Proceedings of the
NATIONAL ACADEMY OF SCIENCES

Volume 41 · Number 3 · March 15, 1955

*ISOELECTRIC SPECTRA AND MOBILITY SPECTRA: A NEW APPROACH TO ELECTROPHORETIC SEPARATION**

BY ALEXANDER KOLIN

UNIVERSITY OF CHICAGO

Communicated by R. S. Mulliken, January 31, 1955

Introduction.—Fluids which are of interest to the biochemist and microbiologist are often complicated mixtures containing some components in solution and others in suspension. The separation and classification of these components constitute a problem of basic importance. Physical methods, ultracentrifugation and electrophoresis, are the most widely used general methods for accomplishment of these aims. The moving-boundary method employed in electrophoresis has the drawback that only the slowest and fastest ions can be isolated from the other components. Only a small fraction of the separable components can be isolated. The amounts of fluid required are relatively large, and the time required for analysis may be as long as 2 days.[1] The observation of the moving boundaries requires rather elaborate optical equipment, and special precautions must be taken to eliminate thermal convection. Filter-paper electrophoresis obviates some of the disadvantages of the moving-boundary method. The problem of thermal convection is eliminated, and the components of a mixture can, in principle, be separated from each other. Certain new drawbacks are introduced, however. For instance, there are proteins which are adsorbed by the filter-paper fibers and thus do not migrate and cannot be separated. The detection and quantitative determination of colorless components is cumbersome, requiring staining. This is a disadvantage in preparative work.

The time required in the analysis of a protein mixture by the method of filter-paper electrophoresis is approximately 12 hours.[1] For comparison, the method described below accomplishes a complete separation of a mixture, as a rule, in less than 10 minutes. In the standard methods the different components are characterized by their electrical mobilities. The new method offers the prospect of making it possible to identify the individual protein components by their isoelectric points.

The purpose of this paper is the presentation of a simple electrokinetic method of analysis which is applicable to suspensions as well as to ionized solutions. The components of a given mixture are separated from each other simultaneously and are sorted in a spatial arrangement to which we shall refer as an "electrophoretic line spectrum." The term "spectrum" is used here to designate a sorting in the same sense in which one speaks of "mass spectra," "energy spectra," and "frequency spectra."

We shall consider two kinds of electrophoretic line spectra: (1) "mobility spectra" and (2) "isoelectric spectra." In the spectrum of the first kind the components are separated according to their differences in electrophoretic mobility, whereas in the spectrum of the second kind the components are separated according to differences in their isoelectric points. Both types of spectra may be superimposed upon each other; in this case we shall speak quite generally of an "electrophoretic line spectrum."

Mobility Spectra.—The aim of the following procedure is to condense the ions of the mixture into a thin layer (often a small fraction of a millimeter in thickness) whose ionized components, migrating with different velocities, finally separate into several layers. The side view of these layers offers the aspect of thin lines reminiscent of an optical line spectrum. Since, to achieve separation, two interpenetrating layers do not have to move apart more than the small distance equal to the thickness of the layer, great speeds of separation can be achieved.

Figure 1 shows the electrophoresis cell in which the electrophoretic line spectra are produced. The central U-tube section communicates at the top with the electrode compartments A and B. The bottom of the U-tube is filled with a buffer solution nearly saturated with sucrose to increase its density. The upper portions of the U-tube, as well as the electrode compartments A and B, contain a sucrose-free buffer solution. The layer M (whose density is adjusted by addition of sucrose or glycerol so as to stabilize it between the light and heavy buffers) contains the mixture to be analyzed. The mixture is very dilute (about 0.01–0.1 per cent of dissolved or suspended solids), and its electrical conductivity is very small as compared to the conductivity of the buffer solution.

