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Electrokinetic Separation Methods

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ELECTROKINETIC SEPARATION METHODS

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I. INTRODUCTION

Electrophoresis is the transport of dissolved molecules or suspended particles in a homogeneous polar liquid (such as water) under the influence of an electric field. Most molecules or particles acquire a surface electric charge when dissolved or suspended in buffered water (or other polar liquids), owing to ionization or adsorption of ions present in the water. The sign of the surface charge of molecules or particles determines whether they will migrate towards the positive or the negative electrode of the applied electric field, and the velocity of migration depends on the surface potential of the molecules or particles, as well as on the potential of the electric field.

In porous plugs or gels of which the capillaries or pores are electrically charged, or in channels or chambers with electrically charged walls, a polar liquid (such as water) will itself be transported under the influence of an electric field. The transport will be in the direction of the electrode with the same sign as the sign of surface the charge of the inside walls of the capillaries or pores of the porous plugs or cells, or of the inner walls of the channels or chambers. This process is called electroosmosis. As it is extremely difficult to find porous plugs or gels or chambers of which the inner pores or capillaries or walls are uncharged, electroosmosis almost invariably accompanies electrophoresis. Thus the total movement of molecules or particles in an electric field comprises their electrophoretic movement in a polar liquid and the electroosmotic movement of the liquid itself (which frequently is in a direction opposite to that of the particles).

Electrophoretic transport of an amphoteric molecule or particle in a pH gradient leads to its movement to a pH level that is equal to its isoelectric point; from that point on the amphotere will remain immobile in the electric field. This technique is called isoelectric focusing. Electric transport of molecules or particles in columns consisting of discrete groups of ions with different mobilities, resulting in a redistribution of these molecules or particles according to their mobilities, is called isotachophoresis.

Electrophoresis and related phenomena have been studied since the beginning of the nineteenth century; the early history has been outlined by Abramson et al.[1] The earliest methods for obtaining electrophoretic mobilities of dissolved macromolecules, in particular, proteins, used the technique of particle microelectrophoresis, with the expedient of adsorbing the dissolved proteins onto the surfaces of small particles.[2] For particle and cell electrophoresis microelectrophoresis is still the most important analytical technique, but for dissolved macromolecules two enormous advances were made in about 1930 and in the early 1950's with the introduction of, respectively, moving boundary electrophoresis[3] and zone electrophoresis.[4] From the early 1960's on, zone electrophoresis has virtually completely superseded moving boundary electrophoresis.

Various aspects of electrokinetic separation methods have been treated in extenso in earlier issues of this Series. These aspects of electrophoresis and related methods are therefore discussed more succinctly in the present chapter. The subjects that have previously been elaborated upon in these volumes are:

Zero Gravity Electrophoresis Vol. 2, 259, (1973); Vol. 5, 361, (1976); Vol. 6, 1, (1977); Vol. 6, 61, (1977); Vol. 7, 221, (1978).

Continuous Flow Electrophoresis Vol. 2, 153 (1973); Vol. 8, 1 (1978).

Influence of Size and Shape on Electrophoretic Mobility Vol. 2, 259 (1973); Vol. 4, 167 (1975).

Preparative Isoelectric Focusing Vol. 4, 23 (1975).

Isotachophoresis Vol. 6, 287 (1977).

II. ELECTROKINETICS

1. The Electrical Double Layer and the ζ -Potential. Particles suspended in a polar liquid acquire a net electrical surface charge by:[5]

a) their own (possibly partial) ionization

b) adsorption of ions present in the liquid, and

c) (possibly partial) excess dissolution of ions of one sign of charge

A surface charge by these mechanisms attracts oppositely charged ions not only from the liquid in the immediate vicinity, but also from the bulk of the liquid. Thus every charged particle in a polar liquid is surrounded by a diffuse ionic (or electrical) double layer. The only surface potential that is measurable is the potential at the surface of shear, i.e., at that part of the diffuse ionic double layer that forms the boundary between the layer of ions that travel with the charged particle (when it moves with respect to the liquid) and the ions that stay behind in the liquid when the particle moves away from them. That potential is called the electrokinetic or ζ-potential.[5-7]

The thickness of the part of the ionic double layer closest to the particle is approximated by the value $1/\kappa$, introduced by Debye and Hückel[8]

$$\kappa = \sqrt{\frac{4\pi e^2 \Sigma n_i z_i^2}{\varepsilon k T}} \qquad [1]$$

in which e is the elementary charge ($=1.6022 \times 10^{-20}$ emu), k the Boltzman constant ($=1.3806 \times 10^{-16}$ erg per degree K), T the absolute temperature in degrees K, n_i the average concentration and z_i the valency of each of the small ions in the double layer and ε the dielectric constant of the liquid surrounding the particle. The diffuse double layer has a thickness $1/\kappa$ of about 10 Å in 10^{-1} M NaCl, 100 Å in 10^{-3} M NaCl, 1,000 Å in 10^{-5} M NaCl, and $\simeq$ 1 μm in distilled water.[9] The ζ-potential of a given particle also tends to increase (with the thickness of the diffuse double layer $1/\kappa$), when the ionic strength of the suspending medium decreases.

2. Electrophoresis, Electroosmosis, Electroosmotic Counter Pressure. The five main electrokinetic phenomena and the connection between them are schematically indicated in Figure 1. Electrophoresis is the transport of charged particles, in an electric field, toward the oppositely charged electrode, in a polar liquid (Fig. 1A). Electroosmosis is the transport of the liquid medium

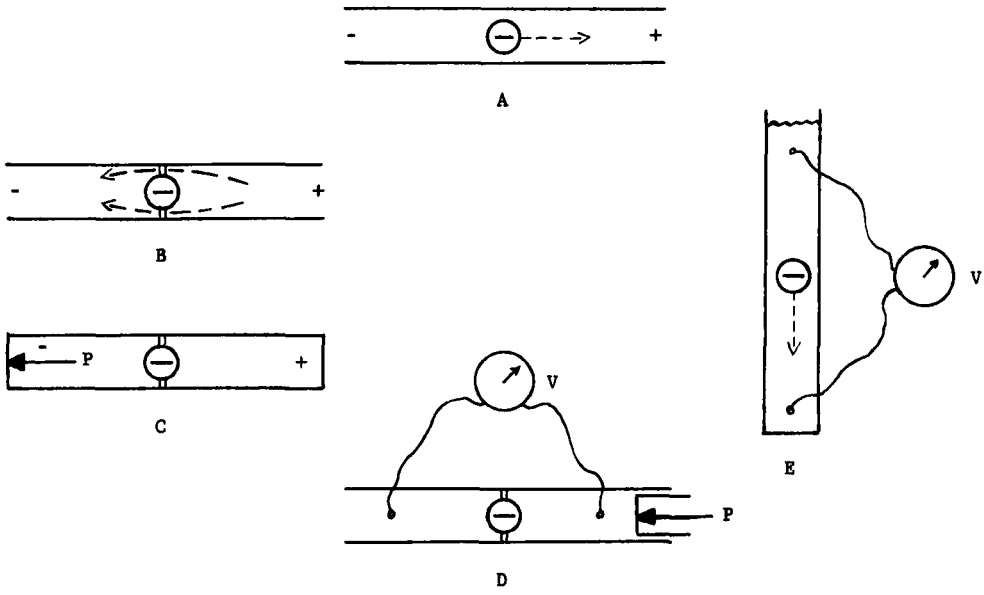


FIGURE 1

Schematic representation of five electrokinetic phenomena: A) Electrophoresis, B) Electroosmosis, C) Electroosmotic counter-pressure, D) Streaming potential, and E) Migration potential; for details see text, Section II, 2. In a clockwise as well as in a counter-clockwise direction each one of these five electrokinetic phenomena depicted above stands in a simple relationship to its closest neighbors; — and + indicate the polarity of an applied electric field; P indicates pressure; ⊖ stands for a particle with a negatively charged surface; ⊖ indicates that such a particle is immobilized by attachment inside the chamber and V designates a voltmeter indicating an electrical potential difference resulting from the last two electrokinetic phenomena (D and E). (Not drawn here is a model of streaming current, which is closely related to streaming potential and has analogies with electroosmosis).

alongside a surface that is electrically charged but immovable, in the direction of the electrode with the same sign of charge as that of the immovable surface (Fig. 1B). Electroosmotic counter-pressure is the pressure that builds up against one extremity of a chamber when electroosmotic flow is prevented by enclosure of the chamber (Fig. 1C). Streaming potential is the electrical po-

tential difference caused by forcing a polar liquid to stream under pressure alongside an electrically charged surface (Fig. 1D). Finally, migration potential is the electrical potential difference caused by the migration (in practice virtually always through sedimentation) of charged particles in a polar liquid (Fig. 1E).

From Figure 1, it is easily seen how these five electrokinetic phenomena are interrelated: Electrophoresis (Fig. 1A) reverts to electroosmotic flow (Fig. 1B) when the charged particles are made immovable. Electroosmotic flow (Fig. 1B) clearly can only manifest itself as electroosmotic counter-pressure when liquid flow alongside a charged surface is forcibly prevented (Fig. 1C). Conversely, when a pressure is applied on the liquid, making it stream alongside a charged surface, an electrical potential difference called streaming potential is created (Fig. 1D). Finally, conversely to electrophoresis (where a charged particle migrates under the influence of an electric field, Fig. 1A), when a charged particle migrates through a polar liquid with, e.g. sedimentation as a driving force, an electrical potential results, called a migration potential (Fig. 1E). Thus a simple relationship can be perceived between any of these five electrokinetic phenomena and its closest neighbor, when classed in the order:

Electrophoresis,
Electroosmosis,
Electroosmotic counter-pressure,
Streaming potential,
Migration potential,
Electrophoresis, etc., (see Fig. 1).

For a more formal treatment of the interrelationship of a number of these phenomena, see Mazur and Overbeek.[10] Not discussed here is, e.g., streaming current, which is closely related to streaming potential, and has analogies with electroosmosis.[10]

TABLE I

Values of ζ for an Electrophoretic Mobility in Aqueous Medium of 1 $\mu m\ sec^{-1}\ V^{-1}\ cm$, as a Function of Temperature [a, b]

Temperature (in °C)	ζ after Hückel; eq. (2), in mV	ζ after von Smoluchowski's eq. (3), in mV
0	34.5	23.0
5	30.0	20.0
10	26.4	27.6
15	23.6	15.7
20	21.2	14.1
25	19.3	12.9
30	17.7	11.8
35	16.3	10.9

a Adapted from ref. 9.

b The values of η and ϵ for water at the various temperatures, needed for the computations, have been obtained from the Handbook of Chemistry and Physics, 51st edition, Chemical Rubber Co., Cleveland, 1970.

3. Influence of the Size and Shape of Molecules and Particles on their Electrophoretic Mobility. Generally speaking the electrophoretic mobility of molecules and particles depends only on their ζ -potential, and not on their size or shape. However, two circumstances may intervene to qualify that general rule, i.e.:

1) when the ratio κa between the radius (a) or dimension of the particle or molecule and the thickness of the diffuse ionic double layer ($1/\kappa$) surrounding it has a value intermediate between very small and very large, and

2) when particles or molecules of a given size range made to electromigrate inside a porous medium with pores sufficient-

ly small to slow down larger particles or molecules more than small ones.

Only the first situation will be discussed in this section; the second will be treated more appropriately in section 5. For the following treatment to be generally valid a number of limitations must first be imposed:[5, 7, 9, 11, 13]

1) only rigid particles or molecules will be considered,
2) the particles' surface charge should be uniformly distributed,
3) only non-conducting particles or molecules will be considered,
4) the particles' movement inside the liquid remains laminar,
5) The viscosity and the dielectric constant remain constant throughout the liquid,
6) the influence of the Brownian movement of particles will be neglected,
7) the influence of electroosmotic liquid flow due to characteristics of the chamber's wall will be dissociated from the true electrophoretic mobility of particles and molecules.

There are two limiting cases of the ratio of κa of particle radius (a) or dimension and thickness of double layer ($1/\kappa$) to be considered: small κa, or a particle or molecule with a thick double layer with respect to its diameter, and large κa, or a particle with a thin double layer, compared to the size of the particle

$\kappa a < 1$ (small particle and/or thick double layer)

In the first case, with $\kappa a < 1$, it can easily be shown[5, 7, 9, 11-13] that Hückel's[14] equation for the electrophoretic mobility (u) applies:

$$u = \frac{\zeta \varepsilon}{6 \pi \eta} \qquad [2]$$

where η is the viscosity of the medium.

