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CHAPTER

5

Electrophoresis

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5.1 Principles

Whether in solution or suspension in water, in aqueous solutions or other liquids, macromolecules (proteins, nucleic acids), particles, emulsion grains, or even bacteria all tend to be displaced under the effect of an electric field. This is the phenomenon of *electrophoresis*, discovered in 1892 by Linder and Picton, which was developed as much as an analytical as a preparative method in the 1930s by Tiselius (Nobel, 1949).

The importance of the electrophoretic phenomenon, other than its theoretical interest, resides in its wide use in analytical and preparative processes, and more particularly, for the study and fractionation of proteins and nucleic acids.

Electrophoresis is certainly the *most commonly used technique of macromolecule fractionation by biologists and biochemists today*. There are several reasons for this popularity. It is a high resolution technique, but can also be used at the preparative level. It is easy to perform, and its cost is relatively low. It can furnish data on the charge, conformation, and shape of molecules. And finally, it is a polyvalent technique that can be used in parallel or in series with numerous other biochemical techniques. This method has been responsible for the discovery, characterization, and purification of several animal and plant tissue components, as well as the study of pathological serums and the testing of quality and composition of several food products.

Using electrophoresis assumes first that the molecules or particles in question possess an electric charge. For ionizable macromolecules, the origin of the charge is obvious, but in general, numerous substances can acquire an

electric charge upon contact with certain liquids. In such an instance, silica or glass acquire a negative charge in contact with water by losing H^+ (silica) or Na^+ (glass) ions. In addition, molecules can be given charge when bound to ionized ligands (e.g., sodium dodecylsulfate).

The electric charges are also assumed to be localized on the surface of a particle, which (according to the principle of electroneutrality) imposes the presence of a layer of ions of the opposite charge around the particle which exactly compensates for the charge of the particle.

The classical theories of electrophoresis are based on the structure of the double electric layer and are derived from the theories of *electroendosmosis*. Electroendosmosis corresponds to the displacement of a liquid along the length of a solid surface under the effect of an electric field parallel to the surface. This displacement occurs because of the fact that when a solid surface acquires charge, for example, a negative charge when in contact with water, the water is found, in return, to have a positive charge in relation to the surface, and since the solid is immobile, it is the water that migrates (in the opposite direction) when an electric field is applied.

In an actual electrophoresis, a solid or liquid particle can have its own charge or acquire a surface charge when in contact with an aqueous solution, just as the solid surface in electroendosmosis. However, contrary to the preceding example, the particle is not immobilized. In an electric field, it is carried by the attraction its global surface charge has toward the electrode of oposite charge. The particle can sometimes be slowed by mobile counter-ions that accumulate in front of the moving particle and move by drawing the liquid the length of the particle surface in the direction opposite to the particle migration before being dispersed behind the particle.

Electrophoresis differs from *electrolysis*, although in both cases the charge transfer causes the displacement of ions. However, in electrophoresis, one of the ions is a macro-ion of greater importance than its counter-ion, and above all, with electrophoresis, the separation of charged particles is studied by comparing their speed, whereas electrolysis only involves obtaining products at the electrodes by discharging ions.

There are *four principal modes of electrophoretic techniques*, each with numerous variables:

1. mobile front electrophoresis (today it is practically abandoned)
2. zone electrophoresis or support electrophoresis (presently the most widely used)
3. isoelectric focusing
4. isotachophoresis

Regardless of the technique, electrophoretic separation rests on the principle of ion or charged molecule migration in an electric field. At the end of a support electrophoresis, ions or molecules that have different migration speeds will have separated themselves and are detected at different locations. A natural protein mixture, like blood serum for example, generally separates into a large

number of polypeptides seen in the form of a "band" diagram, or a card of "spots," characteristic to the sample.

5.2 Fundamental Laws and Concepts

5.2.1 Protein Ionization

All protein separations based on their electric charge depend on their acid or basic character, which is essentially determined by the number and type of ionizable R-groups in their polypeptide chain and the pH of the environment. Since all proteins differ in their composition and amino acid sequence, each has its own acid/base character.

Remember that *amino acids* are amphoteric substances that can be ionized, as the classic schematic in Figure 5.1 illustrates.

In peptides and proteins, none of the alpha-amino and alpha-carboxylic groups involved in peptide bonds can be ionized. The N- and C-terminal and R-groups (diacidic, and dibasic residues) of the chain can be ionized, contributing to the overall charge. The protein's *net electric charge* thus corresponds to the algebraic sum of the electric charges found on the surface of the molecule. This net charge is obviously a function of the pH of the medium and the denaturation state of the protein. It is a very important parameter for all types of electrophoretic separation.

A protein's *isoelectric* pH (pH_i) is defined as the pH at which the molecule carries no net charge, preventing it from migrating in an electric field. The pH_i is determined by the number and pK of the ionizable R-groups. It can be high (>7.0) if the protein has a high content of basic amino acids (lysine, arginine), such as is found for ribonuclease (pH_i 9.6). On the contrary, the pH_i is lower if the protein contains predominantly acidic residues (aspartic and glutamic acids), such as is found for pepsin (pH_i 1.0). Most globular proteins have pH_i values between 4.5 and 6.5.

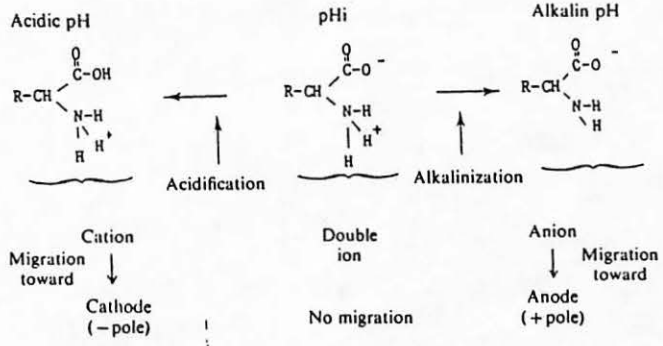

Figure 5.1. Dissociation reaction of amino acids.

For all pH values higher than its pH_i, the protein has a net negative charge, and therefore migrates toward the anode, with the negative charge increasing at higher pH values. Likewise, for all pH conditions less than the pH_i, the protein has a net positive charge, and therefore migrates toward the cathode. To better understand the principles behind protein electrophoretic separation, and to predict their behavior in an electric field, it is recommended to determine their complete *titration curve.*

However, the shape of the titration curve, and the pH_i can change significantly in the presence of neutral salts, which influence the degree of ionization of the R-groups. Proteins can also bind ions like Ca^{2+}, Mg^{2+}, and HPO_4^- in such a way that the observed pH_i can be influenced by the medium in which it is dissolved.

5.2.2 Other Protein Properties that Influence Electrophoretic Separation

Electrophoretic separation of macromolecules also depends on their shape (using its Stokes radius). However, particles with a pronounced ellipsoid shape and an asymmetric distribution of surface charges tend to align their wide axis perpendicular to the field, and therefore migrate more slowly than predicted by their known molecular mass. The *shape and size of the macromolecule* are particularly important when using starch or polyacrylamide gels, which work with a strong molecular sieve effect.

