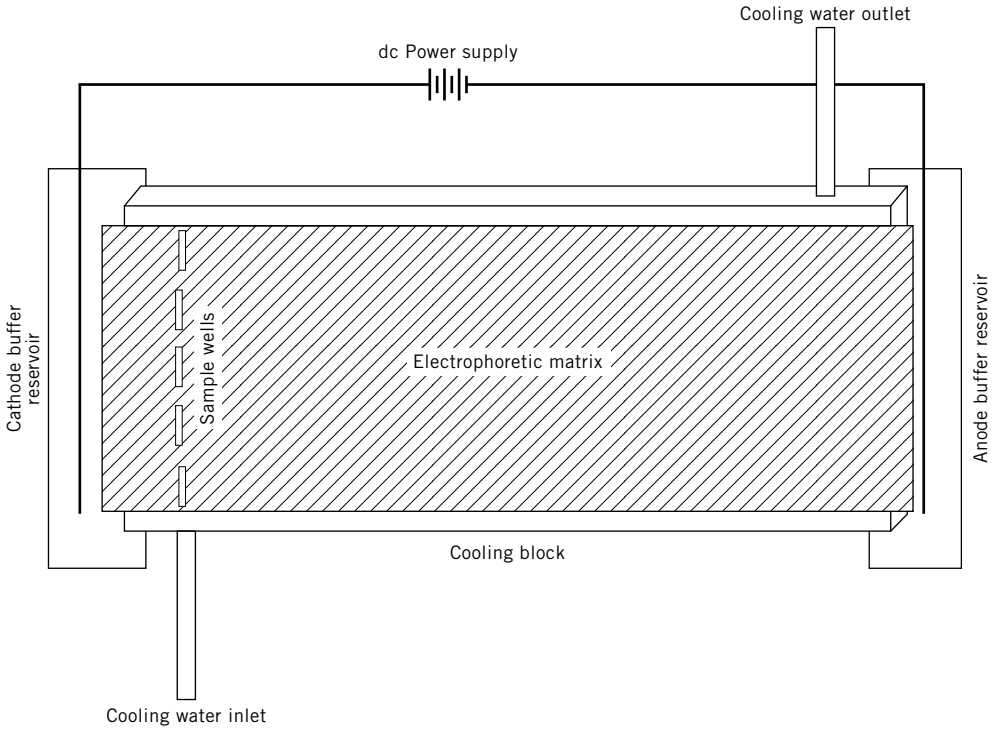

Fig. 1. Electrophoresis equipment.

Under certain conditions, the sample is clearly visible throughout the process. Other times it is necessary to stain the matrix to visualize the components. In cases where a final staining procedure is required, a small amount of dye is often added to the sample before the analysis. The dye typically migrates faster than any sample component. The position of the dye in the matrix indicates the speed of the resolution of the components of the sample. Typically, the electrophoretic medium is discarded after use. Good resolution can be obtained from 1 to 20 h, using applied voltages of 10–2000 V and currents of 5–100 mA.

3.2. Zone Electrophoresis. In zone electrophoresis, multicomponent samples are applied to an electrophoretic medium, most commonly a gel, an electric field is applied, and after a predetermined length of time or after a certain level of power, current, or voltage has been applied, the electrophoretic medium is inspected for resolution of the sample components. A typical zone electrophoresis result is shown in Figure 2, which indicates how closely related molecules can be separated from each other and visualized on a gel. Each band represents a highly enriched substance, has essentially the same shape and width, and is separated from the other bands because each type of substance has a slightly different property. As shown, several samples are typically analyzed side by side on a gel. Bands that migrate the same distance in different sample mixtures typically represent the same substance. Standards are usually run concurrently with samples that are to be compared. In zone electrophoresis, proteins or sample

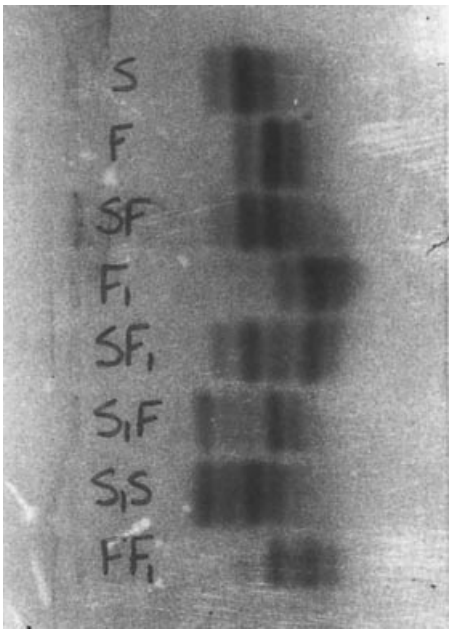

Fig. 2. Zone electrophoresis separation where S, F, S_1, and F_1 are different materials.

components are completely separated into discrete zones as they migrate through the media onto which they are applied.

