[CONTRIBUTION NO. 81 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. VI. Influence of *p*H and Chemical Modification on the Electrophoretic Behavior of Proteins in Acidic Media[1]

BY JOHN R. CANN AND ROBERT A. PHELPS

RECEIVED MARCH 14, 1959

Electrophoretic experiments on ovalbumin and bovine serum albumin in acetate- and formate-containing media at different *p*H values support our previous conclusion that the electrophoretic behavior of these proteins in acidic media is determined by interaction of the macromolecular ions with un-ionized buffer acid. These experiments as well as others on chemically modified proteins indicate that macromolecular structural parameters are important in determining the effect of buffer acids on electrophoretic patterns.

Introduction

A new effect of acetate (NaAc–HAc), formate (NaF–HF) and other carboxylic acid buffers on the electrophoretic patterns of ovalbumin, bovine γ-pseudoglobulin and bovine serum albumin (BSA) in acidic media has been described previously.[2–6] The nature of the electrophoretic patterns of these proteins at *p*H 4 depends upon the concentration of buffer in the supporting medium. Increasing the concentration of buffer at constant *p*H and ionic strength results in progressive and characteristic changes in the patterns, notably in the appearance and growth of fast-moving peaks at the expense of slow ones. (See, for example, Figs. 1 and 2 of ref. 3 and Fig. 2 of ref. 5). This behavior has been interpreted in terms of complexing of the protein with undissociated buffer acid with concomitant increase in electrophoretic mobility. The extremely non-enantiographic nature of the patterns is a reflection of such interaction and is not due to the relatively high protein concentration (1 to 1.3%) *per se*.

It has also been shown[6] that the various peaks in the electrophoretic patterns of BSA and ovalbumin in NaAc–HAc at *p*H 4 correspond neither to single stable protein components nor to single components involved in a slowly adjusted equilibrium. Resolution of the patterns into multiple moving boundaries is intimately related to the production of conductance and *p*H gradients during electrophoresis. However, it should not be inferred from this that BSA and ovalbumin do not undergo some reaction during electrophoresis. Rather, it would appear that resolution of the patterns results from a coupling of such reactions with electrophoretic transport of the ions of the supporting medium. An idealized qualitative explanation of the patterns has been proposed.[6] This explanation assumes that fast-moving protein–acid complexes exist in instantaneous equilibrium with the undissociated buffer acid as well as other constituents of the supporting medium and predicts that during electrophoresis the *p*H in the Tiselius cell will change in such a manner as to induce a type of non-enantiography typical of that actually observed under a variety of conditions of buffer composition.

The present communication presents a study of the influence of *p*H and chemical modification on the electrophoretic behavior of ovalbumin, BSA and ribonuclease (RNAase) in acetate- and formate-containing media. In addition to supporting our previous conclusion concerning the role of undissociated buffer acid in determining the electrophoretic behavior of proteins in acidic media, the results of these studies indicate that macromolecular structural parameters are important in determining the effect of these acids on electrophoretic patterns.

Experimental

The methods used for electrophoretic analysis in the Tiselius apparatus have been described in detail previously.[2–6] In all schlieren patterns shown in the figures the apparent mobilities u were positive. The values of $10^5 \times u$ cm.^{2} sec.$^{-1}$ volt^{-1}, are given in the figures. In general, these values cannot be placed in correspondence with mobilities, since it has been shown that very large changes in conductance and *p*H occur in the electrophoresis cell during passage of the current.[6] Crystalline ovalbumin were prepared by the method of Sorensen and Høyrup.[7] The BSA was Armour's crystallized plasma albumin. The RNAase was Pentex's 5× crystallized material. BSA was esterified in acidic methanol according to the method of Fraenkel-Conrat and Olcott.[8] Esterified-BSA was acetylated by slow addition of acetic anhydride to a solution of the protein derivative in 0.1 *M* NaAc cooled to 0°.[9] RNAase was oxidized with performic acid according to the method used by Harrington and Schellman[10] to prepare their sample 3.

Results and Discussion

Influence of *p*H.—A complex dependence of the electrophoretic behavior of ovalbumin and BSA upon the *p*H of their acidic solutions was revealed by preliminary electrophoretic analyses carried out on protein solutions equilibrated by dialysis against 0.04 *M* NaF buffers containing varying concentrations of HF. Representative electrophoretic patterns of ovalbumin at several *p*H values are shown in Fig. 1. At *p*H 4.50 the descending pattern shows a large and sharp slow-

(1) Supported in part by research grant No. E-1482 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.

