[CONTRIBUTION NO. 54 FROM THE DEPARTMENT OF BIOPHYSICS, FLORENCE R. SABIN LABORATORIES, UNIVERSITY OF COLORADO MEDICAL CENTER]

Effect of Binding of Ions and Other Small Molecules on Protein Structure. II. Isomerization of Ovalbumin, γ-Globulin and Serum Albumin in Acidic Media[1]

BY JOHN R. CANN AND ROBERT A. PHELPS

RECEIVED APRIL 10, 1957

Electrophoretic studies indicate that in acidic media ovalbumin, γ-globulin and serum albumin are equilibrium mixtures of electrophoretic components, the equilibrium composition depending upon the composition of the supporting medium, particularly the concentration of un-ionized carboxylic acids. A reaction scheme which accounts for some of the features of the electrophoretic behavior of these proteins is proposed.

Introduction

In the first paper of this series[2] we reported a new effect of acetate buffer (NaAc–HAc) on the electrophoretic behavior of bovine serum albumin at *p*H values acid to its isoelectric *p*H. It was concluded that at these *p*H values serum albumin is an equilibrium mixture of electrophoretic components, the electrophoretic composition depending upon the composition of the supporting medium, particularly the acetate concentration. Similar findings can now be reported for ovalbumin and bovine γ-pseudoglobulin in several different buffers. These new experiments indicate that the un-ionized carboxylic acid component of the buffer and not its anion determines the equilibrium composition of the protein. An attempt is made to interpret the complex electrophoretic behavior of ovalbumin in terms of a set of simultaneous and consecutive, reversible, unimolecular isomerization reactions.

Experimental

Materials.—Crystalline ovalbumin was prepared by the method of Sorensen and Høyrup.[3] The bovine γ-pseudoglobulin was the water-soluble fraction of Armours' Fraction II of bovine plasma, and the serum albumin was Armours' crystallized bovine plasma albumin.

Methods.—The electrophoretic analyses were carried out in the usual manner using the Perkin–Elmer Tiselius apparatus, fitted with a current-regulating power supply and a potentiometer to measure the voltage drop across a standard resistance placed in series with the cell. The protein solution was equilibrated against cold buffer either by static dialysis with at least one change of dialysate or by means of a rocking dialyzer with flowing dialysate. Donnan equilibrium was established within 24 hours by the static method and there was no difference between the electrophoretic patterns of protein solution dialyzed for 24 and 48 hours. In all the schlieren patterns shown in the figures the electrophoretic mobilities, μ, were positive. The values of $10^5 \times \mu$cm.^{2} sec.$^{-1}$ volt^{-1}, are given in the figures. Unless otherwise stated, the protein concentration was 1.3%. The maximum variation in *p*H of a series of media of constant ionic strength, but containing varying concentrations of buffer, *e.g.*, NaAc–HAc, was 0.03–0.04 *p*H unit.

Sedimentation velocity experiments were performed in the Spinco Model E electrically driven ultracentrifuge. A sedimentation cell with a Kel-F centerpiece was used. Runs were made at room temperature on 1.3% protein solutions at 59,780 r.p.m.

Results

Electrophoretic Behavior of Ovalbumin.—The electrophoretic patterns presented in Fig. 1 show that, as in the case of bovine serum albumin,[2] the electrophoretic behavior of ovalbumin at *p*H 4.0 depends upon the concentration of NaAc–HAc in the supporting medium. (Since this *p*H is acid to the isoelectric point, the protein is positively charged and the boundaries migrated toward the cathode.) It can be seen that the patterns are very non-enantiographic and that progressive changes occur as the concentration of NaAc–HAc is increased from 0.002 *M* NaAc–0.010 *M* HAc to 0.04 *M* NaAc–0.200 *M* HAc. At the lowest concentration the descending pattern shows a large and very sharp, slow moving boundary and a diffuse, more rapidly moving one; whereas the rising pattern shows two diffuse and poorly resolved peaks and a very small, faster moving, sharp peak. Increasing the acetate concentration at constant ionic strength results in an increase in the area under the faster moving boundaries, at the expense of slower moving ones. Thus, at the highest concentration, the descending boundary is now diffuse and suggestive of heterogeneity, while the rising pattern shows a very sharp, rapidly moving boundary and a smaller, slower moving one. In addition to these changes, the mobility of the slowest boundary in the rising pattern decreases as the concentration of NaAc–HAc is increased. The protein disappearing across the rapidly moving, rising boundary in 0.01 *M* NaAc–0.050 *M* HAc–0.030 *M* NaCl, was removed from the electrophoresis cell and analyzed for nitrogen. It was found that about 60% of the protein disappears across this boundary, which shows that the slower rising peak is indeed a protein boundary. This same electrophoretic behavior has been shown by several different preparations of ovalbumin.