The use of standards with samples makes zone electrophoresis particularly useful as an analytical tool. However, when samples cannot be analyzed on the same gel, differences in the experimental conditions from experiment to experiment make direct comparison more difficult. To make comparisons from experiment to experiment, a relative mobility, R_f, is often measured by measuring the distance a component travels down the gel compared to some reference or standard component.

$$R_f = \frac{\text{distance migrated by component}}{\text{distance migrated by reference}} \tag{7}$$

Disc Electrophoresis. Resolution in zone electrophoresis depends critically on getting sample components to migrate in a focused band, thus some techniques are employed to concentrate the sample as it migrates through the gel. The most common technique is referred to as discontinuous pH or disc electrophoresis. Disc electrophoresis employs a two-gel system, where the properties of the two gels are different.

Disc electrophoresis was first introduced in the early 1960s (11–13) as various techniques using polyacrylamide gels were being explored and designed. Original work employed several buffer systems and different polyacrylamide gels in order to first concentrate and then separate compounds (14).

The way proteins behave in disc electrophoresis systems depends primarily on differences in the pH in the two gels. The pH of the buffers in both the second

gel (the separating gel) and the electrode reservoir are similar, whereas the buffers of the first gel (the stacking gel) and the sample itself are of a lower pH. This difference in pH allows for different sample–gel interactions. The stacking gel is only ~5 cm, including distance for the sample wells, and is stacked on top of the separating gel which is ~20 cm in length. As with most electrophoretic methods, a current is applied and the molecules in the sample wells begin to migrate anodally. Here, the migration is electrochemically different because the buffer in the upper reservoir chamber has a higher pH than that of the samples and stacking gel. This difference in pH allows the molecules in the samples to migrate rapidly through the stacking gel. When the sample compounds enter the separating gel, movement is slowed because of the pH change. This focuses the molecules into narrow bands and allows more bands to be resolved from one another.

Another difference between other types of electrophoresis and disc electrophoresis is that the molecules in a sample do not start to significantly separate until entering the separating gel. A discontinuous gel system may be used with almost any type of zone electrophoresis application.

Native Zone Electrophoresis. In some cases, good resolution between sample species can be obtained with little or no sample pretreatment. In these cases, the gels are said to be native gels. In this method, the charge on the individual sample component is primarily responsible for its differential migration. The relative mobility, R_f, measured is proportional to the charge when the pore size in the electrophoretic medium is large compared to the component. When the pore size and molecular size are about the same, the size of the molecule becomes important in the electrophoretic mobility. Because of this ambiguity, the absolute meaning of R_f from this technique is useful primarily for component identification and comparison, and not for estimating the properties of a molecule.

When separating proteins, native zone electrophoresis leaves the component proteins in folded, globular states, with all subunits intact. This compact size makes for a faster running molecule. Native electrophoresis is useful for resolving components that may differ by a small size and/or a small charge. For example, this method was used to isolate genetic variants of some proteins in cow's milk (15), which differ by one charge unit and $<0.1\%$ of their total molecular weight. This mode of electrophoresis can also be used to isolate proteins in an active state, so that their activities can either be used for their detection, or so that they can be recovered from the gel and further studied.

Reduced SDS Electrophoresis. The combination of sodium dodecyl (lauryl) sulfate (SDS) [151-21-3], $CH_3(CH_2)_{10}CH_2OSO_3Na$, treatment of samples and polyacrylamide gel electrophoresis was first described in the late 1960s (14,16). The SDS is an ionic surfactant that solubilizes and denatures proteins (see SURFACTANTS). The surfactant coats a protein through hydrophobic interactions with the polypeptide backbone, effectively separating most proteins into their polypeptide subunits. The majority of proteins to which SDS binds then unfold into linear molecules having a similar surface potential. Nonreduced proteins bind ~0.9–1.0 g of SDS per gram of protein (17).

SDS–polyacrylamide gel electrophoresis (SDS–page) allows separation of molecules strictly on the basis of size, ie, molecular weight. When SDS-treated samples migrate into a gel and are electrophoresed, the principal difference is size or length. Smaller molecules travel through the matrix more quickly than

those that are larger. The rate at which molecules migrate through a polyacrylamide gel is inversely linear with the logarithm of their molecular weight. Thus denatured samples can be analyzed alongside standards of known molecular weight to aid in the interpretation of a substance's physical size.

Other dissociating agents may be used to further break down a protein. Urea [57-13-6] may be used to disrupt hydrogen bonds. When urea is the only dissociating agent added (no SDS), a protein's intrinsic charge is not affected and separation on the basis of size and charge may be achieved. If a protein contains internal disulfide bonds, a thiol reagent such as β-mercaptoethanol [60-24-2] may be used in order to reduce the sample and break the disulfide bonds. Proteins having reduced disulfide bonds bind ~1.4 g of SDS per gram of protein, compared to ~1 g/g for nonreduced. Typically, both a reduced and nonreduced sample are run in order to evaluate protein disulfide content.