(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).

(4) R. A. Phelps and J. R. Cann, *ibid.*, **79**, 4677 (1957).

(5) J. R. Cann, *ibid.*, **80**, 4263 (1958).

(6) J. R. Cann, *J. Phys. Chem.*, **63**, 210 (1959).

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

(8) H. A. Fraenkel-Conrat and H. S. Olcott, *J. Biol. Chem.*, **161**, 259 (1945).

(9) F. W. Putnam in H. Neurath and K. Bailey, "The Proteins," Vol. IB, Academic Press, Inc., New York, N. Y., 1953, Chapter 10, p. 925.

(10) W. F. Harrington and J. A. Schellman, *Compt. rend. lab. Carlsberg, Sér. chim.*, **30**, 21 (1956).

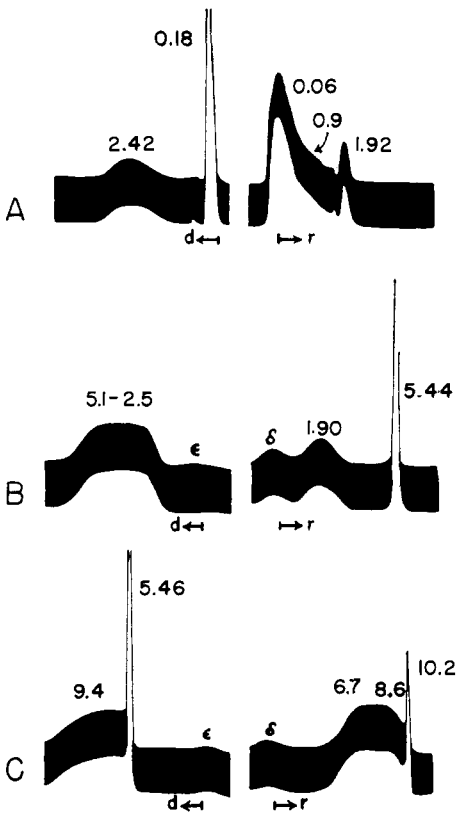

Fig. 1.—Electrophoretic patterns of ovalbumin in 0.04 *M* NaF buffers: A, *p*H 4.50; B, *p*H 4.00; C, *p*H 2.69. Protein concentration, 1.3%.

moving boundary and a small and diffuse fast-moving one. The rising pattern is somewhat more complex and shows at least three boundaries. When the *p*H is lowered from 4.50 to 4.00, the fast-moving boundaries grow at the expense of the slower-moving ones. However, when the *p*H is lowered still further to a value of 2.67, the slow-moving boundaries now grow at the expense of faster ones. (Similar results were obtained with BSA.) This behavior indicates that there are at least two factors which operate to determine the proportions of the various peaks in the electrophoretic patterns of these proteins in the *p*H range 4.5 to 2.7. Since both the *p*H and the concentration of HF were of necessity varied in these experiments, it is reasonable to suppose that the nature of the electrophoretic patterns is sensitive to both parameters. This supposition is consistent with our previous conclusion concerning the role of undissociated buffer acid in determining the nature of the electrophoretic patterns of proteins at *p*H 4.0. It thus seemed advisable to evaluate the influence of *p*H upon electrophoretic behavior by studying its influence upon the effectiveness of undissociated buffer acid in producing the changes in electrophoretic patterns described in the introduction.

In experiments designed with this in mind, a set of electrophoretic patterns was obtained at each of several *p*H values. The concentration of formate, and in some cases acetate, buffer in the supporting medium was varied over a wide range at each *p*H value, the ionic strength being maintained constant with NaCl. The several sets of patterns were then examined in order to estimate the influence of *p*H upon the effectiveness of either formic or acetic acid in producing the described changes in electrophoretic patterns. The concentrations of acid required to give different distributions of area between the fast- and slow-moving peaks in the electrophoretic patterns at a given *p*H were compared with the concentrations required to give the same distributions at *p*H 4.0. (When the fast-moving rising peak and the slow descending one were too sharp to be completely recorded on the photographic film, it was necessary to compare concentrations of acid required to give the same area enclosed by the slow rising peaks or the fast descending ones.) When, for example, the concentration of acid required to give a certain distribution at a given *p*H was found to be five times that required at *p*H 4, it was concluded that the effectiveness of the acid at the first *p*H is 0.2 that at *p*H 4.0. The estimated effectiveness at a given *p*H value was arbitrarily referred to that at *p*H 4.0 taken as 100 and is therefore called the relative effectiveness. These data are summarized in Fig. 2

