Effect of Binding of Ions and Other Small Molecules on Protein Structure

VII. THE ROLE OF ALIPHATIC ACID BINDING AND OF CONVECTION IN THE ELECTROPHORESIS OF SERUM ALBUMIN AT LOW pH*

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Bovine serum albumin undergoes two electrophoretically distinguishable types of interaction in acidic media (1). The first (1-6), which is also shown by ovalbumin, bovine γ-pseudoglobulin and oxidized ribonuclease, is observed only in media containing acetate buffer (NaAc-HAc) or other carboxylic acid buffers. The various peaks shown by the electrophoretic patterns obtained in these media correspond neither to single stable protein components nor to single components involved in a slowly adjusted equilibrium as evidenced by the results of fractionation experiments (3, 6). Resolution of the peaks is intimately related to changes in conductance and pH produced in the Tiselius cell by the electrophoretic process. This should not be interpreted to mean that bovine serum albumin and other proteins do not undergo some interaction with these media; rather, it would appear that resolution of their electrophoretic patterns into multiple peaks results from coupling of such interactions with electrophoretic transport of the small ions of the solvent medium for the protein. The electrophoretic patterns have been interpreted qualitatively in terms of reversible binding of undissociated buffer acid by the protein with the assumption that the resulting protein-acid complexes have more positive electrophoretic mobilities than the uncomplexed protein molecules (3, 6). This interpretation predicts that during electrophoresis the pH at certain levels in the Tiselius cell will change in such a manner as to induce a type of nonelectroantigraphy in the two patterns typical of that actually observed under a variety of experimental conditions. Furthermore, observed changes in pH are in the direction predicted by the model. Since it is assumed that protein-acid complexes exist in instantaneous equilibrium with uncomplexed protein molecules and undissociated buffer acid, the various peaks in the patterns should constitute a single reaction boundary in which a homogeneous phase cannot be generated between any two peaks. Consequently, the protein gradient should not become zero between the two peaks.

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The second type of interaction of BSA (but not ovalbumin) with acidic media has been described by Aoki and Foster (7-11). These workers found that the electrophoretic patterns of 0.2% BSA in 0.02 M NaCl-HCl and in 0.02 M NaAc-HAc show two peaks whose proportions depend upon pH. These observations have been interpreted in terms of a reversible, pH-dependent transformation (isomerization) of BSA between two states in which the protein molecule has different electrophoretic mobilities. Although the experiments of Aoki and Foster have been restricted to conditions of low protein concentration, Cann (1) has shown that with proper choice of electrolyte composition the isomerization reaction can also be observed at higher protein concentrations (0.4 to 1%). The fact that both the interaction of BSA with acetate buffer and the isomerization reaction can occur simultaneously in acetate-containing media of appropriate compositions has apparently led to a certain amount of confusion concerning interpretation of the electrophoretic patterns obtained in such media. During experiments designed to clarify this situation we observed for the first time that convective disturbances in the Tiselius cell play an important role in the electrotransport of serum albumin at acidic pHs.

EXPERIMENTAL PROCEDURE

Electrophoresis was carried out in the standard 11-cc Tiselius cell with the Spinco model H electrophoresis-diffusion instrument. Schlieren patterns were recorded photographically with the cylindrical lens optical system. In all schlieren patterns shown in the figures the apparent mobilities, u, were positive.

* The abbreviations used are: BSA, bovine serum albumin; HSA, human serum albumin.
Values of $10^5 \times u$ cm$^2$ sec$^{-1}$ volt$^{-1}$, which at pH 4 vary in a complex fashion with the composition of the solvent medium (1), have been calculated but are given in only a few of the figures. In general these values cannot be placed in correspondence with mobilities, since large changes in conductance and pH occur in the electrophoresis cell during passage of the current (6). The BSA was Armour's crystallized bovine plasma albumin, Lot No. T6820 U01. The human serum albumin (HSA) was Merck Sharp and Dohme's 25% solution of HSA containing 0.02 M sodium caprylate and 0.02 M sodium acetyltryptophanate. Crystalline ovalbumin was prepared by the method of Sorensen and Høyrup (12). For experiments in buffered solutions, samples of protein were dissolved or diluted in buffer and then dialyzed against buffer in the cold for about 17 hours with one change of dialysate. In the case of unbuffered systems, samples of protein in 0.02 M NaCl were adjusted to pH 4.0 with HCl and then dialyzed in the cold against 0.02 M NaCl-HCl, pH 4.0, with one change of dialysate.