$\kappa a > 300$ (large particle and/or thin double layer)

The second case, with $\kappa a > 300$ can be shown (6) to be best described as von Smoluchowski's[14] equation:

$$u = \frac{\zeta \varepsilon}{4 \pi \eta} \qquad [3]$$

Henry's equation

All values of κa, from very small to very large, are described by Henry's generalization:[16]

$$u = \frac{\zeta \varepsilon}{6 \pi \eta} f(\kappa a) \qquad [4]$$

When $\kappa a > 300$, $f(\kappa a) = 1.5$, and Henry's equation [4] becomes that of von Smoluchowski [3]. At $\kappa a < 1$, $f(\kappa a) = 1.0$, so that then Henry's equation [4] equals Hückel's equation [2]. For all values of κa a curve can be constructed that approximates $f(\kappa a)$.[6, 9] In the case of long cylindrical particles, perpendicular to the electric field, $f(\kappa a)$ varies from 0.75 to 1.5 with, respectively, low and high values of κa. For long cylinders, parallel to the electric field, $f(\kappa a) = 1.5$ for all values of κa.[7, 9]

Relaxation effect

However, at κa values that are intermediate between 0.1 and 25 another important disturbing effect (that was not taken into account by Hückel, von Smoluchowski or Henry) makes itself felt, i.e., the relaxation effect, elucidated by Overbeek.[7, 17] Briefly, double layers of intermediate thickness tend ro remain somewhat behind the particles they surround, if they are being transported rather rapidly through a liquid under the influence of an electric field. The resulting deformation of the ionic double layer generally causes a retardation in the electrophoretic velocity of the particles it surrounds. That effect is called relaxation. However, at low values of ζ ($\zeta < 25$ mV), as well as at $\kappa a > 0.01$ and $\kappa a > 1000$ the relaxation effect may be neglected. As soon as $\zeta > 25$ mV then in all cases where $0.01 < \kappa a < 1000$, tables and/or graphs showing the variation of electrophoretic mobilities as a function of ζ and κa should be consulted.[7, 9, 11, 13] For values of $1/\kappa$ in various electrolyte solutions, see [9, 16], see also section 1, above. The κa of most biopolymers (e.g., proteins, nucleic acids, polysaccharides, glycoproteins, etc.), in the commonly used buffers, varies between 1 and 10, so that in all cases where their ζ-potential >25 mV, the relaxation effect can play a considerable role (nucleic acids in particular tend to have an elevated

negative surface potential). Even with $\zeta > 25$ mV (and thus in the region where the relaxation effect is insignificant), a graph for the Henry equation should be consulted[6, 9] for the correlation between ζ -potential and electrophoretic mobility. Living cells virtually all have a $\kappa a > 1000$, so that relaxation is no problem, and their electrophoretic mobility is entirely ruled by von Smoluchkowski's equation [3]. The pronounced differences in electrophoretic mobility induced by κa differences, particularly in the range $0.1 < \kappa a < 300$ has been utilized for the analysis of size distribution of latex particles,[18] as well as for the separation of two different sizes (0.23 μm and 0.80 μm diameter) of polystyrene latex particles (with, however, quite similar ζ -potentials) under conditions of weightlessness, during the flight of Apollo 16 to the moon.[19, 20]

The influence of the size and shape of particles and molecules on their electrophoretic mobilities has recently been treated by Overbeek and Bijsterbosch.[21]

4. Calculation of ζ -potential from Electrophoretic Mobilities. It should be emphasized that the ζ -potentials that can be obtained from equations [2, 3, 4], or from tables or graphs[7, 9, 11, 13] based upon observed electrophoretic mobilities, in the cgs system, are expressed in electrostatic units (esu) of potential difference.[5, 9] For instance, in Hückel's equation [2] it should be kept in mind that the electrophoretic mobility

$$u = \frac{U}{X} \qquad [5]$$

where U is the electrophoretic velocity and X the electric field strength. Thus, from [2] and [5]:

$$\zeta = \frac{6 \pi \eta U}{X \epsilon} \qquad [6]$$

and in order to convert esu potential differences to volts ζ as well as X should be multiplied by 300, so that the right hand side of equation (6) must be multiplied by 90,000, if one is to express ζ in volts and if the electro-

phoretic mobility is given in cgs units ($cm^2\ sec^{-1}\ V^{-1}$). In Table I values for ζ according to equations [2] and [3] are given, in mV, for an electrophoretic mobility of 1 $\mu m\ sec^{-1}\ V^{-1}$ cm at various temperatures. As the viscosity η of water decreases much more strongly with an increase in temperature than the dielectric constant ϵ, the temperature effect is considerable, and to achieve the same electrophoretic mobility, a particle or molecule needs to have twice as high a ζ-potential at 0°C as at 35°C (Table I).[9]

III. MOVING BOUNDARY ELECTROPHORESIS

For many years electrophoretic mobilities of proteins were determined by adsorbing the proteins onto small particles and subjecting these to microelectrophoresis.[22] However, a breakthrough in our knowledge of electrophoretic mobility distributions of proteins was made possible by the development of moving boundary electrophoresis by Tiselius [3,6,23-25] and by the development of "schlieren optics" for the Tiselius apparatus by Philpot[26] and Svensson.[27] As moving boundary electrophoresis has now virtually become an obsolete technique, only a brief description will be given of it here.

The apparatus is basically a U-tube in which the protein-solution occupies the lower half, and an ultrafiltrate (or dialysate) of it the upper part of both branches. A major contribution of Tiselius has been to devise a sliding arrangement by which the upper (ultrafiltrate or dialysate)branches can be brought into contact with the lower (protein solution) part of the U-tube, which creates a sharp starting boundary between solution and solvent.[3,6,23-25] When an electric potential difference is applied across the two branches of the U-tube, the component with the highest ζ-potential will migrate fastest, up one branch, and down in the other. The component with the next lower ζ-potential will migrate less rapidly, also up in one branch, and down in the other. Thus, when a mixture of components is being electrophoresed, only some of the fastest component (in the branch in which it moves upward), and some of the slowest component (in the other branch) can be obtained in a pure

state; components with ζ-potentials intermediate between the highest and lowest cannot be easily separated for preparative purposes by moving boundary electrophoresis.

For analytical purposes, however, the method has been put to great advantage, thanks to several optical systems[26-28] that yield an image of the function of the change in refractive index (which is proportional to the change in concentration) versus distance of migration. The use of schlieren[26, 27] and scale[28] optical methods have been described in various places[6, 24, 25] and treated thoroughly by Svensson and Thompson;[29] a more recent analysis of these and related optical methods used in the visualization of concentration gradients was given by the present author.[30]

The necessity for equilibrating the protein solution with the supernatant buffer, the relative complication of assembling a Tiselius-cell, the large volumes of solution needed (11-24 ml), the requirement of taking a series of photographs of the electrophoresis in progress, the need for precise temperature control (at +4°C) of the waterbath, and the great expense of the equipment, in combination, have rendered the method obsolete as soon as extremely simple and cheap zone electrophoresis methods were developed. Such Tiselius apparatus as are still in use probably now are mostly used for the determination of diffusion coefficients, and even that nowadays is done more simply and with less liquid ($\approx$0.5 ml), with an analytical ultracentrifuge, at relatively low rpm in a synthetic boundary cell,[31] or by immunochemical means.[31, 32] Nevertheless, it should not be forgotten that with moving boundary electrophoresis, inter alia, the fact that antibodies are γ-globulins was discovered.[33-34] The results obtained with moving boundary electrophoresis were an important incentive in the development of simpler and more accessible zone electrophoresis methods.

Also, as moving boundary electrophoresis (at least at ambient gravity) shows no significant effects of electroosmotic backflow, the method is still useful for the determination of absolute electrophoretic mobilities of control sub-

stances of low ζ-potential, such as dextran.[35, 36] A simplified form of moving boundary electrophoresis can also be used for colloidal suspensions, such as latices with a sucrose density gradient to stabilize the suspension against convective disturbances.[37,38]

IV. SIMPLE ZONE ELECTROPHORESIS

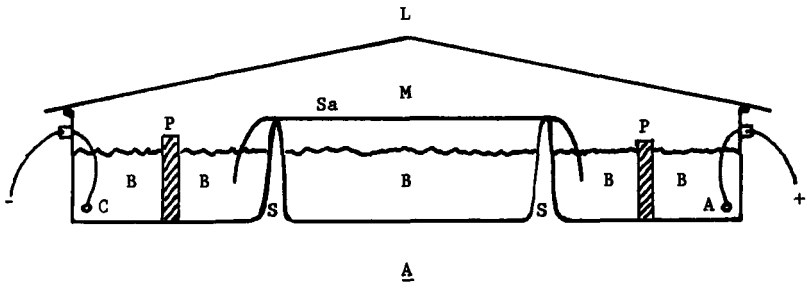
In moving boundary electrophoresis the migrating substances occupy, typically, all of the lower half of a U-shaped column, the fractions are only slightly displaced with respect to one another, and are only mutually distinguishable with the help of a sophisticated optical system. Thus moving boundary cannot easily be used for the separation of fractions from one another (see above). For that reason, from the early 1950's on, partly under the influence of the fast-growing art of chromatography, and partly spurred on by the remarkable results coming from a few privileged laboratories possessing a Tiselius apparatus, zone electrophoresis developed with astonishing speed. In contrast to moving boundary electrophoresis, in zone electrophoresis less than 1% of the column is occupied by a narrow band of the solution to be electrophoresed. In an electric field all components originally present in that band will then migrate, each at its own speed, so that (ideally) all fractions become completely separated from one another. Once that is accomplished further migration will only lead to band-spreading and the electrophoresis should be stopped and the individual bands separated and analyzed.

1. Paper Electrophoresis. Under the influence of the great advances in paper chromatography that were taking place at that time, almost all the first work on zone electrophoresis was done with filter paper as a carrier. Grassman has given an excellent review of these developments in the early 1950's.[4] The early paper electrophoresis devices tended to keep the filter paper strips in as vertical a position as was feasible,[39] by analogy with the usage in paper chromatography, but it soon became apparent that for electrophoresis the horizontal position is more practical than the vertical, particular-

ly when electrophoretic mobilities had to be determined.[39, 40] A variety of factors play a role in the actual electrophoretic velocity of charged compounds in filter paper: electroosmosis, the tortuosity of the liquid channels,[39, 40] and solvent evaporation and diffusion.[41] The quantitative determination of the various protein fractions after electrophoresis, drying and staining is further complicated by the tendency of proteins to adsorb to paper to various degrees (albumin adsorbs particularly strongly),[42] and also by the fact that the dye-binding capacity of different proteins varies considerably.[43] Techniques for paper electrophoresis have been thoroughly described by Smith.[44] However, for all the reasons mentioned above, paper as a carrier for electrophoresis has now largely been superseded by other porous carriers or gels.

2. Cellulose Acetate Electrophoresis. Cellulose acetate as a carrier for analytical zone electrophoresis was first described in 1957 by Kohn,[45] and the method has been more recently reviewed in detail by Chin,[46] as well as by Kohn.[47] Cellulose acetate has enormous advantages over cellulose (paper) for zone electrophoresis, e.g.; protein adsorption is very slight, separation is rapid, only small samples are needed, electroosmotic backflow is minimal. In addition, the strips can be made completely transparent after staining, so that a properly screened, stained and cleared cellulose acetate electropherogram of normal human serum[47] is indistinguishable from one obtained by moving boundary with schlieren optics. After staining and drying, cellulose acetate strips can be cleared with oil or, preferably, with acetic acid.[47, 48] Lists of manufacturers of cellulose acetate strips and ancillary equipment may be found in references 46 and 47. See Figure 2A for a diagram of the cellulose acetate electrophoresis method. Platinum or graphite electrodes are usually employed. Buffers used generally range in pH from 7.0 to 8.8, and in ionic strength (μ) from 0.02 to 0.06. Samples are applied (in quantities of $\approx 5\ \mu l$) by means of a micropipette or a special parallel wire sample applicator.

3. Large-pore Gel Electrophoresis. Gels made from agar or agarose are also used as inert stabilizing carriers in zone electrophoresis.[49-51] They



A

FIGURE 2A

Cross section of a cellulose acetate electrophoresis device. M is a cellulose acetate membrane, soaked in buffer, of which both ends are immersed in the buffer B. Sa indicates the place where the sample is deposited. Each buffer compartment is divided into two sections by means of a porous (or perforated) barrier P, to prevent the membrane from being directly exposed to electrolysis products emitted by the electrodes C (cathode) and A (anode). The entire chamber should be closed by a lid L, when in use; it is desirable for that lid to have slightly sloping surfaces, so that droplets of condensed water will slide away to the sides of the chamber, without dripping on the membranes. Care should be taken that the levels of buffer B be equalized between the cathodal and the anodal side, to prevent untoward liquid migration inside the membranes through hydrostatic forces. Devices of this type are also used for paper electrophoresis, agar and agarose electrophoresis, and immunoelectrophoresis. For the latter applications the gel is confined to a tray that reposes with both ends on the supports S. Filter paper wicks steeped in buffer are then used to establish electrical contact between both sides of the gel and the buffer B.

have much the same advantages as cellulose acetate, but, as they cannot be obtained ready-made, require much more work to prepare. Agar manifests strong electroosmotic backflow, but with agarose the electroosmosis is greatly reduced.[52, 53] These gels nowadays are used principally in electrophoresis in combination with immunoprecipitation (see below), although some laboratories continue to use agarose electrophoresis for clinical research.[52-54]

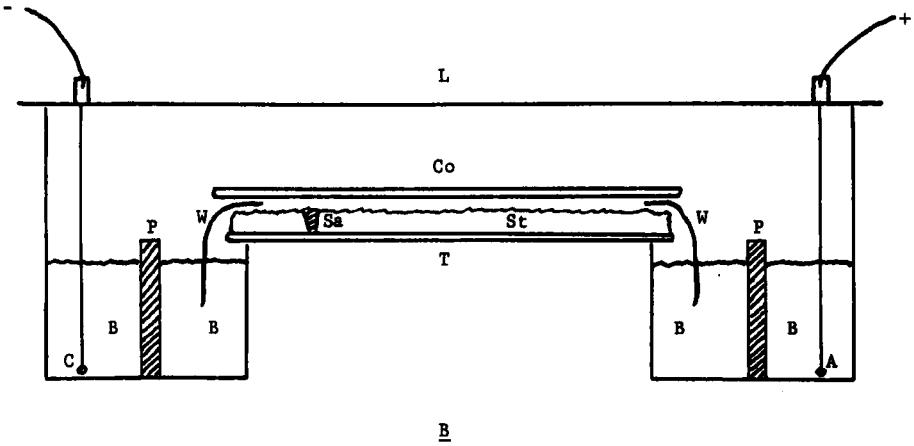
An interesting development was the very dilute agarose gel electrophoresis method (Spectrophor), introduced by Bausch and Lomb.[55] Here

serum electrophoresis was done in very dilute semi-liquid agarose gels, with subsequent scanning of the patterns at a wave length close to the maximum absorptivity of the peptide band (205 nm). This method also yields patterns that are similar to those obtainable by Tiselius moving boundary electrophoresis. However, probably on account of the costly instrumentation, the apparatus has been discontinued and the method has been replaced in most clinical laboratories with the simpler, cheaper and less cumbersome cellulose acetate approach.