Whereas the nature of the electrophoretic patterns are very sensitive to the concentration of NaAc–HAc, they are surprisingly insensitive to ionic strength at constant acetate concentration. Thus, the patterns obtained at ionic strength 0.1 (adjusted by addition of NaCl to the buffer) were essentially the same as those shown in Fig. 1 for ionic strength 0.04. The greatest difference was found at the lowest concentration of NaAc–HAc where the rising pattern at ionic strength 0.1 showed a single boundary of electrophoretic mobility 3.06×10^{-5} with a suggestion of a faster peak; however, the descending patterns at the two ionic strengths were essentially the same. It was also found that substitution of NO_3^- for Cl^- in the supporting medium containing the lowest acetate

(1) Supported in part by research grant No. E-1482(C5) from The National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Public Health Service; and in part by the Damon Runyon Fund and the American Cancer Society. Presented in part at the Miami meeting of the American Chemical Society, April 7–12, 1957.

(2) R. A. Phelps and J. R. Cann, THIS JOURNAL, **78**, 3539 (1956).

(3) S. P. L. Sorensen and M. Høyrup, *Compt. rend. trav. lab. Carlsberg*, **12**, 12 (1916).

concentration had no effect on the electrophoretic patterns, except to decrease the mobilities of the two fastest moving, rising boundaries.

The effect of NaAc–HAc on the electrophoretic behavior of ovalbumin is reversible. Thus, protein equilibrated against buffer of composition 0.01 *M* NaAc–0.050 HAc–0.030 *M* NaCl and then redialyzed against buffer of composition 0.002 *M* NaAc–0.010 *M* HAc–0.038 *M* NaCl before electrophoresis, gave the same pattern as protein never exposed to the higher acetate concentration. Furthermore, protein equilibrated against buffers containing either high or low acetate concentrations at *p*H 4.0 before electrophoresis in phosphate buffer at *p*H 7.0, gave the same patterns and mobilities as protein never exposed to the acidic media.

Electrophoretic analyses of ovalbumin were also carried out in media containing formate buffer (Naf–Hf) at *p*H 4.0. The resulting patterns, which are shown in Fig. 2, not only eliminate the possibility that the complex patterns found in acetate buffers arise as a result of poor buffering action

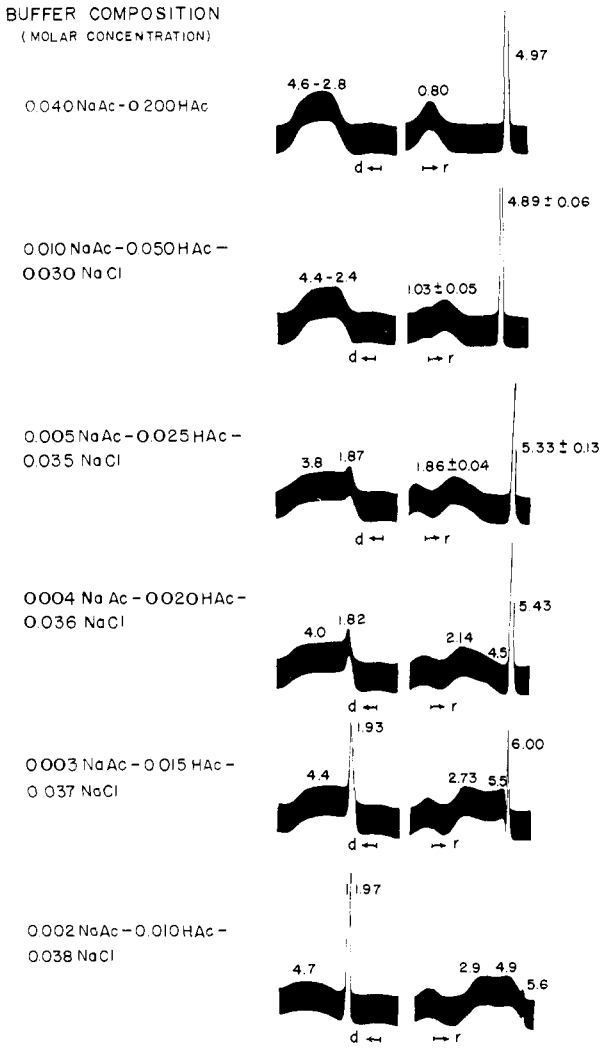

Fig. 1.—Electrophoretic patterns of ovalbumin in media containing varying concentration of acetate buffer (NaAc–HAc); *p*H 4.0.