Proteins also differ by their solubility, which must be taken into account when choosing an electrophoretic technique, since a protein cannot be studied unless it has been solubilized. In principal, water soluble proteins, such as those found in serum, do not pose problems in any of the types of electrophoresis. On the other hand, other proteins that are associated to membranes by hydrogen bonds, hydrophobic or ionic interaction, require particular treatments for their solubilization (such as high concentrations of salt or other dissociating agents, like urea, guanidinium chloride, dimethylformamide, diluted acids, detergents, or disulfide reducers). Ionizable agents whose action is only efficient at high concentrations (Gu-HCl, salts) cannot be used in electrophoresis.

Certain proteins are particularly *hydrophobic* due to a predominance of hydrophobic amino acids (phenylalanine, tryptophane, leucine, etc.) or surface lipids. These species can only be solubilized in alcohol solvents (ethanol, butanol) or detergents. Detergents possess a strongly polar hydrophilic (e.g., Triton, Tween, Nonidet P40, Brij 35) or ionic (SDS, CTAB) end, and a hydrophobic chain that interacts at the appropriate sites on the protein by hydrophobic interaction. Detergents associate with hydrophobic proteins rendering them water soluble. Nonionic detergents do not significantly affect the charge of the protein, while ionic detergents break all noncovalent bonds in the protein. Ionic detergents, when associated with disulfide reducers (β-mercaptoethanol, dithiothreitol [DTT]), can totally destroy a protein's tertiary structure.

Since there is a formation of *protein-detergent micelles* around the unfolded polypeptide chain, the native charges of the molecule are lost through the covering of the ionic detergent. These micelles have an ellipsoidal shape, so that the long axis of the complex is related to the molecular mass of the incorporated polypeptide chain (considering that the charge density per unit length is constant). In these conditions, the sign and density of the charges on the surface of the micelle are uniform, and the mobility of the micelle only a function of the molecular size of the protein.

5.2.3 Electric Field, Current Intensity, and Density in an Electrolyte

Without becoming preoccupied with chemical transformations that occur at the electrodes when passing a current through an electrolyte, the transport of the current within the electrolyte will be discussed.

This transport occurs through the electrolyte ions, not electrons. Its study involves what can be called *ion kinetics.*

When a potential U is applied between the electrodes of an electrolytic cell of length l, it creates an *electric field*, by definition: $E = U/l$ (with E in volts/m).

In the simplest case (flat, parallel, identical, large surface area electrodes), the field is uniform, and the current lines are straight, and parallel. The *density* of current i in the electrolyte, and on the electrodes is then defined as the intensity crossing a unit surface area: $i = I/s$. This is expressed in amperes/m^2 or mA/cm^2, depending on the units chosen for I and s.

5.2.4 Protein Migration: Electrophoretic Mobility

During displacement in an electrolyte, ions are subjected to two opposing forces. One is the electrostatic force (F), which is equal to $q \cdot E$ or $z \cdot e \cdot E$, where q designates the ion's charge, z the electrovalence of the ion, e the elementary charge, and E the electric field (V/m) at the point in question. The other is the frictional force (f), which depends on the ion velocity v, its size (r = ion radius), and the viscosity of the medium η, expressed as:

$$f = 6 \cdot \pi \cdot r \cdot v \cdot \eta$$

After a short time, f becomes equal to F, therefore the ion or protein reaches a maximal velocity v defined by the relation:

$$F = f = 6 \cdot \pi \cdot r \cdot v \cdot \eta$$

therefore, $q \cdot E = z \cdot e \cdot E = k \cdot v$.

The relationship μ between the protein's velocity v in the direction of the current (in relation to a reference, usually the solvent) and the electric field E

which directs the migration, is then called the *protein mobility*: $\mu = v/E$ (or $v = \mu \cdot E$), also expressed as:

$$\mu = \frac{v}{E} = \frac{1}{6 : \pi \cdot \eta} \cdot \frac{q}{r}$$

From this relationship, the mobility of a particle migrating in a uniform field can be deduced depending on three factors:

- that it is proportional to its charge
- that it is inversely proportional to its radius (and is influenced by the porosity and sieve strength of the support in which the electrophoresis takes place)
- that it is inversely proportional to the medium's coefficient of viscosity

Therefore, there should not be any confusion between mobility, which is a constant characteristic of the particle in a given medium, with the migration velocity, which depends on the electric field. In fact, when the mobility of a protein is measured, only an apparent mobility is found, a quantity that varies according to the nature of the electrophoretic support, and which is only identical to the theoretical mobility if the viscosity of the support is the same as water.

Mobility possesses signs, which are positive for cations, and negative for anions. An ion's mobility can also be expressed as its velocity in m/s in an electric field of 1 V/m. The units for mobility are therefore $m^2/V \cdot s$.

Diverse relationships can be established between the conductivity γ and the concentration C of the electrolyte, the electric field E, the current I and the area s of the electrophoretic cell, as well as Faraday's constant $\mathfrak{F}$ and the ionic valence z (for more details, see the work of Maurer, 1971 or Simpson and Whittaker, 1983), for example: $I = E \cdot s \cdot \gamma$, or $I = E \cdot \mu \cdot C \cdot s \cdot \mathfrak{F} \cdot z$.

For a strong binary type A^-/B^+ electrolyte ($C_A = C_B = C; z = 1$), I becomes:

$$I = E \cdot \mathfrak{F} \cdot C \cdot s(\mu_A + \mu_B)$$

In cases concerning weak electrolytes (incomplete dissociation, with coefficient of dissociation x), it becomes:

$$I = E \cdot \mathfrak{F} \cdot C \cdot x \cdot s(\mu_A + \mu_B)$$

In general cases (mixture of n ions of concentrations C_i, having valences z_i, and mobilities μ_i), the total current intensity carried by the group of ions is expressed as:

$$I = E \cdot \mathfrak{F} \cdot s \Sigma C_i \cdot z_i \cdot \mu_i$$

A solution's conductivity is therefore constituted of the sum of the contributions of each of the constituent ions, with the conductivity contribution of ion i, written as (the Kohlrausch equation):

$$\gamma = \mathfrak{F} \cdot C_i \cdot z_i \cdot \mu_i$$

It thus appears that the contributions of ions' conductivity to the solution's conductivity are related to the individual mobilities of each of the ions.

Electrophoretic analysis is precisely founded on the differences in mobility between charged macromolecules (also on the manner in which the mobility varies as a function of the pH).

5.2.5 Theory of Concentration Effects in Discontinuous Electrophoresis and Isotachophoresis

Kohlrausch observed in 1897 that by subjecting two solutions (A and B) superimposed in a cylinder to an electric field (solution A, by hypothesis, containing substances a of higher mobility but the same sign of substances b in solution B, as well as a counter-ion c in common) a boundary appears between A and B. This boundary maintains itself while being displaced in the electric field (from which the term *moving boundary* comes), showing that substance a and b, from each of the sides of the boundary, "arranged" themselves for migration, without mixing, at the same velocity $v_a = v_b$.

