ELECTROPHORESIS OF PROTEINS BY THE TISELIUS METHOD[1]

L. G. LONGSWORTH AND D. A. MACINNES

Laboratories of The Rockefeller Institute for Medical Research, New York, New York

Received January 30, 1939

INTRODUCTION

The recent improvements introduced by Tiselius (15) in the electrophoretic method have greatly increased its importance in the study and preparation of proteins. Our contribution to this Symposium will consist of a discussion of the principles underlying the Tiselius method, together with a description of the experimental procedure involved in the use of the method, including such modifications as we have introduced. The topics to be considered are (*a*) the apparatus, (*b*) the optical principles involved in the observation of electrophoretic boundaries, (*c*) the effects of thermal convection, (*d*) some typical applications of the method, and (*e*) "boundary anomalies". These will be treated in the order given.

APPARATUS

To study electrophoresis of proteins and related materials by the moving boundary method it is necessary, in the first place, to form a boundary between a solution of the material in a suitable buffer and the buffer itself. Passage of current then causes the boundary to move. For this movement to be a measure of the mobility of the protein it is desirable, first, that the electric field and pH in the neighborhood of the boundary be substantially constant, second, that the electrode processes do not involve the evolution of gas or other uncertain volume changes, and third, that the electrode products do not reach the regions in which the boundaries are moving. These conditions have been admirably met in the electrophoretic apparatus developed by Tiselius, which, with the modifications that we have made, will be described below.

The cell in which the boundaries are formed and observed is shown in cross section in figure 1A and consists of the sections I, II, III, and IV.

[1] Presented at the Symposium on the Physical Chemistry of the Proteins, held at Milwaukee, Wisconsin, September, 1938, under the auspices of the Division of Physical and Inorganic Chemistry and the Division of Colloid Chemistry of the American Chemical Society.

These may be slid over one another along the planes a-a′, b-b′, and c-c′. Through the cell runs a U-shaped channel d-d′ of rectangular cross section. Figure 1B is a top view of one of the center sections. To form a boundary, the channel is filled with the buffer solution of protein to a level slightly above the plane b-b′, about 10 ml. of solution being required. Section III is then pushed to one side, the excess solution in section II is removed, and this section is rinsed with buffer. The remainder of the cell and the attached electrode vessels to be described are then filled with the buffer.

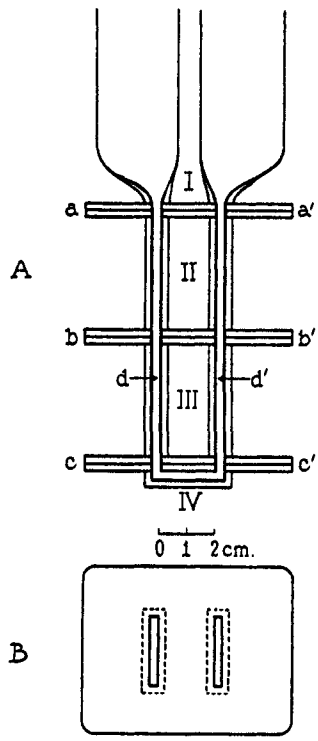

FIG. 1. A, electrophoresis cells in cross section; B, top view of one of the center sections.

The support for the cell and the electrode vessels, together with the mechanism for moving the sections of the cell in relation to each other, are shown in figure 2. In our apparatus a rack-and-pinion system replaces the pumps utilized by Tiselius for this purpose. Turning the knurled knobs k-k′ attached to the concentric shafts (s) operates the bevel gears (g), which in turn cause a horizontal motion of the racks r. Each rack presses against a metal insert e, which communicates the pressure

to the edges of the horizontal glass plates. The sections II and III may therefore be shifted in either direction by manipulation of the appropriate knurled knob.

After filling the apparatus as described above, the silver–silver chloride electrodes (E-E′ of figure 2) are inserted. For effective operation of these electrodes, described more fully later, they must be immersed in a strong chloride solution. This is accomplished by carefully introducing the solu-