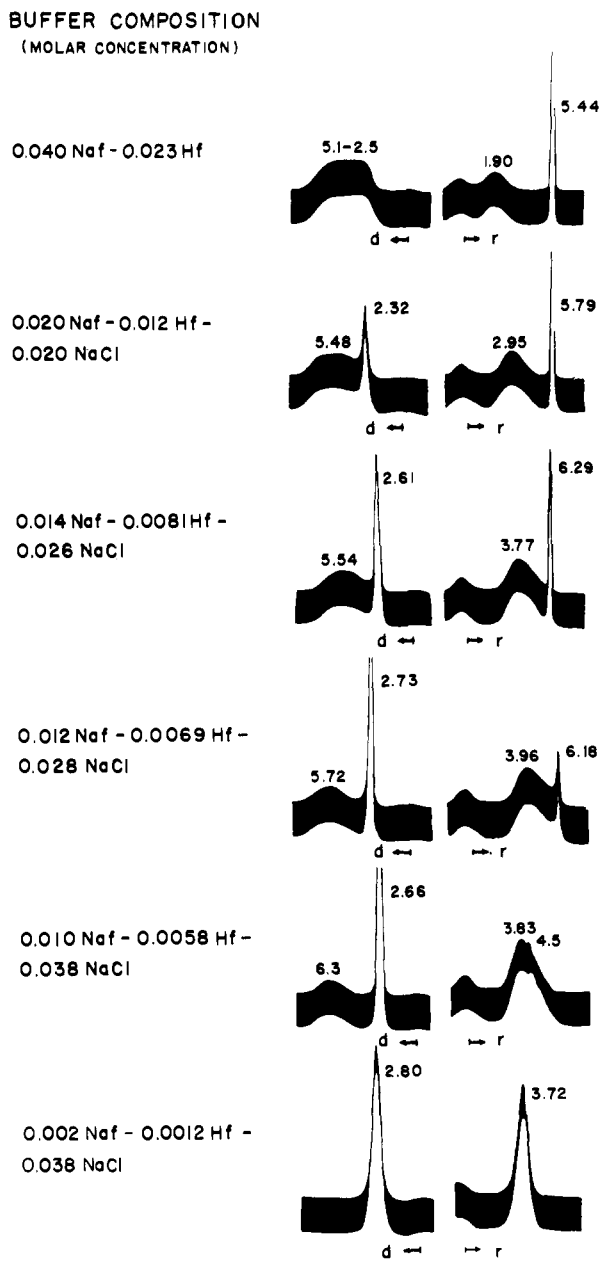

Fig. 2.—Electrophoretic patterns of ovalbumin in media containing varying concentrations of formate buffer (Naf–Hf); *p*H 4.0.

but also contribute to our understanding of these patterns. Thus, at the lowest concentration of formate buffer, ovalbumin shows a single moving boundary. In addition, the rising and descending patterns are fairly enantiographic. However, as the formate concentration is increased, the patterns change in the same remarkable manner as in acetate buffers, although formate is not as effective as acetate buffer in producing these changes. Comparison of Figs. 1 and 2 shows that the relative effect of the two buffers in producing these changes correlates rather well with the concentration of unionized carboxylic acid in the supporting medium and very poorly with the concentration of acid anion. This correlation is also shown by other

buffers at *p*H 4.0. Thus, the electrophoretic patterns in propionate and valerate buffers (0.01 *M* Na salt of acid–0.03 *M* NaCl) were essentially the same as in acetate buffer. However, the effectiveness of methoxyacetate[4] over a wide range of buffer concentrations is less than acetate and formate; and substitution of chloroacetate for chloride in medium containing the lowest concentration of acetate buffer, had no effect on the electrophoretic patterns. Thus, the order of decreasing effectiveness of the various buffers is: acetate, propionate and valerate > formate > methoxyacetate > chloroacetate. This is also the order of decreasing concentration of the respective un-ionized acids in buffers[5] of *p*H 4.0.