From the Kohlrausch equation above, it is possible to express the relationship between the total concentrations of molecules a and b in order to create and maintain a moving boundary between solutions A and B in the following manner:

$$\frac{[A]}{[B]} = \frac{x_b \cdot C_a}{x_a \cdot C_b} = \frac{\mu_a \cdot z_b(\mu_b - \mu_c)}{\mu_b \cdot z_a(\mu_a - \mu_c)}$$

This "moving boundary equation" shows that, due to the judicious choice of the ionic nature, and pH of the medium, it becomes possible to control the relationship between the ion or protein concentrations on both sides of the boundary. In creating a discontinuity in the pH and/or the gel concentration, Kohlrausch's principle, a *concentration effect* can be used to form very fine protein bands, and therefore improve the resolution of the electrophoretic technique. In the classic case where a, b, and c are, respectively, glycine, Cl^-, and K^+ ions with $z_a = -1$ and $z_b = -1$, it can be calculated (Ornstein, 1964; Davis, 1964; Maurer, 1971) that:

- for the glycine ion: $\mu_a = -15$ mobility units (assuming $x_a = 1$)
- for the Cl^- ion: $\mu_b = -37$ mobility units
- for the K^+ ion: $\mu_c = +37$ mobility units

where $[gly]/[Cl^-] = [A]/[B] = 0.58$.

In reality, the mobility of glycine is not μ_a, but $\mu_a \cdot x_a$, where the dissociation coefficient x_a is a function of pH. Otherwise, above pH 8.0, numerous proteins (serum) have mobilities from -0.5 to -7.5 units. If the net mobility of glycine $\mu_a \cdot x_a$ became less than -0.5, the net mobilities of the proteins would fall to between those of glycine and Cl^- (-37). This condition can be satisfied if $x_a = 1/30$ (because $\mu_a = -15$). The pH at which this degree of dissociation

exists can be calculated by:

$$pH = pKa - \log[(1/x_a) - 1]$$

Therefore, in these conditions, pH = 8.3.

If, for example, a protein with a mobility of -1 is placed in a glycine solution at pH 8.3 at 1 cm above the gly/Cl^- boundary, when the boundary advances 1 cm (net mobility $\mu_a \cdot x_a = -0.5$), the protein will have migrated 2 cm (since its mobility $= -1$), and therefore will have reached the boundary. It then continues to migrate to the boundary because the mobility of the Cl^- is greater than that of the protein.

If a large number of albumin molecules in solution (μ approximately -6.0 units) are placed in the glycine (pH 8.3), they concentrate at the gly/Cl^- border at the concentration predicted by the Kohlrausch formula, this time using *a* for the albumin serum. In this case:

$z_a = -30$ (approximate charge of an albumin molecule at pH 8.3)
$z_b = -1$
$\mu_a = -6$ units
$\mu_b = -37$ units
$\mu_c = +37$ units

therefore, $[\text{albumin}]/[Cl^-] = 9.3 \times 10^{-3}$. If $[Cl^-] = 0.06$ M, then [alb] = 0.00056 M. That means that the albumin (68,000 g/mol) will concentrate to about 3.8% behind the Cl^-, and then stay at a constant concentration. If the initial albumin concentration had been 0.01% in the glycine buffer, and if 1 ml of mixture had been placed on top of a Cl^- solution in a cylinder with a 1 cm^2 cross-sectional area, then after a 1 mm displacement from the boundary with the Cl^-, all the albumin molecules would have been concentrated in a zone directly behind the Cl^- that would only have been 25 mμ thick. In this manner, a 380-fold increase in concentration can be achieved in a few minutes, with the volume occupied by the protein found in an extremely thin disk. If the concentration had been 0.0001%, the same final concentration would have been reached, with a concentration factor of 38,000.

If, in place of a single protein, 1 ml of a mixture of proteins with mobilities ranging from -1.0 to -6.0 units is placed behind the Cl^-, when the boundary advances 1 cm in the electric field, all the proteins will have been concentrated into very thin bands, stacked one on top of another in decreasing order of mobility, the last being immediately followed by glycine. This procedure is performed in a large pore gel layer called a *stacking gel* (see Figure 5.2).

If the border with the Cl^- (and the stack of protein bands that follow) is then made to pass through a region of smaller pore diameter (which causes a sieving effect or higher friction, both of which can change the order of mobility of the various ions), the mobility of the fastest protein can then fall behind that of glycine. The glycine would then overtake all the protein bands and migrate to just behind the Cl^-. The group of proteins would then find themselves in a uniform voltage gradient, and therefore would begin to migrate as though in

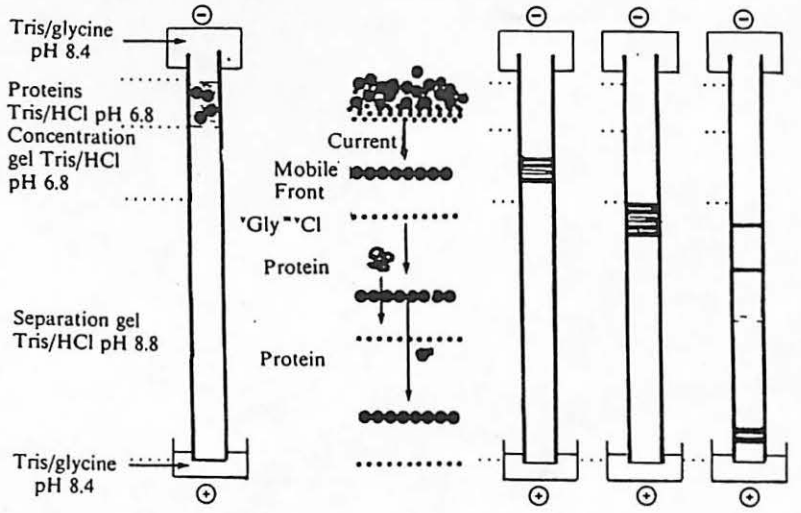

Figure 5.2. Principle of discontinuous electrophoresis.

a normal electrophoresis (in a second gel called a *separation gel*), but starting from a very thin departure zone.

The preceding phenomenon can be further enhanced if the proteins pass through a zone of higher pH. Take pH 9.8, for example, the pK of glycine, where x_{gly} is equal to 0.5 by definition and its mobility is thus -7.5 units. These conditions also make glycine pass the proteins and migrate to just behind Cl^-, leaving the proteins in a uniform voltage gradient as before. The same principle intervenes in the discussion of isotachophoresis (see Section 5.4.4).

5.3 Practical Considerations

5.3.1 Equipment for Electrophoresis

All types of electrophoresis require certain basic equipment: a stable source of continuous current that can deliver a few hundred volts at several dozen milliamperes, a cooling system that absorbs the heat due to the Joule effect while the current is being applied, extraction and centrifugation equipment for the protein or nucleic acid extracts, microsyringes for depositing the samples, etc.... none of which will be discussed here. This section will discuss the materials that are specifically designed for protein migration.