Almost every manufacturer of electrophoresis equipment markets his own DC power supply (see, e.g., one of the current "Lab Guides" published by Analytical Chemistry, Science, etc.). One inexpensive high-quality power supply is the Heath Model SP-17 A (Heath-Schlumberger, Benton Harbor, Michigan).

4. Block Electrophoresis. The electrophoretic separation of samples of several milliliters volume (instead of the 10^{-3} to 10^{-2} ml samples to which the earlier mentioned zone methods are limited), requires porous carriers of a larger volume and different composition. Porous blocks or slabs can be made from wet slurries of granular materials such as potato starch, poly (vinyl chloride), polystyrene, glass powder, etc.[56-58] For most purposes starch block electrophoresis is still by far the simplest and the best method,[58] see Figure 2B. But in those cases where polysaccharides, glycoproteins or other carbohydrates are to be separated, the analysis of the various fractions is much facilitated by using more inert material as a carrier, such as "Pevikon" beads, made of a copolymer of vinyl chloride and vinyl acetate,[57,59] or styrene-divinylbenzene copolymer beads.[58] See Bloemendal's monograph[56] for an extensive list of media and working conditions for the purification of a large variety of biopolymers.

Many of the materials used in block electrophoresis have a fairly pronounced negative ζ-potential, which causes a significant electroosmotic flow towards the cathode. The sample should therefore not be deposited too



B

FIGURE 2B

Cross section of a starch block electrophoresis device. St is the starch block, soaked in buffer, both ends of which are connected by means of paper wicks W, steeped in the buffer, with the buffer B. Sa indicates the place where the sample is deposited. The starch block rests on a plastic tray T, and is covered with a plastic cover Co. Each buffer compartment is divided into two sections by means of a porous (or perforated) barrier P, to prevent electrolysis products emitted by the electrodes C (cathode) and A (anode) from reaching the wicks, and thus the starch block St. The entire chamber should be closed by a lid L when in use; the electrical outlets of the electrodes A and C can only be connected through the lid when it is closed, to prevent inadvertent electrocution of laboratory personnel. The levels of buffer B should be equalized between the cathodal and anodal side. Devices of this type can also be used for other types of horizontal block or gel electrophoresis. This type of block electrophoresis should be performed in a well-ventilated cold room, to dissipate most of the Jouleian heat engendered by the electric field.

closely to the cathodal edge of the block but rather, as a first approximation, one-third of the length of the tray from the cathodal edge.[58] The same types of electrodes and buffers as those indicated above for cellulose acetate electrophoresis are applicable to block electrophoresis. Block (e.g., starch block) electrophoresis is best done in a well-ventilated cold room, at +2 to +6°C. In most cases, at those temperatures, and with blocks of about 1.5 cm

thick, 25 cm long and 8 cm wide, one should not apply more than 20 W to the block. Application of 3 to 10 V/cm, overnight, generally will result in good separation. The granules are best applied as a slurry (suspended in the electrophoresis buffer), deposited in a plastic tray. With a knife or spatula a channel is formed in the block, into which the sample mixture (5-15 ml, dissolved in the sample buffer) is poured carefully. The channel, after the solution has been completely adsorbed, is pressed close again. Both ends of the tray should be provided with wicks made of thick filter paper and the top surfaces of the block may be closed off with a thin hydrophobic sheet (e.g., saran wrap), or with a plastic lid. The wicks should dip into the electrode vessels (see Figure 2B).

Once the electrophoretic run is finished, the block should be sliced up into, e.g., 5 or 10 mm thick slices, each of which is then eluted (and/or further extracted)with the help of a fritted glass Büchner funnel. In this author's laboratory, fairly long and narrow Büchner funnels are used that fit into centrifuge tubes, which allows the extraction of 12 slices at a time, by centrifuging the funnels, each with one starch block slice inside it. The contents of the extract or eluate of each slice are analyzed by colorimetry, ultraviolet spectroscopy, or by immunochemical means (see below).

Electrophoresis in packed slabs of granular material need not be limited to horizontal block electrophoresis, but can also be done with the help of vertical blocks (e.g., of sponge rubber or foam plastic),[58] and also with hollow vertical cylindrical columns, packed with granular material.[60]

Block electrophoresis is one of the simplest and most useful methods, best used as a first step in the isolation of proteins from a mixture.[56,58]

5. High Voltage Electrophoresis. At high voltage gradients (40-200 V/cm), electrophoretic separations of small molecules (e.g., aminoacids, sugars, indoles, purines, pyrimidines, phenolic acids, keto acids, imidazoles, steroids) can be carried out with a resolution impossible to achieve with the lower, more conventional voltage gradients.[61] However, as high voltage

electrophoresis requires potentials as high as 10,000 V and uses up to 2.5 kwh for a separation, special measures for safety as well as for cooling are necessary.[62] The carrier that is most suited for this method is filter paper (preferably Whatman 3 MM). Various kinds of apparatus have been described. One type (capable of up to 10,000 V and 200 V/cm comprises water-cooled metal plates between which paper insulated with polyethylene sheets is clamped.[61-63] Another method, rarely capable of reaching more than 5,000 V, or 100 V/cm, uses a cooled, water-immiscible liquid (such as toluene) that surrounds the wet filter paper.[64, 65] Both types of apparatus must be provided with adequate safeguards against the possibility of any kind of manual contact while the current is on. With the latter immersion-type apparatus, a graduate student was electrocuted in 1965, and it is urgently recommended that safety measures developed since that occurrence[61, 66] be rigidly followed.

High voltage electrophoresis of small molecules is frequently used in one dimension, on paper, with prior or subsequent partition chromatography in the dimension perpendicular to it. Usually high voltage electrophoresis is the first step, followed by chromatography.[61] However, this author has obtained better results with aminoacid separations by utilizing the two steps in the reversed order.[62]

6. Staining Methods. It is beyond the scope of this chapter to review the many staining and other detection methods for all the compounds that one might wish to characterize and determine quantitatively after electrophoretic separation; instead only a few major methods will be described. For the quantitation of proteins on paper, agar gel or cellulose acetate strips, Ponceau-S is one of the preferred stains,[47, 58] as is amido black.[44] For lipoproteins, Sudan black is a reliable stain,[44, 58] although with cellulose acetate electrophoresis Kohn's ozone-Schiff method is more suitable.[47] For glycoproteins, Schiff's reagent is generally recommended,[44,47] but better results are obtained when, instead of Schiff's reagent, its isomer, pararosaniline is used.[58, 67] For the determination of protein concentrations in eluates a

number of methods are used. Ultraviolet spectrometry (at 280 nm) is practical, but it should be remembered that the absorptivity of various proteins differs widely, being dependent on their tryptophane and tyrosine content.[58, 68] The same drawback exists with the Folin-Ciocalteu (or Lowry) staining method, but not with the simple biuret procedure[68, 69] which detects mainly the peptide bands. Ninhydrin is a useful stain for the characterization of aminoacids.[44]

When photometric scanning of stained strips is practiced, it is essential to realize that the protein concentration at a given point is not proportional to the decrease in light transmission at a given wavelength but, according to Beer's law, to the logarithm of that decrease, i.e., to the optical density (OD).[69] Thus only scanners that record OD versus distance yield graphs in which the total amount of a given protein fraction is proportional to the surface area under its peak.

7. Buffers. Many different buffers are used in zone electrophoresis and it is not feasible to recommend any particular one. In each case descriptions of appropriate buffers can easily be found in the "Methods" sections of the applicable references quoted in this chapter. In general, it is useful to remember that the lower the ionic strength of the buffer, the smaller the Joule heating effect, the higher the electrophoretic mobility and the stronger the electroosmotic backflow. But notwithstanding the higher mobilities at low ionic strength, a decrease in ionic strength of the buffer does not necessarily improve the resolution between fractions.

V. MOLECULAR SIEVE ZONE ELECTROPHORESIS

In 1953 Tiselius and Flodin observed that by means of electrophoresis in gels with very small pores or in very viscous polymer solutions, molecules may be separated according to their size or shape. They named the method "electrokinetic ultrafiltration".[70] Electrophoresis through multiple layers of very dense gels has been used by the present author and his collaborators for the separation of stable lithium isotopes.[71] However, the most prolific ad-

vances with this type of method occurred in the 1960's, after the development of starch gel electrophoresis,[72-75] with the widespread adoption of polyacrylamide gel electrophoresis[76] and especially of "disc" electrophoresis.[77-80]

1. Starch Gel Electrophoresis. In 1955 Smithies demonstrated that electrophoresis in starch gels could yield a remarkable multitude of different protein fractions.[72] Smithies subsequently demonstrated that the separating effect was mainly due to differences in molecular size.[73] He observed that the denser the starch gel, the slower the migration of proteins in it.[74] Starch gel electrophoresis quickly became an important and powerful technique in the characterization and separation of proteins.[64, 75] However, the method is rather tedious,[64] and became superseded by the much more convenient technique of polyacrylamide gel electrophoresis, see below.

2. Polyacrylamide Gel Electrophoresis. The use of concentrated *polyacrylamide gels in electrophoresis* was first described by Raymond and Weintraub in 1959; they initiated the use of these gels as horizontal slabs.[76] Ornstein[77] and Davis[78] subsequently developed "disc electrophoresis", with vertical cylinders of polyacrylamide gel. With that method exquisite resolution can be attained, in which the starting material can be resolved into manifold concentrated fractions, in the shape of "discs", often no thicker than 10 μm each.[79, 80] Thus, disc electrophoresis has become and has remained to this day, one of the most powerful analytical (and preparative) tools for obtaining maximum resolution with mixtures of biopolymers. The very popularity of the method, however, necessitates an important caveat: molecular sieve electrophoresis is essentially based on two entirely different mechanisms that act simultaneously and in the same direction, one of them separating molecules according to their size and shape, and the other according to their ζ-potential (which itself under common electrophoretic conditions, may vary according to their size and shape, see Section II, above). Thus even in the simplest cases, the situation is analogous to one algebraic equation with two unknown vari-

ables. In other words, it is impossible to learn either the size and/or shape or the ζ-potential of a compound from its mobility in a dense gel.[58] Of any given biopolymer isolated or characterized by such a method it is not only impossible to know (without further tests) if it has migrated to a certain point on account of its size, its shape or its charge (or any of these properties combined), but it is also impossible to know (without further tests) whether it is accompanied by other compounds that may have migrated to the same point on account of another combination of properties.[58] What is worse, it is not only possible for two different compounds to migrate to the same point in a gel, a single homogeneous compound may, under favorable conditions, produce more than one band.[81, 82]

Polyacrylamide gel electrophoresis has been reviewed by several authors.[83] Polyacrylamide gel electrophoresis of larger molecules and viruses can be carried out by using more diluted gels,[84] or by using mixed gels of polyacrylamide and agarose.[85]

3. Polyacrylamide Gel Electrophoresis of Nucleic Acids. The difficulties pointed out above, of having both size and charge contribute to the electrophoretic transport in dense gels, do not apply to molecules with a constant charge/mass ratio, such as RNA. Thus different RNA molecules have been successfully separated, according to molecular weight only, by polyacrylamide electrophoresis.[86]

4. Sodium Dodecyl Sulfate Polyacrylamide Electrophoresis. The advantage of having a constant charge/mass ratio, which is a natural attribute of RNA (see above), can be artificially endowed upon other biopolymers (e.g., proteins), by treatment with a strongly negatively charged surfactant such as sodium dodecyl sulfate (SDS). This technique was first published in 1967 by Shapiro et al.[87] The reliability of the method has since been tested on a large number of polypeptide chains.[88] The physicochemical conditions under which the molecular weights of SDS-polypeptides can be subsequently checked by

analytical ultracentrifugation were elaborated upon by Barnett and Spragg.[89]

5. Pore Limit Gel Electrophoresis. Electrophoresis in gels of graded porosity allows macromolecules to migrate into a gel of which the pores continuously become smaller, until a pore size is reached that approaches their molecular size, which causes them to become trapped and prevents them from migrating any farther.[90] Thus this method of gel electrophoresis also separates solely according to the size of macromolecules and is independent of their charge, as long as they have enough surface charge to enable them to be transported electrophoretically.[83, 91]

IV. ELECTROPHORETIC METHODS COMBINED WITH IMMUNODIFFUSION

Thanks to the extraordinary specificity of antigen-antibody interactions, the combination of electromigration with immune precipitation has brought about an expansion of literally orders of magnitude in our powers of electrophoretic protein characterization. For a complete understanding of the principles underlying these techniques, the laws of immune precipitation in gels must first be elucidated.

1. Immune Precipitation in Gels. It must be realized that specific antigen-antibody reactions are non-stoichiometric: antigens and antibodies belong to the class of complex-forming compounds which can interact with one another in a widely varying range of proportions.[92-95] Antigen-antibody complexes tend to be of low molecular weight and soluble when they have been formed under conditions of excess antibody. Intermediate concentration ratios of antigen to antibody will give rise to insoluble complexes of very high molecular weight. The equivalence or optimal antigen-antibody ratio is the ratio at which they form the largest amount of precipitate. At the optimal ratio neither all antigen-specific nor all antibody-specific binding sites are occupied, but at the optimal ratio the formed complexes precipitate most rapidly.[96]

In 1949 Ouchterlony reported a new technique for obtaining antigen-antibody precipitates by double diffusion in gels.[97] With this two-dimensional method it became possible to dinstinguish between antigen-antibody systems that are related (identical), unrelated (non-identical) or partially related[98] (see Figure 3). Closer analysis of the manner in which antigen and antibody molecules, upon meeting and interacting after diffusion towards one another from different wells in a gel, form a precipitate line and not a lens-shaped precipitate patch (compare ref. 99) on the intersecting area between the two diffusion circles (see Fig. 4), together with other data, allows some important conclusions to be made about the nature of the precipitate lines. The precipitate "lines" seen in the gel from above are, of course, little vertical walls standing upright inside the gel. These precipitate walls actually are barriers that are specifically impermeable for just the specific antigens and antibodies that formed them, but they are premeable to all other antigens, antibodies and other compounds.[93-96] The reason for the specific impermeability of these barriers lies in the fact that they are continually self-repairing, with the help of free antigen and free antibody molecules remaining in solution on either side of them. Completely related systems therefore must give rise to precipitate barriers that fuse with one another (see Fig. 3A), and unrelated systems will form two entirely independent precipitate barriers that cross one another (see Fig. 3B). It is the crossing of lines of unrelated systems that makes it possible to distinguish and characterize whole multitudes of different proteins present in a mixture such as blood serum by means of immunoelectrophoresis, where by simple electrophoresis only a few fractions are apparent; see below; see also Table II.