RESULTS AND DISCUSSION

The electrophoretic patterns of 0.4% BSA in a series of pH 4.0 media containing varying concentration of NaAc-HAc, the ionic strength being maintained constant at 0.02 M with NaCl, are presented in Fig. 1. It will be noted that the nature of the patterns depends upon the concentration of acetate buffer in the solvent medium for the protein. Those obtained in 0.02 M NaCl-HCl are similar to the patterns of Aoki and Foster except for the poor resolution of the descending boundary. (The enantiography of the patterns was not improved to any great extent by lowering the protein concentration to 0.2%. Evidence will be presented later in the paper for interpretation of these patterns in terms of a reaction boundary in which the gradient of protein concentration does not become zero between any two peaks.) Substitution of increasing amounts of NaAc-HAc for NaCl-HCl resulted in striking and progressive changes in the electrophoretic patterns, a behavior which has been described in detail previously (1-6). Of immediate concern to this discussion is the evidence which these experiments afford for the importance of convective disturbances to the interpretation of the electrophoretic behavior of serum albumins at acidic pH's. Whereas convective circulation in the Tiselius cell did not appear to occur during electrophoresis of BSA in 0.02 M NaCl-HCl, electrophoresis in acetate-containing media was complicated by such disturbances. Consider, for example, the patterns obtained in 0.001 M NaAc-0.005 M HAc-0.019 M NaCl; C, 0.002 M NaAc-0.01 M HAc-0.018 M NaCl; D, 0.006 M NaAc-0.03 M HAc-0.014 M NaCl; E, 0.01 M NaAc-0.05 M HAc-0.01 M NaCl; F, 0.02 M NaAc-0.1 M HAc. Temperature of electrophoresis was 1º. Electric field strength was about 8 volts cm$^{-1}$ except for F and A. In these latter two cases, the field strength was 12 and 4 volts cm$^{-1}$, respectively. Bar angle was 50º except for A, in which case the bar angle was 33º.

![Fig. 1. Electrophoretic patterns of 0.4% BSA in various solvent media at pH 4.0: A, 0.02 M NaCl-HCl; B, 0.001 M NaAc-0.005 M HAc-0.019 M NaCl; C, 0.002 M NaAc-0.01 M HAc-0.018 M NaCl; D, 0.006 M NaAc-0.03 M HAc-0.014 M NaCl; E, 0.01 M NaAc-0.05 M HAc-0.01 M NaCl; F, 0.02 M NaAc-0.1 M HAc. Temperature of electrophoresis was 1º. Electric field strength was about 8 volts cm$^{-1}$ except for F and A. In these latter two cases, the field strength was 12 and 4 volts cm$^{-1}$, respectively. Bar angle was 50º except for A, in which case the bar angle was 33º.](image)

1. Convection in the Tiselius cell may manifest itself in one or more of the following ways (13, 14): the appearance of small, often transient spikes or pips in the electrophoretic patterns; the distortion of the normally smooth contours of broad boundaries into irregular, sawtooth contours; the hypersharpening of either the leading or trailing edges of boundaries; the erosion of the sides of boundaries as revealed by examination of the schlieren bands; or the production of small, often transient, spikelike phenomena in the Rayleigh interference fringes. Hypersharpening of either the leading or trailing edges of boundaries by convection should not be confused with sharpening due to gradients of pH and/or conductance across protein boundaries. This latter phenomenon and not convection accounts (6) for the sharpness of major, fast moving rising peaks and major, slow moving descending peaks shown by the patterns of BSA and other proteins in certain acetate-containing media. Consider, for example, the descending pattern in Fig. 1B (2). Whereas the hypersharpness of the trailing edge of the broad, fast moving peak is due to convection, the sharpness of the slow moving peak is due to gradients of pH and conductance.

2. The composition of the solvent medium for the protein. Those obtained in 0.02 M NaCl-HCl are similar to the patterns of Aoki and Foster except for the poor resolution of the descending boundary. (The enantiography of the patterns was not improved to any great extent by lowering the protein concentration to 0.2%). Evidence will be presented later in the paper for interpretation of these patterns in terms of a reaction boundary in which the gradient of protein concentration does not become zero between any two peaks.) Substitution of increasing amounts of NaAc-HAc for NaCl-HAc resulted in striking and progressive changes in the electrophoretic patterns, a behavior which has been described in detail previously (1-6). Of immediate concern to this discussion is the evidence which these experiments afford for the importance of convective disturbances to the interpretation of the electrophoretic behavior of serum albumins at acidic pH's. Whereas convective circulation in the Tiselius cell did not appear to occur during electrophoresis of BSA in 0.02 M NaCl-HCl, electrophoresis in acetate-containing media was complicated by such disturbances. Consider, for example, the patterns obtained in 0.001 M NaAc-0.005 M HAc-0.019 M NaCl; C, 0.002 M NaAc-0.01 M HAc-0.018 M NaCl; D, 0.006 M NaAc-0.03 M HAc-0.014 M NaCl; E, 0.01 M NaAc-0.05 M HAc-0.01 M NaCl; F, 0.02 M NaAc-0.1 M HAc. Temperature of electrophoresis was 1º. Electric field strength was about 8 volts cm$^{-1}$ except for F and A. In these latter two cases, the field strength was 12 and 4 volts cm$^{-1}$, respectively. Bar angle was 50º except for A, in which case the bar angle was 33º.