In the experiments described so far, the supporting medium contained NaCl, except at the highest buffer concentrations, and the possibility remains that the observed effect of increasing buffer concentration is actually an effect of decreasing chloride concentration. Also, one of the multiple boundaries observed at low buffer concentrations could conceivably be a false boundary at the type predicted by the moving boundary theory of Dole.[7] However, both these possibilities have been eliminated by electrophoretic experiments carried out in media containing varying concentrations of buffer but no NaCl. The patterns obtained in illustrative experiments are shown in Fig. 3. Comparison of Figs. 2 and 3 shows that the effect of buffer concentration on electrophoretic patterns is obtained even in the absence of NaCl and that as many as three moving boundaries can be obtained in a supporting medium containing only one salt anion.[8]

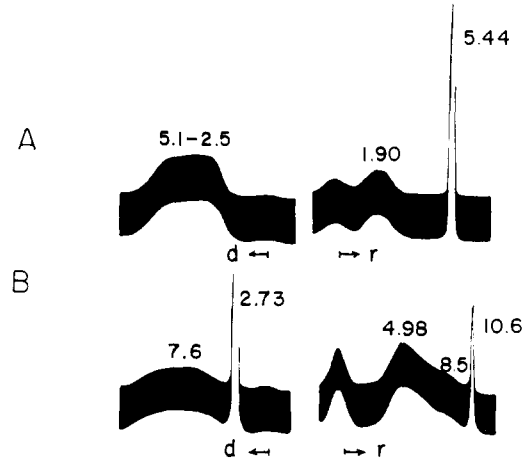

Fig. 3.—Electrophoretic patterns of ovalbumin in formate buffers at *p*H 4.0 and containing no NaCl: A, ionic strength 0.04; B, ionic strength 0.01.

The electrophoretic patterns of ovalbumin at *p*H 8 to 11 and low buffer concentrations seemed about normal, although in barbital buffer (0.002 *M* NaB–0.038 *M* NaCl) at *p*H 8.4 the descending pattern did show a small, rapidly moving boundary which disappeared when NaAc was substituted for NaCl.

Electrophoretic Behavior of γ-Globulin.—As shown in Figs. 4 and 5, the electrophoretic patterns of bovine γ-pseudoglobulin at *p*H 5.7 and 4.0, at which *p*H's γ-globulin is positively charged, depend on the concentration of acetate buffer in the supporting medium in much the same manner as shown at *p*H 4.0 by serum albumin[2] and ovalbumin. Indeed, at the lower *p*H the electrophoretic patterns of γ-globulin are strikingly similar to those of ovalbumin.

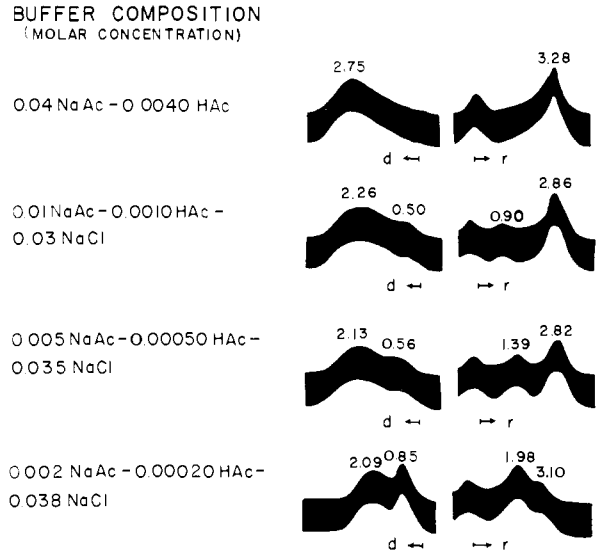

Fig. 4.—Electrophoretic patterns of bovine γ-pseudoglobulin in media containing varying concentration of acetate buffer; *p*H 5.7.

Evidence of an Isomerization Equilibrium.—Several different types of experiments indicate that ovalbumin and serum albumin are equilibrium mixtures of electrophoretic components at *p*H 4.0. Each experiment will be discussed separately.

(a) Perhaps the most convincing experiment is the one in which the protein is fractionated in the electrophoresis cell. As shown in Fig. 1, the rising pattern of ovalbumin in a supporting medium of composition 0.010 *M* NaAc–0.050 *M* HAc–0.030 *M* NaCl, shows a sharp, rapidly moving boundary and a somewhat diffuse, slower moving one. About

(4) The electrophoretic mobilities of the various peaks were greater in methoxyacetate than in acetate-containing media.