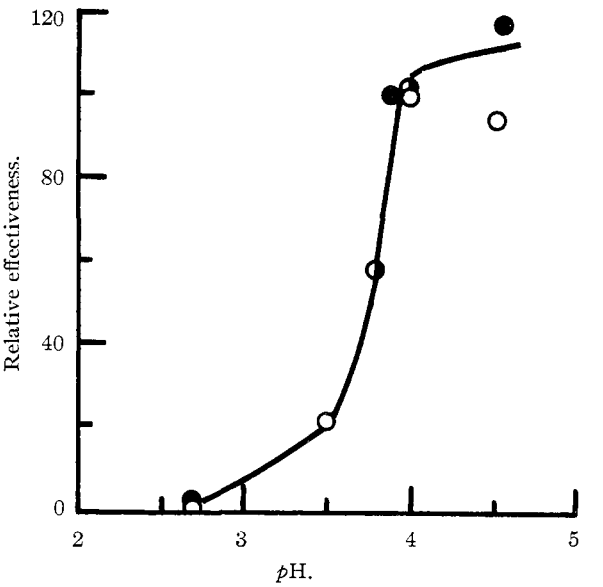

Fig. 2.—The relative effectiveness of buffer acids in altering the electrophoretic patterns of proteins in acidic media as a function of *p*H: ●, BSA in 0.04 ionic strength formate buffers; ◑, BSA in 0.01 ionic strength acetate buffers; ○, ovalbumin in 0.04 ionic strength formate buffers.

which is a plot of relative effectiveness *vs.* *p*H. This plot shows that the relative effectiveness of an undissociated buffer acid in causing the growth of fast-moving boundaries at the expense of slow-moving ones is about the same at *p*H 4.0 as at *p*H 4.5 but that when the *p*H is lowered still further there is a marked and progressive decrease in relative effectiveness. This affords an explanation of the results of our preliminary experiments illustrated in Fig. 1. When the *p*H in these experiments was lowered from 4.5 to 2.7 holding the concentration of NaF constant, the proportion of fast peak at first increased due to the increase in the concen-

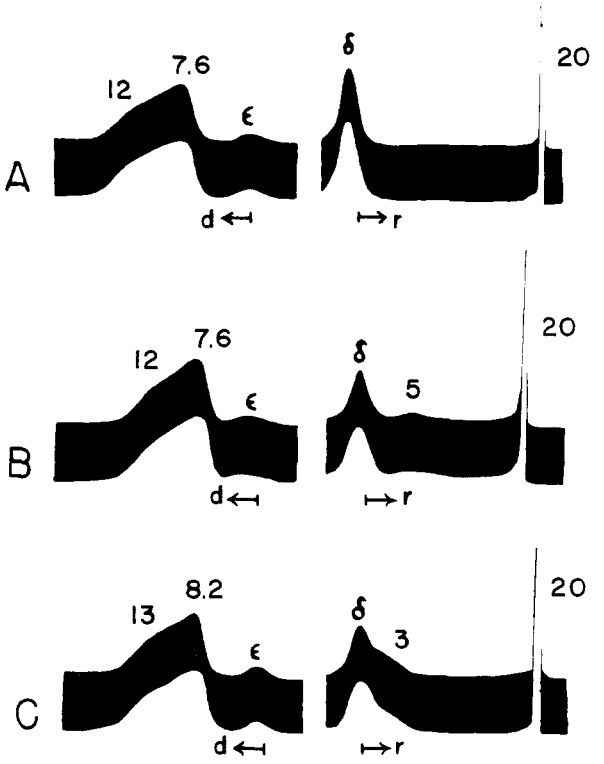

Fig. 3.—Electrophoretic patterns of esterified-BSA at *p*H 4.0: A, 0.01 *M* NaCl–HCl; B, 0.002 *M* NaAc–0.01 *M* HAc–0.008 *M* NaCl; C, 0.01 *M* NaAc–0.05 *M* HAc. Protein concentration, 1%.

tration of HF but then decreased due to the decrease in effectiveness of the acid.