Each apparatus possesses a number of advantages and disadvantages, and is more or less adapted, usually in its design, to resolve one fractionation problem or another. There are, for example, designs for vertical gels, horizontal plates (see Figure 5.3) and vertical plates. Putting aside certain cases of first dimension separations in two-dimensional electrophoreses, today, plate gels

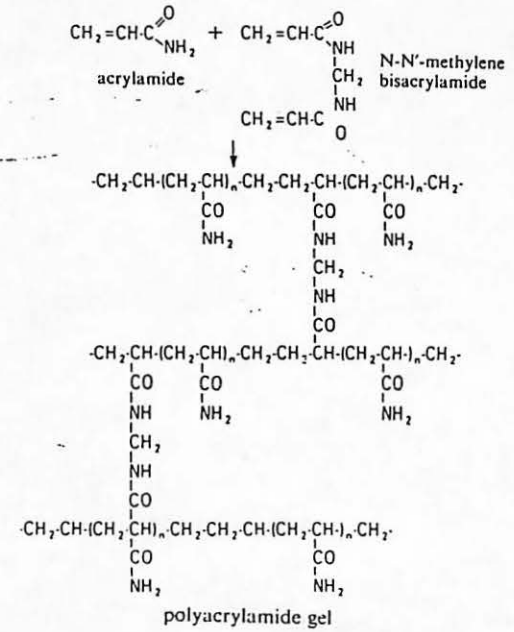

Figure 5.3. Acrylamide polymerization.

are preferred over tube gels because of their following advantages:

- better heat reduction (especially in current ultra-thin plates) and reduced band distortion caused by heat
- much easier densimetric and photographic analysis; more precise band mobility comparisons between different samples
- reproducible gel preparation, allowing simultaneous analyses of several samples under the same conditions

Gel plates are generally 12 × 14 cm, 7 × 7 cm (minigels), or more recently 3 × 3 cm (Phast-System), contrasted to 20 × 20 cm gels for bidimensional analyses, or 20 × 80 cm gels for nucleic acid sequencing. Their thickness ranges anywhere from 0.75 to 3 mm, but ultra-thin gels (400 or 200 μm) are becoming more popular. The number of samples that can be analyzed simultaneously in the same apparatus ranges from 10 to 48, or even up to 96. Depending on the shape and thickness of the gel, as well as on the type (size and charge) of protein, optimal separations can be obtained either at low voltage for 4, 6, 8, or 24 hours, or at high voltage (1,000 to 3,000 volts) for only 40 or even 20 minutes. At higher voltages, an efficient temperature regulated water cooling system is absolutely essential.

5.3.2 Choice of Support

In zone electrophoresis, differently from liquid electrophoresis, proteins move in a hydrated solid support (limiting diffusion) that is reticulated (producing the molecular sieve effect). There is high resolution in this technique because of the proteins separating themselves as a function of their two most important characteristics: net electric charge and molecular bulk.

Since the birth of electrophoresis, various types of supports have been used: paper, cellulose acetate, agar gels, starch gels, polyacrylamide gels, polyacrylamide-agarose gels, and more. A list of each of the respective advantages and disadvantages of starch and polyacrylamide gels has been compiled by Autran (1984). Depending on the nature of the protein being studied and the type of problem to resolve, a support judged as favorable following empirical trials is chosen. It is desirable to consider the following factors in this choice:

- resolving power
- mechanical resistance (the greatest drawback for starch gels)
- simplicity of preparation (polyacrylamide gels)
- electroendosmosis problems (agar gels)
- structure reproducibility (gels possibly sold ready to use)
- cost and purity of commercially available products
- toxicity (acrylamide)

The most frequently used gels for protein analysis are currently *polyacrylamide gels* (PAGE or SDS-PAGE). Some of these gels are available in a ready-to-use form. They sometimes can be preserved dehydrated, and rehydrated in the desired buffer at the time of use. Agarose gels are used in immunoelectrophoresis (see Section 5.4.6). Starch gels are being used less, but can offer very good resolution with certain proteins. Agarose gels are also used for nucleic acid electrophoresis.

5.3.3 Continuous and Discontinuous Systems

Zone electrophoresis systems in which the same buffer ions are present in the gel as in the electrode reservoirs and sample solutions are called *continuous systems*. In these systems, the protein sample is applied directly to the gel in which the separation will take place. This separation gel must have sufficiently small pores to cause a sieving separation of the sample components.

Advancements in polyacrylamide gel electrophoresis (PAGE) were made by Ornstein and Davis (1964) with the *discontinuous method of electrophoresis* (or "disc-electrophoresis"). Note that the word *disc* could have referred to the disk-like appearance of the separated protein bands in the cylindrical tubes that were used at the time. It is also an abbreviation of discontinuous, from the fact that the method is characterized by discontinuities in the buffer system that is used. The disc-electrophoresis method revealed itself as having remarkable

resolution, rendering all previous electrophoretic methods obsolete. Only isoelectric focusing, developed at about the same time, has a comparable resolving power.

Unlike continuous systems, discontinuous (or polyphasic) buffer systems use different buffers between the gel and the electrode reservoir. Most discontinuous systems exhibit discontinuities both in the pH and concentrations. Samples are placed on a large-pored gel ("concentration gel" or "stacking gel") that has been polymerized on top of the actual separation gel (see Figure 5.2).

The main advantage of discontinuous systems is that they can be used with high and relatively dilute volumes of protein solutions while still providing high resolution. As indicated in Section 5.2.5, this results from the concentration effect caused by the inverse relationship between conductivity (or mobility) and the electric field. In a zone where the buffer has a low conductivity, the ions are less mobile, while in a highly conductive buffer, the ions are very mobile. But, since both ion groups are forced to move at the same velocity v on either side of their border, the electric field takes on higher values behind the boundary and lower ones in front, in order to satisfy the relation: $v = \mu \cdot E$. If a protein's mobility is between those of the two zones, it will rapidly become concentrated in an extremely fine layer before entering the separation gel. This provides higher resolution than is obtained without a concentration gel. The buffer pH and ionic strength are naturally calculated so that the mobilities of their ions encompass those of the sample proteins, thus creating the concentration effect.

5.3.4 Importance of Buffer Ionic Strength and pH

Zone electrophoresis (starch gel, polyacrylamide gel) can be performed in a theoretical pH range of 2.5 to 11, restricted in practice from only 3 to 10 due to hydrolysis effects that can intervene at extreme pH levels.

In sodium dodecyl sulfate (SDS) electrophoresis (see Section 5.4.2), SDS-protein complexes are negatively charged in a wide pH range, in such a way that the exact pH value (which especially comes into play in the concentration effect) is generally not critical. On the other hand, the pH value is highly critical in continuous systems in nondissociating media because the proteins separate on the basis of their net charge (and size). A change in pH generally causes a change in net charge of the proteins, and therefore in the final results obtained. It is therefore fundamental to work with perfectly controlled pH values, as well as to use buffer solutions to prepare the gels and electrode solutions, because the sample charge will then be constant. The following are classic buffers: acetic acid/acetate (pK = 4.7), lactic acid/lactate (pK = 3.2), Tris-glycine/HCl ($pK_{Tris} = 8.08$, $pK_{gly} = 2.35$), and so on.

When choosing a continuous system, the first condition should be the consideration of the pH range in which the sample proteins are stable. This range can be very narrow if it is desirable to maintain biological activity (such

as in electrophoreses detecting enzymatic activity in the gel (Section 5.4.8), or in preparative electrophoreses intended as purification steps of native enzymes or proteins). The pH range can be broader if the proteins are detected simply by staining.

Within the pH range, the exact choice of pH results from a compromise between two opposing considerations:

- The farther the buffer pH is from the pH_i of the proteins, the higher the proteins charges will be, shortening their migration time (and less band broadening caused by diffusion).
- On the other hand, the closer the buffer pH is to the pH_i of the proteins, the larger the charge differences between the proteins will be, and the greater their chance of separating.