The precipitate lines, formed by double diffusion, always start in the same position between the starting points dividing the distance d (where $d = a + b$) between them in the ratio

$$a/b = \sqrt{(D_{Ag}/D_{Ab})} \qquad [7]$$

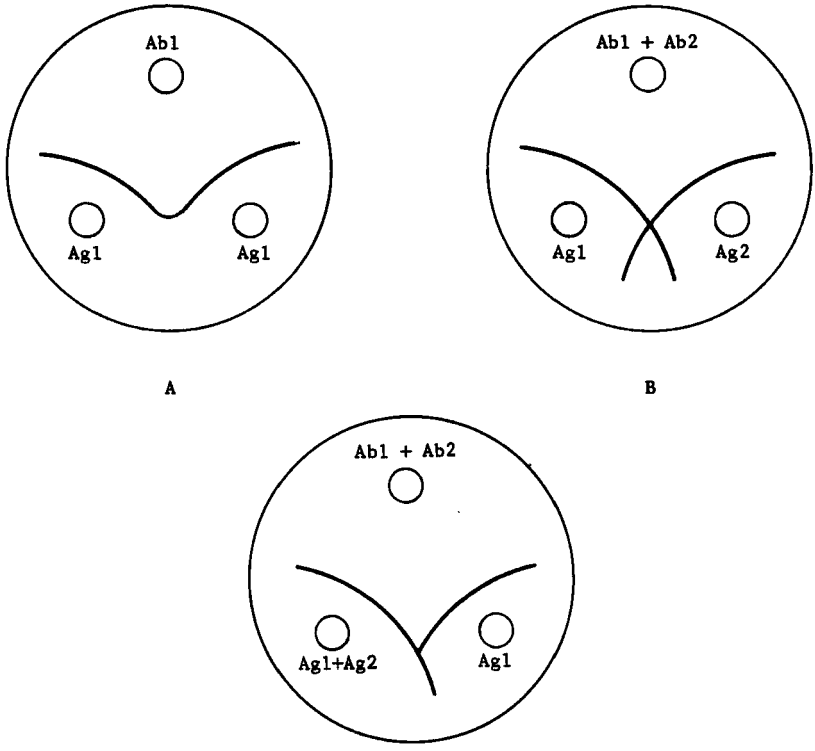


FIGURE 3

Three possible patterns of double diffusion of antigen and antibodies in gel.

A. Fusing of precipitate lines formed between two related (or identical) antigens (Ag1), diffusing from two different wells, and their corresponding antibody (Ab1).

B. Crossing of precipitate lines formed between two completely unrelated (non-identical) antigens (Ag1 and Ag2) in two different wells, and their corresponding antibodies (Ab1 and Ab2) both deposited in the same well.

C. Spur formation of precipitate lines formed between two partially related antigens (Ag1 and Ag1 + Ag2) in two different wells, and their corresponding antibodies (Ab1 and Ab2) both deposited in the same well. For further explanation, see text.

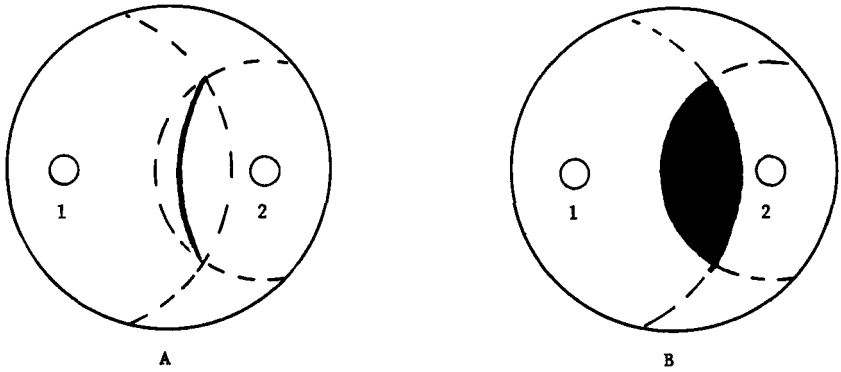


FIGURE 4

Double diffusion of antigens and antibodies in gels.

A. Formation of a precipitate line in a gel double diffusion plate, as a result of the interaction between the two reagents 1 (antigen) and 2 (antibody) diffusing towards one another from their starting wells. This precipitate line is actually a little vertical wall, seen from above, that is specifically impermeable barrier to the substances that formed it.

B. If the interaction product were not specifically impermeable to the reagents (1 and 2) that formed it, a lens-shaped zone of reaction in the intersecting area between the two diffusion circles would result. In precipitating systems this never occurs, but in certain non-precipitating immunological reactions such lens-shaped fields of interaction have been described.[99]

virtually regardless of the initial concentrations of Ag and Ab;[95] D_{Ag} and D_{Ab} are the diffusion coefficients of, respectively the antigen (Ag) and the antibody (Ab) molecules . Once the precipitate lines are formed, they will remain sharp and immobile only if the two interacting reagents are present in solution in their starting wells in equivalent (or optimal) ratios (and thus are continually available to furnish material for the self-repair of the barriers). If one of the reagents is present in excess, the precipitate line will decay soon after its formation and reform further on, then thicken, multiply, or appear to move in the direction of the other reagent that was present in the lower concentration.[94-96, 100] By making use of that property and by taking the sharp-

ness and immobility of a precipitate line as the indicator of equivalence, a double diffusion titration method in gels for antigen-antibody systems was developed;[94-96, 101] see also ref. 98. As all points on the precipitate line

TABLE II

List of the Principal Human Serum Proteins Distinguishable by by Immunoelectrophoresis, in the Order of Decreasing Electrophoretic Mobility; (adapted from ref. 106)

Electrophoretic Mobility Group	
Albumin	Prealbumin Albumin
α_1 Globulins	α_1-Fetoprotein α_1B-Glycoprotein α_1T-Glycoprotein α_1-Lipoprotein α_1-Acid Glycoprotein α_1-Antitrypsin α_1-Antichymotrypsin Gc-Globulin Inter-α-Trypsin-Inhibitor
α_2 Globulins	Ceruloplasmin Zn-α_2-Glycoprotein α_2-HS-Glycoprotein α_2-Antithrombin (III) α_2-Macroglobulin Haptoglobin Cholinesterase

	β_1C/β_1A-Globulin
	C4 (β_1E-Globulin)
	Transferrin
	β_2-Glycoprotein I
	β_2-Glycoprotein II (C 3 Activator)
	β_2-Glycoprotein III
	Hemopexin
	Plasminogen
. β Globulins	β-Lipoprotein
	Retinol-Binding Protein
	Prothrombin
	Factor XIII
	Fibrinogen
	C-Reactive Protein (CRP)
	C 1 s-Inactivator
	C 3 Proactivator
	Carboanhydrase B
	Carboanhydrase C
	Immunoglobulin E (IgE)
	Immunoglobulin D (IgD)
	Immunoglobulin M (IgM)
	Immunoglobulin A (IgA)
	Immunoglobulin G (IgG)
γ Globulins	Immunoglobulin Light (L) Chains ( )
	Immunoglobulin Light (L) Chains ( )
	IgG-γ-H-Chain
	IgG-Fab-Fragment
	IgG-Fc-Fragment
	IgG-Fd-Fragment

(when first formed) have the same distance ratio

$$a'/b' = \sqrt{(D_{Ag}/D_{Ab})} \qquad [7']$$

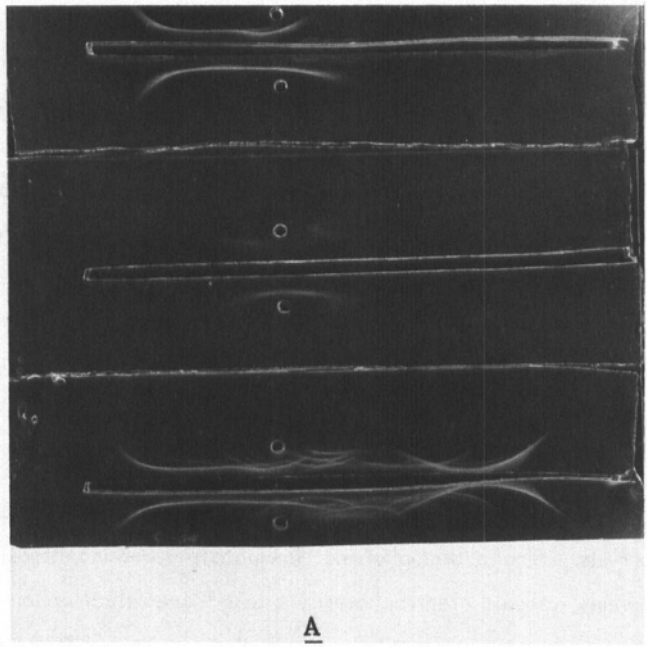
with respect to the two starting points, it can easily be shown that the precipitate line will be a circle, with a radius $R = ab/(a-b)$,[94, 96] which becomes a straight line only when $a = b$ (or $D_{Ab} = D_{Ag}$), e.g., in the case of a γ-globulin-anti-γ-globulin system. Precipitate lines resulting from two line-shaped sources will be straight lines and those resulting from a point-source and a straight line (such as a trough) will have the shape of a parabola.[94,96] The last two considerations are of special relevance to immunoelectrophoresis.

2. Immunoelectrophoresis. Grabar and Williams initiated the two-step combination of agar gel electrophoresis of protein mixtures followed by double diffusion immune precipitation with an antiserum to the protein mixture in a direction perpendicular to the electrophoretic path in 1953.[102] Figure 5 shows diagrams and photographs of immunoelectrophoresis plates. All of the multiple intersecting arcs are formed by different proteins, each having been precipitated by its corresponding specific antibody contained in the antiserum. The technique has been extensively described by, e.g., Ouchterlony,[103] Grabar and Burtin[104] and Arquembourg et al.[105] The five major protein fractions hitherto recognizable in human serum by means of electrophoresis have now, thanks to immunoelectrophoresis, been expanded into about four dozen easily characterized serum proteins, see Table 2.[106] Protein concentrations can be semi-quantitatively estimated from immunoelectropherograms by keeping in mind that (other parameters being equal) the closer the precipitate arc to the antiserum trough, the higher the concentration of the electrophoresed fractions.[96, 107] However, once a protein is qualitatively characterized, the quantitative estimations of its concentration is done more precisely by single radial immunodiffusion.[96] Changes in the shape of the precipitate line can be especially important in interpreting possible immunoglobulin abnormalities.[96, 104-107] Immunoglobulin G (IgG), which normally is electro-

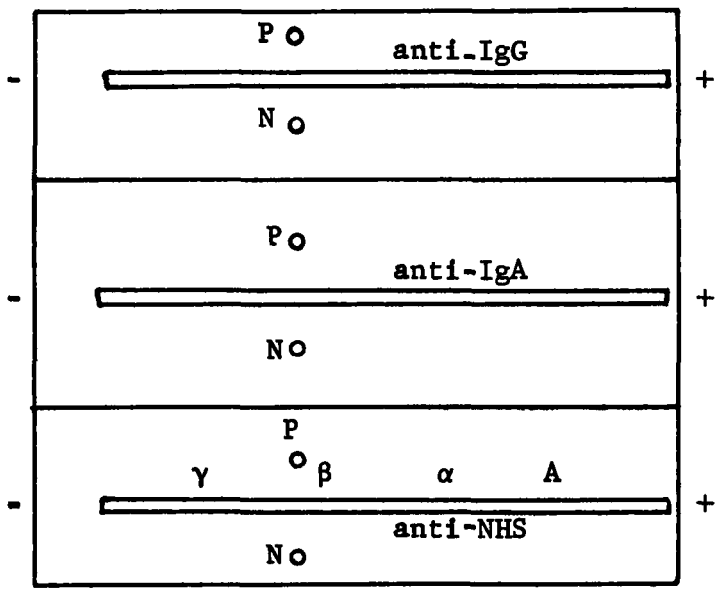
phoretically quite heterogeneous and thus migrates in a rather long drawn out field, yields by immunoprecipitation with antiserum from the trough that runs parallel to that long field, an arc that rather closely resembles a long, barely bent, almost straight line (see Fig. 5). But a homogeneous, monoclonal, IgG abnormality that migrates as a visual point source gives rise to a parabola-shaped precipitate arc;[96, 104-107] see Fig. 5. Albumin, on the other hand, which naturally occurs as an electrophoretically homogeneous protein, always forms a parabola-shaped precipitate arc in immunoelectrophoresis; see Fig. 5. The identity of any given arc among the many arcs in an immunoelectropherogram can be established by demonstrating its capability to fuse with an arc made by immunoprecipitation of a known antigen-antibody system.[103-105]

3. Counterelectrophoresis. Counterelectrophoresis, counterimmunoelectrophoresis, immuno-osmophoresis, immunoelectro-osmophoresis, cross-electrophoresis, crossed electrophoresis, crossed-over electrophoresis, electrophoretic immunoprecipitation, electrosyneresis, or immunofiltration are names that have been used to describe a technique that used electrophoresis to transport antigen toward antiserum in a gel, resulting in immune precipitation. The advantages of counterelectrophoresis over immune precipitation in gel, by double diffusion are two-fold: 1) almost all the antigen can be brought into contact with almost all of the antibody, whereas by double diffusion at least 75% of each of the reagents diffuses away from each other without ever having the possibility to react; and 2) a visible reaction is obtained in a much shorter time, not only because of the higher concentration of the reagents at the place where they meet (see above), but also because by means of electrophoresis the reagents can be brought together faster than by diffusion.