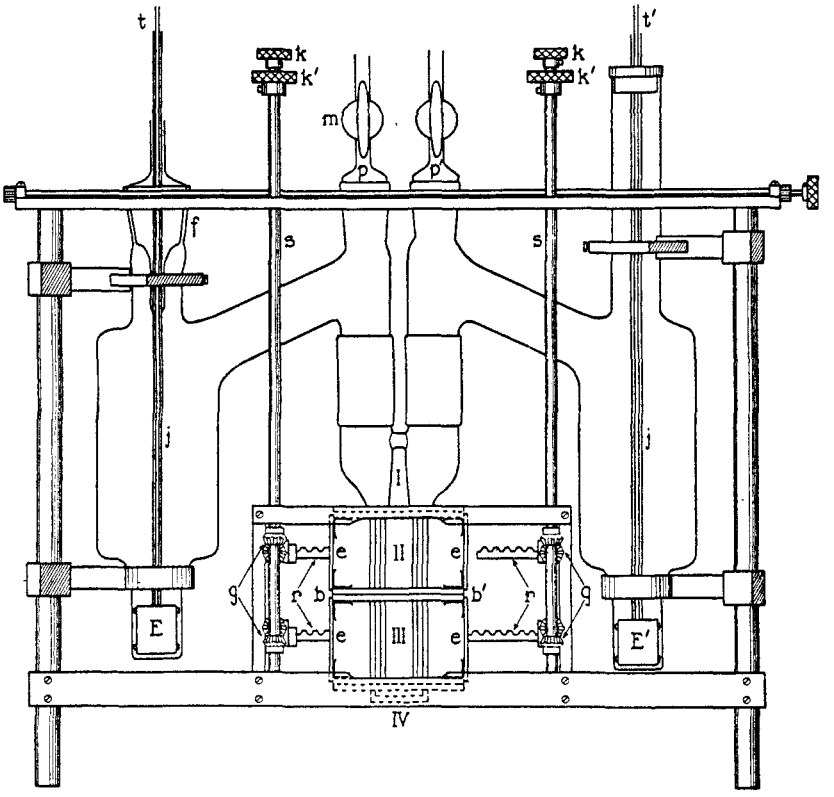

FIG. 2. Electrophoresis cell, electrode vessels, and support

tion through the silver tubes t-t′. These silver tubes, which are insulated by the glass tubes (j), also serve as current leads to the electrodes E-E′. We have modified the procedure of Tiselius and our earlier procedure, in that one side of the apparatus is closed, care being taken to exclude air bubbles. This is accomplished with the ground-glass stopper f and the stopcock m. Closing one side is more convenient than having both sides open, as the latter procedure involves equalizing the liquid levels on the

two sides of the system before forming the boundaries. It also permits the use of an improved type of compensation, to be described below.

With the cell and electrode vessels filled as described, the two boundaries are formed in the plane b-b′ by returning section III to the position shown. A potential from a battery or "power pack" applied to the terminals t-t′ will then, in general, cause the boundary in one side of the cell to rise and that in the other to fall.

With the design of the apparatus shown in figure 2, filling of the cell and electrode vessels, as described, may be carried out in the low-tem-

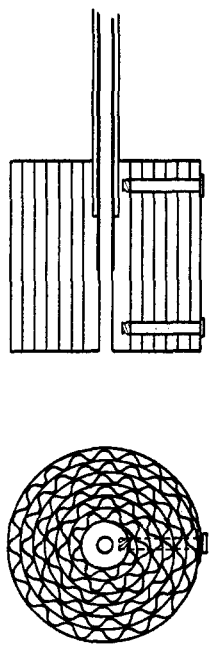

FIG. 3. Diagram illustrating the electrode construction

perature thermostat. Since the stoppers p and p′ are directly above the cell, the protein solution and buffer may be introduced with pipets. For this purpose we have found a syringe, provided with a long stainless-steel needle, serviceable.

Further details of the electrodes E-E′ are as follows. They must be capable of carrying currents of at least 30 milliamperes for long periods of time without evolution of gas. Sufficient capacity has been obtained by winding a flat and a corrugated strip of sheet silver together into a tight spiral, as shown in figure 3. The ends of the spirals are anchored to a hollow silver core with silver screws. The silver tube is also threaded

into this core. This type of construction, suggested by Professor H. P. Cady, exposes a large electrode surface to the electrolyte. Air bubbles trapped by the electrodes may be easily dislodged.

OBSERVATION OF THE BOUNDARIES

Another important contribution to the electrophoretic method made by Tiselius was the adaptation of Toepler's "schlieren" (shadow) method (9) for the observation of the boundaries. A diagram of the optical system is shown in figure 4. The image of the slit S, illuminated by the lamp L and condenser C, is brought to focus in the plane P by the lens D. The schlieren diaphragm, a screen with a sharp, horizontal upper edge, is placed in the plane P and may be moved vertically, a micrometer adjustment being used. The cell E, in which the electrophoresis is carried out, is placed as near the lens D as the thermostat construction permits. The

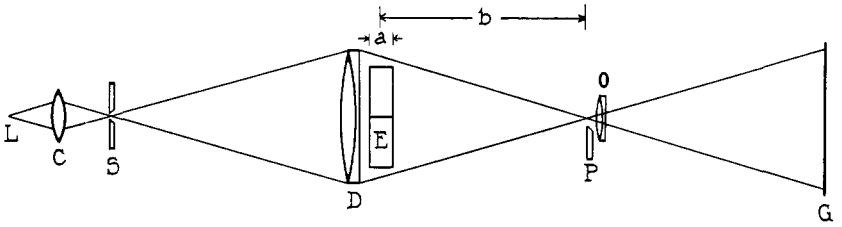

FIG. 4. Arrangement for observation of the boundaries with the schlieren method. L, 5-volt, 18-ampere ribbon filament lamp; C, projection condenser; S, 0.3 × 15 mm. horizontal slit; D, Dallmeyer portrait lens 6 D, 4 in. diameter, 24 in. focal length; E, electrophoresis cell; P, schlieren diaphragm; G, ground-glass screen or photographic plate; O, Dallmeyer R.R. lens, 3 in. diameter, 24 in. focal length.

camera objective O, placed immediately behind the schlieren diaphragm, is focussed on the cell and forms a full-size image on a ground-glass or photographic plate at G. Further details of the system as used in obtaining the results described in this paper are indicated in the legend of the figure.

In the absence of refraction gradients in the electrophoresis cell all of the light is brought to focus in the image of the illuminated slit at P and enters the camera objective. If, however, a boundary is present in the cell, the refraction decreases with increasing height through the boundary, and the pencils of light through this region are deflected downward. These deflected pencils are intercepted by the schlieren diaphragm and fail to reach the screen. Thus the region at G conjugate to the boundary appears as a dark band on a light background. This is shown in figure 5. The horizontal dark lines are the schlieren bands of the boundaries between a

0.5 per cent egg albumin solution in a 0.02 normal sodium acetate buffer at pH 5.2 and the pure buffer. The upper and lower photographs are of the boundaries migrating in the anode and cathode sides of the channel, respectively. Exposures were made at 30-min. intervals, and after four exposures the current was reversed. It is of great interest that the reversal brought the boundaries accurately back to their original positions.