Let us consider one ionic component migrating in the M layer under the influence of the electric field. Due to the very low conductivity of this layer, the potential gradient in M is very much larger than in the buffer, and hence the ions migrate in this region very much faster than they do in the buffer. Suppose that negative ions are moving from boundary *1* toward boundary *2* (Fig. 1, lower section) with a speed $v_m = uj/\sigma_m$ (where j is the current density, u the ion mobility, and σ_m the electrical conductivity of the M layer). The ion velocity will suffer a change when the ions enter the buffer, where $v_b = uj/\sigma_b$. When the last ions have reached boundary *2*, the front of the ions has traversed in the buffer the distance $d = v_b t$, whereas during the same time the distance M covered in the M layer is $M = v_m t$. Hence

$$d = M\frac{v_b}{v_m} = M\frac{\sigma_m}{\sigma_b}.$$

Since $\sigma_m \ll \sigma_b$: $d \ll M$. Thus the ions, which originally fill the wide belt M, are contracted into a thin layer of width d as they enter the buffer. If the zone M contains a mixture of ions and of charged, suspended particles, practically one single common line is formed at the boundary of M. Ions of opposite sign are condensed at the opposite boundaries of M.

After the formation of the common line, the current is continued until the migrating line splits into as many lines as there are separable components in the mixture.[2]

Figure 2, A, B, C, illustrates a mobility spectrum obtained with a mixture of three dyes. Figure 2, *A*, shows the M layer before passage of current. (The marks *1* and *2* of Fig. 1 [lower section], indicate the positions of the boundaries of the M