Pulsed Field Gel Electrophoresis. The inverse linear relationship between the relative electrophoretic migration of a molecule and the logarithm of its size or length begins to break down for very large molecular weights. Pulsed field gel electrophoresis is a technique that was developed to separate these large molecular weight compounds, represented by large pieces of DNA in agarose gels. In pulsed field electrophoresis, the direction of the field is intermittently changed, either forward and backward or from side to side. A small molecule notices no real difference in its electrophoresis because it can completely reorient in each field direction. However, the redirectioning of the electric field causes larger molecules to travel in a zigzag pattern, putting kinks into the length of the molecule. The longer the molecule, the more kinks in its length, and the slower it travels down the length of the gel. The larger molecule finds itself traveling backward along some sections of its length with respect to the direction of the electric field. This has allowed resolution of megabase size strands of DNA, and is one analytical advance that has made the Human Genome Project feasible (18–23).

3.3. Isoelectric Focusing. Isoelectric focusing is a technique used for protein separation, by driving proteins to a pH where they have no mobility. Resolution depends on the slope of a pH gradient that can be achieved in a gel.

Ampholytes or Zwitterions. An ampholyte is a molecule that can be either positively or negatively charged, depending on the pH. These molecules are also called zwitterions. All amino acids (qv) and proteins are ampholytes, or amphoteric. Not only does the sign of the charge of an ampholyte change with pH, but the magnitude of the charge can also vary. The charge on a protein, for example, may vary from +10 or more at low pH, to −10 or more at high pH. For a protein, the pH at which its mobility is zero is called the isoelectric point. This is sometimes slightly different from the pH where the protein has zero charge (point of zero charge or p.z.c.), which is found by titration. The reasons for this difference are complex, and not fully understood.

A special class of ampholytes has been synthesized for the purpose of isoelectric focusing (24). These ampholytes have an amino end and a carboxyl end that are separated by varying numbers of methylene groups. The further apart the amino and carboxyl groups, the less one affects the ionization of the other; thus a different isoelectric point (pI) is established for each molecule. These ampholytes, which may be added to an electrophoretic medium, migrate

in one direction or another, under the influence of an applied electric field, until they reach a zone in which the pH is the same as that ampholyte's isoelectric point. The ampholyte molecules buffer themselves and establish the local pH as they migrate through the gel. As the ampholytes reach an isoelectric pH, they establish a stationary spatial pH gradient in the electrophoretic medium.

Isoelectric Focusing. Isoelectric focusing (ief) is an electrophoretic technique in which amphoteric samples are separated according to their isoelectric points along a continuous pH gradient. Ief analyses are carried out in various matrices: in acrylamide, agarose, and capillaries. The agarose or acrylamide gels that are used must be prepared with carrier ampholytes bracketing a specific pH range. After some time, the ampholytes separate and there is a pH gradient which covers the range of all the ampholytes' isoelectric points. Initial research on ief (25) was primarily directed toward evaluating the properties of synthetic ampholytes in solution. Later work refined the technique to apply gel matrices (12) and provided the basis for ief methodologies.

Problems associated with gel-to-gel variability have been rectified with the advancement of ampholyte mixtures. One commonly used mixture of ampholytes, called Immobilines, is an improved ampholyte mixture that produces no gradient drift or unequal pH gradient (26) and can be used in a gel matrix reproducibly from one day to the next.

Because protein samples are actually ampholytes, when samples are loaded onto the gel and a current is applied, the compounds migrate through the gel until they come to their isoelectric point where they reach a steady state. This technique measures an intrinsic physicochemical parameter of the protein, the pI, and therefore does not depend on the mode of sample application. In ief, the highest sample load of any electrophoretic technique may be used, however, sample load affects the final position of a component band if the load is extremely high, ie, high enough to titrate the gradient ampholytes or distort the local electric field.

Isoelectric focusing takes a long time (from $\sim$3 to 30 h) to complete because sample compounds move more and more slowly as they approach the pH in the gel that corresponds to their isoelectric points. Because the gradient ampholytes and the samples stop where they have no mobility, the resistivity of the system increases dramatically toward the end of the experiment, and the current decreases dramatically. For this reason, isoelectric focusing is usually run with constant voltage. Constant current application can lead to overheating of the system.

Some forms of agarose are specifically designed to work with large (mol wt $>$500,000) molecules (27,28). The types of samples for which the agarose ief system are utilized are larger plasma proteins such as immunoglobulins, or actual tissues, such as organ samples and tumors.

Another form of ief is a method called direct tissue isoelectric focusing (dtif) (29) where isoelectric focusing in agarose is used to evaluate tissues. The tissue to be analyzed is placed directly onto the gel. Using the tissue itself and not tissue extracts has advanced the study of proteins that are difficult to extract from tissue, or are damaged by the extraction procedure. Dtif is an important advancement in the area of sample handling and application where direct application of a solid to a gel matrix may actually enhance resolution.