Some of the characteristic features of the schlieren method may be considered with the help of figure 4. The angular deviation of a pencil of light in the boundary is proportional, under appropriate conditions, to

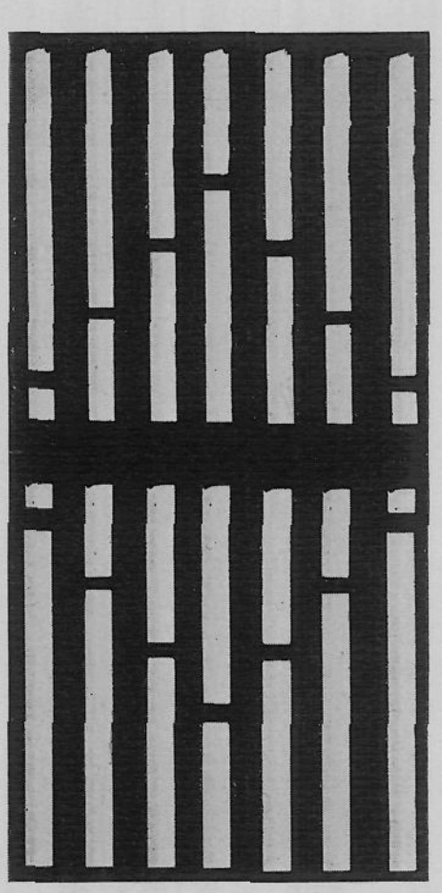

FIG. 5. Schlieren bands of a single protein. Exposures were made at 30-min. intervals and the current was reversed after the fourth exposure.

the gradient, dn/dx, of the refractive index, n, and the horizontal breadth, a, of the boundary. The displacement, Δ, of the schlieren diaphragm necessary to intercept the deflected pencil is also proportional to the "optical lever arm," b. We therefore have the expression

$$\Delta = ab\frac{dn}{dx} \qquad (1)$$

in which a and b are constants of the apparatus, and dn/dx varies vertically through the boundary. As the schlieren diaphragm is raised, the first pencils of light to be intercepted are those which have passed through

the steepest gradients of refractive index, i.e., the center of the boundary if the diffusion and spreading of the latter have been normal. Thus a series of photographs of the boundary with decreasing displacement of the diaphragm give an indication of the variation of the refractive index through the boundary. Such a series of photographs of the boundaries formed in a mixture of rabbit and guinea pig hemoglobins is shown in figure 6, the upper half of the figure referring to rising boundaries and the lower to descending boundaries.

Since the refraction of the solution is substantially proportional to the concentration of protein, the schlieren method may be used to obtain

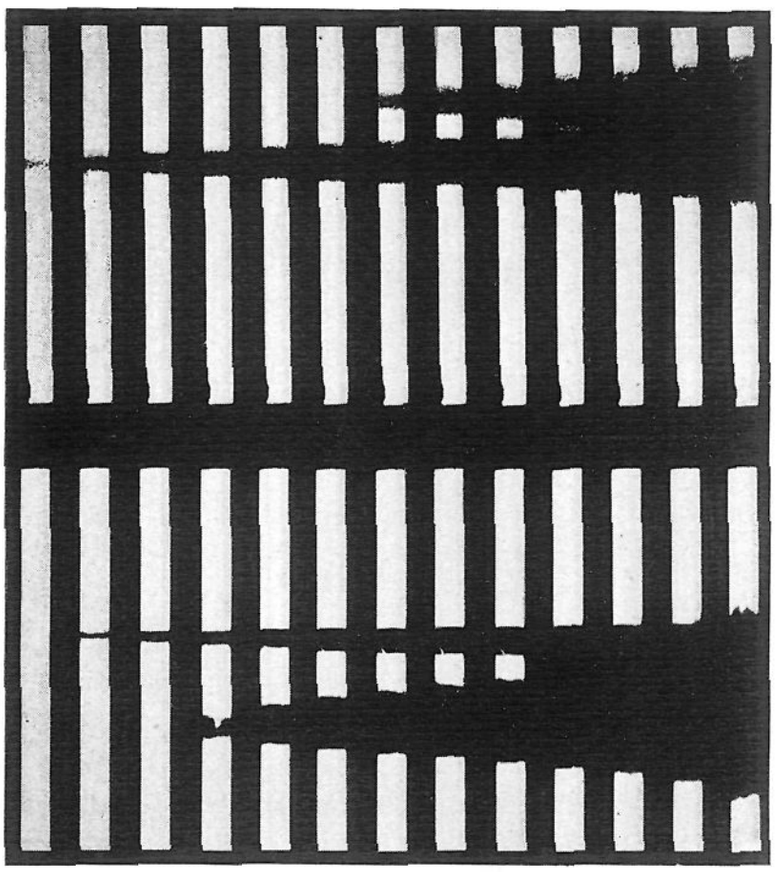

FIG. 6. Schlieren bands, for different schlieren diaphragm settings, of a mixture of rabbit and guinea pig hemoglobins.

quantitative estimates of the concentrations of the various constituents. Thus, if the cell image at the photographic plate is masked by a narrow vertical slit, a slow horizontal movement of the plate simultaneously with the vertical displacement of the schlieren diaphragm produces automatically on the plate a transparent area proportional to $\int \Delta \, dx$. Reference to equation 1 shows that

$$\int \Delta \, dx = \int ab \frac{dn}{dx} \, dx = ab\Delta n$$

in which Δn is the refractive index increment due to the protein constituent causing the boundary in question. This modification of the schlieren method for quantitative analysis has been described in two papers from this laboratory (6, 7).