(5) Interpretation of this result could be complicated by differences in affinity of the various acids for ovalbumin. Steinhardt, Fugitt and Harris[6] have shown that the fibrous protein wool can combine with very large quantities of weak acids in their un-ionized form and that there are large differences in affinity of the undissociated acids for the protein. Thus, for example, the affinities of aliphatic acids at 0° increase in the order propionic < acetic < glycolic < formic < monochloroacetic. Possible differences in affinity of various acids for ovalbumin might afford an explanation for our observations with potassium acid phthalate. The electrophoretic patterns in media containing potassium acid phthalate were similar to those shown in the other buffers except for a slightly greater complexity of the descending patterns and lower mobilities. In effectiveness potassium acid phthalate falls between formate and methoxyacetate, which indicates that some factor other than concentration of un-ionized acid is operative in this case. A small amount of precipitate formed during dialysis against potassium acid phthalate, indicating that some denaturation had occurred.

(6) J. Steinhardt, C. H. Fugitt and M. Harris, *J. Research Natl. Bur. Standards*, **30**, 123 (1943).

(7) V. P. Dole, THIS JOURNAL, **67**, 1119 (1945).

(8) The effect of ionic strength on the patterns in formate buffers is consistent with our experience with acetate-containing media.

60% of the protein disappears across the rapidly moving boundary. The protein was fractionated by withdrawing from the electrophoresis cell the materials disppearing across the fast boundary. (The peaks were separated in the electric field with back compensation, so that at the end of electrophoresis the rising channel contained only the protein disappearing across the faster boundary.) A composite of such fractions was redialyzed against buffer and analyzed electrophoretically. The resultant patterns were identical with those of the unfractionated ovalbumin. The fraction was then refractionated in the same manner to yield a second fraction which also gave electrophoretic patterns identical to those of the original unfractionated protein.

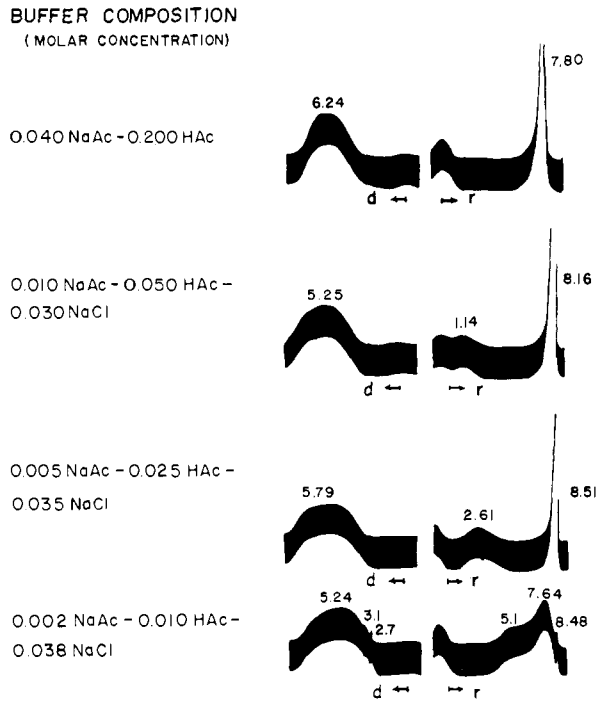

Fig. 5.—Electrophoretic patterns of bovine γ-pseudoglobulin in media containing varying concentration of acetate buffer; *p*H 4.0.

(b) In the second type of experiment which indicates that ovalbumin and serum albumin are equilibrium mixtures, reequilibration was allowed to take place in the electrophoresis cell. The results of typical experiments are shown in Fig. 6. Serum albumin was chosen for purposes of illustration because of the great interest in this protein. In a chloride-free acetate buffer of ionic strength 0.01 and *p*H 4.0, serum albumin shows two rising boundaries similar to those shown by ovalbumin at the same buffer concentration but ionic strength 0.04, Fig. 1. These two boundaries were separated by electrophoresis to give the pattern shown in Fig. 6A. The electric current was then turned off and the boundaries allowed to sit in the cell for varying lengths of time before turning on the current again. The only changes in the patterns which occurred during this time were simply those expected of diffusion. When the electric field was reapplied,