Counterelectrophoresis can be practiced in all cases where the isoelectric point of the antigen differs from that of the antibody. In all such cases it suffices for the reagent with the highest (e.g., negative) ζ-potential to be placed closest to the cathode so that it can overtake (and react with) the other reagent (with the lower negative ζ-potential) on its way to the anode.



A



B

Contrary to explanations advanced or implied by a number of authors,[108-110] the process of counterelectrophoresis is fundamentally independent of electro-osmosis and it is therefore preferable to leave any allusion to electro-osmosis out of the name for this technique. Electro-osmosis causes the entire liquid to flow, but it does not influence the relative mobilities of two different solutes with respect to each other within that flowing liquid. Electro-osmosis must only be taken into account when deciding on the location of the starting points for the two different reagents in the gel, so that electroosmotic flow will not cause the place of interaction between the reagents to fall outside the gel.

Crossed electrophoresis is a term that has also been used for the somewhat different technique of bidimensional electroimmunodiffusion (see below), so that the least ambiguous and most aptly descriptive term for the present technique is the one we adopted: counterelectrophoresis.

Techniques that were essentially counterelectrophoresis were first described in 1953, independently by Macheboeuf et al.[111] and by

FIGURE 5

Immunoelectropherogram of a normal serum (N) and a serum of a patient (P) with multiple myeloma of the IgG class. A is a photograph of the unstained gel slides, taken against a black background, with side illumination. B is a diagram of the slides shown in A. As indicated, in all three slides normal serum (N) was put in the top wells, and patient's serum (P) in the bottom wells. In the troughs, were, from top to bottom: rabbit anti-human IgG, rabbit anti-human IgA, and rabbit anti-whole human antiserum. In the bottom slide the approximate locations of γ, β and α globulins, and albumin (A) are indicated. In the case of the myeloma patient, note the extra dip formed with anti-IgG (close to the patient's well) in the top slide; in the middle slide note the patient's decrease in IgA (hardly visible precipitate line as compared to the normal serum), while in the bottom slide the extra dip in the patient's IgG line is also visible. In general, note the multiplicity of lines made visible with anti-whole serum (see Table II).

Grassman and Hübner.[112] Descriptions were also given later by Crowle,[113] and by Bussard;[114] see also ref. 103. Nakamura wrote a monograph on certain aspects of the technique, using mainly filter paper,[115] but by far the most important use of counterelectrophoresis in the 1970's has been for the detection of Australia (hepatitis B-virus-associated; HB_sAg) antigen in units of blood intended for transfusion,[109] which may not be transfused when the presence of that antigen can be demonstrated. The technique has also been used for the diagnosis of mycotic diseases,[108] as well as for the detection of other viruses,[110, 116] and for the diagnosis of bacterial infections.[117]

Electromigration is not the only method, apart from diffusion, to effect an encounter between antigens and antibodies in a carrier such as filter paper or a gel; forced evaporation of water at the middle region of a carrier will, by capillary, cause a transport of buffer (carrying the reagents) from both ends towards the middle.[111, 118, 119] This method, called immunorheophoresis[119] is as sensitive as counterelectrophoresis, but requires approximately twice as much time. This method is also used for the detection of Australia antigen (HB_sAg) associated with hepatitis B virus.[120]

4. Mono- and Bi-dimensional Electroimmunodiffusion. In gels that are uniformly impregnated with low concentrations of a specific antibody, the corresponding antigen, when deposited into a well, will diffuse outward and form a precipitate disc that will continue to grow until the antigen has become diluted to a concentration equivalent to that of the antibody in the gel, at which time the precipitate ring at the periphery of the disc will have reached its ultimate size. The surface area of the completed precipitate disc is then proportional to the initial concentration of the antigen, for a given antibody-containing gel-plate.[96, 121] This method, called radial single diffusion, or radial immunodiffusion in gels, is much used clinically for the quantitative determination of numerous protein components of serum and other body fluids. With most proteins, however, it is not practical, in a hospital, to wait the necessary length of time (often one or more days) for the reactions to come to

its ultimate completion. It may then be possible to plot the diameters the precipitate discs have attained, after a predetermined time lapse (of e.g., 16 hours), versus the logarithms of the antigen concentrations, in order to obtain a reasonably straight line.[122] However, a more accurate method for accelerating the precipitation in those cases where diffusion is too slow, is to transport the antigen molecules through the antibody-containing gel plates by an electromigration process called monodimensional electroimmunodiffusion, or the Rocket, or Laurell method.[96, 123]

Monodimensional Electroimmunodiffusion. In this method, developed by Laurell,[123] antigen is transported through a gel that is uniformly impregnated with a low concentration of antibody by means of an electric field. Rocket-shaped precipitates form in a manner analogous to radial immunodiffusion (described above); their surface areas, after development to their ultimate sizes, when plotted versus the initial concentrations of the antigen, yield a straight line; see Fig. 6A. With this method (as well as with the counterelectrophoresis method), one can only analyze those antigens that have an isoelectric point different from that of the antibody to be used in the gel. The monodimensional electroimmunodiffusion method is particularly recommended for the precipitative assay of antigens of a molecular weight higher than 40,000.[124] The method has been used for the determination of the normal concentrations of the plasma proteins in adults, pregnant women, and newborns;[125] its sensitivity can attain as low a value as 0.01 mg/l, when the gel plates are impregnated with radioiodinated antibody, and the precipitate rockets are visualized by autoradiography.[126]

Bidimensional Electroimmunodiffusion. This technique is analogous to immunoelectrophoresis (see Section 2 above), but with a second electrophoresis into an antiserum-containing gel, perpendicular to the first, separative electrophoresis, instead of the immunodiffusion step. The result is a collection of overlapping precipitate rockets, the surface area of each of which is proportional to the concentration of the particular antigen that formed it (see

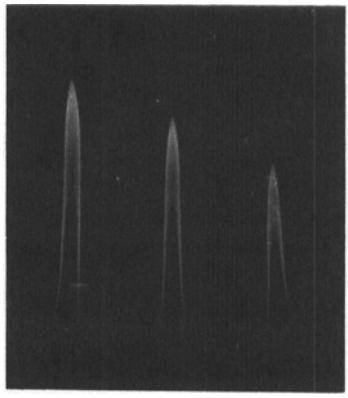


FIGURE 6A

Monodimensional electroimmunodiffusion of bovine serum albumin into an agarose gel containing 25 X diluted rabbit anti-bovine serum albumin antiserum. The wells contained, from left to right: 2.0, 1.5 and 1.0 mg/ml bovine serum albumin. After completion of the electrophoresis in the gel (the anode was at the top), the surface areas of the precipitate rockets (or, as a first approximation, the heights of the rockets) are proportional to the initial concentration of the bovine serum albumin deposited in the wells.

Fig. 6B). By this method the quantitative determination is possible of each one of a multitude of different antigens in a mixture.[96, 127, 128] This technique is also alluded to as Crossed Immunoelectrophoresis[127, 128] which resembles some of the terms used for Counterelectrophoresis (see Section 3 above). Although the origins of both techniques were conceptually related (see e.g., ref. 115), it should be stressed that these two procedures as they have evolved now are totally different, and are used for entirely different purposes.

5. Visualization of Immune Precipitates in Gels. It normally suffices to photograph the gel plates after the precipitate patterns have fully developed. The best method is to photograph the gel plates against a dark background (e.g., a black cloth), with side illumination (see Fig. 5). The quality of the photograph will improve if the slide is covered with a thin layer of

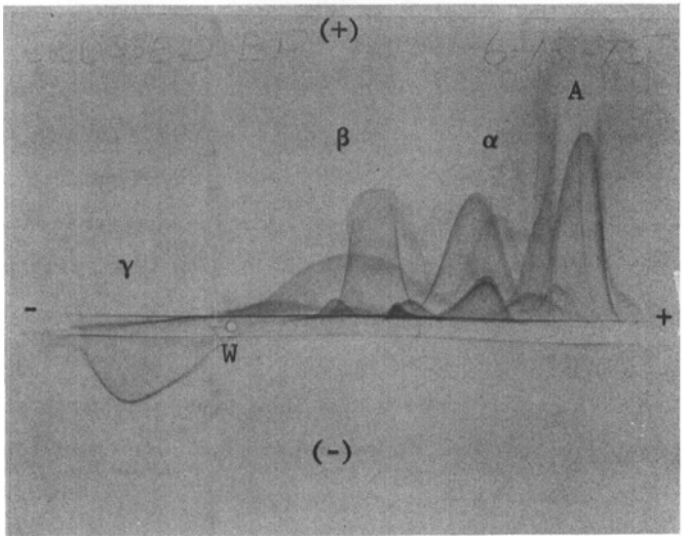


FIGURE 6B

Bidimensional electroimmunodiffusion of whole human serum. First a small amount of human serum was deposited in well W, and electrophoresed (polarities indicated by — and +). Once the first electrophoresis is completed the gel strip containing the separated proteins (along the — to + axis) is cut out and inserted in a gel containing goat antiserum to whole human serum. A second electrophoresis is then done in a direction perpendicular to the first one (polarities indicated by (—) and (+)), until all peaks have reached their final size. The locations of albumin (A), α, β and γ globulins are indicated. The areas under each of the peaks (which are proportional to the total amount of antigen making them up) can be measured by planimeter. Note that, in both directions, γ globulin migrated (apparently) cathodally, due to electro-osmotic backflow. By courtesy of Dr. W.B. Bartholomew of the Department of Microbiology, State University of New York at Buffalo, and Erie County Laboratories, Buffalo, NY.

water before photographing it. A good photograph taken in that manner, at actual size, will show at least as many lines as are visible to the naked eye. Gel slides may also be stained, and conserved in the dry state. But to that effect they must be washed in saline for at least 24 hours, to remove all dis-

solved protein that has not taken part in immune precipitation, and then air-dried, after having been covered with a sheet of wet filter paper.[107] Only dried slides can be stained (for stains see Section V, 6 above, and also refs. 51, 52, 105). It must be stressed that on account of the necessity for thorough washing to remove all dissolved protein, some of the precipitated protein will also be removed, so that a stained immunodiffusion plate (or immunoelectropherogram, etc.) may divulge less information than a good photograph of the unstained slide.

A powerful manner for enhancing the characterizing potential of immunoprecipitation procedures is to combine them with specific enzyme staining methods,[128] which when applied to immunoelectrophoresis is called enzymoimmunoelectrophoresis.[129] Autoradiography is another method, allowing the visualization of exceedingly minute amounts of antigen or antibody.[126,130]

VII. CONTINUOUS FLOW ELECTROPHORESIS

Continuous flow electrophoresis is a procedure that started as continuous zone or curtain electrophoresis (see below) and that through many technical improvements and refinements outgrew the carrier and curtain stage, and is now developing in several different directions as a free flowing liquid technique. In all cases the electric field is perpendicular to the direction of liquid flow. The method has three theoretical advantages of considerable importance:

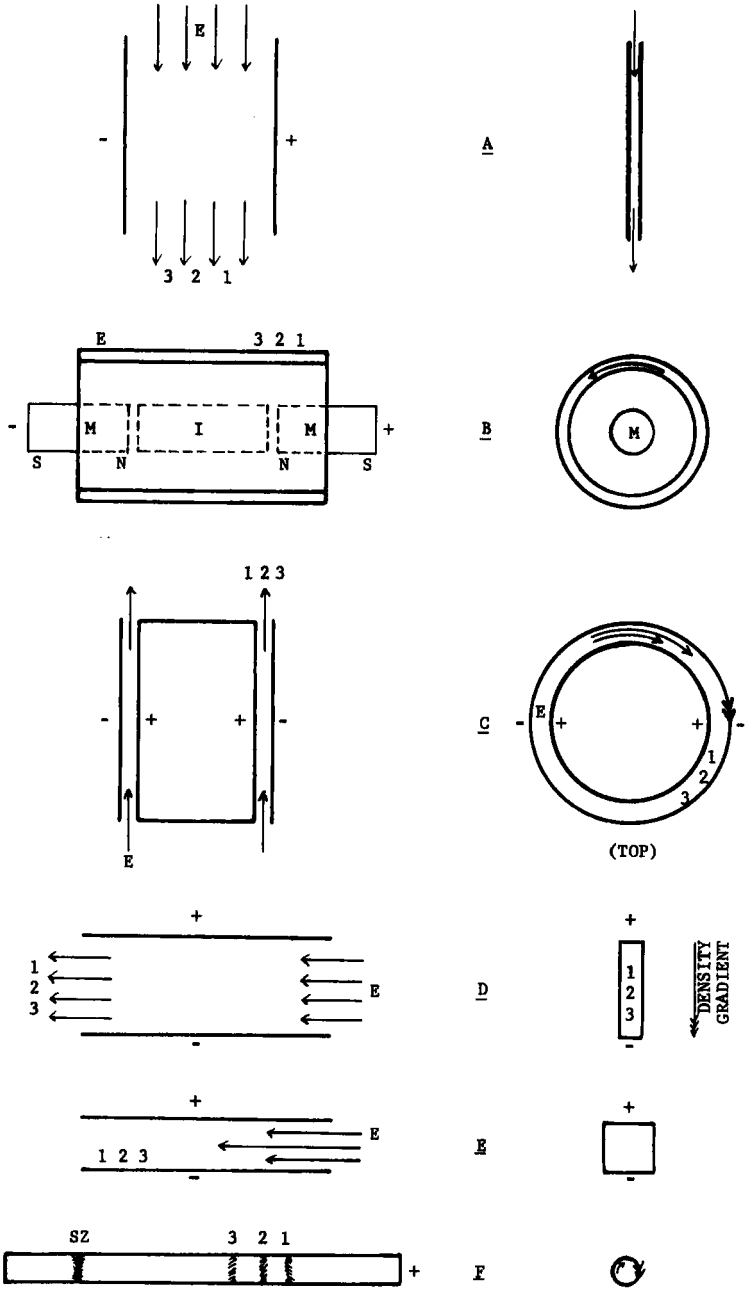
1) It presents the simplest built-in possibility for continuous and automatic fraction collecting;

2) It allows the electrophoresis of rather large quantities of solute that are directly proportional to the duration of electrophoresis, which as long as a steady state is maintained, is virtually unlimited;

3) In the free flowing liquid version it affords a possible solution to the intractable problem of sedimentation, which makes it especially attractive to cell electrophoresis.