3. Convection in the Tiselius cell may manifest itself in one or more of the following ways (13, 14): the appearance of small, often transient spikes or pips in the electrophoretic patterns; the distortion of the normally smooth contours of broad boundaries into irregular, sawtooth contours; the hypersharpening of either the leading or trailing edges of boundaries; the erosion of the sides of boundaries as revealed by examination of the schlieren bands; or the production of small, often transient, spikelike phenomena in the Rayleigh interference fringes. Hypersharpening of either the leading or trailing edges of boundaries by convection should not be confused with sharpening due to gradients of pH and/or conductance across protein boundaries. This latter phenomenon and not convection accounts (6) for the sharpness of major, fast moving rising peaks and major, slow moving descending peaks shown by the patterns of BSA and other proteins in certain acetate-containing media. Consider, for example, the descending pattern in Fig. 1B (2). Whereas the hypersharpness of the trailing edge of the broad, fast moving peak is due to convection, the sharpness of the slow moving peak is due to gradients of pH and conductance. Gradients of pH and conductance also account for the sharpness of the major, slow moving descending peak in the patterns presented in Figs. 1, B and C, and 2, B and C of this paper and of the major fast moving rising peak in Figs. 1C, 2, C, D, and E, and 3.

4. Figure 1. Electrophoretic patterns of 0.4% BSA in various solvent media at pH 4.0: A, 0.02 M NaCl-HCl; B, 0.001 M NaAc-0.005 M HAc-0.019 M NaCl; C, 0.002 M NaAc-0.01 M HAc-0.018 M NaCl; D, 0.006 M NaAc-0.03 M HAc-0.014 M NaCl; E, 0.01 M NaAc-0.05 M HAc-0.01 M NaCl; F, 0.02 M NaAc-0.1 M HAc. Temperature of electrophoresis was 1º. Electric field strength was about 8 volts cm$^{-1}$ except for F and A. In these latter two cases, the field strength was 12 and 4 volts cm$^{-1}$, respectively. Bar angle was 50º except for A, in which case the bar angle was 33º.

5. Convective circulation in the Tiselius cell may manifest itself in one or more of the following ways (13, 14): the appearance of small, often transient spikes or pips in the electrophoretic patterns; the distortion of the normally smooth contours of broad boundaries into irregular, sawtooth contours; the hypersharpening of either the leading or trailing edges of boundaries; the erosion of the sides of boundaries as revealed by examination of the schlieren bands; or the production of small, often transient, spikelike phenomena in the Rayleigh interference fringes. Hypersharpening of either the leading or trailing edges of boundaries by convection should not be confused with sharpening due to gradients of pH and/or conductance across protein boundaries. This latter phenomenon and not convection accounts (6) for the sharpness of major, fast moving rising peaks and major, slow moving descending peaks shown by the patterns of BSA and other proteins in certain acetate-containing media. Consider, for example, the descending pattern in Fig. 1B (2). Whereas the hypersharpness of the trailing edge of the broad, fast moving peak is due to convection, the sharpness of the slow moving peak is due to gradients of pH and conductance. Gradients of pH and conductance also account for the sharpness of the major, slow moving descending peak in the patterns presented in Figs. 1, B and C, and 2, B and C of this paper and of the major fast moving rising peak in Figs. 1C, 2, C, D, and E, and 3.
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are not so evident in the patterns obtained in media containing still greater concentrations of acetate buffer, nevertheless, such disturbances were present. Thus, for example, examination of the Rayleigh interference fringe pattern revealed that during the first half of the electrophoresis experiment in 0.006 M NaAc-0.014 M NaCl there was a gradient of protein concentration between the constellation of fast moving peaks and the broad, slow moving peak shown by the rising pattern presented in Fig. 1D. Eventually, however, this gradient was obliterated by convection. It is interesting that the gradient never became zero between the broad, slow moving peak and the δ-boundary. (The Rayleigh fringe pattern also indicated that there was a small gradient of concentration over much of the region between the descending moving boundary and the δ-boundary, but this could have been caused by traces of slow moving protein contaminants in our BSA preparation.) The rising pattern obtained in 0.01 M NaAc-0.05 M HAc-0.01 M NaCl (Fig. 1E) shows evidence of convection in the form of a series of very small pips along the trailing edge of the constellation of fast moving peaks and the leading edge of the broad, slow moving peak. Finally, we have consistently observed, with both the cylindrical lens and Rayleigh interference optical systems, a gradient of protein concentration between the constellation of fast moving peaks and the broad, slow moving peak during early but not later stages of electrophoresis in 0.02 M NaAc-0.1 M HAc buffer (Fig. 1F) at both 12 and 6 volts cm⁻¹. Very small convective pips sometimes developed in the extreme trailing edge of the constellation of fast moving peaks during the later stages of electrophoresis. These results indicate that the electrophoretic patterns of 0.4% BSA at pH 4 should be interpreted in terms of reaction boundaries, modified in most cases by convection.

