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

[CONTRIBUTION NO. 55 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. III. Influence of Amino Acids on the Isomerization of Proteins[1]

BY ROBERT A. PHELPS AND JOHN R. CANN

RECEIVED APRIL 10, 1957

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

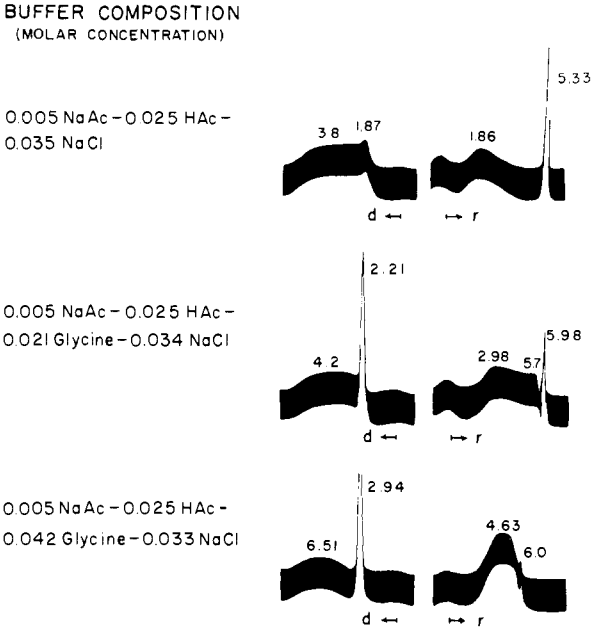

Fig. 1.—Electrophoretic patterns of ovalbumin in media containing constant concentration of acetate buffer but varying concentration of glycine; *p*H 4.0.

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(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).

with that obtained in 0.003 NaAc-0.015 HAc-0.037 NaCl. The nature of the patterns obtained when 0.042 M glycine was added to the supporting medium is what one would expect of a medium containing less than 0.002 NaAc-0.010 HAc and no glycine. Other amino acids also showed this effect, but of those tested none was as effective as glycine. The orders of decreasing effectiveness are: (a) glycine > sarcosine > proline > betaine; and (b) glycine > leucine and alanine > tryptophan > phenylalanine. The L-isomers of the optically active acids were used, but no difference was found between D- and L-alanine. Comparisons of the various amino acids were made at constant concentration of the zwitterion form. The electrophoretic patterns obtained in media containing proline and phenylalanine are presented in Fig. 2. In contrast to glycine, ethylamine has no effect on the electrophoretic patterns of ovalbumin.[4]

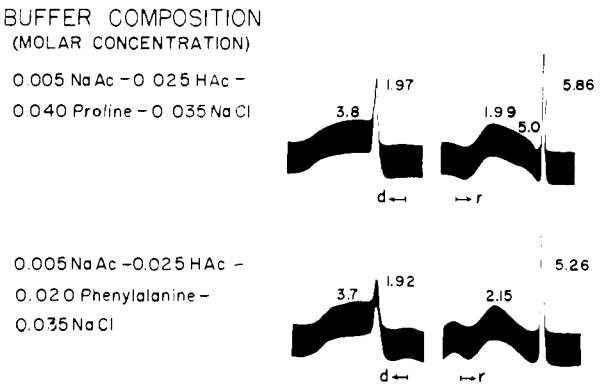

Fig. 2.—Electrophoretic patterns of ovalbumin in media containing proline and phenylalanine; pH 4.0. The concentrations of the acid form of proline and phenylalanine are 8×10^{-4} and 8×10^{-5} M, respectively.

As shown in Fig. 3, the effect of 0.042 M glycine can be practically overcome by a twofold increase in the concentration of NaAc-HAc in the supporting medium, ionic strength maintained constant. This shows that the inhibition of the effect of acetic acid by glycine is competitive in nature.

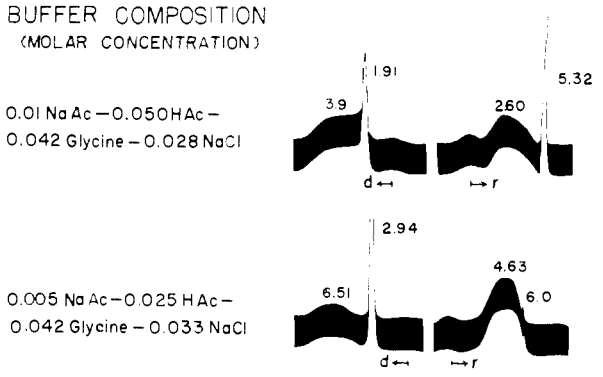

Fig. 3.—Electrophoretic patterns of ovalbumin in media containing constant concentration of glycine but varying concentration of acetate buffer; pH 4.0.