Although all continuous flow electrophoresis methods are closely interrelated, it is convenient to subdivide them into the following sections: Curtain Electrophoresis, Free Flow Electrophoresis, Endless Belt Electrophoresis, Cylindrical Rotating Continuous Flow Electrophoresis, Stable Flow Electrophoresis, Electrophoretic Field Flow Fractionation, and Horizontal Rotating Cylindrical Electrophoresis (see Fig. 7). Some of these methods may possibly fit as well under one of the other headings for reasons of taxonomical propinquity, but they all seem to have developed far enough into the direction of a well-defined separate method to merit treatment of their own.

1. Curtain Electrophoresis. Continuous zone electrophoresis was developed independently by Grassman and Hannig[131] and Svensson and Brattsten[132] in 1949 (see 133). The method quickly proved to be most practical when a vertical sheet of thick filter paper or cardboard was used as a carrier, hence the designation Curtain Electrophoresis.[133-136] The free hanging sheet of filter paper carries a continuous downflow of buffer, while an electric field is applied in the plane of the filter paper, perpendicular to the buffer flow. The solute mixture that is to be separated is continuously applied to a point on the top part of the filter paper. The bottom of the filter paper is cut out in a pattern of multiple triangles, culminating in points at the bottom, under each of which a test tube is placed for the collection of the individual fractions (see Fig. 7A). Apparatus of this type can, once stable flow and field conditions are reached, be run for many days continuously, and fractionate, e.g., 200-1,000 mg of mixed proteins per 24 hours. The process should be done in a well-ventilated cold room, at +4°C. No curtain devices have been commercially available since 1971, although in some cases, parts may still be obtainable from the original manufacturers. In the present author's laboratory two Spinco Model CP Curtain Electrophoresis devices (Beckman Instrument Co., Palo Alto, CA) are still frequently employed in the first fractionation step of protein isolation from whole sera; their yield is considerably higher than that obtainable with starch block electrophoresis (see above). The major draw-



back of curtain electrophoresis is the aspecific adsorption of proteins onto the curtain material. However, once a steady state is reached and a certain load of protein has been adsorbed, no further protein will accumulate and the separation becomes quite efficient when continuous runs of several days or more can be sustained.

2. Free Flow Electrophoresis. Continuous electrophoresis across freely flowing liquid between two closely spaced parallel flat plates is treated here (see Fig. 7A; endless belt electrophoresis is discussed in the next section, and cylindrical continuous flow electrophoresis, stable flow (Staflo) electrophoresis, electrophoretic field flow fractionation, and horizontal rotating cylinder electrophoresis are grouped in the sections following it, see Fig. 7.

The development of free flow electrophoresis, or "nonstabilized continuous deflection electrophoresis" has been and remains linked with the pioneering and continuing work of Hannig.[137-139] One of the most important advantages of this method is that cell separations can be achieved that begin to be on a preparative scale.[140-143] The method has also been used success-

FIGURE 7A - F

Schematic representation of the various continuous flow electrophoresis methods. Front views on the left and side views on the right (except for 7C, where the right hand view is a top view). Polarities of the electric field are indicated by + and —. The direction of liquid flow is indicated by an arrow with a single arrowhead. The direction of rotation of a solid cylinder is indicated by an arrow with a double arrowhead, see C and F. The vertical (downward) direction of the density gradient in D is indicated by an arrow with a triple arrowhead. The approximate place of continuous entry of the sample mixture is indicated by E, and the place of three continuously emerging fractions are marked 1, 2 and 3, in the order of decreasing electrophoretic mobility.

A: Curtain Electrophoresis and Free Flow Electrophoresis; B: Endless Belt Electrophoresis (M = magnets, N = north, S = south, I = soft iron cylinder); C: Cylindrical Rotating Continuous Flow Electrophoresis; D: Stable Flow Electrophoresis; E: Electrophoretic Field Flow Fractionation; F: Horizontal Rotating Cylinder Electrophoresis (SZ = starting zone).

fully for the isolation of biopolymers that, due to adsorption and other problems, are not easily purified by means of carrier electrophoresis, such as acid mucopolysaccharides[144] and blood clotting factor VIII.[145] Polystyrene latex particles of different sizes (and thus of different κa, see Section II, 3 above) have also been separated by free flow electrophoresis.[18, 146]

The method still labors under many difficulties of electrokinetic as well as of hydronamic nature. Electroosmosis causes the distortion of the cross-section of fraction streams to a crescent shape,[147] and hydronamic considerations hamper the scaling-up of the process, particularly due to the thermal convection created by Joule-heating. The latter problem is still so severe that operation at 0 gravity has been considered as one of the better solutions (see below under Section X, 7). Hannig et al., treated these and other theoretical and experimental aspects of free flow electrophoresis extensively.[146] To obviate particle or cell sedimentation during continuous electrophoresis, Hannig placed the separation chamber vertically with the liquid flow in the downward direction.[138, 139, 146]

3. Endless Belt Electrophoresis. Kolin attained the same advantage by combining continuous flow with fluid rotation in a most ingenious manner: a continuously moving fluid belt is maintained by rotating it in a thin cylindrical or flattened cylindroid torus around a horizontal axis, by means of a double axial magnet that creates a radial magnetic field, and an electric field perpendicular to it (parallel to the axis). The electric field at the same time is the driving force for the electrophoresis;[148-151] see Fig. 7B. The method, of course, labors under the same difficulties as regular free flow electrophoresis between flat plates, with the added drawbacks of somewhat greater complexity of the apparatus, and of diminished freedom to regulate the liquid flow velocity independently of the electric field used for the electric transport.

4. Cylindrical Rotating Continuous Flow Electrophoresis. This type of continuous flow electrophoresis is relatively new. Like Kolin's endless belt electrophoresis (see above) it uses a rotating cylindrical torus of liquid, but its

axis is vertical, and in addition to rotating, the entire cylindrical liquid vein also is forced to flow upward. The rotary liquid movement is maintained by rotating at (150 rpm) the outer of the two cylinders that confine the liquid, which much enhances the cooling efficiency and thus allows high electric fields; see Fig. 7C. Also contrary to Kolin's method, the electric field is radial, which, of course, allows a very high field strength over a thin layer of liquid. Thus very large quantities of material may be continuously separated with this method, of the order of up to 10 grams of protein/hour.[152]

5. Stable Flow Electrophoresis. Stable flow (Staflo) electrophoresis was first proposed by Mel in 1959.[153-155] This is horizontal fluid flow electrophoresis, with a vertical electric field, stabilized by a (sucrose) density gradient included in the fluid flow; see Fig. 7D. This method has been applied to a variety of separations (most of them done at the originating laboratory), e.g., of rat bone marrow cells,[156] spinach chloroplasts,[157] lipoproteins,[158] diploid yeast cells and spores,[159] and sperm cells.[160]

6. Electrophoretic Field Flow Fractionation. Based on general theories of field flow fractionation (FFF) developed by Giddings,[161, 162] electrophoretic FFF (EFFF) was proposed in 1972 and applied to simple protein separations.[163] EFFF is (like Staflo, see above) horizontal free flow electrophoresis, with a vertical electrical field (but without a density gradient), spacer membranes, and with collection of fractions by their simple piling up in a given spot on the lower spacer membrane, once a steady state has been reached; see Fig. 7E. No significant novel and/or useful separations obtained with this method have as yet been reported.

7. Horizontal Rotating Cylinder Electrophoresis. In 1967 Hjertén described a free zone electrophoresis method in a slowly rotating (at $\approx$ 40 rpm) horizontal tube, with the electric field in the direction of the axis;[164] see Fig. 7F. In the same manner as Kolin's method (see above), rotating the fluid primarily serves to obviate sedimentation, and thus allows the electrophoresis

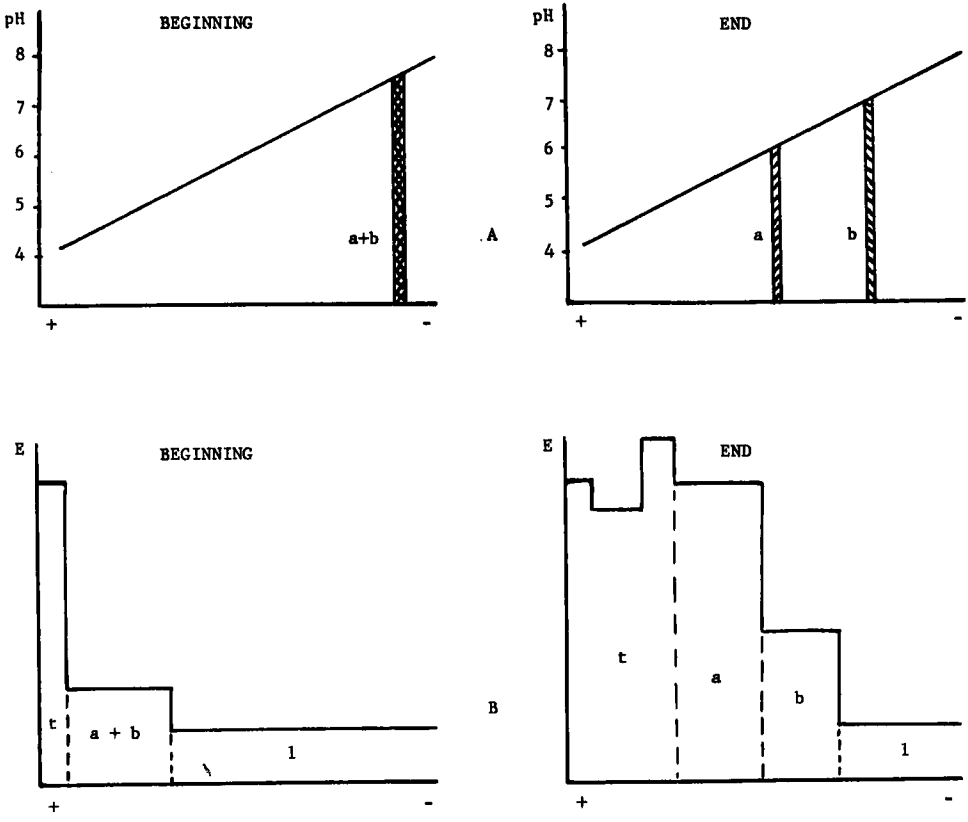
of suspended particles and cells as well as of dissolved solutes. When a quartz tube is used, the method can be used for analytical purposes with the help of a UV scanning device. As in a number of the other methods described above, zone deformation by electroosmosis occurs, but it can to a certain extent be alleviated with the help of low ζ-potential coatings, e.g., with methyl cellulose. This procedure is essentially a micro-method, because thermal convection due to Jouleian heating makes homogeneous cooling possible only with tubes of fairly small internal diameter, hence the advantage of working with toroid-shaped or flat liquid veins that can be cooled from both sides, as described above. Nevertheless the scale on which this type of micro-electrophoresis can be applied surpasses that of microscope-electrophoresis (see below, Section IX, 1) by an order of magnitude, so that determinations could be done with the rotating tube method that were not possible earlier. It could, for example, be demonstrated that the highest insulin content within various fractions of β-cells of mouse pancreas islets of Langerhans are to be found in the fraction richest in spherical granules.[165] Virus isolation was also studied by this method.[166, 167]

VIII. ISOELECTRIC FOCUSING AND ISOTACHOPHORESIS

Both isoelectric focusing and isotachophoresis can be considered as electromigration in buffers of which the composition is non-constant with respect to location within the applied electric field (see Fig. 8). Hence these two methods are frequently treated together,[168, 169] as they will be here.