As shown in Fig. 2, similar results have been obtained with HSA. Convection was never observed during electrophoresis in 0.02 M NaCl-HCl at 11.3º, although minor disturbances were observed between the δ-boundary and the moving boundary in the experiment at 1º. The resultant patterns (Fig. 2A) definitely appear to be interpretable in terms of reaction boundaries as evidenced by the fact that the gradient curve is elevated well above the base-line between the two major peaks shown by both patterns. In all of the experiments carried out in acetate-containing media (with the one exception of 0.02 M acetate buffer (Fig. 2E)) the gradient curves were elevated above the base-line all the way from the δ- or ε-boundary to the leading edge of the fastest moving rising peak or the front of the broad descending boundary. Serious convective disturbances were observed in media of low acetate concentration (Fig. 2, B and C) but not at high concentrations. (There was an indication of convection between the two fastest moving peaks shown by the rising pattern in Fig. 2E.) The rising pattern obtained in 0.004 M NaAc-0.02 M HAc-0.016 M NaCl (Fig. 2D) is particularly striking in that the whole pattern appears to be a reaction boundary apparently undisturbed by convection in which a homogeneous phase was not generated between any two peaks of the pattern during the electrophoretic process.

In contrast to the results obtained in the present study with 0.4% BSA, our previously published patterns of 1% BSA in various acidic media gave little, if any, evidence of convection (see, for example, Fig. 2 (1)) except in the case of two patterns obtained at pH 4.7 (Fig. 1, A and B (2)). It should be noted that the present work was done with the Spinco electrophoresis cell whose cross-sectional area is greater than the Perkin-Elmer cell used in our previous work. These cells must certainly have different characteristics of heat dissipation and electro-osmotic flow and, consequently, it may not be justifiable to compare convection in the two cells. In any event, the rising pattern shown by 1% BSA in 0.01 M NaAc-0.05 M HAc, pH 4.0, in the Perkin-Elmer cell is curious in that the gradient of protein concentration becomes zero between the sharp, fast moving peak (which eventually resolves into two or three sharp peaks of similar apparent mobility) and the broad, slow moving one; despite the fact that fractionation experiments established that the two peaks constitute a reaction boundary in which the gradient should not become zero (3, 6). The results presented above suggest that although the gradient curve may be elevated above the base-line between the peaks during early stages of electrophoresis, this gradient eventually is obliterated by mild convective circulation in that region. Indeed, this has now been shown to be the case. Electrophoresis in the Spinco cell revealed that as the two peaks in the reaction boundary separate and the refractive index gradient in the intervening zone decreases, a situation is reached in which the accompanying vertical density gradients can no longer support convection-free electrophoresis and localized circulation thus ensues. With a protein concentration of 1%, circulation appeared at a relatively early stage of electrophoresis and obliterated the gradient of protein concentration between the two peaks. In contrast, circulation ap-

![Fig. 2. Electrophoretic patterns of 0.4% HSA in various solvent media at pH 4.0: A, 0.02 M NaCl-HCl; B, 0.001 M NaAc-0.005 M HAc-0.019 M NaCl; C, 0.002 M NaAc-0.01 M HAc-0.018 M NaCl; D, 0.004 M NaAc-0.02 M HAc-0.018 M NaCl; E, 0.02 M NaAc-0.01 M HAc. Temperature of electrophoresis, 11.3º; electric field strength in the range 2.9 to 4.3 volts cm⁻¹. Bar angle was 50º. Experiments A, C and D were repeated at 1º at a field strength of 3.9 to 4.2 volts cm⁻¹ with results practically identical with those obtained at 11.3º.](image-url)
peared at a relatively late stage of electrophoresis of a 3% protein solution and was not sufficiently strong to obliterate the gradient, although it did cause erosion of the schlieren bands. (Elevation of the gradient curve above the base-line between the peaks was barely discernible in the cylindrical lens pattern during later stages of electrophoresis, but the Rayleigh fringe pattern left no doubt that such a gradient did indeed exist.) It is concluded, therefore, that the peaks shown by the rising pattern of BSA in 0.01 M NaAc-0.05 M HAc in both the Spinco and Perkin-Elmer cells constitute a reaction boundary but that the gradient of protein concentration between the peaks may become zero as a result of convective circulation of solution in this region of the cell. Similar results have been obtained with ovalbumin in the same solvent.

As mentioned at the beginning, these results resolve the apparent discrepancy which had existed between our explanation of the electrophoretic patterns of BSA and other proteins in acetate-containing media and the nature of the rising patterns obtained in certain of these media. They also permit a more detailed description of the patterns than has previously been possible. Thus the bracket, - -, above a given pattern in Figs. 1 and 2 indicates that portion of the pattern which is a reaction boundary, modified in many instances by convective disturbances. The reaction boundary arises as a result of interaction of the protein with undissociated buffer acid and, in the case of some but not all rising patterns, possesses a fine structure resulting from the superimposed isomerization reaction. In the case of the rising patterns shown in Fig. 1, B and C, and Fig. 2D, the gradient curve is elevated above the base-line over the entire reaction boundary despite the convection shown by the first two patterns. However, the gradient curves obtained in other acetate-containing media are sufficiently perturbed by convection to coincide with the base-line between some of the peaks comprising the reaction boundary. Consider, for example, the rising pattern shown in Fig. 1E. Although the whole pattern is without question a reaction boundary, the gradient of protein concentration becomes zero in the region between the constellation of three fast moving peaks and the broad, slow moving peak. In the absence of convective circulation in this region of the cell, one would expect to obtain a pattern similar to that shown by the solid-line curve in Fig. 3; but because convective circulation does occur, the gradient becomes zero and the pattern takes on the appearance of the broken-line curve of Fig. 3. Similar arguments apply to other patterns of Figs. 1 and 2.