REDUCTION OF THERMAL CONVECTION

The chief problem in electrophoretic methods is the elimination of mixing due to thermal convection. If a current of electricity is passing through a conducting solution in a tube, heat is generated in each volume element of the solution but flows to the thermostat only through the wall of the tube. The solution along the axis of the tube is thus hotter than at the wall. Normally, therefore, the solution at the wall will be heavier, and in falling will give rise to convection currents. We shall consider, as a typical example, a current of 0.006 ampere passing through a solution the specific conductance of which is 0.0038 mhos (0.1 N sodium acetate at 0°C.) in a cylindrical tube of 5 mm. internal and 10 mm. external diameter. The formula[2] which describes the temperature of the solution, t_s, in the steady state as a function of the distance, r, from the axis is

$$t_{r=0} - t_s = \frac{I^2 \epsilon}{4\kappa K_s} r^2 \tag{2}$$

The corresponding formula for the temperature of the glass, t_g, is

$$t_g - t_0 = \frac{a^2 I^2 \epsilon}{2\kappa K_g} \ln \frac{b}{r} \tag{3}$$

In these equations a and b are the inside and outside radii, respectively, of the tube, K_s and K_g are the thermal conductivities of the solution and glass, I is the current density, ϵ is the electrical equivalent of heat, and t_0 is the thermostat temperature. Using equation 2, the solution along the axis of the tube is 0.65°C. hotter than at the wall, and from equation 3 the drop in the wall is 0.67°C. The computed temperature distribution is given in figure 7a, in which the temperature increase, Δt, over that of the thermostat is plotted against the distance from the axis of the tube. If the thermostat is regulating at 25°C., this temperature gradient in the buffer solution is accompanied by the density variations shown in figure 7b and, as has been stated, it is these differences which cause mixing, by convection currents, of the solution in the tube. If, on the other hand,

[2] These equations are from a private communication from Dr. Melvin Mooney. The authors wish to acknowledge gratefully this aid, which has been of service to them both in this work and in their earlier work on the determination of transference numbers by the moving boundary method.

the thermostat is regulating at 0°C., the density differences in the solution are much less and the variation is in the opposite direction, as shown in figure 7c. The contrast between the curves of 7b and 7c arises from the fact that this buffer solution has a maximum density at 2.85°C. (2). If, in this particular example, the thermostat temperature were regulated at 1.85°C., the average temperature in the tube would be 2.85°C., and the density gradient would be a minimum, as indicated by the horizontal

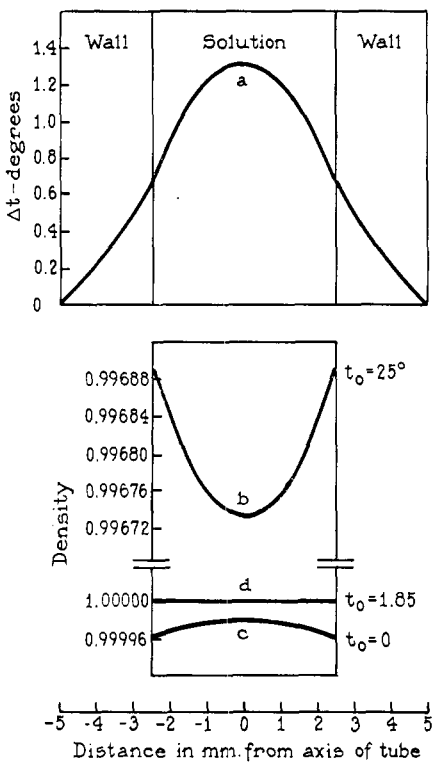

FIG. 7. Distribution of temperature and density in a salt solution, in a cylindrical tube, during passage of electric current.

line in figure 7d. Some preliminary measurements of the temperature variations in a rectangular channel indicate that they are of the same order of magnitude as those indicated in the example just given for a cylindrical tube.

In spite of the fact that the density differences produced by temperature gradients are, as indicated in figure 7, very small, the recent great advances in the electrophoretic method are due to the important observation of

Tiselius that convection can be largely eliminated by working at temperatures near that of maximum density. The computations suggest that further progress may be made by additional knowledge of the temperature of maximum density of the buffer solutions used and of the actual temperature distribution in the electrophoresis cell. Investigations along both these lines are in progress by Dr. T. Shedlovsky of this laboratory.