3.4. Isotachophoresis. Isotachophoresis takes advantage of the fact that electroneutrality must be maintained in an electrophoretic system in order to support an electric field. If a current passes through a medium, that current must be constant from one electrode to the other, regardless of the local ion concentration or mobility; ie, dilute ions must move faster to keep up with a zone of more concentrated ions. Electric fields compensate for this because the electric field strength does not have to be constant along the length of the medium. The electric field strength is lowest where the ions are most concentrated and most mobile. Isotachophoresis takes advantage of this phenomenon by lining up the ions of interest, fastest (most mobile) to slowest. This is a highly specialized technique that requires detailed knowledge of the properties of the sample to be separated, and is generally not applicable to analytical separations of unknown constituents.

An electrophoretic medium, such as a gel or a capillary tube, is cast or filled with an electrolyte that has a higher mobility than any components in the mixture of interest. This electrolyte, called the leading electrolyte, also fills the anode buffer reservoir. A sample is applied on top of the leading electrolyte, and another electrolyte with lower mobility than the sample fills the cathode reservoir. This essentially envelopes the sample. As the electric field is applied, the leading electrolyte moves rapidly toward the anode. The highest mobility component in the sample is drawn toward the lead electrolyte to fill in the conductivity gap left by the quickly migrating lead electrolyte. The electric field driving the first ion in the sample must increase to allow the sample ion to follow at the same speed as the leading electrolyte. The current through the whole media is constant, so the electric field strength increases because the resistivity increases. The resistivity increases because the first ion in the sample to follow the lead electrolyte dilutes so the electric field within the first ion sample zone is increased to give the two zones the same speed. Progressively, each ion in the sample follows at lower conductivity, higher electric field, constant current density, just behind an ion of higher mobility and just ahead of an ion of lower mobility. Finally, the "trailing" electrolyte, of lowest mobility, terminates the separation.

This separation technique has been employed primarily for preparative types of separations because detailed knowledge of the properties of the sample is required. Also, because this separation results in discrete zones of sample ions that are virtually pure, it makes sense to use this technique when the sample size is large. This technique is ineffective when the levels of impurities are small with respect to the target compound; small amounts of sample ions do not form zones well and tend to mix with the target compound. Information on this technique is available (30).

4. Electrophoretic Materials and Matrices

Various support media may be employed in electrophoretic techniques. Separation on agarose, acrylamide, and paper is influenced not only by electrophoretic mobility, but also by sieving of the samples through the polymer mesh. The finer the weave of selected matrix, the slower a molecule travels. Therefore, molecular

weight or molecular length, as well as charge, can influence the rate of migration.

In addition to polymeric support media, capillaries and flowing buffers have been used as support media for electrophoresis. Although these are not used as frequently, there are definite advantages for certain types of samples and applications.

4.1. Agarose Electrophoresis. Agarose is produced from the processing of red seaweed. When agar is extracted from the seaweed it is in two components, agaropectin and agarose. The agarose portion is nearly uncharged and is therefore the portion desirable for use as an electrophoretic matrix. Charge on the agarose leads to electroosmotic flow through the gel. The chemical composition of agarose is alternatively repeated residues of 1,3-linked-β-D-galactopyranose and 1,4-linked-3,6-anhydro-α-L-galactopyranose (31).

To prepare a gel for electrophoresis a combination of agarose and buffer is heated until the agarose solid is dissolved and boiling. The solution is cooled sufficiently and then poured into a warmed gel casting apparatus which forms the shape of the gel as it cools. After the cooled solution is poured into the casting apparatus, it is allowed to gel and can then be used in an agarose electrophoresis method. Because the composition of agarose is a network of residues that hold water molecules, the extra agarose solution may be stored and used at a later time. The concentration of agarose mixtures typically varies between 0.5 and 2% in a weight/volume ratio, although more extreme concentrations have been evaluated. The varying concentrations depend on the desired application. The higher the concentration of agarose, the smaller the pore size.

The use of agarose as an electrophoretic method is widespread (32–35). An example of its use is in the evaluation and typing of DNA both in forensics (see FORENSIC CHEMISTRY) and to study heritable diseases (36). Agarose electrophoresis is combined with other analytical tools such as Southern blotting, polymerase chain reaction, and fluorescence. The advantages of agarose electrophoresis are that it requires no additives or cross-linkers for polymerization, it is not hazardous, low concentration gels are relatively sturdy, it is inexpensive, and it can be combined with many other analytical methods. Premade agarose gels are now commercially available and quite convenient.

4.2. Polyacrylamide Electrophoresis. Polyacrylamide gels are synthesized through the combination of acrylamide [79-60-1] (qv), $CH_2{=}CHCONH_2$, monomer and a cross-linking comonomer (see ACRYLAMIDE POLYMERS). Typically, the cross-linking comonomer of choice is *N,N′*-methylenebisacrylamide [110-26-9] (bisacrylamide), $(CH_2CHCONH)_2$, although others are available, such as ethylenediacrylate (EDA) and *N,N′*-diallyltartardiamide (DATD) [58477-85-3] (37). The cross-linking of polymerized monomer with the comonomer is what controls the pore size of the gel polymer mesh. This level of pore size control makes polyacrylamide gel electrophoresis an effective analytical tool.