Let us now turn our attention to the fine structure of the reaction boundary attributable to the isomerization reaction studied by Aoki and Foster (7-11) and indicated by a brace, - -, in Figs. 1 and 2. The simplest situation, of course, is the one presented by the electrophoresis of BSA and HSA in 0.02 M NaCl-HCl, pH 4.0. The pattern obtained in this medium consists of several moving peaks which constitute a reaction boundary arising solely from the isomerization reaction. In contrast, the electrophoretic behavior shown in acetate-containing media is considerably more complex. In fact, it is impossible to ascribe any one portion of the patterns obtained in 0.001 M NaAc-0.005 M HAc-0.019 M NaCl and 0.002 M NaAc-0.01 M HAc-0.018 M NaCl to a given type of interaction, the whole pattern reflecting the interplay of the interaction of protein with undissociated acetic acid and the isomerization reaction. On the other hand, when the concentration of acetate buffer in the solvent medium for the protein is increased, it becomes possible to ascribe the fine structure of a portion of the pattern to the isomerization reaction. Thus, for example, although the entire rising pattern shown in Fig. 1E is a reaction boundary arising from the interplay of the interaction with acetate buffer and the isomerization reaction, the constellation of the three fast moving peaks indicated by the brace is within itself interpretable in terms of the isomerization reaction alone. In the case of a protein such as ovalbumin which does not show the isomerization reaction, the pattern obtained at the same concentration of acetate buffer is a reaction boundary, which arises solely from the interaction of ovalbumin with undissociated acetic acid and is comprised simply of a single, sharp, fast moving peak and a broad, slow moving peak. In other words, the pattern is similar to that of BSA but lacks the fine structure which we have come to associate with isomerization. (Compare the pattern of ovalbumin presented in Fig. 7A (3) with the pattern of BSA obtained at the same protein concentration in Fig. 1 (1).) At much higher concentrations of acetate buffer, e.g. 0.1 M NaAc at pH 4, the pattern of BSA is a reaction boundary attributable entirely to the isomerization reaction, all of the protein apparently being in the form of protein-acid complexes (9). These considerations should clarify the apparent confusion which has arisen concerning interpretation of the electrophoretic patterns of serum albumin in acetate-containing media.

It is asserted above that the constellations of fast moving peaks indicated by the braces in Figs. 1 and 2 are within themselves interpretable as isomerization-reaction boundaries and that the peaks shown by the patterns of serum albumin in 0.02 M NaCl-HCl, pH 4.0, constitute an isomerization-reaction boundary in acetate-containing media.

The finding that the reaction boundaries of BSA in acetate-containing media may be modified by convection in no way invalidates our previous interpretation of these patterns. Convection can account for neither the bimodality of the reaction boundaries nor the growth of the area sustained by the fast peak at the expense of the slow one when the concentration of acid is increased. This is clear from our experiment on 1% BSA in 0.01 M NaAc-0.05 M HAc, in which the sharp, fast moving peak and the broad, slow moving one were already well separated before convection ensued and obliterated the protein gradient in the intervening zone. This observation together with measurements on the pH and conductance of the intervening phase (8) show that the fast moving peak is sharpened by pH and conductance gradients and not by convection. Similar arguments apply to our descending patterns.

In two of our early papers (3, 4) the term, "isomerization," was used incorrectly to describe the interaction of BSA and ovalbumin with acetic acid.

Unpublished experiments of the author.
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Fig. 4. Electrophoretic patterns of BSA at pH 4.0: A, 0.2% protein in 0.02 M NaAc-0.1 M HAc after 235.4 minutes of electrophoresis at a field strength of 5.8 volts cm⁻¹; B, pattern shown by the constellation of peaks indicated by the brace in A after an additional 480.2 minutes of electrophoresis at 11.6 volts cm⁻¹ with back-compensation; C, pattern shown by proteins in 0.02 M NaCl-HCl after electrophoresis at 4.0 volts cm⁻¹ for 773.8 minutes, the last 402.3 minutes of which were conducted with back-compensation. (Compare with Fig. 1.4 which is the pattern obtained after 371.4 minutes of electrophoresis without back-compensation.) Pattern A was photographed at a bar angle of 28°; B, 19.6°; and C, 33°. Temperature of electrophoresis was 1°C.