With many protein pH_i values between pH 4–7, the most common compromises are to use buffers in the pH 8.0–9.5 range for more acidic proteins, and pH 3.0–3.5 for more basic ones. It is recommended to perform a systematic preliminary study by progressively varying the pH in the pH_i ranges until finding the optimal separation and resolution for the protein mixture. This can also be done by constructing a titration curve for the proteins.

Remember that the *ionic strength* refers to the ability of neutral salts to influence protein solubility, and that it is a function of both concentration and the number of electric anionic and cationic charges in the solution, as expressed in the classic formula:

$$\text{Ionic force} = 1/2 \, \Sigma \, C_i \cdot z_i^2,$$

where C_i is the molarity of each of the ionic species, and z_i is the valence of the ionic species in a solution containing n ions.

The *choice of buffer and its ionic strength* is extremely important, because the majority of the current crossing the electrophoretic cell is carried by the buffer's ions. Highly mobile ions (metallic ions: Na^+, K^+, or simple anions: F^-, Cl^-), which carry a lot of current due to their mobilities, often must be avoided and replaced by organic ions of lower mobility, particularly those with buffering capacities.

The ionic strengths that are habitually employed are on the order of 0.05 to 0.25. This corresponds to a compromise between (1) low conductivity, which allows the use of high voltages but causes protein-protein interactions, and (2) fewer interactions, but in an environment of higher conductivity where overheating problems can be encountered.

Remember that the buffering capacity increases with the concentration of buffering species, and that it reaches a maximum at the pK of these species (practically speaking, the range of efficient buffering is in a pH interval of $pK \pm 1$).

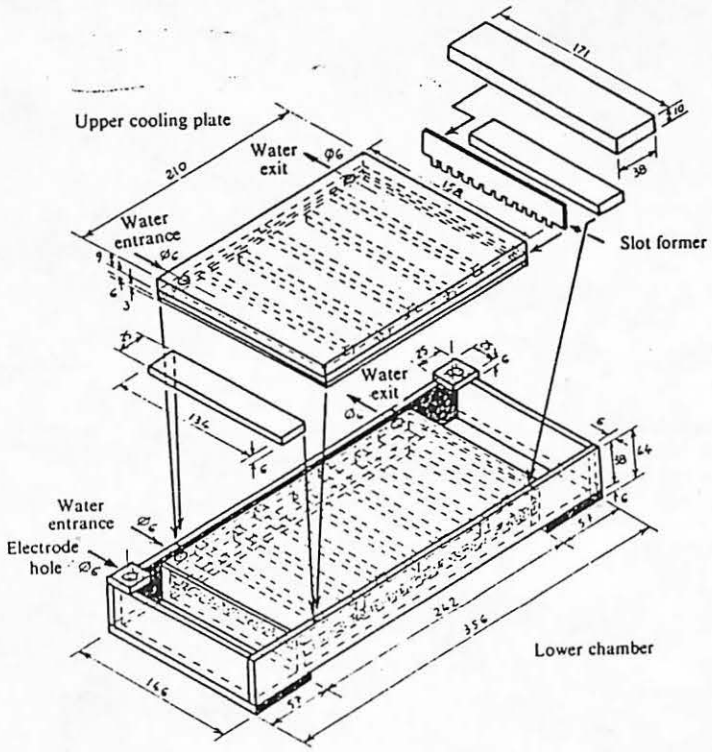

Figure 5.4. Diagram of a horizontal PAGE electrophoretic chamber (Bushuk and Zillman, 1978).

5.4 Current Principal Electrophoretic Techniques

5.4.1 Polyacrylamide Gel Electrophoresis (PAGE)

5.4.1.1 Mechanism of Polymerization

Polyacrylamide gel is the product of polymerization of the acrylamide monomer, CH_2=CH-CO-NH_2, and a bifunctional reagent that cross-links the polymer chains. This "bifunctional cross-linking agent" is usually N,N′-methylene-bis-acrylamide, CH_2=CH-CO-NH-CH_2-NH-CO-CH=CH_2, or "bis." It can be replaced by N,N′-bis-acryllylcystamine (BAC), an analog with a disulfide bridge, or also by diallyltartardiamide (DATD). The three-dimensional network in the gel is created by a vinyllic polymerization mechanism (see Figure 5.4). This leads to the formation of several randomly crossed chains with a high entropy state, meaning, with the most irregular form possible. This structure therefore characterizes the resulting ensemble of microchannels and

pores, through which the protein molecules must move in the electric field while experiencing the sieve effect.

The principal catalyzers that furnish free radicals to the polyacrylamide polymerization reaction are:

- ammonium persulfate / N,N,N′,N′-tetramethylethylenediamine (TEMED)
- ammonium persulfate/3-dimethylaminopropionitrile (DMAPN)
- peroxide/ferrous sulfate/ascorbic acid
- riboflavin/TEMED

The first three are chemical systems, the last is a photochemical system.

Priming the *chemical polymerization* requires the presence of a reduction-oxidation type donor–acceptor system consisting of a promoter or initiator (such as persulfate), and a reaction propagator that must be a base like TEMED. This system forms highly reactive peroxide free radicals that attach to the carbon–carbon double bonds of the acrylamide monomer to start a chain reaction regenerating new free radicals that attack other monomers, thus forming the three-dimensional lattice characteristic to polyacrylamide.

Since bases like TEMED or DMAPN are necessary, it seems that polymerization can be retarded or prevented at acidic pH levels. Molecular oxygen, the cold, or impurities can also prevent this effect. Since slowed polymerizations rarely produce reproducible gels, it is recommended that proper conditions are chosen, that limit gel setting to less than 30, or even 15, minutes.

In photochemical polymerization, the initiator is riboflavin. In the presence of traces of oxygen and TEMED, as well as a source of UV light (which can be provided by a simple neon lamp), it undergoes photodecomposition and reduction, under the action of TEMED, to form the uncolored leuco-derivative, or leucoflavin, which, quickly reoxidized by oxygen, produces acrylamide free radicals that interact to form the lattice.

It is important to consider that by-products may result from the interaction between the protein sample and excess persulfate or riboflavin.

Note: It is also important to note that the term *catalyzer*, which is often used to describe the initiators and propagators used, is not totally accurate, since the substances are either transformed or destroyed by the polymerization reaction.

5.4.1.2 Characteristics of Polyacrylamide Gel

The monomer concentration, in comonomers, as well as the degree of polymerization (chain length) and cross-linking (amount of incorporated N,N′-methylene-bis-acrylamide), determines the density, viscosity, elasticity, and mechanical resistance of the gel. The density and concentration of the gel can be defined by two indices T and C, T being the total acrylamide and "bis" concentration, and C being the concentration of "bis" in relation to the total

acrylamide + "bis":

$$T = \frac{a+b}{V} \cdot 100\% \qquad C = \frac{b}{a+b} \cdot 100\%$$

a = acrylamide (g)
b = N,N′-methylene-bis-acrylamide (g)
V = buffer volume (ml)

The acrylamide concentration directly influences the pore size of the gel. Thus, gels with less than 2.5% acrylamide, which are necessary for sieving proteins of molecular mass on the order of a million, are semi-liquid. At the other extreme, 30% acrylamide gels are used for polypeptides with molecular masses only up to 2,000.