The most commonly used combination of chemicals to produce a polyacrylamide gel is acrylamide, bisacrylamide, buffer, ammonium persulfate, and tetramethylenediamine (TEMED). Both TEMED and ammonium persulfate are catalysts to the polymerization reaction. The TEMED causes the persulfate to produce free radicals, causing polymerization. Because this is a free-radical driven reaction, the mixture of reagents must be degassed before it is used.

The mixture polymerizes quickly after TEMED addition, so it should be poured into the gel-casting apparatus as quickly as possible. Once the gel is poured into a prepared form, a comb can be applied to the top portion of the gel before polymerization occurs. This comb sets small indentations permanently into the top portion of the gel which can be used to load samples. If the comb is used, samples are then typically mixed with a heavier solution, such as glycerol, before the sample is applied to the gel, to prevent the sample from dispersing into the reservoir buffer.

Maximum resolution of proteins is the main consideration when determining the acrylamide concentration of a gel. Two parameters define the composition of gel: the wt% of total monomer, % T (acrylamide plus bisacrylamide in grams per 100 mL), and the proportion by weight of monomer that is the cross-linking agent, % C (bisacrylamide) (38). Gels having concentrations between 5 and 15% are typically used to achieve the most desirable separation of the components of interest. Unlike the agarose matrix, once the mixture polymerizes, it cannot be reused. These gels are popular because of their durability. They can be dried and saved, although they are typically photographed or scanned electronically for archival purposes these days. Prepoured polyacrylamide gels of various %T and %C are also commercially available.

Polyacrylamide gel electrophoresis is one of the most commonly used electrophoretic methods. Analytical uses of this technique center around protein characterization, eg, purity, size, or molecular weight, and composition of a protein. Polyacrylamide gels can be used in both reduced and nonreduced systems as well as in combination with discontinuous and ief systems (39).

An example of the use of polyacrylamide gels in an ief system in combination with immunoblotting is given in (40), where this method is used to detect low quantities of group specific component (GC) subtypes. Another example of polyacrylamide in combination with ief is given in (41), where the technique is used as a screening tool for inheritance of a certain polymorphic protein.

Both the ease of use of this method for characterization of proteins and nucleic acids, and the ability to analyze many samples simultaneously for comparative purposes, have led to the prevalence of this technique. The drawbacks of a polyacrylamide matrix is that acrylamide is a neurotoxin, the reagents must be combined extremely carefully, and the gels are not as pliable as most agarose gels.

4.3. Paper Electrophoresis. Paper (qv) as an electrophoretic matrix was employed in some of the first electrophoretic techniques developed to separate compounds. Paper is easier than a gel matrix because the paper matrix requires no preparation. Besides being easy to obtain, paper is a good medium because it does not contain many of the charges that interfere with the separation of different compounds. Two types of paper employed in this type of electrophoresis are Whatman 3 MM (0.3 mm) and Whatman No. 1 (0.17 mm).

In paper electrophoresis, the sample is placed directly onto chromatographic or filter paper and then exposed to a buffer solution at each end and an electric field is applied. As in most electrophoretic techniques, charged dyes are combined with samples and standards to see the progress of the electrophoresis. The movement of samples on paper is best when the current flow is parallel to the fiber axis in the paper. The paper has high resistance so voltages are

typically much higher than in agarose and polyacrylamide matrices. Like agarose and polyacrylamide matrices, paper is combined with other analytical tools to enhance separation and identification of sample components. For example, paper electrophoresis has been combined with chromatography (42).

The difference between paper electrophoresis and paper chromatography is that electrophoresis separates by charge, whereas chromatography separates by polarity. This combined technique was used to evaluate polymorphisms of the hemoglobin molecule, ie, normal A-type versus the sickle cell S-type. It has been called peptide fingerprinting. This method was later modified (43) to further evaluate peptides and is one technique known as peptide mapping. The peptide mapping technique also uses high voltages to obtain the desired resolution.

Some advantages of paper in electrophoresis are that paper is readily available, easy to handle, and new methodologies can be developed rapidly. The disadvantages of paper electrophoresis are that the porosity of paper cannot be controlled, the technique is not very sensitive, and it is not easily reproducible.

4.4. Capillary Electrophoresis. Capillaries were first applied as a support medium for electrophoresis in the early 1980s (44,45). The glass capillaries used are typically 20–200 μm in diameter (46), may be filled with buffer or gel, and are frequently coated on the inside. Capillaries are used because of the high surface/volume ratio that allows rapid heat dissipation and thus higher voltages than traditional methods. The only limitations associated with capillaries are limits of detection and clearance of sample components.