The shape of the plot shown in Fig. 2 is of considerable interest since it indicates that the degree of ionization of the carboxyl groups on the protein which are being titrated over this *p*H range, is of major importance to the mechanism underlying the effect of acetate and other carboxylic acid buffers on the electrophoretic patterns of proteins in acidic media. The experiments described below are concerned with the questions raised by this conclusion

Influence of Chemical Modification.—The results of the above experiments indicate that as the carboxylate groups on the protein are converted to uncharged carboxyl groups by titration with H^+, the effectiveness of un-ionized buffer acid in changing the electrophoretic patterns of the protein decreases. This suggests that if the carboxyl groups on the protein were converted to ester groups, then the electrophoretic patterns of the esterified protein should be insensitive to the concentration of buffer in the supporting medium at constant *p*H and ionic strength. Indeed, this has been found to be the case.[11] The electrophoretic behavior of BSA esterified by reaction with acidified methyl alcohol was studied at *p*H 4, ionic strength 0.01, in supporting media containing varying concentration of acetate buffer. (Titration data indicated that at least 65% of the carboxyl groups were esterified.) Representative electrophoretic patterns of esterified-BSA are presented in Fig. 3. Comparison of these patterns with those of BSA in the same media, Fig. 2 of ref. 5, shows that the electrophoretic behavior of esterified-BSA is quite different from that of BSA In fact, acetate buffer has very little effect upon the electrophoretic patterns of esterified-BSA. For example, the rising patterns show essentially a single, rapidly-moving boundary over a wide range of acetate concentration. This result strengthens our conclusion that caroxylate groups on the protein are essential for the effect of acetate buffer on electrophoretic patterns.

It is of considerable interest to determine whether or not reversal of esterification by hydrolysis restores the effect of acetate buffer on electrophoretic patterns. Since alkaline hydrolysis of esterified-BSA either occurs to only a limited extent or leads to an insoluble derivative,[12] it was decided to attempt acid hydrolysis. In control experiments 1% solutions of BSA in 0.8 *M* HCl were incubated at 42° for 23 hr. This treatment resulted in precipitation of the protein, but the precipitated protein redissolved during dialysis of the acidic suspension against 0.1 *M* NaAc. (The precipitate dissolved by the time the *p*H had increased to a value of about 1.5.) The solution of redissolved protein was then dialyzed exhaustively against buffer at *p*H 4.0 followed in some cases by dialysis against 0.1 *M* NaCl. Titration data on the acid-modified BSA indicated that about 18 peptide bonds per 65,000 g. of protein were hydrolyzed by this treatment. The electrophoretic patterns of the acid-modified BSA are presented in Fig. 4A. While these patterns are somewhat different than those of native BSA and reflect the damage done to the protein by the acid-treatment (compare with Fig. 2 of ref. 5), they nevertheless show that acetate buffer still has a large effect upon electrophoretic behavior of the protein. When esterified-BSA was treated in an identical manner, about 47 titratable carboxyl groups per 65,000 g. of protein were released. At least 30 of these presumably resulted from hydrolysis of ester groups. The electrophoretic patterns of the hydrolyzed esterified-BSA, Fig. 4B, are strikingly similar to those of the control and reveal once again a profound effect of acetate buffer on electrophoretic behavior. Furthermore, when the hydrolyzed esterified-BSA was re-esterified with acidified methyl alcohol, the electrophoretic patterns of the re-esterified derivative were identical to those of the original, unhydrolyzed esterified-BSA, *i.e.*, the effect of acetate buffer disappeared once more as a result of re-esterification. Although these experiments suffer from obvious complications, they clearly demonstrate that carboxylate groups on the protein play an important role in the mechanism underlying the effect of acetate buffer on the electrophoretic behavior of proteins in acidic media.

The question naturally arises whether this role is simply the control of net electrical charge on the protein molecule. The following experiment indicates that this is the case: a sample of esterified-

(11) Saroff and his co-workers[12] first showed that esterified-BSA shows only a single moving boundary in 0.1 *M* acetate buffer, *p*H 4–6.

(12) H. A. Saroff, N. R. Rosenthal, E. R. Adamik, N. Hages and H. A. Scheraga, *J. Biol. Chem.*, **205**, 255 (1953).