(4) In these experiments ethylamine hydrochloride was substituted for NaCl in the supporting medium.

In Fig. 4 the electrophoretic patterns of serum albumin in media containing 0.01 and 0.002 M NaAc buffer, pH 4.7, are compared with the electrophoretic patterns obtained in a medium con-

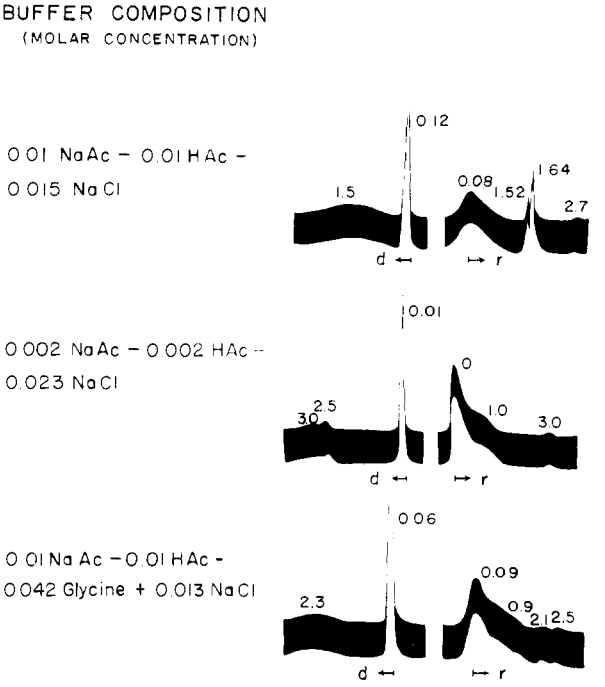

Fig. 4.—Electrophoretic patterns of bovine serum albumin in media containing varying concentration of acetate buffer and glycine; pH 4.7. The moving boundaries migrated toward the cathode.

taining 0.01 M NaAc buffer and 0.042 M glycine. It will be noted that the effect of glycine on the electrophoretic patterns of serum albumin is quite similar to that observed with ovalbumin. In contrast to these results, glycine had only a small effect on the electrophoretic composition of bovine γ-pseudoglobulin at pH 4.0.

Discussion

Some of the features of the electrophoretic behavior of ovalbumin and serum albumin in acidic media appear to be accounted for by a set of reversible, isomerization reactions.[3] While acetic acid appears to displace the postulated equilibria in one direction, amino acids have the opposite effect. The orders of decreasing effectiveness of the various amino acids in inhibiting the action of acetic acid seem to correlate with both increasing molecular size and increasing acidity of the carboxyl group, but neither correlation is perfect. For example, the order glycine > leucine and alanine > tryptophan correlates well with increasing molecular size. However, tryptophan which is a larger molecule than phenylalanine is nevertheless more effective than phenylalanine and is also a weaker acid. It is concluded, therefore, 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. Since amino acids competitively inhibit the effect of acetic acid, it seems likely that the two molecules compete for the same

site on the protein. If acetic acid is bound to this site, perhaps through hydrogen bond formation, then subtle structural changes resulting in change in net charge may occur. However, if the amino acid is bound, then the positively charged amino group might interact electrostatically with a neighboring negatively charged group on the protein, thereby preventing the structural changes from occurring. In this event, steric factors would be expected to influence the strength of the electrostatic interaction and thus the action of the amino acid. The smaller effect of glycine on the electrophoretic composition of γ-globulin at pH 4 as compared to ovalbumin at the same pH can be understood in terms of difference in electrostatic interaction between the net charge on the protein and the amino acid. Since at this pH γ-globulin carries a much greater net positive charge than ovalbumin, the electrostatic repulsion between the positively charged protein and the acid form of the amino acid would also be greater and the extent of glycine binding would be less.

It is interesting to compare the effect of acetic acid and amino acids on the electrophoretic behavior and on the kinetics of heat denaturation of proteins. Gibbs[5] has found that the rate of denaturation of human plasma albumin at 55–65° is greater in the presence than in the absence of acetate buffer. Also, the heat of activation is considerably lower when acetate is present. In contrast, glycine[6] has no influence on the denaturation of albumin in the presence of chloride but absence of acetate. It would appear that at least some of the isomeric states of serum albumin in acetate buffer are more heat labile than the state of the protein in the absence of acetate. Ballou, Boyer, Luck and Lum[7] have found that fatty acid anions increase the heat stability of relatively concentrated solutions of albumin at nearly neutral pH in the restricted sense of inhibiting aggregation of presumably denatured protein. The effectiveness of the acid anions increases with increase in chain length.