SOME TYPICAL APPLICATIONS OF THE ELECTROPHORETIC METHOD

One of the most interesting uses of this electrophoretic method is the determination of the mobilities of proteins as a function of the pH and the ionic strength of the buffer solution in which they are dissolved. The following data for a typical experiment were obtained in collaboration with Drs. Karl Landsteiner and van der Scheer (4). The protein was crystalline albumin from guinea hen eggs. A 0.5 per cent solution was prepared in a 0.02 normal acetate buffer at pH 5.19. The specific conductances, at 0°C., of the buffer and protein solutions, as measured on the bridge described by Shedlovsky (10), were essentially the same and equal to 0.000832 mho. The current, measured potentiometrically, was practically constant throughout the experiment and equal to 0.00679 ampere. From a calibration with mercury the cross-sectional area of the channel in the cell was known to be uniform and equal to 0.815 sq.cm. The current density was, therefore, 0.00833 ampere and the electric field 10.01 volts per centimeter. The schlieren bands were photographed, using unit magnification, at 30-min. intervals and are shown in figure 5. As mentioned earlier in connection with this figure, the current was reversed after the fourth exposure and the boundaries returned accurately to their original position. The displacement of the boundary moving upward into buffer during the 90-min. interval before reversal of the current was 2.05_5 cm., giving $3.80_5 \times 10^{-4}$ cm. per second as the velocity and -3.80×10^{-5} cm. per second per volt per centimeter as the mobility of the protein, the migration being anodic. The corresponding displacement of the boundary moving into protein was 1.99_5 cm., giving a mobility of -3.70×10^{-5}. The average from the two boundaries is thus -3.75×10^{-5}. The greater displacement of the boundary moving into buffer has been quite generally observed and will be discussed later in this paper.

In addition to the measurement of mobilities the method furnishes information as to the purity and homogeneity of the protein solution and also may be adapted to separation of the components of a mixture. This latter application, called "electrophoretic analysis" by Tiselius, is illustrated in figure 8. Suppose a mixture of proteins A, B, and C, for which the (positive) mobilities are $u_A > u_B > u_C$, is placed in the cell as shown in figure 8a. Movement of the bottom center section of the cell to the

right will bring the protein and buffer solutions into contact in the plane α. On passage of a current three boundaries will appear in the upper cathode and in the lower anode sections, as is shown in figure 8b. It is evident from the figure that, in the ideal case assumed, there is a separation of pure component A in the region between the two leading boundaries on the cathode side and of pure component C between the two slowest boundaries on the anode side. However, if the electrolysis is continued as indicated, before any large proportions of A and C have been separated, the boundaries will have migrated out of the cell in one case and into the bottom section in the other. However, if the second boundary could be given an apparent velocity of zero, as indicated in figure 8c, while the leading boundary moves through the length of one section, isolation of this section

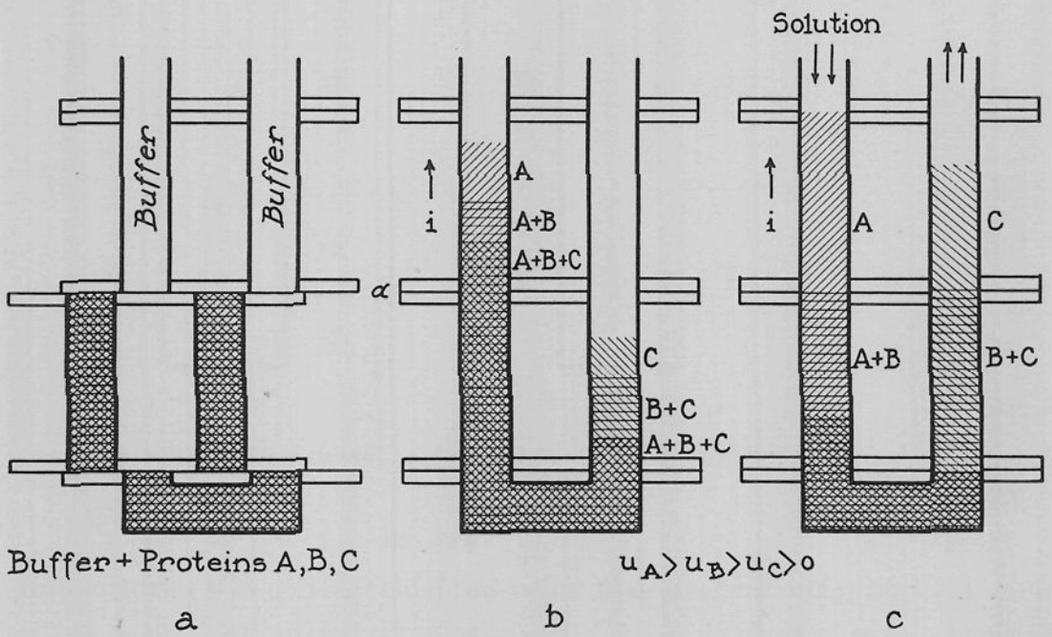

FIG. 8. Ideal electrophoresis of a protein mixture

from the others would make possible the recovery of a solution of pure A from the upper cathode portion of the cell. Simultaneously, as indicated, the slowest component would have an apparent negative velocity, and pure C could be recovered from the upper anode section. As a matter of fact, a boundary can be given any apparent velocity desired by a displacement of the entire solution of the cell. Such displacements have been accomplished in a variety of ways. In Smith's (11) moving boundary apparatus this was done by the withdrawal of mercury, while Collie and Hartley (1) used a piston driven by clockwork. In Tiselius' apparatus he displaces the boundaries by withdrawing, with clockwork, a loosely fitting plunger in one of the electrode vessels. We have used this method also, but in the more recent apparatus described in this paper we have

kept one electrode vessel closed and have displaced the solution in the cell by forcing buffer into this side from a syringe, the piston of which is displaced, at the desired rate, by a threaded rod operated by a synchronous clock motor.