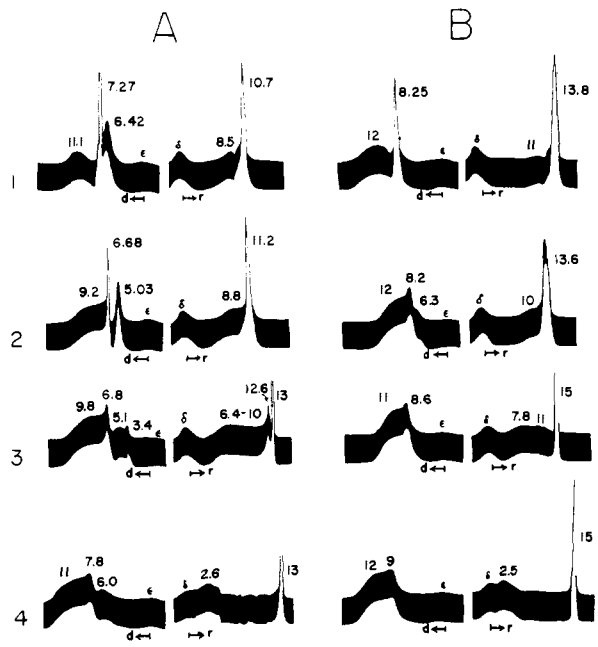

Fig. 4.—Electrophoretic patterns at *p*H 4.0–4.1: A, acid-modified BSA; B, acid hydrolyzed esterified-BSA. Buffers: 1, 0.0006 *M* NaAc–0.003 *M* HAc–0.0094 *M* NaCl; 2, 0.001 *M* NaAc–0.005 *M* HAc–0.009 *M* NaCl; 3, 0.002 *M* NaAc–0.01 *M* HAc–0.008 *M* NaCl; 4, 0.01 *M* NaAc–0.05 *M* HAc. Note evidence of convection in rising pattern of acid-modified BSA in 0.01 *M* NaAc–0.05 *M* HAc buffer.

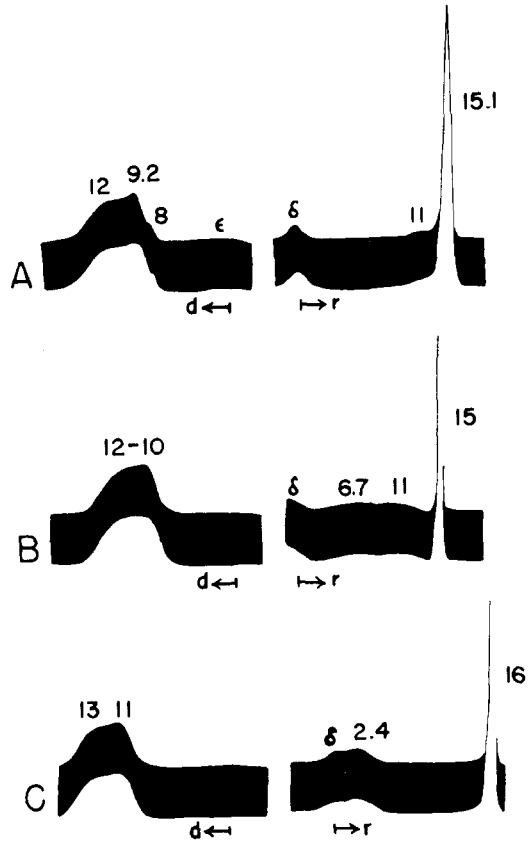

Fig. 5.—Electrophoretic patterns of acetylated esterified-BSA at *p*H 4.0: A, 0.0008 *M* NaAc–0.004 *M* HAc–0.0092 *M* NaCl; B, 0.002 *M* NaAc–0.01 *M* HAc–0.008 *M* NaCl; C, 0.01 *M* NaAc–0.05 *M* HAc.

BSA was acetylated with acetic anhydride to bind free amino groups, thereby decreasing the net charge on the protein. Measurement of the apparent electrophoretic mobility demonstrated this change in charge. Furthermore, the rising electrophoretic patterns of the acetylated esterified-BSA, Fig. 5, differ from those of esterified-BSA, Fig. 3, and are qualitatively similar to those of hydrolyzed esterified-BSA, Fig. 4B, indicating that the net charge on the protein definitely influences its entire electrophoretic pattern rather than just its mobility. However, the net charge probably is not directly involved in the effect of acetate buffer but more likely exerts its influence on the stability of some structural feature of the protein molecule necessary for interaction with un-ionized acetic acid. A large net positive charge would modify this structure and thereby decrease the effectiveness of the buffer acid. Apparently this structure is disrupted by esterification of BSA while at the same time the net positive charge on the molecule increases. The structure is largely restored by subsequent hydrolysis and is partially restored by acetylation, both of which lower the charge to a value close to that of the native protein.