which the gradient of protein concentration does not become zero between the peaks. This assertion is supported by experiments in which BSA in either 0.02 M NaCl-HCl or 0.02 M NaAc-0.1 M HAc was subjected to prolonged electrophoresis with back-compensation of the solution in the rising limb of the Tiselius cell in order to increase the effective length of the electrophoresis channel. The resulting patterns are presented in Fig. 4. (Only the constellation of fast moving peaks is observed after prolonged electrophoresis in acetate buffer, the small slow moving peak having been compensated out of the rising limb of the cell.) It will be noted that the gradient of protein concentration never became zero between any two peaks despite prolonged electrophoresis. Although this is strong evidence for a reaction boundary, it must be borne in mind that electrophoresis with back-compensation is not entirely comparable to the usual static method of electrophoresis. In particular, incomplete drainage and mixing within the pattern of peaks may be of importance in the former method. Mixing of solution within the pattern could conceivably prevent the gradient from becoming zero between the peaks.

Quantitative interpretation of the electrophoretic patterns shown by proteins in acetate-containing media at pH 4.7 and below presents a difficult problem. However, Gilbert and Jenkins (15) have recently made an important contribution to its eventual solution in their theoretical study of the electrophoretic behavior of the interacting system of macromolecular ions, A + B ⇋ C. They mention that their theory can be readily extended to the case in which one of the reactants is a constituent of the solvent medium for the macromolecular ion, e.g. undissociated buffer acid; and from my correspondence with Dr. Gilbert, I conclude that it is his intention to apply their theory to our results. Another possible approach to this problem is to use the concepts of the theory of weak-electrolyte moving boundaries (16, 17) to compute the equilibrium constants for binding of undissociated buffer acids to proteins. In applying these ideas it will be assumed that the protein molecule, Pₙ, binds undissociated acid, HA, according to the reaction equation,

\[ P + nHA \rightleftharpoons P(HA)ₙ \]  

From the mathematical definitions (17) of the constituent concentration and constituent mobility of the protein in the dialyzed solutions used in our electrophoretic experiments, the following expression was derived for the equilibrium constant, K, of Reaction 1:

\[ K = \frac{\bar{u}_P - u_P}{n_{HA} \bar{u}_{HA}} \]  

where \( n_{HA} \) is the molar concentration of free buffer acid; \( \bar{u}_P \), the constituent mobility, i.e. average mobility, of the protein; \( u_P \), the mobility of the uncomplexed protein molecules; and \( u_{HA} \), the mobility of the protein-acid complex. Thus, the equilibrium constant can be evaluated from appropriate mobility data if it is assumed that the free concentration of acid in the dialyzed protein solution is equal to the concentration of acid in the buffer. The value of n is chosen so as to make K constant.

The various electrophoretic mobilities were evaluated as follows:

Consider the systems of boundaries which should be obtained on electrophoresis of an interacting protein solution such as that described above. As previously, the mobility of the protein-acid complex is assumed to be greater than that of the uncomplexed protein. When the electric field is applied the complexes in the ascending limb of the Tiselius cell will migrate into the original equilibrium mixture. The uncomplexed protein molecules left behind will react instantaneously with acid to re-establish equilibrium thereby increasing the pH in that region. Since the rear portion of the boundary migrates in the original, lower pH buffer, the back of the boundary will sharpen. Thus, the descending reaction boundary should be bimodal. The slow moving peak will be sharp and the fast one, broad. In the rising limb of the cell, the rapidly migrating complexes move out of the original equilibrium mixture into fresh buffer. In order to re-establish equilibrium, a fraction of the complexes will dissociate instantaneously to liberate acid which lowers the pH behind the leading edge of the boundary so that the front of the boundary will sharpen. The slower moving protein molecules at the rear of the reaction boundary migrate into the zone of lower pH and are accelerated. Consequently, the rising reaction boundary should also be bimodal. In this case, however, the fast moving peak is sharp whereas the slow moving one is broad. This type of behavior is typical of the patterns shown by proteins in media containing the appropriate concentration of buffer. At higher concentrations of buffer, the descending reaction boundary is broad although usually still bimodal whereas the rising boundary shows either a sharp, fast moving peak or a constellation of fast peaks and a broad, slow moving one, e.g. Fig. 1F. These latter conditions are suitable for determination of the mobilities required for evaluation of the equilibrium constant for the binding of undissociated buffer acid by protein. Assuming that the centroids of the reacting constituents remain congruent as the descending boundary spreads, the mean mobility of this boundary can be taken as equal to \( \bar{u}_P \). Since the leading edge of the rising reaction boundary advances at a rate determined by the mobility of protein-acid complexes, the apparent mobility of the sharp rapidly moving peak (or the average mobility of the constellation
of fast peaks) can be placed into correspondence with \( u_{p(\text{HA})} \) after correction for the change in conductance across the peak (or constellation of peaks). The value of the apparent mobility of the broad slow moving rising peak should be a good approximation to \( u_p \).