Figure 9 is the electrophoretic pattern, obtained in collaboration with Dr. Karl Landsteiner, of a water-insoluble fraction of a naturally occurring mixture of plant proteins. The three bands indicate the presence of three well-defined constituents. Having demonstrated the complexity of the protein material, it was of importance to locate a certain biological activity which one of the components possessed. To this end the leading components were separated, using the compensation method outlined

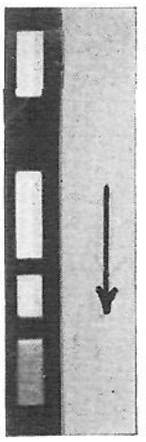

FIG. 9

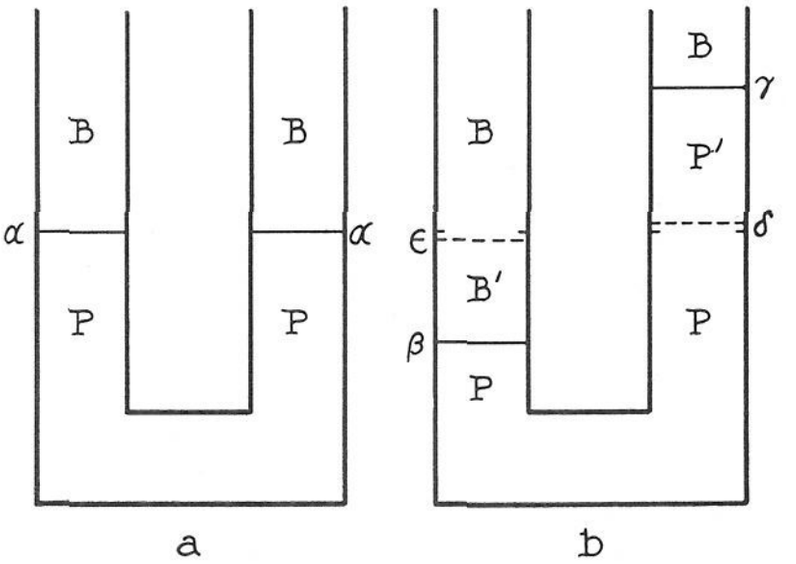

FIG. 10

FIG. 9. Electrophoretic pattern of water-insoluble fraction of a mixture of plant proteins.

FIG. 10. Diagrammatic representation of boundary formation and migration during electrophoresis.

above. They exhibited no activity, whereas the slow component did possess the activity. An electrophoretic study of the water-soluble fraction, which was also active, indicated the presence of a single protein with a mobility near that of the active material in the water-insoluble fraction. The possibility remained that the two active proteins were identical, the ammonium sulfate separation having possibly been incomplete. This question was answered unambiguously by analyzing electrophoretically a mixture of the two active proteins. Two bands were obtained, indicating the separate identity of the two proteins.

In electrophoretic analysis, using compensation, it is frequently necessary to pass relatively heavy currents through the cell for long periods of

time. Under these conditions either very large electrode vessels must be used, or a layer of concentrated buffer solution must be introduced (17) between the concentrated chloride solution around the electrode and the dilute buffer solution above. Tiselius (15) has given an excellent discussion of the size of electrode vessel required when no concentrated buffer solution is used.

BOUNDARY ANOMALIES

An electrophoresis experiment with a single protein may be represented diagrammatically by figure 10. Initially boundaries are formed at the level α in the two sides of the cell between the buffer solution at a concentration B and the buffer solution of protein at a concentration P (in which the protein is assumed to be negatively charged). Passage of a current causes one of these boundaries to descend through a volume V_P to a new position β (figure 10b), and the other to rise through a volume V_B to the position γ. Under ideal conditions, realized closely with a dilute solution of a homogeneous protein in a buffer of sufficient strength to control the pH and conductance of the system, the volumes V_P and V_B will be equal. With more concentrated protein solutions, however, so-called "boundary anomalies" are observed. These are illustrated in figures 11 to 13. For instance, the schlieren bands shown in figure 11a for different settings of the schlieren diaphragm indicate a boundary that has risen into buffer and 11b the boundary that has descended into the protein solution in the other side of the cell. It is quite evident that the boundary moving into buffer is the sharper of the two. This is quite generally observed. A more disturbing anomaly is shown in figure 12, in which the relative displacement of the schlieren bands indicates that the rising boundary is moving more rapidly than the descending one; this is also, apparently, a general rule. Another anomaly is illustrated in figure 13. In this experiment with a 2.5 per cent solution of a single protein the boundaries were formed in the usual manner. They were then shifted in the cell with the compensation device to the positions shown in column 1, so that a very slowly moving boundary, which might normally be obscured by the horizontal glass plates, could become visible. On passage of a current the protein boundaries migrated as usual to the new positions indicated in column 2 of the figure. However, on raising the schlieren diaphragm a second, and relatively faint, boundary, δ (column 3), appeared in the protein solution but slightly removed from the original position of the boundary. In the discussion to follow it will become evident that this is not due to a second protein, but arises from a gradient of concentration left behind by the advancing protein boundary. It is similar to the "δ globulin" boundary of Tiselius (16, 13). On raising the diaphragm still further, at a

somewhat later time, a very faint boundary appeared in the buffer solution at ϵ (column 4) and was similar in nature to the δ-boundary.