The idea that macromolecular structural parameters are important in determining the effect of acetic and other carboxylic acids on electrophoretic behavior is supported by experiments on RNAase. There is little, if any, effect of acetate-buffer concentration on the electrophoretic patterns of RNAase at *p*H 5.6 and 5.2. However, an effect was found at *p*H 4.0, Fig. 6. This effect is weak and rather different than that shown by ovalbumin

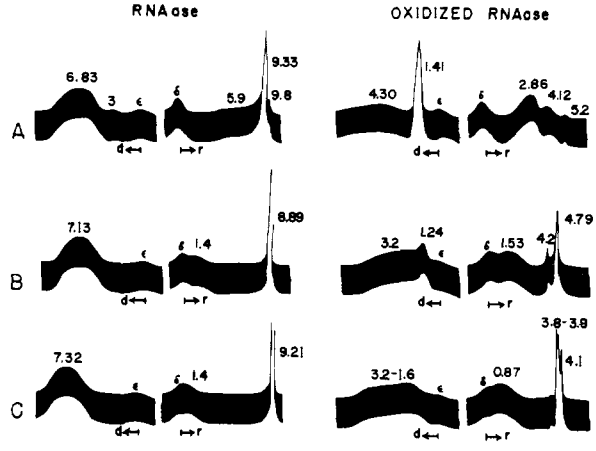

Fig. 6.—Electrophoretic patterns of RNAase and oxidized RNAase at *p*H 4.0: A, 0.002 *M* NaAc–0.01 *M* HAc–0.038 *M* NaCl; B, 0.01 *M* NaAc–0.05 *M* HAc–0.03 *M* NaCl; C, 0.02 *M* NaAc–0.1 *M* HAc–0.02 *M* NaCl.

and BSA.[13] However, when RNAase was treated with performic acid, the electrophoretic patterns of the resultant oxidized RNAase, Fig. 6, were found to be almost identical with those of ovalbumin in the same media (see Fig. 1 of ref. 3) and

(13) At very low acetate concentrations the descending pattern of RNAase did show a sharp, slow-moving boundary and a diffuse, fast-moving one; but the rising pattern showed only a single boundary.

very similar to patterns shown by BSA in appropriate media (see Fig. 2 of ref. 5 and Fig. 7 of ref. 6). This change in electrophoretic behavior is undoubtedly related to the change in macromolecular structure accompanying oxidation. When the disulfide cross-linkages of the very compact RNAase molecule are oxidatively cleaved with performic acid, the molecule unfolds and appears to assume a flexible or random-coil type of structure rather than a hydrogen-bonded structure.[10] This suggests that the flexibility conferred upon the molecule by cleavage of the –S–S–bonds is responsible for the change in its electrophoretic behavior in acetate-containing media.

Furthermore, these results indicate that there is some structural feature of oxidized RNAase which is also present in ovalbumin and BSA and which is presumably related to a certain degree of flexibility of the polypeptide chain. Although ovalbumin and BSA are compact molecules in the *p*H range from about 4 to 10, it is conceivable that portions of these molecules may be flexible. A large net positive charge acquired by binding H^+ evidently modifies this structural feature (perhaps through internal electrostatic repulsions) such that the protein molecule interacts less strongly with buffer acid at low *p*H values than near the isoelectric *p*H. Indeed, BSA undergoes profound changes in molecular configuration below about *p*H 4 as revealed by changes in viscosity,[14,15] optical rotation[14] and sedimentation constant.[16,17] These changes in properties have been interpreted in terms of reversible expansion or unfolding of the protein molecule. We find that there is a striking relationship between the change in configuration of BSA with *p*H and the change in effectiveness of un-ionized buffer acid in altering its electrophoretic patterns. Thus, the viscosity of BSA increases progressively on going from *p*H 4.0 to 2.7; and, as shown in Fig. 2, the effectiveness of buffer acid decreases progressively over the same *p*H range. It would appear that expansion of the BSA molecule decreases the strength of its interaction with the buffer medium. There would also seem to be a correlation between a small charge in the molecular configuration of ovalbumin and the *p*H-dependence of the effectiveness of buffer acid in altering its electrophoretic patterns. Small changes in optical rotation[14,18] and a barely perceptable but significant change in sedimentation constant[17] indicate that ovalbumin underoges some change in molecular configuration below *p*H 4 but to a much smaller extent than with BSA.