(5) R. J. Gibbs, *Arch. Biochem. Biophys.*, **52**, 340 (1954).
(6) R. J. Gibbs, *ibid.*, **51**, 277 (1954).
(7) G. A. Ballou, P. D. Boyer, J. M. Luck and F. G. Lum, *J. Biol. Chem.*, **153**, 589 (1944).

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[CONTRIBUTION FROM THE ROBERT W. LOVETT MEMORIAL LABORATORIES FOR THE STUDY OF CRIPPLING DISEASES, MASSACHUSETTS GENERAL HOSPITAL, AND THE DEPARTMENT OF MEDICINE, HARVARD MEDICAL SCHOOL]

Characterization of an "Electrophoretically Homogeneous" Human Serum Albumin[1]

BY K. SCHMID

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A hitherto unknown form of albumin has been isolated from Cohn's Fraction VI of pooled normal human plasma. It was crystallized and partially characterized. Its most outstanding property is the homogeneity by electrophoresis at pH 4.0 which is lost after treatment with cysteine and deionizing with ion-exchange resins.

In the course of a systematic study of the very soluble proteins of normal human plasma,[2,3] a new form of albumin was discovered. It is distinguished by special solubility properties and, particularly, by its electrophoretic homogeneity in pH 4.0 acetate buffer.

Experimental

The starting material for these investigations was the supernatant solution of Fraction V derived from pooled normal human plasma which had been fractionated according to Method 6.[4] Throughout the fractionation work, the temperature of the solutions was kept at −5°. The protein of the starting material was concentrated with zinc hydroxide, and the resulting protein–zinc hydroxide paste was decomposed at pH 5.8.[2] Insoluble material, the protein of which consisted essentially of albumin identical to that of Fraction V,[4] was removed. Precooled 1M $BaAc_2$ was added to the supernatant solution to give a concentration of 0.02 M. The formed barium precipitate was centrifuged, dissolved by addition of a precooled, aqueous solution of neutralized ethylenediaminetetraacetic acid, and subsequently dialyzed and lyophilized.

The resulting protein fraction designated as Fraction VI-2,[2] was comprised of approximately 90% albumin as judged by electrophoresis in pH 8.6 diethyl barbiturate buffer. The corresponding subfraction obtained from any other runs so far investigated was essentially free of albumin. The major component of our fraction crystallized easily at −2° as a Pb^{++}-complex[5] from a 7% protein solution which, in addition, contained 10% methanol and 10% acetone. The pH of the solution was not controlled. The crystals were washed with ice-water, dissolved by adding sodium citrate, dialyzed and lyophilized. The recrystallized protein appeared homogeneous on electrophoretic and ultracentrifugal analyses: the electrophoretic mobility in pH 8.6, $\Gamma/2$ 0.1 diethyl barbiturate buffer was 6.2×10^{-5} cm.^{2}/volt. sec. and the sedimentation constant of a 1.4% solution in 0.15 M NaCl was 4.6 S. The protein was devoid of a component with a sedimentation constant of 6 S corresponding to that of the albumin dimer and did not seem to contain free sulfhydryl groups judging from the negative nitroprusside reaction and its inability to form the mercurial dimer.[6,7] Immunochemical determination using an antiserum against human Fraction V-albumin indicated essentially pure serum albumin. The carbohydrate content of the crystallized protein proved negligible. In contrast to the albumin of Fraction V or human mercaptalbumin, the above-described albumin remained homogeneous

(1) This is publication No. 213 of the Robert W. Lovett Memorial Laboratory for the Study of Crippling Diseases, Department of Medicine, Harvard Medical School, and the Massachusetts General Hospital. Grants in support of these investigations have been received from the Helen Hay Whitney Foundation, New York, and from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service (A-509-C2).
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(3) K. Schmid, *ibid.*, **77**, 742 (1955).
(4) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, Jr., J. N. Ashworth, M. Melin and H. L. Taylor, *ibid.*, **68**, 459 (1946).
(5) It is worth noting that Lewin crystallized over 200 derivatives of albumin, but did not report the crystallization of its Pb^{++}-complex; J. Lewin, *ibid.*, **73**, 3906 (1951).
(6) W. L. Hughes, Jr., *ibid.*, **69**, 1836 (1947).
(7) J. T. Edsall, in "Ion Transport Across Membranes," H. T. Clark, Ed., Academic Press, Inc., New York, N. Y., 1954.