1. Isoelectric Focusing. While the electrophoretic transport of charged molecules in an electric field in a homogeneous buffer of a given pH is continuous and has to be stopped if one wishes to avoid losing the molecules through migration into the electrode compartment of the opposite charge, electromigration of a charged amphoteric molecule through a continuous pH-gradient automatically ceases when that amphoteric molecule has reached the place in the gradient where the pH is the same as its isoelectric point. Thus,

isoelectric focusing is the sorting-out of amphoteric molecules according to their different isoelectric points, by electromigration through a pH-gradient (see Fig. 8A). Generally speaking, prolonged electromigration of a mixture of amphoteric molecules in a pH-gradient tends to enhance the resolution of each of its constituents, hence the designation of isoelectric focusing. The historical background has been given by Righetti and Drysdale,[170] who outlined the contributions by Kolin, Svensson (now called Rilbe) and Vesterberg, all of whom also recently described the origins of this method from their own particular viewpoints.[171-175] The development that finally made the general application of isoelectric focusing a practical reality was the synthesis of many different "carrier ampholytes", each having several acidic and basic groups with closely spaced pK values per molecule, with many different pI values;[176] see also ref. 175. Since the early 1970's, when these carrier ampholytes became generally available, a veritable publication explosion in the fields of analytical as well as preparative isoelectric focusing has taken place (see e.g., refs. 169-170; see also a recent review by Righetti.[177] This is due to the fact that for, e.g., protein separation, isoelectric focusing allows an improvement in resolution of about an order a magnitude, compared to electrophoresis. This is largely because, with time, bands separated by isoelectric focusing reach and maintain an optimal sharpness (due to the focusing effect) while with electrophoresis separated bands continuously tend to broaden because of diffusion. For analytical purposes, and in particular for the separation of proteins by isoelectric focusing, one must be mindful of the fact that the carrier ampholytes most used are isomers and homologues of aliphatic polyamino-poly-carboxylic acids,[176] and are thus difficult to distinguish from proteins by the most commonly used colorimetric or spectrophotometric methods. However, as these carrier ampholytes have an average molecular weight of about 800, while that of most proteins is above 10,000, proteins generally can be easily separated from the ampholyte molecules by means of gel filtration (with Sephadex^{R} G-50).[175, 178]



Various isoelectric focusing methods in completely free solution have recently been described by Quast[179] and Bours,[180] but more generally stabilization by means of density gradients,[170, 180] gels[83, 170, 181, 183] or granulated gels[184] is practiced. Isoelectric focusing can also be combined with continuous flow of the ampholine system.[185-187] It is not always necessary to attain equilibrium for isoelectric focusing to be useful. Catsimpoolas studied isoelectric focusing phenomena prior to reaching equilibrium, which he called "transient state isoelectric focusing",[188, 189] by means of optical scanning.[190]

2. Isotachophoresis. The history and background of isotachophoresis has been well described by Haglund.[192] Like isoelectric focusing, isotacho-

phoresis is practiced in a buffer system of which the composition is non-constant with respect to location (see above), but while isoelectric focusing is best done in a buffer system consisting of an essentially continuous pH gradient, *isotachophoresis needs a buffer system* of which the components have markedly discontinuous properties. In isotachophoresis the sample mixture is placed in the sample compartment, "terminating electrolyte" in this cathode compartment, and the "leading electrolyte" in the anode compartment (see Fig. 8B).[193] The "effective mobility"[193] of the leading electrolyte is the highest, and that of the terminating electrolyte the lowest; the effective mobilities of he components in the sample mixture should be intermediate between those extremes. Because of the possibility of achieving a total separation between different ionic species, once a steady state is reached, isotachophoresis affords an

FIGURE 8

Schematic representation of the principles of isoelectrofocusing (A) and isotachophoresis (B). The Beginning and End situations are depicted for both techniques.

In isoelectric focusing (A) the pH is plotted vs. the path of migration. The pH gradient formed by the carrier ampholytes is ideally constant, although this is not always the case in actual practice.[191] A mixture of two components a and b with different isoelectric points may be inserted at any place in the migration path, but is here shown to start near the cathodal end (a + b); (A, Beginning). When the separation is completed (in principle the separation continues to become sharper with time), a and b have reached the locations in the migration path where the pH's correspond to their respective isoelectric points (6 for a and 7 for b); (A, End).

In isotachophoresis (B) the electric field strength E (in V/cm) is plotted vs. the path of migration; see Everaerts et al.[193] The sample mixture (a + b) is placed between the terminating electrolyte (t) in the cathode compartment, and the leading electrolyte (l) in the anode compartment. The effective mobility[193] of the leading electrolyte (l) is the highest, and that of the terminating electrolyte (t) the lowest; the effective mobilities of materials a and b should be intermediate between these two. Once a steady state has been reached, the separation between a and b is complete, and all four zones (t, a, b, l) move with equal velocity.

extremely high resolution. The method is therefore increasingly employed for analytical purposes,[194-196] e.g., by using capillary tubes.[197-200] At the same time isotachophoresis is becoming useful for preparative separations,[201, 202] which also can include the capillary tube approach.[203, 204]

IX. PARTICLE AND CELL MICRO-ELECTROPHORESIS

The methods described in this section permit the determination of electrophoretic mobilities of $\approx 10^2$ cells, and generally require 10^3 - 10^5 cells per determination.

1. Classical Microelectrophoresis. Microelectrophoresis of particles and cells antedates the other electrophoretic methods,[1] and even the determination of electrophoretic mobilities of dissolved proteins initially was possible only by first coating small particles with them, and then practicing microelectrophoresis on these particles;[2] see above, Section I. The classical technique of particle and cell microelectrophoresis has been well described by Abramson et al.,[2] and more recently by Seaman for cylindrical chambers,[205] and by Fuhrmann and Ruheustroth-Bauer for rectangular chambers.[206] The classical technique is complicated by the effect of electroosmotic backflow in the vicinity of the inner wall of the microelectrophoresis chamber, which causes the observed mobilities of particles to vary considerably, according to their position in that chamber. To cope with this effect, the observed mobilities must either be plotted vs. location perpendicular to the electric field inside the chamber, or obtained by focusing on the "stationary level",[2, 205] provided the particles do not tend to sediment, and remain long enough at that level to have their velocity measured.

2. Microelectrophoretic Methods with Lasers. Ware developed a method with which the mobilities of particles can be detected by means of measurement of the Doppler shift of coherent light (perpendicular to the electric field), scattered by the moving particles.[207] Disturbances due to electroosmotic backflow are reported to be circumvented by using an open U-tube

of the Tiselius type (see Section III above), although of a very much smaller size.[208] With this method Ware observed that polystyrene latex particles manifest a leveling off in electrophoretic mobility, with decreasing ionic strength.[207] Ware advances this as the first experimental observation of that effect, and professes that the effect has not yet been explained.[207] However, this is a classical example of relaxation at a relatively high ζ-potential ($\approx$100 mV) and rather low κa (between 0.1 and 10), first elucidated by Overbeek in 1943,[17] see above, Section II, 3 and also refs. 7, 9, 11-13, 19, 20. Little is known about the reliability of the method in other respects, as few comparisons as yet seem to have been published between the results obtained with Ware's method, and conventional microelectrophoresis.

Uzgiris has devised a laser light scattering electrophoresis cell of very simple design, consisting mainly of an optical cell with two parallel plate electrodes inside it.[208, 209] This arrangement may partly obviate various problems, such as electroosmotic backflow, Joule heating, etc., but replaces them with a variety of possible disturbances created by the presence of unshielded electrodes. Nevertheless, the electrophoretic composition of human peripheral lymphocytes has been analyzed rather accurately by this method.[209]

Finally, Goetz and Penniman developed a new technique, applied to classical microelectrophoresis, but using a rotating prism, and laser light.[210] Illumination by a polarized thin laser beam allows focusing virtually solely on the stationary layer (see the preceding section). The electrophoretic motion is optically counterbalanced by an opposite motion caused by the rotating prism; at that null condition the particle image appears stationary. With this method the mobility of single particles as well as of groups of many particles can be determined in a very short time, in the conventional manner, as well as automatically. The mobilities of particles as small as 15 nm can be measured with this method (provided they have a high refractive index differential with the medium).

3. Microelectrophoretic Measurements by a Resistive Pulse Technique. Recently DeBlois and Wesley described a method for the measurement of electrophoretic mobilities of sub-micron particles (especially viruses), inside single pores of Nuclepore® membranes, based on the resistive pulse techniques of the Coulter counter.[211] Apart from electrophoretic mobilities, size distributions and particle concentrations can also be determined with that method. A typical pore diameter is ≈ 340 nm. The electroosmotic correction for the ζ-potential of the inner pore wall postulates plugflow for the electroosmotic backflow through the open pore, which appears reasonable. This method is likely to be useful for the analysis of various properties (including electrophoretic mobility) of various sub-micron particles.

4. Microelectrophoresis with Uniformized Electroosmotic Backflow. The paraboloid velocity distribution resulting from electroosmotic backflow in closed capillaries can be completely obviated by reducing it to a uniform plugflow. This can be done by coating the inner surface of glass capillaries with a gel layer (e.g., of 2.5% agarose gelled in the electrophoresis buffer), and once the capillaries contain the sample cells or particles, by plugging them at both ends with the same gel.[35, 36] The observed electrophoretic velocity of particles and cells inside these capillaries is then the same at all depths of focusing; cells may be followed by keeping them in focus while they sediment, and sedimentation of very large cells can be circumvented by occasional rotation of the capillary that contains them, around its cylindrical axis.[36] The characteristic electroosmotic backflow of the agarose must be determined for each buffer used, and that value must be added to the electrophoretic mobilities found for the cells.[212, 213] The method has been compared with classical microelectrophoresis and the same results were obtained with various cells under a variety of conditions.[35] With low or medium ionic strength buffers, air cooling of the capillary suffices to obviate the Joule effect, however, for ionic strengths $\mu > 0.1$, water cooling is desirable.[35]

Recent results with the electroosmotic plugflow method showed bimodal distributions in histograms of normal human granulocytes.[36] A widely ranging histogram of guinea pig pancreas cells has also been given, as well as a demonstration of the effect of neuraminidase treatment on the electrophoretic mobility and other properties of human granulocytes.[36] A slight difference was found between the average electrophoretic mobilities of guinea pig alveolar and peritoneal macrophages.[36] The electrophoretic mobilities of macrophages are especially difficult to measure with the classical method, due to the pronounced tendency of these cells to sediment, on account of their large size. Recently the macrophage electrophoretic mobility (M.E.M.) test for the detection of cancer[212] has been studied with this simplified method.[213] It was found that alveolar (guinea pig) macrophages give more reproducible results than peritoneal macrophages, that cryopreserved alveolar macrophages give better results than fresh macrophages, and that significant changes in the electrophoretic mobility of lymphocytes of cancer patients could be obtained directly, which might simplify this test by eliminating the need for guinea pig macrophages entirely.[213]

X. PREPARATIVE PARTICLE AND CELL ELECTROPHORESIS

It should be realized that for the determination of any of the myriads of biological properties living cells possess, one usually needs to have $\approx 10^6$ cells per isolated fraction at one's disposal. Thus to be able to determine which one of, say, ten different cell fractions contains cells capable of synthesizing compound X, at least 10^7 cells of the original mixture have to be fractionated. The methods described in Section IX, above, clearly are quantitatively at least four orders of magnitude removed from the capability of directly determining which biological properties accompany which electrophoretic mobility fraction in a given mixture of cells.

For the electrophoretic separation of $>10^7$ cells, a total separation distance between the slowest and the fastest group of cells of at least

several cm is generally required. Given the usual ζ-potentials of cells (generally a few times 10-31 mV), in electric fields that create no excessive Joule effects, times of the order of $\approx 10^5$ seconds are required, and during such time lapses most mammalian cells sediment $\approx$ 1 cm in water. This means that, in the time required to separate mammalian cells in numbers sufficient to give separated fractions large enough for analysis, all the cells will have sedimented to the bottom of most electrophoresis chambers, unless a way is found to prevent that occurrence. The following sections treat a number of approaches that have been devised to deal with that problem.

1. Free Flow Electrophoresis of Particles and Cells. Of the continuous flow electrophoresis methods described in Section VII above, free flow electrophoresis and stable flow electrophoresis appear to be the most suited for preparative cell electrophoresis.

With free flow electrophoresis (see Section VII, 2) separations of cell mixtures consisting of several times 10^7 cells have now become possible.[141, 142, 214, 215] Fractionations have been reported of T and B lymphocytes,[141, 142, 214, 215] rabbit kidney cells,[214] rat liver lysosomes,[214] as well as complete separation between inside-out and outside-in vesicles from human erythrocyte ghosts.[214]

For continuous flow isoelectric focusing of cells, see Section X, 7, below.

2. Stable Flow Cell Electrophoresis. With stable flow (Staflo) electrophoresis (see Section VII, 5) separations of mixtures up to 10^8 blood cells are feasible.[160, 215] Mel obtained complete separation between rabbit and chicken erythrocytes,[215] and partial but significant separation of rat bone marrow cells.[216] Pistemma et al. obtained a significant enrichment of viable and fertile cells by means of stable flow electrophoresis of fowl sperm cells.[160]

As one of the most intractable problems of free flow electrophoresis is stability (mainly against disturbances caused by thermal convection

and flow), stable flow electrophoresis in which stability is manitained by means of a flowing density gradient may well be one of the most promising of all continuous flow methods for cell separation on a preparative scale. The one drawback, i.e., the use of high concentrations of sugars for the stabilizing gradient (which may adversely affect living cells), can possibly be obviated by the use of D_2O instead of sugar gradients (see Section X, 4, below).