It is conceivable that un-ionized buffer acid may interact with the protein molecule by forming double hydrogen bonds either with two different segments of the same peptide chain or with two different chains in the molecule. If this were the case, then such interaction would be sensitive to the relative orientation of the chains and/or their various side groups. Such orientation might in turn be sensitive to electrostatic repulsions within the molecule and most certainly to gross configurational changes of the kind observed with BSA below *p*H 4.

Acknowledgments.—The authors wish to thank Drs. T. T. Puck and Serge N. Timasheff for their critical review of the manuscript.

(14) J. T. Yang and J. F. Foster, THIS JOURNAL, **76**, 1588 (1954).
(15) C. Tanford, J. G. Buzzell, D. G. Rands and S. A. Swanson, *ibid.*, **77**, 6421 (1955).
(16) P. Bro, S. J. Singer and J. M. Sturtevant, *ibid.*, **77**, 4921 (1955).
(17) P. A. Charlwood and A. Ens, *Can. J. Chem.*, **35**, 99 (1957).
(18) P. Doty and E. P. Geiduschek in H. Neurath and K. Bailey, "The Proteins," Vol. IA, Academic Press, Inc., New York, N. Y., 1953, Chapter 5, Fig. 2, p. 410.

DENVER, COLORADO

[CONTRIBUTION FROM THE DEPARTMENTS OF MICROBIOLOGY AND NEUROLOGY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY AND THE NEUROLOGICAL INSTITUTE, PRESBYTERIAN HOSPITAL]

Immunochemical Studies on Dextrans. II.[1] Antidextran Specificities Involving the $\alpha 1 \rightarrow 3$ and the $\alpha 1 \rightarrow 2$ Linked Glucosyl Residues

BY PETER Z. ALLEN AND ELVIN A. KABAT

RECEIVED MARCH 4, 1959

Human antidextran sera with a specificity directed against $\alpha 1 \rightarrow 3$ linked glucosyl residues were obtained by immunization with dextran NRRL B 1355S-4. In addition, sera with a specificity involving the $\alpha 1 \rightarrow 2$ linked glucosyl unit were obtained by immunization with dextran NRRL B 1299S-3. Quantitative precipitin curves determined for various dextran preparations show the immunochemical reactivity of dextrans in $1 \rightarrow 3$ specific sera to be related to their content of $1 \rightarrow 3$ like linkages while reactivity in sera of $1 \rightarrow 2$ specificity is related to the proportion of $1 \rightarrow 2$ linkages. Quantitative oligosaccharide inhibition data show nigerose to be the best inhibitor of precipitation of $1 \rightarrow 3$ specific antidextran and kojibiose the most effective inhibitor of precipitation of $1 \rightarrow 2$ specific antidextran by their respective homologous dextrans.

Immunochemical studies with human antidextran based on quantitative precipitation and oligosaccharide inhibition[2-5] have thus far demonstrated two kinds of antidextran. One type shows a specificity directed against a terminal non-reducing sequence of up to six or seven glucose residues in

(1) This investigation was carried out under the Office of Naval Research, Navy Department, Washington, D. C., the William J. Matheson Commission and the National Science Foundation (G-5208). Reproduction in whole or in part is permitted for any purpose of the United States Government.
(2) E. A. Kabat and D. Berg, *J. Immunol.*, **70**, 514 (1953).
(3) E. A. Kabat, THIS JOURNAL, **76**, 3709 (1954); (b) *J. Immunol.*, **77**, 377 (1956); (c) *J. Cell. and Comp. Physiol.*, Supp. #1, **50**, 79 (1957); (d) In: "Chemistry and Biology of Mucopolysaccharides," Ciba Foundation Symposium, Little, Brown and Company, Boston, 1958; (e) E. A. Kabat (in preparation).
(4) P. Z. Allen and E. A. Kabat, THIS JOURNAL, **78**, 1890 (1956).
(5) E. A. Kabat and A. E. Bezer, *Arch. Biochem. Biophys.*, **78**, 306 (1958).