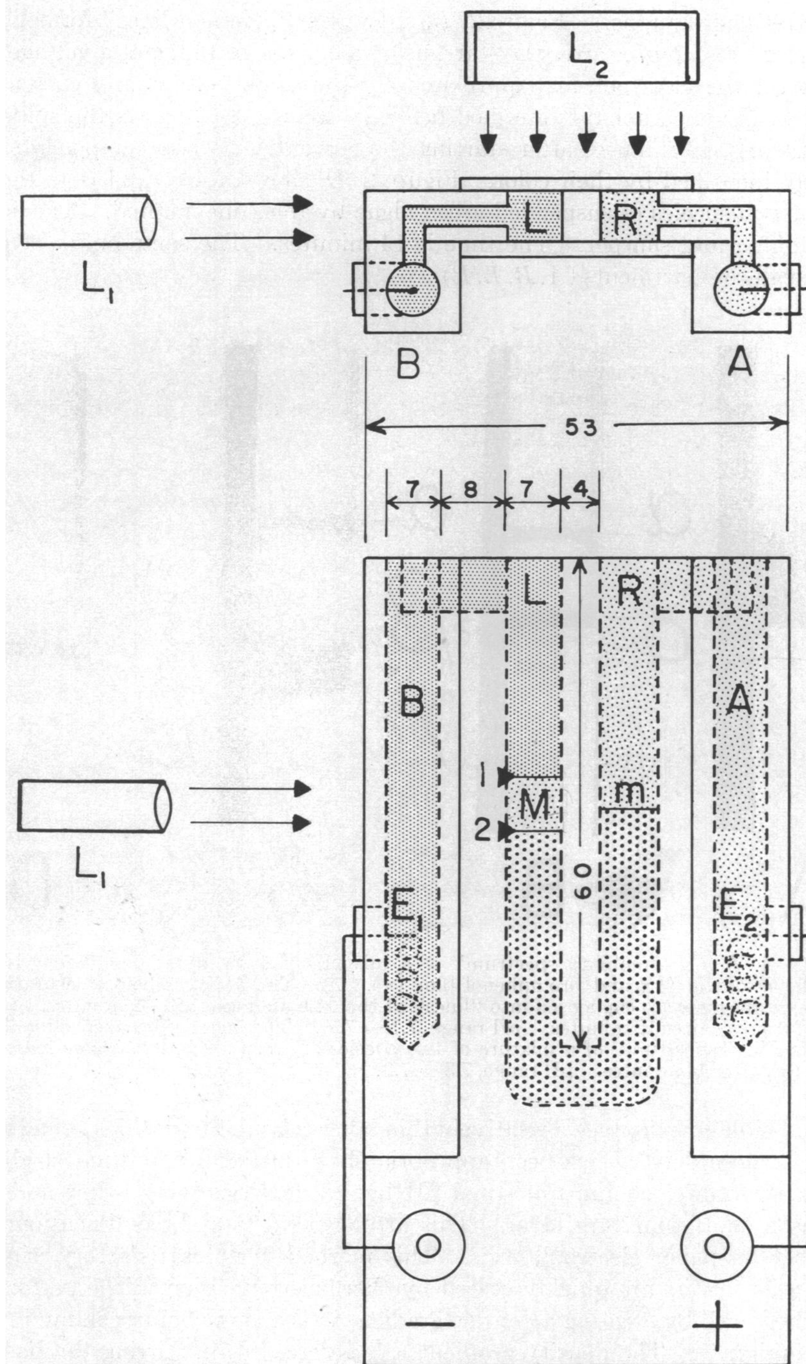

FIG. 1.—L_1 and L_2: sources of light; E_1 and E_2: electrodes; *A* and *B:* channels filled with buffer; *L* and *R:* legs of the U-tube; *M:* mixture to be analyzed; *m:* boundary between light and heavy acid buffer; *1* and *2:* marks indicating the positions of the boundaries of the mixture layer before separation. *Upper section:* top view; *lower section:* front view. The dimensions are in mm.

layer in all photographs.) Figure 2, *B*, shows the "common lines" formed at the *M* boundaries 2 minutes after the start, using a current of 13 ma at a voltage of 110 volts across the electrodes. (Approximately the same voltage and current were used in all the experiments described below.) Figure 2, *C*, shows the splitting of the lower line, 12 minutes after starting the current, into two components which are easily identified by their color. Figure 2, *D*, shows, for comparison, the separation of two species of suspended green algae by the same method. The lines are much thinner and sharper. The time is 14 minutes. The same buffer of pH 7.3 is used in all compartments (*A*, *B*, *L*, *R*).

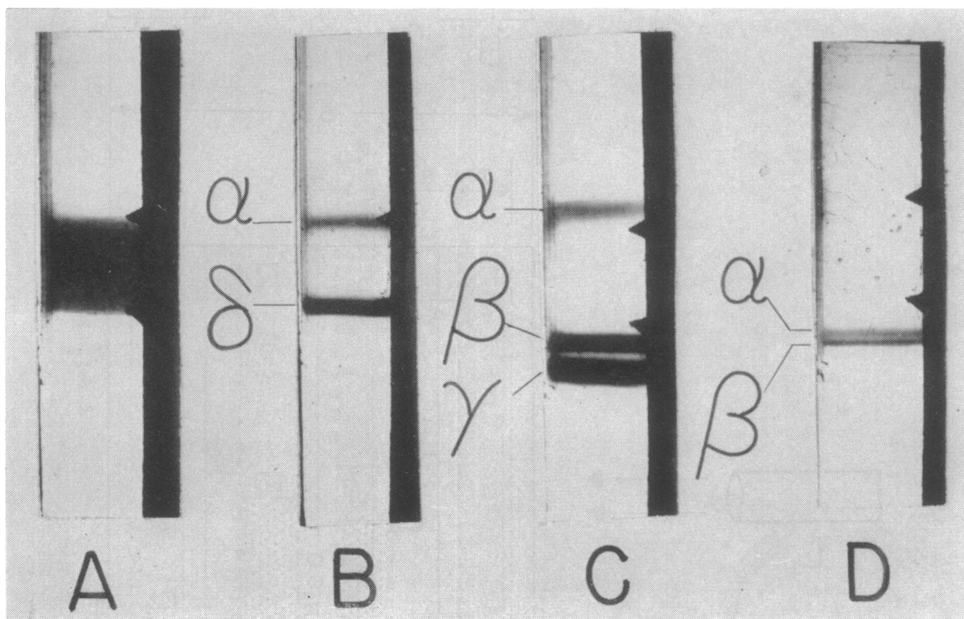

FIG. 2.—*A*, *B*, *C*: "mobility spectrum" of a mixture of three dyes: methylene blue (α), chrome hematoxylin (β), and bromphenol blue (γ). *A*: The *M* layer before separation. *B*: Ions of opposite signs are condensed into "lines" at the *M* boundaries after 2 minutes. *C*: Separation of β and γ after 12 minutes. *p*H range, 7.7 → 7.7 . The lines are broadened by diffusion. *D*: "mobility spectrum" of a mixture of two species of green algae, *Ankistrodesmus* (α) and *Chlorella* (β), after 14 minutes. pH range, 7.3 → 7.3.