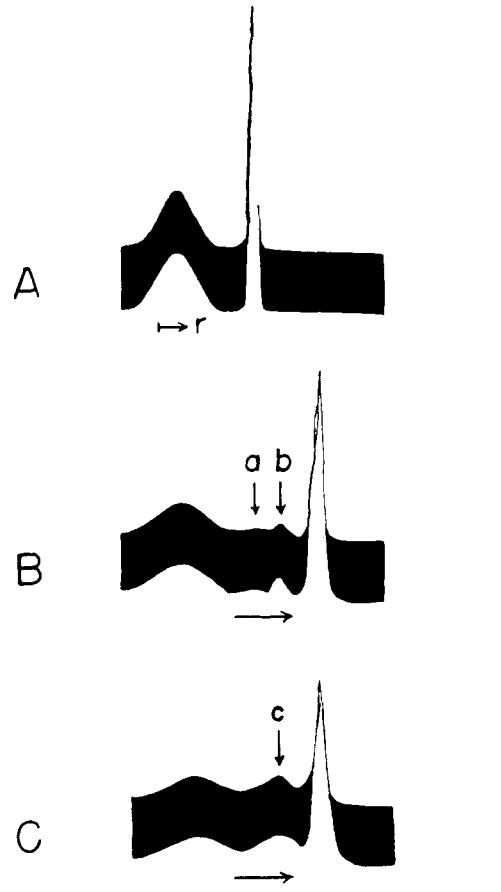

Fig. 6.—Rising electrophoretic patterns of a 1% solution of bovine serum albumin at *p*H 4.0, ionic strength 0.01 acetate buffer. The electrophoretic experiments giving rise to these three patterns are discussed in the text.

the resultant electrophoretic pattern now showed three or four peaks. Thus, when the original two boundaries had been allowed to sit for 4 hours before reapplication of the electric field, two new boundaries, designated as a and b in Fig. 6B, appeared. When the boundaries were allowed to sit 12 hours before reapplication of the field, a single new boundary was observed, designated by c in Fig. 6C. It thus appears that during the time between stopping the first electrophoresis and reapplication of the field, some of the material disappearing across the leading boundary was converted to one or more new electrophoretic components of lower mobilities. The growth of new components is relatively slow, having a half-time of 4–6 hours. Quite similar results have been obtained with ovalbumin.

(c) Ovalbumin gives the same sedimentation pattern and sedimentation constant at low as at high acetate concentration, which indicates that the effect of acetate buffer on the electrophoretic behavior of the protein is not a reflection of aggregation or dissociation of the protein. Thus, it can be assumed that at *p*H 4 ovalbumin undergoes reversible, unimolecular isomerization reactions leading to components of different electrophoretic mobilities but the same molecular weights. If this is the case, then the electrophoretic patterns should

be sensitive to the time of electrophoresis. The theory of isomerization equilibrium and electrophoresis[9] predicts that for a simple, unimolecular, isomerization reaction, $A \underset{k_2}{\overset{k_1}{\rightleftarrows}} B$ and $k_1 = k_2$ (diffusion constants of the two species assumed to be the same), two moving boundaries will be observed for times of electrophoresis less than or of the order of the half-time of the reaction. For longer times of electrophoresis a single moving boundary of intermediate mobility should be observed. As shown in Fig. 7, this has been approximately realized for ovalbumin. For a relatively short time of electrophoresis two rising boundaries are observed; whereas for a very long time, *i.e.*, low field strength, the slower, rising boundary is very small and diffuse. However, the mobility of the major boundary is about 40% greater than expected for a simple, unimolecular, isomerization reaction.

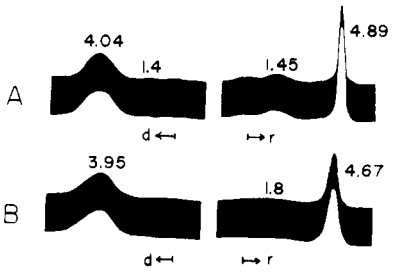

Fig. 7.—Electrophoretic patterns of ovalbumin in media containing 0.010 *M* acetate buffer; *p*H 3.94 and ionic strength 0.049: A, time of electrophoresis was 52 minutes at a field strength of 17.7 volts cm.$^{-1}$; B, time of electrophoresis was 471 minutes at a field strength of 1.97 volts cm.$^{-1}$; protein concentration 0.43%.