In practice the protein solution is prepared by dialysis against the buffer solution. It is important to recall that a difference of buffer salt concentration, between the two solutions, exists when the dialysis is complete,

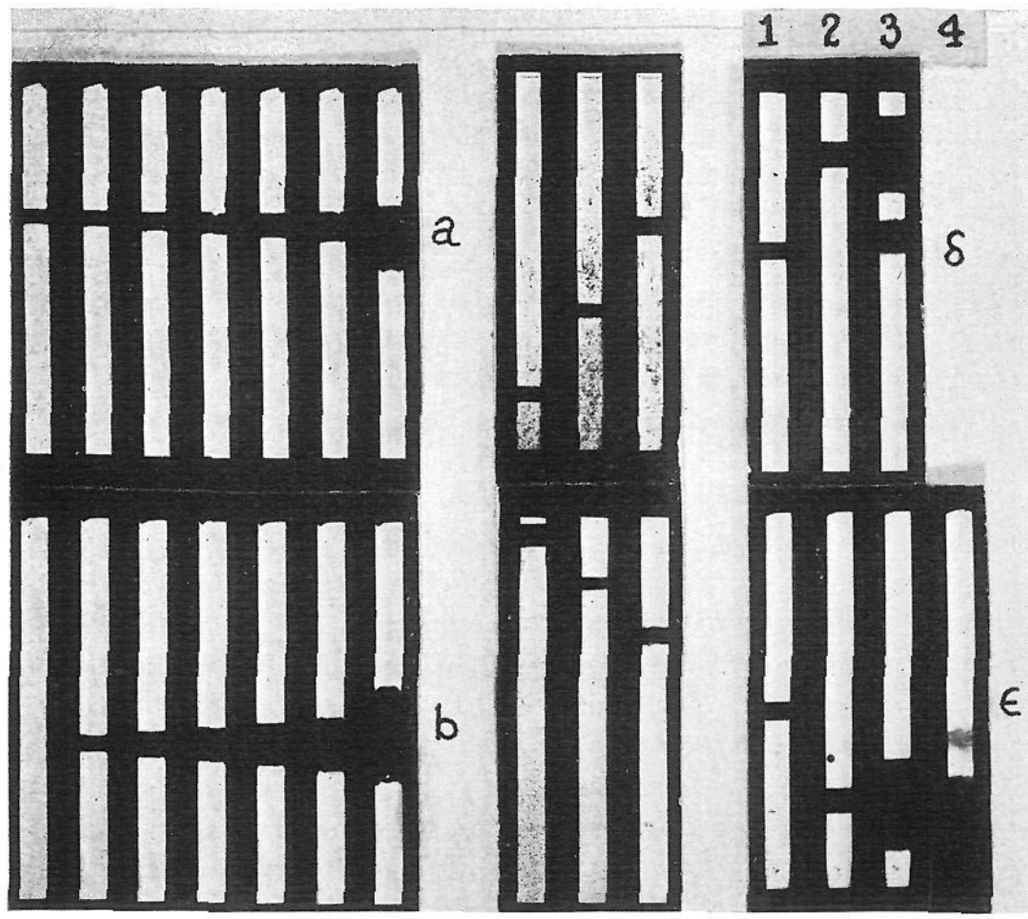

FIG. 11

FIG. 12 FIG. 13

FIG. 11. Schlieren bands, for different diaphragm settings, of a single protein. The boundary (a) moving into buffer is sharper than that (b) moving into protein.

FIG. 12. Schlieren bands of a single protein. The velocity of the boundary moving into buffer is greater than that of the boundary moving into protein.

FIG. 13. Schlieren bands of a single protein. The δ and ϵ bands are due to different concentrations of the same substance.

owing to the Donnan equilibrium. When passage of a current causes a boundary between two such solutions to move (from α to β, figure 10) there is formed in the intervening volume, V_P, a buffer solution of composition B'. This composition has been "adjusted," in general, to a value different from B in such a way that its "regulating function" has the same value as that of the protein solution it has replaced. To quote an earlier

paper by the authors (8), "This (regulating) function defines a property of the solution which, at any given point, retains a constant value independent of changes of concentration caused by electrolytic migration. If, as a result of such migration, species of ions different from those initially present appear at a point, their concentrations will be adjusted to values compatible with the constant determined by the *initial* composition of the solution." It may be noted here that the concentration changes which occur behind the boundary moving into the protein solution are

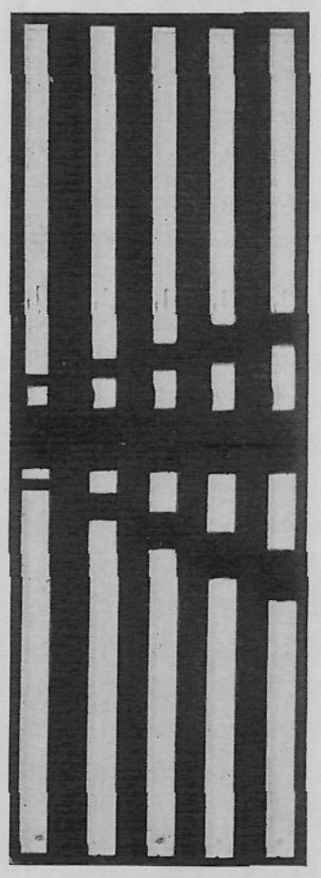

FIG. 14. Schlieren bands of the concentration boundaries between 0.01 and 0.1 normal solutions of lithium chloride.

similar to those encountered by one of the authors (5) in the electrolysis of mixtures of hydrogen and potassium chlorides. The boundary ϵ thus forms between two solutions of the same salt, but at the different concentrations B and B'. It is stable if the lower solution is the more concentrated. Boundaries of this type have been investigated theoretically by Kohlrausch (3) and Weber (18) and experimentally by Smith (12) and ourselves. That such boundaries persist and move on the passage of a current is illustrated in figure 14. Boundaries were formed between 0.01 and 0.1 normal solutions of lithium chloride; these were shifted from behind

the horizontal plates and current was passed. The motion of the boundaries, actually very slow compared with others dealt with in this paper, is indicated by the displacement of the schlieren bands. The motion is due to a change of the transference numbers with concentration.