It is possible to introduce a relatively thin layer (about 1 mm. thick) of a buffered mixture (whose density has been appropriately adjusted by addition of glycerol) between two buffer columns of equal pH but of densities lying below and above the density of the mixture layer. This arrangement is a three-dimensional analogue to filter-paper electrophoresis. This method is not satisfactory, since the traveling ion layers are quickly erased by thermal convection. The performance can be improved by creating a density gradient in the lower buffer column in which the ions migrate. The density gradient can be created by stirring the boundary between two buffer columns containing glycerol or sucrose in different concentrations or by superimposing many short columns whose densities vary in steps. The "ion condensation method" described above, however, is much more satisfactory, since the ion layers can be made exceedingly thin and sharp, so that only short distances have to be traversed for complete separation. Thus the traveling ion layers remain throughout the process sufficiently close to the steep density

gradient at the *M* boundary, where thermal convection is most effectively suppressed.

Isoelectric Spectra.—In the separation of ampholytes, such as proteins, polypeptides, and amino acids, special use can be made of the existence of an isoelectric point, in order to obtain particularly sharp and stable "line spectra." The distance which the ions must traverse in the process of separation can be limited by interposing the mixture between two buffers of suitable pH. The separation time can thus be greatly reduced. Thus far, the method has been applied to proteins only.

The principle of the method is based on the following consideration: Let us imagine a column of electrolyte solution in which a pH gradient has been established. We shall assume that this solution also contains a protein at uniform concentration. The charge on the protein ions, being a function of the pH of the solution, will vary throughout the pH gradient. For every ampholyte there is a characteristic pH at which the net charge of the molecule is zero. As we proceed from this point toward increasing pH values, the protein molecules acquire a negative charge, which increases with increasing pH. On the other hand, as we proceed toward the acid side of the gradient (decreasing pH), the protein molecules acquire a positive charge, which increases as the pH diminishes. An electric field of constant intensity will not move the protein ions in a constant direction with constant speed in our liquid column of varying pH. If the current flows in the direction of increasing pH, the protein ions which are on the acid side of the isoelectric point move with the current toward the isoelectric point, being positively charged. The protein ions on the basic side of the isoelectric point are negatively charged and hence move contrary to the direction of the current, i.e., likewise toward the isoelectric point. Thus the protein ions converge toward the isoelectric point, where all the protein can be eventually concentrated. Since different proteins have, as a rule, different isoelectric points, the idea suggests itself of separating different components of a protein mixture by concentrating them at their characteristic isoelectric points.[3] We shall refer to a sorting pattern obtained on the basis of this principle as an "isoelectric spectrum."

Reversal of the current should produce the contrary effect, namely, a dilution of the protein solution at the isoelectric point due to migration of the protein ions away from this point. The predicted dilution effect, as well as the concentration and separation effects, are illustrated in Figures 3 and 4.

The technique of producing the pH gradient for the isoelectric spectra, shown in the preceding photographs, is very similar to the method of obtaining a conductivity gradient for mobility spectra shown in Figure 1. The protein mixture of low electrolyte content is dissolved in a sucrose or glycerol solution and introduced at *M* into the U-tube (Fig. 1, lower section). The dense sucrose buffer solution filling the lower portion of the U-tube is an acid buffer of appropriate pH nearly saturated with sucrose. The buffer filling the leg *R* above mark *m*, as well as the chamber *A*, is the same acid buffer free of sucrose. A basic buffer of appropriate pH[4] fills the upper half of leg *L* and the chamber *B*. This arrangement establishes a pH gradient in the layer *M* by convection and diffusion. The pH varies from pH_1 at mark *1* to pH_2 at mark *2*. To yield an isoelectric spectrum, the isoelectric points of the proteins must lie between these limits; otherwise, a mobility spectrum of the proteins is obtained.