A constant value of the equilibrium constant for the binding of undissociated acetic acid to BSA, Table IA, was obtained by assigning the fractional value of 2.5 to \( n \). This suggests heterogeneity of the protein with respect to acid binding; and, in fact, the apparent ionization constants of the buffer acids used in these experiments.

The apparent ionization constants were computed from the pH and composition of the buffers used in these experiments.

The measured changes in pH and conductance across the rising peaks shown by ovalbumin in 0.01 M NaAc-0.050 M HAc-0.03 M NaCl have the values, 1.28 ± 0.07 × 10^{-5} and 5.40 ± 0.06 × 10^{-4} cm² sec⁻¹ volt⁻¹.

The following symbols are used to designate the various acid anions: AC, acetate; Pr, propionate; Va, valerate; Fo, formate; MeoAc, methoxyacetate.

The calculated value, 22, is of the same order of magnitude as the values of \( K_s \) for the binding of acetate anion at pH 9.1 as determined by Teresi and Luck (20) with the method of equilibrium dialysis. This, however, is the only similarity between the two reactions. Whereas BSA binds only 2 or 3 moles of acetic acid, it can bind 32 moles of acetate anion.

These calculations assume that the electrophoresis of BSA in acetate buffer is ideal except for the acid-protein interaction. However, the measured changes in pH and conductance across the various peaks in the reaction boundary show that the electrophoresis is actually nonideal. The nonideality also becomes apparent when one compares the measured areas of the rising peaks in the electrophoretic pattern with theoretical areas computed from the equilibrium constant. In the case of the pattern shown in Fig. 1, the computed value of the relative area of the slow moving peak, 12%, is only about two-thirds of the measured value, 19%.

Equilibrium constants have also been evaluated for the binding of various aliphatic acids by ovalbumin. Comparison of the last two columns in Table IB reveals a correlation between the equilibrium constants and the ionization constants of the buffer acids. In general, the stronger the acid, i.e. the less negative the effective charge on the carboxyl oxygens, the greater the tendency to bind. (The general picture is not changed by assuming values of \( n \) greater than 1). This would seem to eliminate the possibility that the undissociated acid binds to the protein by means of formation of double hydrogen bonds of the acetic acid dimer type. This type of bonding would be weakened by any structural change which tends to make the effective charge on the carboxyl oxygens, the greater the tendency to bind. (The general picture is not changed by assuming values of \( n \) greater than 1).
grouping as the acceptor. This type of bond should be strengthened by structural changes in the acid which tend to make the effective charge on its carboxyl oxygens less negative. Our equilibrium constants are perhaps larger than one might expect for such a bond, but factors such as the low dielectric constant of the cavity formed by the protein molecule in the solvent should act to increase the strength of the bond. In any event, the most logical type of binding force to postulate for the combination of a neutral molecule such as formic acid with a protein is a hydrogen bond.

Finally, the question arises as to the cause of the convective disturbances frequently observed during electrophoresis of serum albumin in acetate-containing media, but generally not in NaCl-HCl. To arrive at a satisfactory answer to this question it is necessary to evaluate the relative importance of the several possible causes of circulation of liquid in the electrophoresis channel (13). Although all of our experiments were conducted at an electric field strength less than the value generally accepted as the maximal permissible one for avoidance of serious disturbances due to Joule convection arising from dissipation of heat generated by the passage of current, it may be presumed that some Joule circulation, nevertheless, occurred in the regions of the channel between boundaries. Whether or not such circulation would cause significant perturbations in the electrophoretic pattern depends upon the magnitude of the vertical density gradients accompanying the solute gradients. It must be concluded that these gradients are usually sufficiently strong in the case of albumin in NaCl-HCl to prevent significant disturbances in the electrophoretic patterns. In contrast to other aqueous solutes, acetic acid contributes relatively little to the stabilization of vertical density gradients and, consequently, Joule convection might be more pronounced in NaAc-HAc than in NaCl-HCl. However, before deciding upon the contribution of Joule convection to the disturbances observed in acetate-containing media, it is first necessary to explore the possibility that inverted vertical density gradients are sometimes produced by electrophoresis in these media.

Electrophoresis could conceivably produce inverted density gradients by means of at least three mechanisms. The first would be operative only in those cases in which the solvent for the protein contains the three salt ions, Na+, Cl- and Ac-.