The acrylamide/bis ratio is critical: if it is lower than 10, the gels become brittle; if it is greater than 100, the gels are soft and have a low mechanical resistance. Normal gels, which are elastic and transparent, are made with a ratio of 30 : 1, on the condition that the acrylamide concentration is higher than 3%. Increasing the acrylamide content must normally be accompanied by a drop in "bis" concentration, if the gel's elasticity is to be preserved. The calculations of optimal concentration can be made using formulas in this type: $C = 6.5 - 0.3\,T$ (Maurer, 1971).

It is important to point out that acrylamide and "bis" are neurotoxins. All contact with the skin and mucous membranes must be avoided when dealing with these materials in solution or in crystal form. Contrarily, after polymerization, the polyacrylamide gel is practically nontoxic; however, it is advisable to treat the gel with caution, since it could still contain some nonpolymerized monomers.

5.4.2 Polyacrylamide Gel Electrophoresis using SDS (SDS-PAGE)

Sodium dodecyl sulfate (SDS), $CH_3\text{-}(CH_2)_{11}\text{-}SO_3\text{-}Na^+$, is the most widely used ionic detergent in electrophoresis. Its use serves two functions: (1) to dissociate proteins that are aggregated, hydrophobic, or of low solubility, such as membrane proteins; and (2) to allow the separation of proteins based on their shape, size, and molecular mass.

When there is excess SDS in the medium, it attaches to proteins at a constant mass ratio (1.4 g SDS per gram of polypeptide) and turns them into polyanions. According to the technique's inventors (Weber and Osborne, 1968), the native charges (of the R-groups and the terminal amino acids) of the polypeptide are then insignificant relative to the cloud of negative charges carried by the bound detergent, such that the SDS-protein complexes have, for no matter what protein, essentially identical charge densities, and in principle, migrate in the polyacrylamide gel uniquely as a function of their molecular size.

In reality, this dissociation-denaturation only occurs if the action of the SDS is combined with the action of a disulfide reducer (mercaptoethanol, DTT) at high temperature (1–2 h at 40°C, or 1–2 min at 100°C). In these conditions, the SDS-polypeptide complex assumes a rod-like shape whose length is proportional to its molecular mass.

Since the charge density for all proteins is identical, it logically follows that the Q/r ratio is constant (Q representing the protein's overall negative charge, and r representing its radius), and therefore the electrophoretic mobility ($\mu = k \cdot Q/r$) is the same for each particle. The negative charge causes migration toward the anode, and the particles are separated by the sieving effect.

In addition to the polypeptide composition analysis of the sample, it is therefore possible to get an estimation of molecular mass by comparing the migration distances to those of proteins of known masses that have been calibrated under the exact same experimental conditions, using a formula of this type: log[mol. mass] = $k \cdot$ [distance (cm)].

The simplicity and speed of this technique, besides the fact that it can be used with only microquantities of sample, has made SDS-PAGE the most widely used method for determining molecular masses of polypeptide components of a protein sample. However, it should not be forgotten that it is only an estimation method, because there is a great difference between the masses found by SDS-PAGE and those of other methods (e.g., D-hordeine from barley sugar: 100,000 Da by SDS-PAGE, 50,000 Da by sedimentation equilibrium) due to the shape of the protein (different from the reference), its proline content, or the location of the proline residues in its sequence.

5.4.3 Isoelectric Focusing

The development of this technique near the end of the 1960s by different Swedish teams (Haglund, Rilbe, Svesson, Vesterberg) represented major progress in the field of fine protein separation (see the works of Castimpoolas, 1973 and Arbuthnott and Beeley, 1975). Its procedure involves equipment similar to that used for PAGE; but, contrary to electrophoresis where the separation takes place at a constant pH, isoelectric focusing is performed in a pH gradient that is formed by the migration (preliminary or simultaneous) of amphoteric substances that are commercially available (and are rather expensive) for all possible pH ranges (2.5–4, 4–6, 5–7, 7–9, 3.5–10, etc.) under names like "Ampholines," "Pharmalytes," "Servalytes," and the like. These ampholyte substances have molecular masses of 300 to 700 Da (like peptides), and can be synthesized, for example, by a reaction between acrylic acid and amines like hexamethylene- or pentamethylene- diamine, from which a mixture of hundreds of constituents representing very subtle molecular mass and pH_i variations is created, therefore giving rise to quasi-continuous electrophoretic mobility characteristics. Under the effect of an electric field, these ampholytes then arrange themselves between the anode and cathode in successive layers in the order of their pH_i, thus forming a pH gradient.

When a protein is introduced to such a gradient, for example, at a pH lower than its pH_i, it acquires, by definition, a net positive charge. It will therefore migrate toward the cathode, and also toward the higher pH values, which, in return, influence (reduce) its ionization. Thus, in the course of its migration, the protein's net charge will decrease until it reaches the region of its pH_i, when it has no net charge. It will therefore stop at that point.

Isoelectric focusing is thus a technique that separates proteins by differences in their isoelectric point. The final pattern that is obtained is therefore independent of the place at which the sample was introduced. The protein is assumed to have reached its pH_i whether it began at the cathode or anode, as long as the experiment was allowed enough time. Moreover, when all the proteins have reached their pH_i, the separation pattern does not change, leaving the final result independent of the total experiment time.

It should also be explained that if a protein that has reached its isoelectric point begins to migrate to one electrode or another (due to effects of diffusion), it will acquire the respective ion charge, causing it to migrate [back] in the other direction toward its isoelectric point. *The focusing effect thus acts against the effect of diffusion, so that the separated fractions concentrate in very fine bands with a resolution that cannot be obtained with conventional electrophoresis.*

In theory, it is therefore an equilibrium method that, as long as the pH gradient is stable and well established, is self-correcting and not very demanding in terms of experimental technique. In practice, this is not always true, notably because of the "plateau" phenomenon which tends to reduce the slope of the gradient over time, and "drift" problems, mostly at the basic end. These problems have been overcome by new generations of ampholytes, like the "Immobilines," which covalently bond themselves to the polyacrylamide chains during gel polymerization.

It is also important to note that certain very basic proteins have problems entering electrofocusing gels, or they do not separate very well. A variant procedure, known under the name nonequilibrium pH gradient electrophoresis (NEPHGE), involves placing the sample at the acidic end of the pH gradient and stopping the experiment before the proteins really reach their isoelectric point, and was proposed as a way to overcome these problems, notably for first dimension separations in two-dimensional electrophoresis.

5.4.4 Isotachophoresis

In isotachophoresis, ionic species of the same charge sign having the same counter-ion migrate in an applied electric field. Isostachophoresis involves applying an electric field to a system consisting of:

1. A sample solution containing ions (of the same sign) to separate. The solution must be introduced between:

2. An electrolyte, "leading electrolyte", that must contain only one ionic species, the minor ion (L^-), having the same sign as, and higher net mobility $\mu_a x_a$ than, the sample ions, and
3. A second electrolyte, "terminal electrolyte", that contains one ionic species, the terminal ion (T^-), having the same sign as, and lower net mobility $\mu_b x_b$ than, the sample ions.

In such a system, the Kohlrausch equation applies. There is thus both a concentration effect (seen in very narrow bands and high resolution), an internal rearrangement of the protein layer causing the component proteins to separate based on their net mobilities μx in direct contact with each other, and then, when dynamic equilibrium ("steady state") is reached, *all the proteins migrate at the same speed* (from which comes the term *isotachophoresis*).