Ideally, one would desire the pH distribution in *M* not to be affected by the cur-

rent. Kohlrausch[5] and Dole[6] have shown for the simple case of a strong electrolyte column containing two species of ions whose concentration varies arbitrarily along the axis that the concentration distribution is not affected by the current. They also showed for strong electrolytes that the current does not affect the concentration distribution in a solution containing many species of ions, provided that the concentration ratio of the ions does not vary along the current axis. A concentration distribution satisfying this condition can be obtained by dilution of the electrolyte. These results are, however, not applicable with a sufficient degree of approximation to solutions of weak electrolytes such as are encountered in buffer

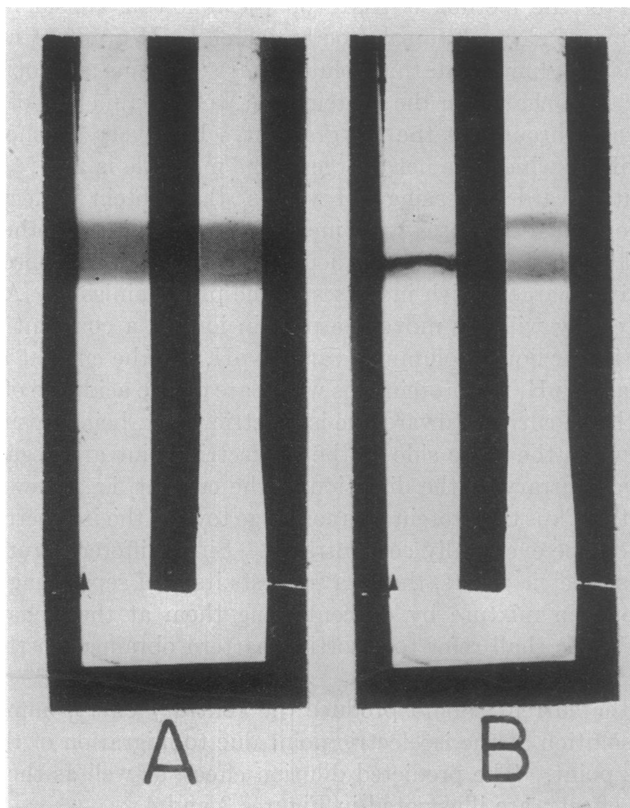

FIG. 3.—Electrophoretic concentration and dilution of hemoglobin in a pH gradient. pH range (2.6 → 9.6). *A:* hemoglobin layers before passage of current (the polarity is as indicated in Fig. 1). *B:* concentration effect in left leg and dilution effect in right leg after 40 seconds.

systems.[7] In the absence of a satisfactory theory from which the required buffer compositions for a stationary or nearly stationary pH distribution could be computed, a suitable combination of buffers was found empirically.[8]

The "isoelectric lines" of proteins are much sharper and more stable than "mobility lines" of proteins and of nonproteins. The greater sharpness of the isoelectric lines is due to the stable equilibrium of protein molecules in the isoelectric plane. Should a protein molecule be deviated from this position by such effects as diffusion or convection, it will acquire a charge of such sign as to be returned to its original position by the electric field. As a rule, an isoelectric spectrum is formed more

rapidly than a mobility spectrum. It is confined mostly to the *M* zone but may project somewhat beyond its boundaries. A mobility spectrum, on the other hand, lies entirely beyond the boundaries of *M*. Figure 5 shows an isoelectric spectrum of proteins. Four proteins were separated between buffers of pH 4.8 and 7.7. The proteins were cytochrome C, hemoglobin, catalase, and dyed collagen ("Azocoll"). The cytochrome C line is not part of the isoelectric spectrum. The isoelectric point of cytochrome C lies at pH 10.6, outside the range of the pH gradient; thus cytochrome C forms a line at the upper *M* boundary due to retardation in the buffer. The isoelectric points of hemoglobin (pH 6.8), catalase (5.7), and collagen (5.3) lie within the range of the pH gradient, so that the three lower lines form an isoelectric spectrum between the *M* boundaries. The separation shown in Figure 5 was accomplished in 4 minutes.[9]

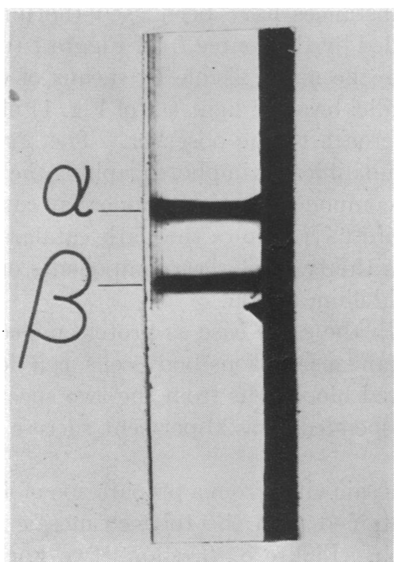

FIG. 4.—Separation of cytochrome C(α) and hemoglobin (β) after 2¼ minutes. pH range, 3.3 → 7.7. The hemoglobin line is produced by "isoelectric condensation," whereas the cytochrome C line is produced by retardation at the buffer boundary.