Finally, mention should be made concerning the denaturing effect of acetic acid on ovalbumin. While there is no evidence for denaturation in acetate buffer of the concentrations used in the experiments described above, denaturation was observed in 0.1–0.5 *M* acetate buffers. When a sufficient quantity of concentrated buffer was added to ovalbumin solution to give a final concentration of 0.1 *M*, only a slight precipitate developed during aging at room temperature for 24 hours. However, when the final concentration was 0.5 *M*, a heavy precipitate developed immediately. This precipitate could not be redissolved in 0.1 *M* NaCl. On the other hand, when aliquots of protein solution, equilibrated against the buffers used in the electrophoretic experiments, were added to 0.2 *M* acetate buffer at *p*H 4.7, no precipitation was observed.

Discussion

While the electrophoretic patterns of ovalbumin, serum albumin and γ-globulin in acidic media are complex and must be interpreted with caution, several things seem clear. Thus, for example, the experiments described above indicate that the proteins are equilibrium mixtures of electrophoretic components, the equilibrium composition depending upon the concentration of un-ionized carboxylic acid in the supporting medium. While solutions of these proteins may well contain molecules which differ chemically, for example, the components, A_1, A_2 and A_3 of ovalbumin,[10] it is concluded that each of these chemically different molecules participates in the equilibrium. In order to be perfectly clear, this conclusion can be restated as follows: each protein molecule in solution can exist in two or more interconvertible states which differ in their electrophoretic mobilities, but not significantly in their molecular weights. The simplest reaction scheme for ovalbumin which serves as our working hypothesis and which accounts, in part at least, for the observed electrophoretic behavior, is that in acidic media, the protein undergoes a set of simultaneous and consecutive, reversible, unimolecular, isomerization reactions. If it is assumed that the ovalbumin molecule exists in the state P in the absence of acetic acid or in solvents containing low concentrations of formic acid, then as the acid concentration is increased at constant *p*H, the protein undergoes the simultaneous reactions

$$P \rightleftarrows P_1 \rightleftarrows P_2$$

The mobilities of the protein in these three states increase in the order $P < P_1 < P_2$; presumably due to an increase in net positive charge. The state P_1 might correspond to the boundary of mobility 4.9×10^{-5} in the rising pattern observed in medium of composition 0.002 *M* NaAc–0.010 *M* HAc–0.038 *M* NaCl, Fig. 1. The state P_2 then corresponds to the most rapidly moving boundary which grows at the expense of slower boundaries when the acetate concentration is increased. Another reaction which gives rise to a component of lower mobility, designated as P′, appears to occur simultaneously

$$P \rightleftarrows P'$$

The slowest moving, rising boundary shown by each of the patterns presented in Fig. 1 may represent an equilibrium mixture of P and P′, but at the higher acetate concentrations it may be P′ only. If the latter is the case, then either the mobility of P′ decreases with increasing acetate concentration, or P′ represents several similar states of the molecule, all in equilibrium and possessing different mobilities. The non-enantiographic nature of the electrophoretic patterns is as yet not understood, but is clearly related to reactions of the protein during electrophoresis. The similarity of the electrophoretic behavior of ovalbumin, serum albumin and γ-globulin at *p*H 4 indicates that a reaction scheme such as that outlined above may be of general applicability.

Calculations of the valence of the protein disappearing across the various moving boundaries indicate that isomerization of ovalbumin may involve changes in net electrical charge as large as 4 to 5 electrons. The differences between the various states of the protein are not known, but they do not appear to be simply differences in the number of salt ions bound to the protein molecules. Since

(9) J. R. Cann, J. G. Kirkwood and R. A. Brown, *Arch. Biochem. Biophys.*, in press.

(10) J. R. Cann, THIS JOURNAL, **71**, 907 (1949); C. F. C. MacPherson, D. H. Moore and L. G. Longsworth, *J. Biol. Chem.*, **156**, 381 (1949); G. E. Perlmann, *J. Gen. Physiol.*, **35**, 711 (1952).

high concentrations of acetate buffer denature ovalbumin, it is conceivable that at lower concentrations binding of acetic and other acids to the protein produces subtle structural changes which involve changes in the ionization constants of acidic groups and/or unmasking of ionizable groups on the protein.

Simultaneously with our first communication,[2] Aoki and Foster[11] reported on the isomerization of serum albumin in NaCl–HCl solutions. It is possible that both of us were observing related reactions and that serum albumin undergoes an isomerization even in the absence of acetic acid. Perhaps in NaCl–HCl solution isomerization is effected by simultaneous binding[12] of H^+ and Cl^-.

Finally, the experiments described in this paper emphasize the need for caution in interpreting electrophoretic experiments designed to test the homogeneity of purified protein preparations such as enzymes.