The isotachophoresis technique therefore uses effects that are very interesting theoretically. These effects are furthermore used, often without knowing it, in concentration gels of disc-electrophoresis. At a practical level, however, isotachophoresis separations remain difficult to execute, and require sophisticated equipment, whether analytical (capillary columns with UV detection and directly recorded output), or preparative. Furthermore, in order to improve the separation of the components in a protein mixture, it is almost essential to add "spacer" ions that can be the same ampholytes used in isoelectric focusing, also contributing to the high cost of this method.

5.4.5 Two-Dimensional Methods

It is possible to combine two of the electrophoretic techniques previously described to significantly increase resolution by associating two different separation principles (Celis and Bravo, 1984; Galteau and Siest, 1986). Some of the following examples of two-dimensional systems can be described: acidic PAGE × basic PAGE, nondissociating PAGE × dissociating PAGE (6 M urea), acidic PAGE × SDS-PAGE, IEF × PAGE, and so on.

The system that currently provides the best resolving power is one combining an IEF in the presence of urea or neutral detergent in the first dimension, and SDS-PAGE in the second dimension. Such a system (IEF × SDS-PAGE) was described by O'Farrell (1975). This separation is based on the combination of two independent protein characteristics: its charge, reflected by its pH_i, and its molecular size, which determines the mobility of the SDS-protein complex in the SDS-PAGE gel. This method has the extraordinary potential of revealing, for example, more than 1,000 polypeptides (of which, some represent less than 0.001% of the total protein) from a cellular extract. This technique has been very widely used for separating protein mixtures from prokaryotic or eukaryotic organisms. Figure 5.5 shows a separation of proteins found in hard wheat.

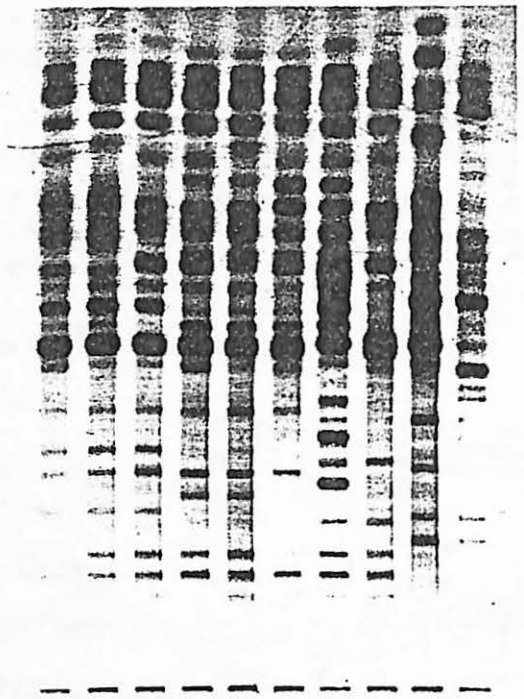

Figure 5.5. PAGE electropherogram of alcohol-soluble proteins (gliadines) in different varieties of wheat (Autran, 1986).

When dealing with certain basic proteins that do not penetrate classic IEF gels, or that do not run well in gels, the NEPHGE × SDS-PAGE method is one of the recommended paths (O'Farrell et al., 1977).

5.4.6 Immunoelectrophoresis

Immunoelectrophoresis techniques exploit both protein mobility in electric fields and their *immunological properties*. In the technique that was initially developed in the 1950s by Grabar, a trough is made in the gel parallel to the direction of the electric field a few millimeters from the edge of the protein migration line. An antibody to the protein is then introduced into the trough. It then diffuses into the gel where it can encounter its corresponding antigen to form (in regions where the antigen/antibody ratio is optimal) insoluble complexes. These complexes can be seen in the form of precipitation arcs having very characteristic shapes and positions (relative to the proteins and their original starting position).

Several variations of immunoelectrophoretic techniques have been described (Daussant and Bureau, 1988; Skerritt, 1988) according to:

1. The principle of the separation: constant pH zone electrophoresis, IEF, isotachophoresis, two-dimensional methods.
2. The electrophoretic support: agarose gel (most common), polyacrylamide (major advantage: no electroendosmosis), mixed gels (polyacrylamide-agarose), cellulose acetate membranes, various other supports (like paper, Sephadex gel, liquid medium).
3. Immunochemical detection: for example,
   - *Antigen/antibody precipitation.* Occurs through double diffusion of antigens and antibodies toward each other. A simple technique, but requires long diffusion times and poses problems in quantitation or interpretation when there are too many arcs.
   - *Immunoelectro-precipitation*, or the "rocket technique." Occurs through electrophoretic antigen migration in a gel containing antibodies. It is quick and provides quantifiable results. It can also occur by electrophoretic migration of antigens that have undergone a preliminary electrophoretic separation (in the perpendicular direction) that are placed in a gel containing antibodies.
   - *Immuno-blotting.* Here, the proteins are first separated by electrophoresis, then transferred to a membrane (cellulose nitrate, nylon, etc.) by diffusion or electrotransfer. A very rapid immunoidentification is then possible using this copy and marked antibodies (radioactive or fluorescent molecules, colloids, or enzymes).

5.4.7 Gel Staining and Quantitation Techniques

Protein bands of sufficiently high concentration can be detected directly by spectrophotometry of unstained gels at 280 nm. This procedure is, however, limited by the relatively low absorption of proteins at 280 nm, light diffraction and also the presence of impurities in commercially prepared acrylamide that absorb in the UV range. Therefore, protein bands almost always have to be identified by using specific stains.

The stain that currently most commonly used for polyacrylamide gels is *Coomasie blue* (G250 or R250) due to its high sensitivity and the simplicity of its use. With this method, 0.2 to 0.5 μg of a protein present in very fine bands can be detected, and the stain can quantitate, in principle, to 15 μg. Other protein-specific stains (amido black, fast green, nigrosine, etc.) are much less sensitive and are not used very often.

In practice, the procedure involves a preliminary staining of the proteins by immersion in a trichloroacetic or perchloric acid bath (12–15%). The actual staining time depends on the thickness and density of the gel. It generally takes a few hours (30–60 min for ultra-thin gels). After staining, it is often necessary to eliminate excess stain and improve the contrast of the gel in a destaining step. This can be done either by immersing and washing a gel in an acidic or alcohol solution, or by another rapid longitudinal electrophoresis.

It would be proper to finally note the recent development of *silver nitrate staining* methods. Several variations have already been described that can be used to attain unequaled detection sensitivity. This method is based on the specific reduction of metallic silver salt in the presence of polypeptides. Following one of the classical procedures, the gels must be incubated in a solution containing methanol and acetic acid, then pretreated in formaldehyde before the addition of a complex mixture containing silver nitrate and copper nitrate. The silver is then selectively reduced in the presence of ethanol, formaldehyde, and citric acid.

Today, this method is by far the most sensitive (comparable to some autoradiography methods), but (although having been greatly simplified) still remains sophisticated and must be performed with great care for cleanliness.

Quantitation of the separated bands in electrophoresis is usually done by densimetry of stained gels, or photography of the gels (Chrambach, 1985). A densitometer consists of an isolated light source that moves across each gel at constant speed. A photomultiplier detects differences in light transmission caused by the protein bands, and sends the signal to a recorder that plots a curve with peaks representing the position and intensity (by area) of the bands. Some densitometers automatically calculate the peak area and construct an analysis report of the relative percentages of each of the peaks, as well as other calculations relating to mobility or concentration calibrations.