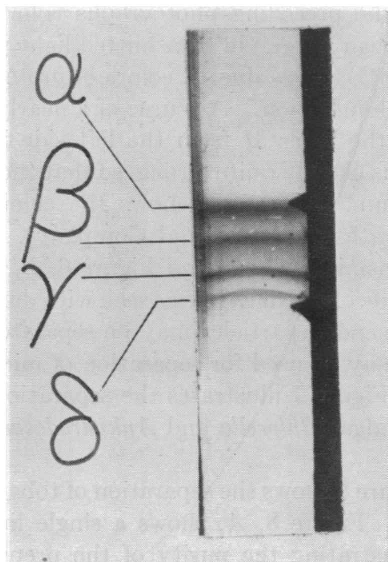

FIG. 5.—Electrophoretic line spectrum obtained in 4 minutes with four proteins: cytochrome C (α), hemoglobin (β), catalase (γ), and dyed collagen ("Azocoll") (δ). pH range, 4.8 → 7.7. α is formed by retardation at the buffer boundary, whereas β, γ, and δ are formed by isoelectric condensation.

Electrophoretic Line Spectra.—Since the arrangement for production of isoelectric spectra combines a conductivity gradient, such as is used for mobility spectra, with a pH gradient, it is evident that the above procedure will be effective in producing simultaneously a mobility spectrum as well as an isoelectric spectrum. The mixture may thus contain ampholytes as well as nonampholytes, and the isoelectric points of some of the proteins may lie outside the range of the pH gradient. This is the most general arrangement for production of "electrophoretic line spectra." In Figure 5 the cytochrome C line, for example, is not formed by isoelectric condensation.

Whenever possible, it is advantageous to accomplish the separation by producing an isoelectric spectrum rather than a mobility spectrum. The lines are thinner,

sharper, and more stable. The isoelectric spectrum is formed more rapidly, since the ions move in the *M* layer, where the potential gradient is much higher than in the buffers in which the mobility spectrum is formed. A wider separation between the lines can be obtained in a short time. The distance traversed by the ions and hence the separation time can be made very short by making the height of the *M* layer small. The sharp density gradient at the adjacent *M* boundaries, combined with the gentle density gradient in the *M* layer, accounts for the effective suppression of disturbances due to thermal convection in the *M* layer, where the isoelectric spectrum is formed. The final prospective advantage of the isoelectric spectrum lies in the possibility that the pH could be measured by means of microelectrodes at the sites of the individual isoelectric lines. This would permit one to identify the ampholytes by a rapid determination of their isoelectric points.

In the preceding photographs colored substances have been used the lines of which can be seen in transmitted light provided by the source L_2 of Figure 1 (upper section). Lines due to colorless proteins can be made visible by means of dark-field illumination. A source of a nearly parallel beam of light (L_1 of Fig. 1) illuminates the layer *M* from the left side in reference to the observer. The Tyndall effect is usually quite strong, so that there is no difficulty in photographing the "line spectrum." Figure 6 shows the colorless components of a commercial catalase preparation (Armour and Company). Figure 6, *A*, shows the dark catalase line in transmitted light, and Figure 6, *B*, shows the two colorless components on the acid side of the dark line as seen with dark-field illumination.

Suspended particles may be separated with the same ease as protein molecules. This may be used for separation of microörganisms, various body cells, cell debris, etc. Figure 7 illustrates the separation of red blood cells from the two species of green algae *Chlorella* and *Ankistrodesmus* suspended in a 20 per cent sucrose solution.

Figure 8 shows the separation of tobacco mosaic virus from a preparation of T6r+ virus. Figure 8, *A*, shows a single line obtained with the tobacco mosaic virus demonstrating the purity of the preparation. Figure 8, *B*, shows two lines obtained with the preparation of T6r+ virus. In Figure 8, *C*, one sees the three components into which a mixture of the T6r + virus preparation with the tobacco mosaic virus is resolved. The separated fractions of a mixture may be extracted by means of a micropipette.