Limits of detection become a problem in capillary electrophoresis because the amounts of analyte that can be loaded into a capillary are extremely small. In a 20-μm capillary, eg, there is 0.03-μL/cm capillary length. This is 1/100–1/1000 of the volume typically loaded onto polyacrylamide or agarose gels. For trace analysis, a very small number of analyte molecules may actually exist in the capillary after sample loading. To detect these small amounts of analyte, some on-line detectors have been developed which use conductivity, laser Doppler effects, or narrowly focused lasers (qv) to detect either absorbance or fluorescence (47,48). The conductivity detector claims detection limits down to 10^6 molecules. The laser absorbance detector has been used to measure some of the constituents in a single human cell (see TRACE AND RESIDUE ANALYSIS).

Clearance of sample components from a capillary is a problem that has not been as well resolved as detection. Usually, capillary electrophoresis is conducted in a glass capillary that is coated on its inner surface. Some applications call for gel-filled capillaries. Capillaries are difficult to coat or fill with gel, and are too expensive to discard after each use, as is done when using a standard gel. Therefore, all the sample has to be cleared from the capillary before the capillary is used for another sample, much like analytical chromatography. This can be a problem when capillaries are sometimes more than 100 cm long. Another problem arises when analytes absorb to the glass (or coated) walls in the capillary, a frequent occurrence. This latter problem has prevented capillary electrophoresis from becoming a practical method for protein analysis. In coated capillaries, electroosmosis along the capillary walls sometimes adds velocity to the analytes, and clearance is less of an issue. A great deal of research has focused on choosing and optimizing capillary coatings that resist protein fouling and absorb sufficient charge from buffer ions to give electroosmotic flow.

Electroosmotic flow in a capillary also makes it possible to analyze both cations and anions in the same sample. The only requirement is that the electroosmotic flow downstream is of a greater magnitude than electrophoresis of the oppositely charged ions upstream. Electroosmosis is the preferred method of generating flow in the capillary, because the variation in the flow profile occurs within a fraction of a micron from the wall (49). When electroosmosis is used for sample loading, differing amounts of analyte can be found between the sample in the capillary and the uninjected sample, because of different electrophoretic mobilities among the analytes (50). Other methods of generating flow are with gravity, with air pressure or with a pump.

Capillary electrophoresis is a commercially available technique, and has been integrated with most automated lab equipment such as autosamplers, computer peak analysis (the charts generated are called electropherograms), temperature control, and recirculating buffers. Capillary electrophoresis separations are rapid (minutes), require high voltage sources (10,000 V), and a small amount of automation, particularly in sample application, to be feasible. The use of capillaries as a support medium for electrophoresis is advantageous because it avoids the effects of heating that occur in a gel, can be very rapid, and produces a chart recording rather than a stained gel for archiving.

4.5. Free-Flow Electrophoresis. Free-flow electrophoresis is the most common technique for scaling up electrophoresis for commercial application. In this technique, sample compounds are injected into a curtain of buffer that flows between two flat plates, with electrodes parallel to the flow at each end. The electric field is then applied perpendicularly to the flow direction, so that as compounds flow down between the electrodes they separate horizontally and exit the flow field at different locations. The main challenge for this technique is stabilizing the flow to both heating and electroosmotic forces. Sometimes this is done by dividing the flow curtain into cells using semipermeable membranes, which allow proteins and other sample compounds to migrate from chamber to chamber, but restrict flow (see MEMBRANE TECHNOLOGY). Another method is to apply a very low electric field so that little heating and electroosmosis occur, but then to recycle the material through coolers. The material is then sent back through the separating cell (51–53).

Most electrophoretic methods have been tried in a free-flow format, including isoelectric focusing, native zone electrophoresis, and isotachophoresis. Most free-flow electrophoresis equipment has very low [$\sim$1 g/(L $\cdot$ h)] capacity, and resolution is reduced by heating and electroosmotic considerations.

5. Detection Techniques

Most sample components analyzed with electrophoretic techniques are invisible to the naked eye. Thus methods have been developed to visualize and quantify separated compounds. These techniques most commonly involve chemically fixing and then staining the compounds in the gel. Other detection techniques can sometimes yield more information, such as detection using antibodies to specific compounds, which gives positive identification of a sample component either by immunoelectrophoretic or blotting techniques, or enhanced detection by

combining two different electrophoresis methods in two-dimensional (2D) electrophoretic techniques.

5.1. Chemical Staining. Staining techniques that help to visualize banding patterns resulting from electrophoresis vary (54–56). The size and type of the compound as well as the electrophoretic matrix dictate and often limit the variety of stains that can be used. Molecules can be lost during the staining process, so most staining procedures incorporate a "fixing" step either before or in conjunction with staining. With paper electrophoresis, proteins are fixed to the medium by drying the paper. With agarose and acrylamide gels, the fixation of proteins requires additives. Acidic solutions, such as trichloroacetic acid [76-03-9], are commonly used to fix proteins of all sizes. Once proteins are fixed, they can be stained without loss of the separated components.

Amido black is a commonly used stain, but it is not very sensitive. It is often used to visualize concentrated proteins or components that are readily accessible to dyes such as proteins that have been transferred from a gel to nitrocellulose paper. Two of the more sensitive and more frequently used stains are Coomassie Brilliant Blue (R250 and G250) and silver stains. Because these stains interact differently with a variety of protein molecules, optimization of the fixative and staining solutions is necessary for each application. The Coomassie stains are approximately five times more sensitive than amido black and are appropriate for both agarose and polyacrylamide gels. The silver stain is ~100 times more sensitive than Coomassie and is typically used for polyacrylamide gels.