It is important to consider all densimetric analyses with prudence. The exactness of the results obtained is dependent on several factors: nonlinear response beyond a certain band intensity, stained bands with different absorptions, protein/stained protein quantity ratio that can vary from one group of proteins or stains to another, problems of gel transparency, background noise making the establishment of a baseline difficult, and more.

5.4.8 Detecting Isoenzymes

After the electrophoretic separation of a complex protein extract, it is possible to reveal those bands within that possess a determined enzymatic activity. The main limitations of these procedures arise from the fact that detection methods exist for only a relatively limited number of enzymes, and that the results are not easily quantifiable. This method is therefore mainly used to identify multiple forms of some enzymes (biological polymorphism) for analytical comparisons between different species, breeds, or varieties.

In general, the gel is first incubated in a bath containing one of the enzyme's substrates and other necessary cofactors, all at the optimal temperature of the enzymatic reaction and the pH and ionic strength most favorable for the reaction. The products of the reaction are then detected using a specific stain that outline in the gel the regions containing enzymes with the activity in

question. For example, to reveal the multiple forms of beta-amylase, the gel is incubated in a starch solution and then stained with iodine. The entire gel is stained, except for the amylase regions (where the starch was hydrolyzed), which appear white on a blue-black background. Likewise, for peroxidases, the gel is incubated in guaiacol or catechol and the bands appear on the addition of peroxide.

5.5 Examples of Electrophoresis Applications in the Agriculture and Food Industries

5.5.1 Identification of Cereal (Barley/Wheat) Varieties in Commercial Lots by Electrophoresis in: Starch Gel (Autran, 1975), Polyacrylamide Gel (Autran et al., 1981; Wrigley et al., 1982; Cooke, 1986), or SDS-PAGE (Montembault et al., 1983; Marchylo, 1987) (see Figure 5.6)

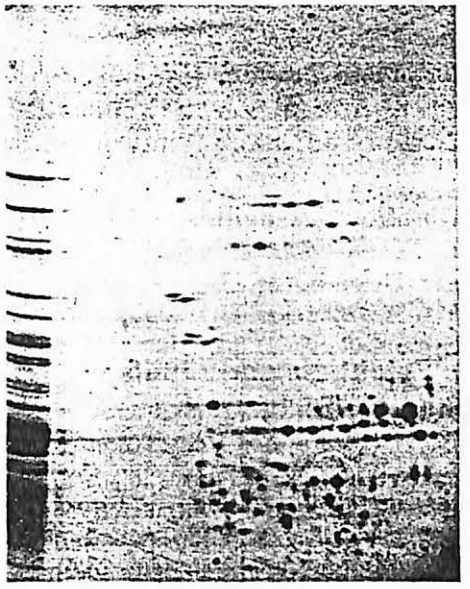

Figure 5.6. Example of a two-dimensional electrophoretic separation of proteins in hard wheat. 1st dim.: IEF in a gradient of pH 4–8.5; 2nd dim.: SDS-PAGE, pH 6.8/8.8 (Morel and Autran, unpublished results).

5.5.2 Predicting the Technological Quality of Wheat Genotypes by Electrophoretic Selection of Gliadins or Glutenins (Autran, 1981; Branlard and Dardevet, 1985; Payne et al. 1987).

5.5.3 Determination of Soft Wheat Flour in Hard Wheat Products and Pasta by Peroxidase Electrophoresis (Kobrehel and Feillet, 1976), or by Isoelectric Focusing of Albumins (Resmini and Bernardi, 1976)

5.5.4 Determination of the Egg Content of Pasta (Kobrehel and Feillet, 1971)

5.5.5 Verifying Fish Species from Sarcoplasmic Proteins (Sobbie and Mackie, 1988; Tao, 1989)

5.5.6 Detection of Cow Milk in Goat Milk or Cheese by Isoelectric Focusing of Beta-Lactoglobulins (Van Eckert, 1988; Addeo et al., 1989)

5.5.7 Detecting Plant Proteins (Cereals, Soy) Added to Products of Animal Origin (Janssen, 1987; Hohlfield, 1988; Nieto Grau, 1988; Windemann, 1988)

5.5.8 Determination of Gliadins or Gluten in Food Products (Coeliac Disease Problems) Using Immunochemical Methods (Dysseler et al., 1986; Freedman et al., 1987)

6 Conclusion: Potentials and Limits of Electrophoretic Techniques

Electrophoresis, under the numerous forms in which it is used today (Hames and Rickwood, 1981; Simpson and Whittaker, 1983; Celis and Bravo, 1984; Jorgenson and Phillips, 1987), is an extremely powerful tool that is universally applicable for analytical purposes, especially for composition and quality testing of numerous food products. It also presents, in relation to other macromolecular methods of analysis, decisive advantages from several points of view, such as: resolving power, sensitivity, speed, and simultaneous and large series analyses of micro-quantities of sample.

However, it is important to be conscious of the limitations of the methods. From a biochemical and genetic point of view, it is obvious that the protein composition and metabolic relationships between the components of living systems are infinitely complex data that cannot be summarized in a few electrophoretic gels. Furthermore, these gels only represent a partial and simplified image of real biochemical polymorphism, because since electrophoretic techniques were founded on charge and size characteristics of the samples, *a very large number of mutations or differences from noncharged amino acid content (70–80% of all units) are not detected.*

It must also be realized that a protein's electrophoretic mobility, although reproducible in established conditions, cannot be considered as a chemical constant of the protein (as density and refraction index can be). Actually, the mobility is a function of both a protein's net electric charge and molecular size. The electric charge, resulting from the ionization of the amino acids at the surface of the molecule, varies in relation to the pH of its surroundings. The size factor, itself influenced by the presence of dissociating agents, more or less affects the separation according to the separating power of the support. The separating power depends heavily on the type of gel used, its degree of cross-linking and pore size, and the catalyzers that are used.

Taking the complexity of natural proteins into account, as well as the fact that components with differences in noncharged amino acids have little chance of being differentiated, it is certain that many apparently pure electrophoretic bands really contain different molecular species. It is therefore surprising that all experimental condition modifications, even when minor or involuntary, can translate into changes in mobility or breaking of bands, thereby yielding different results. For thorough characterizations, one cannot be advised strongly enough to work with rigorously fixed conditions, to combine two or three techniques applying different principles, or to use a two-dimensional technique.

When interpreting any gel, equal mobility of two bands observed under strictly experimental conditions only constitutes a *presumed identification.* If it is found useful to make a simplified reference list of visible bands (by combining bands that are too close to differentiate certainly), the highest care must obviously be observed when trying to establish correlations from these data.

In particular, while the electrophoretic techniques are well-adapted to the study of biochemical polymorphism, it is not evident that they are the best tools for studying a protein's physical, chemical, and functional properties. An electrophoresis gel can be used to construct an inventory and classification list of a number of molecular species. It provides neither information (especially in dissociating conditions after breaking disulfide bonds) on how the components had originally associated, nor on their native structure. For a good number of studies, it is therefore indispensable to use electrophoresis with other techniques that preserve the sample's structure, such as selective extraction, chromatography, or immunochemistry.

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