With the boundary moving into buffer there is a similar but more complicated adjustment of the composition of the protein solution which replaces the buffer as the boundary rises. The resulting concentration boundary, δ (figure 10), between the solutions P and P' moves slowly under the influence of the current and, as mentioned above, is similar to the "δ globulin" boundary of Tiselius. The greater visibility of this boundary, compared with the ϵ-boundary, may be due to the fact that the first involves a gradient of protein concentration whereas the second does not.

In order to account for the boundary anomaly illustrated in figure 11, i.e., the greater diffuseness of the boundary moving into the protein solution, we must consider the effect of the concentration changes, discussed in the preceding paragraphs, upon the specific conductances of the solutions. Owing mainly to its relatively high viscosity, the specific conductance, κ_P, of the protein solution is generally lower than that, κ_B, of the buffer. The conductance differences at the actual boundaries, β and γ of figure 10b, are further increased by the fact that $\kappa_{B'} > \kappa_B$ and $\kappa_{P'} < \kappa_P$. Consequently for a given current the electric field is greater in the protein solution than in the buffer, and variations of the field exist at the boundaries. The dilute uppermost layers of the boundary β moving into the protein solution thus find themselves in weaker fields than do the more concentrated layers and thus tend to lag behind, causing the boundary to become diffuse. In the case of the boundary γ moving into buffer, however, the dilute, slowly moving layers tend to be overtaken by the faster, concentrated ones, with the result that the boundary tends to become sharper. (See also reference 14, page 28.)

If there is a stable δ-boundary, as shown in figure 13, the total concentration below the boundary must be greater than above it. Moreover, it was found in an actual case that the solution P' had a lower conductance than the solution P. The potential gradient above the δ-boundary will therefore, in general, be greater than below, with the result that the protein must move more rapidly in that region than in the main body of the solution. If the displacement of the boundary γ can be assumed to be a measure of the velocity of the protein particles in the solution P', this boundary would be expected to move more rapidly than the β-boundary, as has been true in every case we have observed.

In the preceding discussion of boundary anomalies we have ignored the small differences of pH, required by the Donnan equilibrium, which exist between the buffer and protein solutions. Owing to the change of protein

mobility with pH, these differences at the boundaries can cause anomalies similar to those mentioned. Both theory and experiment indicate, however, that by far the major portion of these anomalies arises from the differences in specific conductance.

Although boundary anomalies are always present to some extent they may be reduced by proper selection of the buffer solution. The buffer used as solvent for the protein should have a high capacity in order to reduce, relatively, the buffer action of the protein itself. It should also have a low specific conductance in order to decrease disturbances due to the heating effect of the current. Since both buffer capacity and conductance increase with the concentration of buffer salts, it is evident that these two conditions are mutually incompatible, and a compromise must be made. As buffer capacity does not depend upon ionic mobilities, buffer salts the ions of which have low mobilities should be selected if possible. We have used sodium salts in preference to potassium salts for this reason. Lithium salts would be still better, if circumstances justified their preparation. High concentrations of weak electrolytes should be avoided in the preparation of buffers, unless allowance is made in the thermostat temperature for the large effect of these components on the temperature of maximum density of the solution.

REFERENCES

(1) COLLIE AND HARTLEY: Trans. Faraday Soc. **30**, 657 (1934).
(2) International Critical Tables, Vol. III, p. 108. McGraw-Hill Book Company, Inc., New York (1929).
(3) KOHLRAUSCH: Ann. Physik **62**, 209 (1897).
(4) LANDSTEINER, LONGSWORTH, AND VAN DER SCHEER: Science **88**, 83 (1938).
(5) LONGSWORTH: J. Am. Chem. Soc. **52**, 1897 (1930).
(6) LONGSWORTH: J. Am. Chem. Soc. **61**, 529 (1939).
(7) LONGSWORTH: Annals N. Y. Acad. Sci., in press (1939).
(8) MACINNES AND LONGSWORTH: Chem. Rev. **11**, 171 (1932).
(9) SCHARDIN: Das Toeplersche Schlierenverfahren. Grundlagen für seine Anwendung und quantitative Anwertung. VDI-Verlag G.m.b.H., Berlin (1934).
(10) SHEDLOVSKY: J. Am. Chem. Soc. **52**, 1793 (1930).
(11) SMITH: J. Am. Chem. Soc. **50**, 1904 (1928).
(12) SMITH: J. Research Natl. Bur. Standards **6**, 917 (1931).
(13) STENHAGEN: Biochem. J. **32**, 714 (1938).
(14) TISELIUS: Thesis, Upsala, 1930. Nova Acta Regiae Soc. Sci. Upsaliensis **4**, 7.
(15) TISELIUS: Trans. Faraday Soc. **33**, 524 (1937).
(16) TISELIUS: Biochem. J. **31**, 1464 (1937).
(17) TISELIUS: Svensk Kem. Tid. **50**, 58 (1938).
(18) WEBER: Die partiellen Differential-Gleichungen der mathematischen Physik, 5th Auflage **1**, 195–203, 503–27. Braunschweig (1910).