The present paper is a preliminary communication. A detailed theoretical and experimental analysis of the method will be presented in a later publication.

The author wishes to thank his colleagues Professor Martin E. Hanke and Dr. Nicholas Nicolaides for numerous biochemical preparations as well as for stimulating chemical discussions. The colored proteins provided by Dr. Nicholaides proved especially helpful. Illuminating theoretical discussions with Dr. Daniel Leenov are much appreciated. The viruses have been kindly furnished by Mr. L. Barrington and the algae by Mrs. H. Gaffron and Drs. F. L. Allen, J. E. Brugger, and E. Kessler. Thanks are also due Mr. John Peters, of the chemistry shop, and Mr. Edward Wolowiec for their able technical assistance.

The support of this work by generous grants from the Office of Naval Research, the Research Corporation, and the Smith, Kline, and French Foundation is gratefully acknowledged.

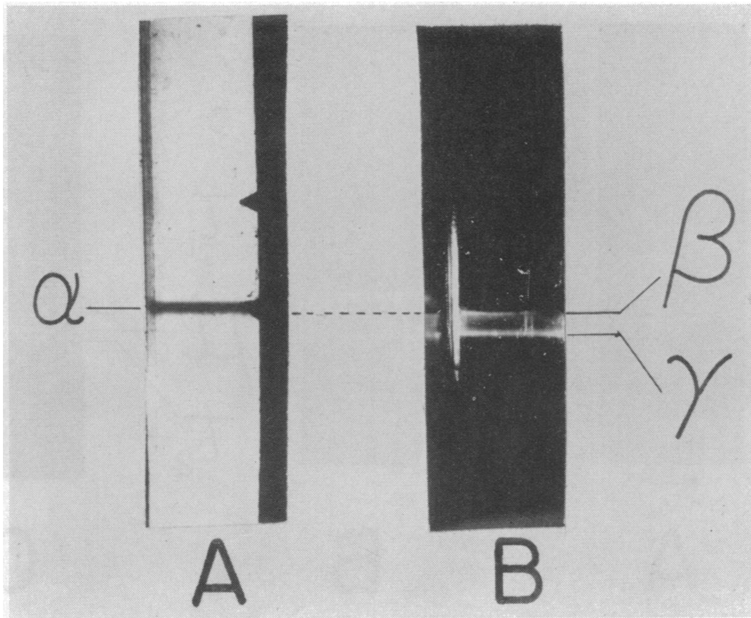

FIG. 6.—Electrophoretic line spectrum of a commercial catalase preparation (Armour and Company). α: the dark line photographed by transmitted light. β and γ: colorless components photographed by dark-field illumination. Separation time, 9 minutes. pH range, 4.8 → 7.7.

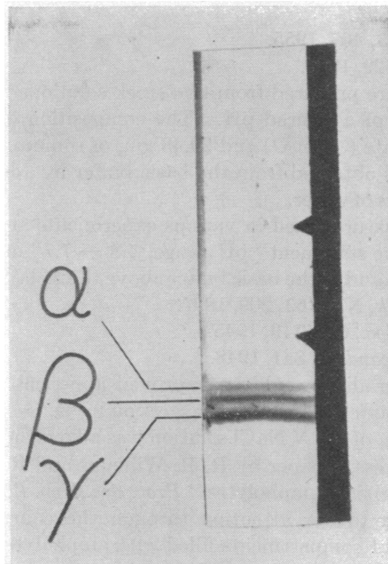

FIG. 7.—Electrophoretic line spectrum of a mixture of the algae *Ankistrodesmus* (α) and *Chlorella* (β), with human red blood cells (γ). Separation time, 9 minutes. pH range, 6.2 → 7.7.