A silver stain is used when proteins exist in a very small quantity or when analysis of as many bands as possible created by separation techniques is desired. One positive application of silver stain is its sensitivity. A drawback of the silver stain, however, is that it is more complex and often requires more troubleshooting to obtain the desired results.

To quantitate proteins from staining, a densitometer aided by computer software is used to evaluate band areas of samples compared to band areas of a standard curve. Amido black, Coomassie Brilliant Blue, and silver stains are all applicable for use in quantification of proteins.

Another method to visualize and identify separation products on a gel is through radioactivity. If a sample is radioactive, the bands that form through migration of a sample are subsequently radioactive. This type of gel may be placed against an X-ray film until the radiation makes a mirror image of the banding pattern on the film. The film is then developed and the resulting autoradiograph displays the bands.

As an alternative to radiation, a stain such as ethidium bromide is used to visualize DNA. The ethidium may be incorporated into the structure of DNA either before or after electrophoresis. The gel is then visualized under a fluorescent lamp.

5.2. Immunoelectrophoretic Techniques. The technique of gel electrophoresis has been successfully combined with immunological techniques in order to further evaluate molecules. Specifically, the concept of double immunodiffusion as described in 1948 (57) and that of single-radial immunodiffusion described in 1963 (58) have been further developed for use with electrophoresis in both the clinical and research settings.

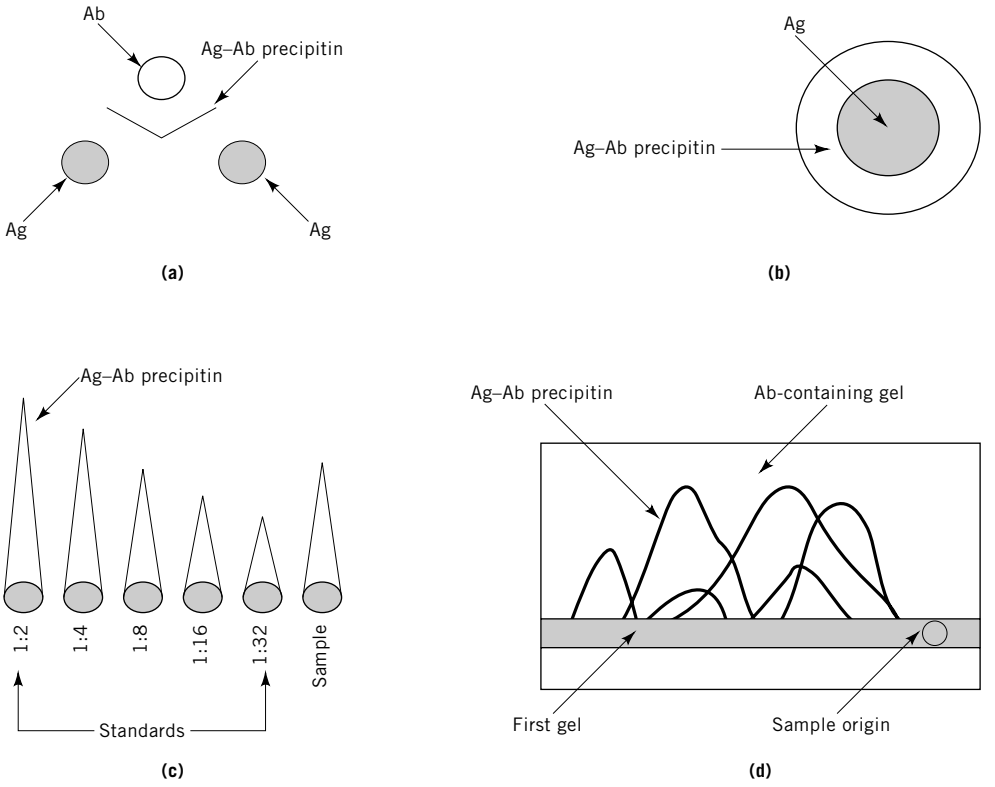

Fig. 3. Immunological reactions, where Ag is antigen and Ab is antibody, for detection in electrophoresis: (**a**) Ouchterlony technique; (**b**) single-radial diffusion; (**c**) rocket immunoelectrophoresis; and (**d**) crossed immunoelectrophoresis.

The double-immunodiffusion technique, often referred to as the Ouchterlony technique, uses an agarose gel as the matrix. Holes are made in the agarose where either sample or antisera is placed. The two solutions are allowed to diffuse into the matrix for a predetermined time. If there is a reaction between the antigen in the sample and the antibody, a precipitate is formed. The Ag–Ab reaction line can be visualized after a stain is used (Fig. 3**a**). Similarly, the single-radial immunodiffusion (Fig. 3**b**) technique is a reaction of equilibrium between antigen and antibody. The difference is that the antigen is added to a warmed agarose solution before it gels. Holes are cut in the gel as for the Ouchterlony technique where samples are placed. The sample diffuses into the gel and, if reactive with the antibody, forms an area of precipitation around the hole that is visualized after staining.