(11) K. Aoki and J. F. Foster, THIS JOURNAL, **78**, 3538 (1956).

(12) J. Steinhardt, *Ann. N. Y. Acad. Sci.*, **41**, 287 (1941); G. Scatchard and E. S. Black, *J. Phys. Colloid Chem.*, **53**, 88 (1949); G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., THIS JOURNAL, **72**, 535 (1950).

DENVER, COLORADO

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Effect of Binding of Ions and Other Small Molecules on Protein Structure. III. Influence of Amino Acids on the Isomerization of Proteins[1]

BY ROBERT A. PHELPS AND JOHN R. CANN

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The influence of amino acids on the electrophoretic composition of ovalbumin and serum albumin in media containing acetate buffer, is interpreted in terms of a competitive inhibition of the effect of acetic acid on the postulated isomerization of these proteins. It appears that the active form of the amino acid is not the zwitterion but rather the acid form and that steric factors are important to its action.

Introduction

The electrophoretic studies presented in the first two papers[2,3] of this series indicate that ovalbumin, serum albumin and γ-globulin in acidic media are equilibrium mixtures of electrophoretic components, the equilibrium composition depending upon the concentration of un-ionized carboxylic acid in the supporting medium. Some of the features of the electrophoretic patterns of ovalbumin appear to be accounted for by a set of simultaneous and consecutive, reversible unimolecular isomerization reactions[3] leading to changes in net charge but not significant changes in molecular weight of the protein. In the present communication it will be shown that whereas acetic acid displaces the postulated equilibria in one direction, amino acids have the opposite effect. Furthermore, the respective effects of acetic and amino acid on the electrophoretic patterns of ovalbumin are competitive in nature.

Methods.—The amino acids used in this study were the purest commercial grades available, *e.g.*, the CfP grade furnished by California Foundation for Biochemical Research. The methods used are described in the second paper of this series.[3]

Results

The electrophoretic patterns of ovalbumin at *p*H 4.0, at which *p*H the protein is positively charged, in media containing a constant concentration of acetate buffer (NaAc–HAc) but varying concentration of glycine are shown in Fig. 1. Comparison of these patterns with those in Fig. 1 of the preceding paper[3] shows that the addition of glycine to the supporting medium results in electrophoretic patterns typical of those observed at lower concentrations of NaAc–HAc and in the absence of glycine. For example, the pattern obtained in a medium of composition 0.005 *M* NaAc–0.025 *M* HAc–0.034 *M* NaCl–0.021 *M* glycine, is almost identical

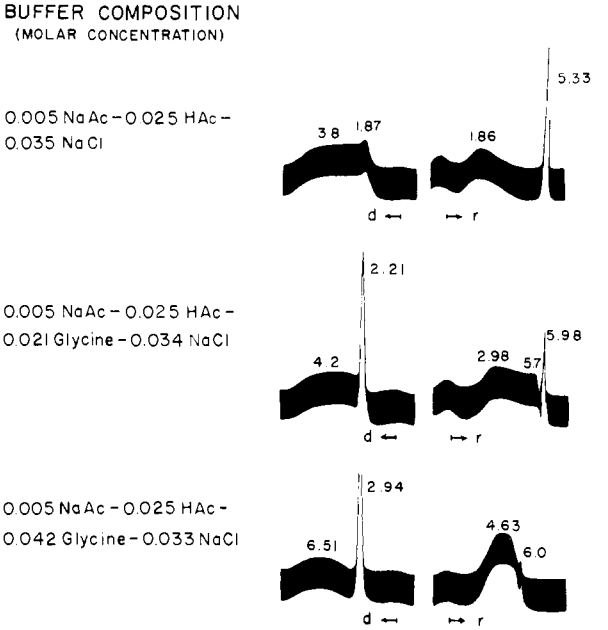

Fig. 1.—Electrophoretic patterns of ovalbumin in media containing constant concentration of acetate buffer but varying concentration of glycine; *p*H 4.0.

(1) Supported in part by research grant No. E-1482(C5) from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Public Health Service; and in part by the Damon Runyon Fund and the American Cancer Society. Presented in part at the Miami meeting of the American Chemical Society, April 7 to 12, 1957.

(2) R. A. Phelps and J. R. Cann, THIS JOURNAL, **78**, 3539 (1956).

(3) J. R. Cann and R. A. Phelps, *ibid.*, **79**, 4672 (1957).