3. Descending Density Gradient Electrophoresis. Up to 4×10^7 cultured Chinese hamster bone marrow cells have been successfully electrophoresed downward into a Ficoll sucrose gradient by Boltz et al.[217] Similar separations were done by Griffith et al. with mixtures of erythrocytes from different species (rabbit and mouse, and human and rabbit); mouse spleen cells; mouse thymus cells and erythrocytes.[218] The present author and his collaborators fractionated 10^8 cultured human lymphocytes by descending electrophoresis into a gradient of low molecular weight dextran (MW = 10,000), which we found the least apt to clump the cells. A considerable enrichment in T cells was obtained in the fastest fractions.[219]

Sucrose density gradient electrophoresis of latex particles in moving boundary cells has been described above (Section III).[37, 38]

4. Ascending Electrophoresis. Cell aggregation by polymers such as Ficoll and dextran,[220, 221] as well as cell deformation and other changes by sugars[222, 223] will remain a problem with all density gradient methods employing these substances. In addition, virtually uncharged polymers nevertheless can cause changes in the ζ-potentials of cells, e.g., dextran tends to bring about an increase in the negative charge of erythrocytes.[220, 224, 225] Attempts to obviate stabilizing gradients in preparative cell electrophoresis by vertical upward electrophoresis into a simple non-gradient liquid column have been described by the present author and his collaborators.[219, 226, 227] By ascending electrophoresis ("levitation") into a non-gradient liquid column, practically pure T-cells were found in the fastest fraction (up to 3×10^7) of

human peripheral lymphocytes;[219, 226, 227] this may result from the fact that human T-cells not only have higher electrophoretic mobilities but are also smaller than B-cells, and thus the least prone to sedimentation. With some of the purest T-cell fractions thus obtained, it proved possible to elicit a rabbit anti-human T-cell antiserum that, without any absorption, appeared specific for human T-cells and showed no cross-reactivity with B-cells.[228] With this method it is nevertheless necessary to form a flat and stable layer of cells, at the start, near the bottom of the vertical tube, by depositing them upon, e.g., a glucose or sucrose cushion.[226] Recently, however, it proved feasible to replace these sugar cushions by a cushion of buffered D_2O.[227] It also proved advantageous to stabilize the entire vertical column, above the pure buffered D_2O cushion, with a shallow D_2O gradient. Various cell types could be separated by ascending electrophoresis into shallow D_2O gradients, e.g., mixtures of $>10^8$ erythrocytes; granulocytes; lymphocytes.[227] However, the fastest lymphocyte fraction always was closer to 100% T-cells, when no such shallow D_2O gradient was used.[227] However, when a purely electrophoretic cell separation is required (without interference of cell size effects, advantageous or otherwise), ascending cell electrophoresis into a shallow D_2O gradient, with a cell-layer of $\approx 10^7 - 10^8$ cells deposited on top of a D_2O cushion as a starting point, appears to be one of the mildest and surest methods available to date. The buffers used must nevertheless contain fairly large amounts of, e.g., glucose, to maintain isotonicity and to avoid high ionic strengths (and thus strong Joule heating). Boltz et al. recently also developed an ascending preparative cell electrophoresis method, into a (Ficoll) density gradient, albeit with relatively few ($10^5 - 10^7$) cells.[229]

5. Packed Column Cell Electrophoresis. In columns packed with glass beads that are all approximately of the same size (0.1 mm diameter) it is possible to separate mixtures of $\approx 6 \times 10^9$ erythrocytes electrophoretically. The interstices between beads are small enough to prevent the migration into the column of erythrocytes by simple sedimentation at ambient gravity, but

large enough to permit virtually unimpeded migration under the influence of an electric field. Complete separation was obtained between human and chicken erythrocytes, and between papain-treated and untreated human erythrocytes.[230] Unfortunately, upon further experimentation with other types of mammalian cells, dimensional monodispersity of the cells proved to be essential for the success of the method. Cells with a fairly wide-spread range of sizes can at best be classified according to size with this method, but not according to charge.[231] This method, therefore, appears to be useful mainly for the electrophoretic separation of erythrocytes, a separation for which there exists no particularly urgent need at present.

6. Cell Isoelectric Focusing. It has been known for over twenty years that at least some blood cells (e.g., lymphocytes, platelets), have true isoelectric points;[232] see also[233]. It would thus seem logical to endeavor to separate them by isoelectric focusing; see Section VIII, 1. One of the first attempts at preparative cell isoelectric focusing was reported by Leise and LeSane,[234] who fractionated up to 5×10^7 lymphocytes (human as well as rabbit). Cell viability (as measured by trypan blue exclusion) of fractions focusing at non-alkaline pH's was fair, but pronounced cell growth after subculturing the various fractions was found to be considerable only with the fraction focusing at pH 4.2. In the second half of the 1970's great improvements in preparative cell isoelectric focusing were obtained in a number of laboratories. Manske et al. separated various kinds of native and modified cells (5×10^6 at a time), with little loss in viability among the electrofocused fractions.[235] Boltz et al. recently studied the electrofocusing of a wide variety of cells (up to 5×10^6 per sample) and retained excellent viability and (with sperm cells) reproductive capacity of the fractionated cells.[236]

Finally, preparative cell isoelectric focusing by the continuous flow method has been achieved with erythrocytes[185] and with rat liver organelles[186] by Just and his collaborators.

7. Zero Gravity Electrophoresis. "Electrophoresis under microgravity conditions" probably would be a more accurate way of describing this section, as during space flights total zero gravity is never achieved, and very low ($< 10^{-6}$ G) gravities only for relatively short periods of time. Nevertheless, during the greater part of most space flights gravity conditions would tend to fluctuate mainly between 10^{-3} and 10^{-5} G, for which electrophoresis experiments still virtually abolishes all sedimentation effects, as well as those fluid disturbances caused by local density differences created by the Joule effect (thermal convection).

The first successful electrophoresis separation at "O G" was done during the flight of Apollo 16. Polystyrene latex particles of two different sizes and thus with different κa ratios, see Section II, 3, (but with the same ζ-potentials) were electrophoresed together and separately, in stationary tubes. The results showed the crucial importance of controlling the effects of electroosmosis (especially marked at O G, because under those conditions no stabilizing gradients can be formed to counterbalance these effects). They also showed that owing to the absence of particle sedimentation and thermal convection, even at high potential fields, preparative electrophoretic separation of particles at O G is indeed feasible.[19, 20]

In the light of the experience obtained with the Apollo 16 experiment, experiments for the Apollo-Soyuz flight were designed, with living cells of various types. Successful electrophoretic separations were achieved during the Apollo-Soyuz flight with red cells of different species, as well as with human kidney cells, separated fractions of which were returned to Earth.

In addition, it was demonstrated during that flight that red cell separation by isotechophoresis at O G also is feasible.[237, 238] One of the most important results obtained was with the electrophoretic kidney cell separation experiment, which seemed to indicate that different subpopulations of cells (as separated by electrophoresis) produce different compounds, i.e., cells from one fraction produced urokinase, and those from other fractions

produced human granulocyte conditioning factor and erythropoietin.[237,238] These results were made possible only by the use of low ζ-potential coatings,[239] developed to obviate the type of electroosmotic backflow encountered on Apollo 16.

On Apollo-Soyuz a free flow electrophoresis experiment was also done, without, however, return to Earth of separated cells. The results were analyzed by optical scanning, the light source of which (due to microgravity conditions) turned out to be too bright for direct analysis of cell distribution. However, subsequent computer analysis of the data returned to Earth appeared to indicate that the apparatus had worked, and that a good separation of spleen cells probably had occurred, thus demonstrating the feasibility of O G free flow cell electrophoresis, allowing the avoidance of thermal convection.[240]

Zero gravity electrophoresis appears to be the only way by which preparative cell electrophoresis can be done without the drawbacks of sedimentation or thermal convection, and without the need for stabilizing gradients or complicated flowing systems.

XI. OTHER ELECTROKINETIC METHODS

For analytical and preparative purposes electrokinetic phenomena other than electrophoresis have been used; these are electroosmosis, streaming potential, sedimentation potential, and as modifications of electrophoresis, electroconvection and electroextraction methods. Electrodialysis, though somewhat related to a number of these methods, is not treated here.

1. Electroosmosis. Synge and Tiselius,[241] and Mould and Synge,[242] first proposed to make use of electroosmotic flow in gel membranes or other porous media, to transport solvents through such porous media and thus to obtain separation according to the site of solutes transported with that solvent, the smaller solute molecules being able to go through smaller pores than the

larger ones (see also 70; Section V, above). More recently Pretorius et al. proposed to use electroosmosis as a means of transporting solvents, in thin-layer and in high-speed chromatography.[243] The advantages of electro-osmosis over other means of transporting solvents are two fold: 1) With a reasonably high ionic strength (obtained by addition of electrolytes), and thus with a compressed electric double layer (see Section II, 1, above), virtually ideal plug-flow can be achieved throughout the column, with a concomitant reduction in theoretical plate height.[243] In open-ended tubes electroosmosis generates no noticeable hydrostatic pressure, and the rate of solvent transport is relatively independent of the site of the pores or channels.[241-243] The author and his colleagues have used electroosmosis and electrophoresis combined and in the same direction through stacks of extremely dense membranes, to achieve enrichment of an Li isotope;[71] see Section V, above.

It has been long established that there essentially is total equivalence between the electrokinetic phenomena, and that ζ-potentials can theoretically be obtained through electroosmosis or streaming potential as well as through electrophoresis determinations;[10, 244] see Section II, 2, above. Among others, Abramson et al. demonstrated that the ζ-potential of gelatin could be measured via the electroosmotic profile in a chamber coated with gelatin as well as through the electrophoretic mobility of gelatin-coated particles in the same chamber.[245] Fike and van Oss have devised a method for the measurement of the ζ-potentials of living mammalian cells by determining the electroosmotic velocity of buffer through glass capillaries, of which the inner walls were entirely covered with monolayers of cultured cells.[246] That method permits the determination of cell surface potentials of cell layers without the need for first dispersing the cells by chemical, enzymatical or mechanical means, required for the measurement of cell electrophoresis. It is known that all of the above-mentioned cell dispersion methods tend to alter the cells' ζ-potential more or less drastically.[246, 247]

Barry and Hope determined membrane potential differences across plant cell membranes *via* electroosmosis,[248] and Fensom *et al.* studied electroosmotic flow across membranes of Nitella.[249]

Thus, electroosmosis can be used as a means of solvent transport, as a method for determining cell surface potentials, and also as a method for determining cell membrane potentials (in a direction perpendicular to the cell surface). Electroosmotic solvent transport *cannot* be used to bring together two solutes; for that purpose electrophoresis only can be used, at a pH intermediate between the isoelectric points of the two solutes; see Section VI, 3, above.

2. Streaming Potential and Sedimentation Potential. ζ-potentials of minerals and other granular materials are most conveniently determined by streaming potential measurements;[250, 251] such measurements are of considerable importance in the mining industry because of a strong correlation between the flotation properties of minerals and their ζ-potentials.[252-255] Other devices for the study of streaming potentials have been developed by Rutgers and de Smet,[256] and by Boumans, who by means of turbulent flow in metal capillaries used streaming potentials to build up extremely high voltages (70,000 V) in a fluid-flow type of van de Graaff generator, developing, however, only 10^{-7} amps.[257] Tuman measured streaming potentials through sandstone (up to several V) at very high pressures (up to 2,000 p.s.i.).[258] Improvement on existing pressure ζ-potential measuring methods have been discussed by Korpi and de Bruyn,[259] and the interference of streaming potentials in the results obtained in automatic potentiometric systems was reported by Van den Winkel *et al.*[260] The present author has described the role of streaming potentials elicited by the potential difference used in ultrafiltration through charged membranes, on the ion retention by these membranes.[261, 262]

Streaming potential measurements have also been used to obtain information on the surface potentials of cells,[263] and of various biopolymers.[264-266] As the pressures (and the concomitant shear forces) used in streaming

potential measurements are considerable, the use of this method, in particular for the determination of cell surface potentials, is open to criticism, and without much doubt the use of the exceedingly gentle method of electroosmosis (see the preceding section) is to be preferred with living cells.

Streaming potentials across membranes (e.g., rabbit gall bladders) can be elicited by establishing differences in osmotic pressure on different sides of the membranes,[267] as well as by mechanically applied pressures.

Sedimentation potentials elicited by the settling of charged particles under the influence of a gravitational field (Dorn effect) has been little studied by direct measurements. The influence of sedimentation potentials on the sedimentation coefficients of polyelectrolytes has been studied by Mijnlieff.[268] "Sedimentation" potentials caused by gas bubbles rising through a liquid are somewhat easier to measure; determinations have been done at the beginning of this century by McTaggart,[269] and were continued by Alty.[270] Recently Usui and Sasaki reported on the "sedimentation" potentials resulting from the rise of small argon gas bubbles through aqueous solutions of cationic, anionic and nonionic detergents.[271]

3. Electroconvection Methods. When a protein in solution is subjected to electrophoresis in a chamber that is separated from the electrodes by membranes that are impermeable to the protein, at a pH different from the protein's isoelectric point, the protein will accumulate in a concentrate layer next to the membrane that is nearest to the electrode of opposite charge to that of the protein. This effect is called electroconvection, or electrodecantation. Clearly, in any protein mixture, only the most anodal and the most cathodal migrating protein can be separated with a single electroconvection chamber. The method was first described by Pauli,[272] who used it for the concentration of ovalbumin, rubber latex and other colloids. In the late 1940's and during the 1950's the method was studied in a number of laboratories,[272-277] but probably due to the incompleteness of the purification attainable with the method, never became universally adopted. The degree

of purification could be improved by means of multimembrane or multicompartment devices,[278-280] but the method is nevertheless still not widely used. Electroconvection has also been advocated as a means of concentrating proteins.[281, 282]

4. Electro-extraction Methods. Recently, Polson devised an extremely simple method for extracting pure plant viruses from infected plants by means of a single-step electrophoretic purification method, which he named electro-extraction.[283] In an H-shaped electrophoresis chamber, an extraction chamber is attached on the anodal side of the infected plant leaves, separated from them by a perforated disc. The chamber is separated from the anode by a membrane that is permeable to chlorophyl and plant proteins but impermeable to viruses. By simple electro-extraction of infected tobacco leaves, pure tobacco mosaic virus could be obtained; however, for the electro-extraction of pure turnip yellow mosaic virus, prior freezing and thawing of the infected Chinese cabbage leaves was required.[283] Polson and his colleagues also adapted this method to serve for the purification of human and animal viruses.[284] Here the same apparatus is used, but in addition, between the unpurified virus compartment, and the pure virus reception chamber anodal to it, a 2% agarose spacer gel, containing antibodies to non-viral antigens, is placed. During electro-extraction the virus passes into the reception chamber, while the various impurities are retained in the spacer gel after having interacted (and precipitated) with the antibodies it contained. In this manner the final purification of Hepatitis-B antigen from human serum could be effected, with the help of rabbit anti-whole human serum antiserum.[284]

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