The Mancini and Ouchterlony techniques are the basis of the techniques employed in immunoelectrophoresis. A technique referred to as rockets (59) is named as such because of the appearance of a rocket-shaped antigen–antibody precipitin formed after an antigenic sample is electrophoresed through a gel-containing antibody (Fig. 3**c**).

Another frequently used method of immunoelectrophoresis is a technique known as crossed-immunoelectrophoresis (Fig. 3**d**) (59). A sample is first run vertically through an agarose gel for a predetermined time. Secondly, the gel area where the sample was electrophoresed is typically cut out and placed horizontally into a similarly sized area of an antibody-containing gel. As an electrical current is applied to the second gel system, the sample in question electrophoreses through the gel and forms an antigen–antibody precipitin over an area that varies from small to large, depending on the banding pattern of electrophoresis from the first gel system.

At a somewhat more basic level, both agarose and acrylamide gel systems have been used for direct immunofixation. In these gels, samples are electrophoresed and then immunofixed by either using strips of cellulose acetate soaked in an antibody or the antibody is placed directly over the sample area of the gel.

All of these techniques are most often, but not exclusively, used in the clinical setting in order to diagnose abnormalities or to evaluate inheritance patterns of polymorphic proteins. Many applications of these techniques exist (60–64).

5.3. Two-Dimensional Electrophoresis. Two-dimensional (2D) electrophoresis is unique, offering an analytical method that is both reproducible and sensitive. It is referred to as 2D because it employs two different methods of electrophoresis, in two different dimensions, to produce one result. Each method separates the sample compounds based on different properties of each compound. The combination of the two methods gives better resolution of the compounds in the sample than could be achieved with either method alone. For example, each method alone may separate up to 100 components of a sample, whereas together they may separate up to 10,000 components.

A pair of electrophoretic techniques commonly employed in 2D analyses are isoelectric focusing (ief) and SDS–polyacrylamide gel electrophoresis (SDS–page). Ief separates sample compounds according to isoelectric point, whereas SDS–page separates the compounds by molecular weight. A 2D analytical technique using ief and SDS–page to separate total protein results in a gel having bands or spots in a random pattern (65). Each spot represents a unique component of a sample. A single charge difference in a component can be identified on the gel by a unique spot. This property of 2D electrophoresis, which allows identification of identical proteins that differ by one charge difference, has made it an invaluable technique for the molecular genetic community. Software is available to identify each spot on a gel and compare abnormalities in samples such as human blood (66), *Escherichia coli*, and yeast proteins.

5.4. Blotting Techniques. Problems encountered when trying to analyze resolved components of a sample mixture on a gel, with techniques such as direct immunofixation or application of a ligand, can be circumvented with the use of blotting techniques followed by staining or autoradiography. It was the inability of some compounds, such as antibodies or ligands, to enter the gel matrix of a specific gel system that led to the development of various blotting techniques which have become widespread since the late 1970s. The nucleic acid and protein blotting techniques have become useful because these combine electrophoretic analyses and sensitive immunological tools. A blotting technique involves the transfer of nucleic acids or proteins, immediately after being separated by electrophoresis, from the gel matrix to another matrix. Typically, the

other matrix is nitrocellulose paper, nylon, or other high affinity membrane and the mode of transfer is electrotransfer for proteins and capillary transfer for nucleic acids. Once nucleic acids or proteins are transferred, further analyses can be performed. On nitrocellulose paper or nylon, nucleic acids and proteins are more accessible than in the original gel matrix. This second matrix is then treated with a ligand to identify a specific component of a sample.

For example, the technique of Southern blotting was developed (67) for use with agarose gel electrophoresis of DNA fragments. Southern blots are designed to detect specific sequences of DNA. After electrophoresis is complete, the DNA is denatured and the single-stranded DNA transferred to the specially prepared nitrocellulose paper. The nitrocellulose is then incubated with radioactive RNA or DNA complementary to those DNA sequences of interest. After the nitrocellulose has been sufficiently incubated with the radioactive complementary DNA, autoradiography is used to identify the fragments of interest.

The Northern blotting technique is similar to the Southern blotting technique with the exception that Northern blots detect specific sequences of RNA, not DNA.

The technique of immunoblotting, often referred to as Western blotting, is frequently used for a variety of applications where protein concentrations are low and staining of the electrophoretic matrix does not produce adequate resolution. In these instances, as with Southern blotting, proteins are transferred to a second matrix like nitrocellulose or nylon and are then treated with antisera specific to a desired protein (68,69).

Blotting techniques may be used in a variety and combination of electrophoretic systems that makes their use widespread and convenient when protein concentrations are minimal and agarose or polyacrylamide is the matrix choice.

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