Electrophoresis in such a solvent may give rise to a "false" boundary across which no ions disappear (23). The possibility that the very small "false" boundary is accompanied by an inverted density gradient which causes convection appears to have been eliminated by computations with the Dole theory (23) on a model system of boundaries. Another source of inverted density gradients might reside in the changes in pH and conductance which occur at certain levels in the channels of the Tiselius cell during electrophoresis. Convection resulting from such inverted gradients is called Kohlrausch convection. This possibility was tested by computing the values of the Kohlrausch regulating function7 for the various electrophoretically generated phases from measured values of their pH and conductivity. In each case examined, the regulating function of the dalyzed protein solution was greater than that of the buffer which indicates that Donnan equilibrium had provided proper solutions for formation of the initial boundary. However, subsequent passage of current may generate phases in the descending channel with inverted values of the regulating function. Thus, in the case of 3% BSA equilibrated against 0.0008 M NaAc-0.004 M HAc-0.0092 M NaCl, pH 4.1 (Fig. 7 (6)) the value of the regulating function for the phase between the descending slow moving sharp peak and the fast moving broad peak is about 25% greater than that of the dalyzed protein solution beneath the fast moving peak. This is a gravitationally unstable condition, and Kohlrausch convection should occur in the zone between the two descending peaks. Convective disturbances have, in fact, been observed in this region of the descending channel at such low acetate concentrations (Fig. 1B) and at higher concentrations at pH 4.7 (Fig. 1B (2)). The situation is different in the rising channel. Thus, with both 3% BSA in 0.01 M NaAc-0.05 M HAc and 0.4% BSA in 0.02 M NaAc-0.1 M HAc, the regulating function of the phase between the slow moving peak and the fast moving peak was less than that of the underlying solution and essentially the same as that of the overlying buffer. Of course, there is the possibility that the electrophoretic process attempts to generate localized inverted protein gradients in the rising channel as a result of the interaction of protein with acetic acid. Gilbert and Jenkins (15) in their theoretical treatment of the electrophoresis of interacting systems have shown that under certain conditions inverted peaks may be generated in the rising channel (Fig. 6 (15)). In the absence of stabilization by a superimposed density gradient of opposite sense, convection would occur in practice and the theoretical patterns would not be realized. Re-examination of the electrophoretic patterns

![Fig. 5. Rising electrophoretic pattern of 1.3% ovalbumin in 0.005 M NaAc-0.025 M HAc-0.021 M glycine-0.034 M NaCl, pH 3.97. The vertical arrow points toward the inverted refraction index gradient. An almost identical pattern was obtained in 0.003 M NaAc-0.015 M HAc-0.037 M NaCl.](image)

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1. The possibility that one of the peaks in the reaction boundaries shown by proteins in acetate-containing media is a "false" boundary has previously been eliminated (3).

7. The Kohlrausch regulating function, $\omega$, is defined as

$$\omega = \sum \frac{C_i}{r_j}$$

where $C_i$ and $r_j$ are the equivalent concentration and relative mobility, respectively, of the $j$th ionic constituent of a given phase (13). The relative mobilities of the various ionic constituents of a phase are obtained by dividing the set of mobilities by the absolute value of any one member of the set. Of two phases with different values of the regulating function the phase having the greater value usually is the more dense one. Whereas a stationary boundary must have unequal values of the regulating function in the two adjoining phases for its existence, a stable moving boundary must have equal values of the function in the adjoining phases.
obtained in our past experiments has revealed two instances in which the refractive index gradient was actually inverted immediately beneath the most rapidly migrating rising peak shown by ovalbumin in media containing moderately low concentrations of acetate buffer (Fig. 5). (A suggestion of an inverted gradient was also found in one experiment with BSA.) A possible explanation of the inverted refractive index gradient is that a localized inverted gradient of protein concentration is stabilized by an opposing gradient of salt concentration. This would be a rare event; and, in general, any attempt by the electric current to generate an inverted gradient of protein concentration will result in convection.

The above considerations indicate that convection in the rising channel of the Tiselius cell is caused to a large extent by the fact that the electric current tries to generate localized inverted gradients of protein concentration. On the other hand, inverted density gradients accompanying gradients of pH and conductance appear to be major causes of the convection observed in the descending channel at low concentrations of acetate buffer. Electrosomotic flow and Joule circulation are undoubtedly superimposed on these phenomena. Since electrosomotic flow is polarized with respect to the direction of the electric field relative to gravity, it would tend to increase Joule circulation in one of the channels and suppress it in the other. This could account for the fact that convection is often observed to occur in the rising but not in the descending channel.

**SUMMARY**

The various peaks in the electrophoretic patterns of serum albumin and ovalbumin in acidic media constitute a single reaction boundary modified in some instances by convective disturbances. In the case of serum albumins in acetate-containing media, the reaction boundaries arise as a result of interaction of the protein with undissociated buffer acid, and, in some but not all rising patterns, possess a fine structure due to the superimposed isomerization reaction. At sufficiently high concentration of acetate buffer, 0.1 M, the pattern of bovine serum albumin is a reaction boundary attributable entirely to the isomerization reaction. The reaction boundaries of ovalbumin in acetate-containing media arise solely from interactions of the protein with undissociated buffer acid. The patterns shown by the serum albumins in NaCl-HCl, pH 4.0, are isomerization reaction boundaries in which the gradient of protein concentration does not become zero between any two peaks.

Equilibrium constants for the binding of undissociated buffer acids by bovine serum albumin and ovalbumin have been computed from mobility data.

**REFERENCES**

1. **Cann, J. R., J. Am. Chem. Soc., 80, 4263 (1958).**
22. **Dole, V. P., J. Am. Chem. Soc., 67, 1110 (1945).**