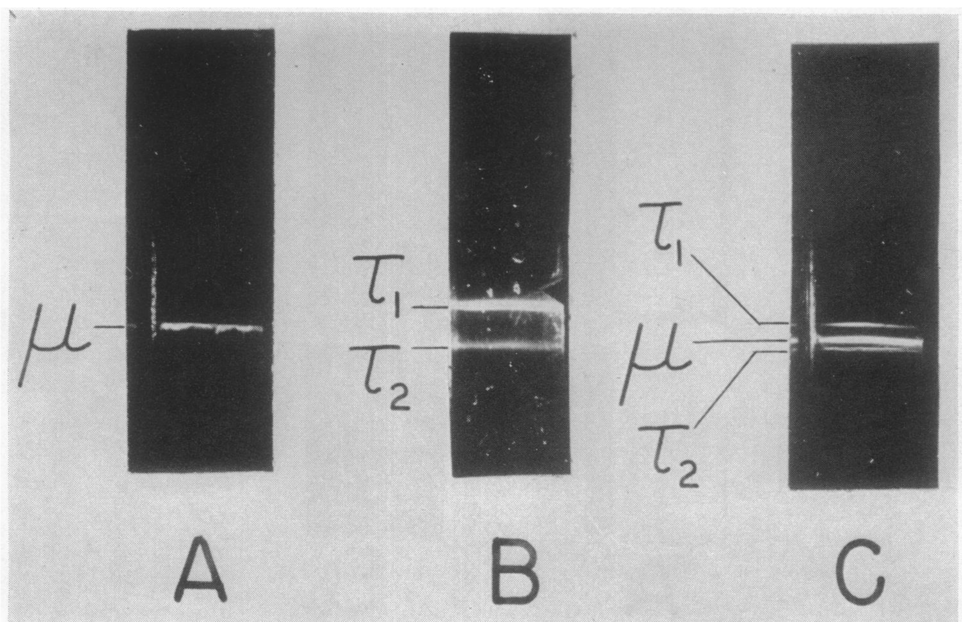

FIG. 8.—Electrophoretic line spectrum of a virus mixture (dark-field illumination). pH range, 4.8 → 7.7. Separation time, 8 minutes. *A:* single line (μ) obtained with tobacco mosaic virus, *B:* the two components (τ_1 and τ_2) of a T6r+ virus preparation. *C:* three components (τ_1, μ, τ_2) obtained with a mixture of tobacco mosaic virus with the T2r+ virus preparation.

* These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Chicago.

[1] H. J. Antweiler, *Die quantitative Elektrophorese in der Medizin* (Berlin: J. Springer, 1952), p. 38.

[2] A. Kolin, *J. Chem. Phys.*, **23**, 407, 1955.

[3] A. Kolin, *ibid.*, **22**, 1628–1629, 1954.

[4] Buffers of different pH were prepared from two stock solutions which were mixed in varying proportions to obtain buffers of a desired pH. The composition of the basic buffer of pH 9.6 was 19.43 gm. of sodium acetate ($+3H_2O$) and 29.43 gm. of sodium barbital per liter of solution. The acid buffer of pH 2.6 was obtained from the basic buffer by adding 1 liter of the latter to 3 liters of 0.1 N HCl and 0.6 liters of water.

The pH values of buffer mixtures used in various experiments are indicated in the legends to the figures. For example, the statement "pH range, 4.8 → 7.7" means that the pH of the acid buffer below the M layer is 4.8 and of the basic buffer above the M layer, 7.7.

[5] F. Kohlrausch, *Ann. Physik*, N.F., **62**, 209, 1897.

[6] V. P. Dole, *J. Am. Chem. Soc.*, **67**, 1119, 1945.

[7] H. Svensson, *Acta Chem. Scand.*, **2**, 841, 1948.

[8] The stability of the pH gradient could be improved appreciably by adding an appropriate amount of NaCl to the basic buffer (following a suggestion by D. Leenov). For the pH 7.7 buffer used in these experiments, 1 cc. of a 5 N NaCl solution was added to 70 cc. of the buffer.

[9] While this paper was in press, a paper by R. R. Williams and R. E. Waterman: "Electrodialysis as a Means of Characterizing Ampholytes," *Proc. Soc. Exp. Biol. & Med.*, **27**, 56, 1929 (and subsequent related work) came to our attention through the courtesy of Dr. M. K. Brakke. Their apparatus consisted of 14 compartments filled with ampholytes in solutions of different pH separated by parchment membranes. After 60 hours the concentration was found to be greatest in compartments having a pH nearest the isoelectric pH of the ampholyte. Some of the later modifications of the apparatus use separate containers connected in series by electrolyte bridges. This method may be considered as an intermediate step between standard electrophoretic technique and the method of "isoelectric spectra."