scribed by Hirs, Moore and Stein.[23] A portion (0.7 g.) of the precipitate was suspended in 0.2 *M* ammonium formate buffer (*p*H 3.11), and concentrated hydrochloric acid was added until the precipitate dissolved. The solution was poured on to a column of resin (5 × 33 cm.) which had been prepared and allowed to equilibrate with 0.2 *M* ammonium formate buffer (*p*H 3.11). The sample was allowed to sink into the resin under atmospheric pressure and rinsed in with 20 ml. of buffer. The buffer reservoir was reconnected to the column which was mounted over a fraction collector. The rate of flow of buffer through the column was adjusted to 45–50 ml. per hour and the effluent was collected in 23 ml. fractions.

Alternate fractions were analyzed qualitatively for disulfide-containing material by means of the cyanide–nitroprusside test.[29] After the elution of the first peak in tube numbers 69–77, the buffer was changed to 0.2 *M* ammonium formate (*p*H 3.28). Tube numbers 150–210 comprised a second peak. At approximately tube 260 the buffer was changed to 0.2 *M* ammonium formate (*p*H 3.40) and at approximately tube 310, to 0.2 *M* ammonium formate (*p*H 4.10). A third peak was contained in tube numbers 350–370. The contents of tube numbers 150–210 were pooled, evaporated in a rotary evaporator to approximately 50 ml., diluted with about 100 ml. of water and evaporated again, to about 20 ml., to remove excess free acid originally present. The *p*H of the suspension was adjusted to 6 by the addition of 2 *N* ammonium hydroxide and the suspension was evaporated to dryness in a rotary evaporator at a temperature below 30°. The white residue was resuspended in water (100 ml.) and the suspension was filtered. The precipitate was washed with water and recrystallized from boiling water (200 ml.) to give 190 mg. of prismatic needles which began to decompose when heated above 250°. Analysis in the amino acid analyzer showed the compound to be chromatographically pure; $[\alpha]^{22}D$ −63.2° (*c* 0.89, 1 *N* HCl).

Anal. Calcd. for $C_7H_{14}O_4N_2S_2$: C, 33.1; H, 5.55; N, 11.0; S, 25.2. Found: C, 33.3; H, 5.55; N, 11.1; S, 25.2.

(29) G. Toennies and J. J. Kolb, *Anal. Chem.*, **23**, 823 (1951).

NOTE ADDED IN PROOF.—Dr. David Yphantis of The Rockefeller Institute, using short column equilibrium centrifugation, has found that 1-(hemi-homocystine)-oxytocin behaves as a monomer at pH 5.6 in 0.15 *M* ammonium acetate. We wish to thank him for this information.

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Effect of Binding of Ions and Other Small Molecules on Protein Structure. IX. The Binding of Acetic Acid to Insulin at *p*H 4[1]

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The electrophoretic behavior of insulin in acetate buffers at *p*H 4.0 is interpretable over a seven-fold range of acetic acid concentration in terms of the binding of a single mole of undissociated acid to the protein. The effect of acid binding on the electrophoretic behavior of proteins is discussed in the context of conformational changes with concomitant changes in the *pK*'s of several carboxyl groups on the proteins.

Introduction

The various peaks in the electrophoretic patterns of a variety of proteins in acidic media containing acetate buffer (NaAc–HAc) or other carboxylic acid buffers constitute a single reaction boundary modified in some instances by mild convective disturbances.[2–5] In the case of serum albumin 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 a sufficiently high concentration of acetate buffer, the pattern is a reaction boundary attributable entirely to the isomerization reaction.) The reaction boundaries of ovalbumin and bovine γ-pseudoglobulin arise solely from interaction of these proteins with undissociated buffer acid. Equilibrium constants for the binding of undissociated buffer acids by bovine serum albumin (BSA) and ovalbumin have been computed[2] from electrophoretic patterns using the concepts of the theory of weak-electrolyte moving boundaries. These investigations have now been extended to insulin whose electrophoretic behavior at *p*H 4 is interpretable over a sevenfold range of acetic acid concentration in terms of the binding of a single mole of undissociated acid to the protein.

Experimental

Electrophoresis was carried out at field strengths of 3 to 8 volts cm.$^{-1}$ 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. Following electrophoretic separation of the peaks in a reaction boundary, portions of solution were removed from various positions in the rising limb of the Tiselius cell for conductance measurements. The crystalline zinc insulin was kindly supplied by the Eli Lilly Company. Samples of the protein were dissolved in $10^{-3}M$ HCl and the resulting solutions dialyzed for 3 hr. against $10^{-2}M$ HCl followed by 24 hr. against buffer with three changes of dialysate. Within experimental error, the *p*H and resistance of the dialyzed protein solution were the same as those of the buffer.

Results

Representative electrophoretic patterns of insulin in a series of *p*H 4.0 media containing varying concentrations 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

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(2) J. R. Cann, *J. Biol. Chem.*, **235**, 2810 (1960).

(3) J. R. Cann and R. A. Phelps, *J. Am. Chem. Soc.*, **79**, 4672 (1957).

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

(5) J. R. Cann and R. A. Phelps, *J. Am. Chem. Soc.*, **81**, 4378 (1959).

nature of the patterns depends upon the concentration of NaAc–HAc in the solvent medium for the protein. Those obtained in 0.02 *M* NaCl–HCl do not differ greatly from the patterns shown by presumably non-interacting proteins at low ionic strengths, *e.g.*, esterified-BSA in 0.01 *M* NaCl–HCl, *p*H 4.0, and BSA in 0.01 ionic strength tris-(hydroxymethyl)–aminomethane chloride buffer at *p*H 8.1. Substitution of increasing amounts of NaAc-HAc for NaCl–HCl results in striking and progressive changes in the electrophoretic patterns, a behavior which has much in common with that described previously for BSA, ovalbumin and bovine γ-pseudoglobulin.[2–4] As with these latter proteins, the peaks in the patterns shown by insulin in acetate-containing media constitute a single reaction boundary. Thus, for example, the Rayleigh interference optical system revealed that the gradient of protein concentration does not become zero between the sharp, fast-moving peak and the broad slow-moving one in the rising pattern presented in Fig. 1. In contrast to BSA in these media, convective disturbances were rarely observed with insulin, although in the case of the rising pattern in Fig. 1C convective circulation in the region between the sharp peak and the broad one became evident in the form of transient pips during later stages of electrophoresis. As a consequence the gradient curve went to the base-line between the peaks.

Following the procedure adopted in the case of BSA and ovalbumin,[2] the electrophoretic behavior of insulin in acetate-containing media can be interpreted in terms of the binding of undissociated acetic acid by the protein. It will be assumed that the protein molecule P binds undissociated acid according to the reaction equation

$$P + n\text{HAc} \rightleftharpoons P(\text{HAc})_n \qquad (1)$$

where the mobility of $P(HAc)_n$ is assumed to be greater than P. Using the concepts of the theory of weak-electrolyte moving boundaries,[6,7] the following expression was derived for the equilibrium constant K of reaction 1

$$K = \frac{\bar{u}_P - u_P}{m^n_{\text{HAc}}\,(u_{P(\text{HAc})_n} - \bar{u}_P)} \qquad (2)$$

where m_{HAc} is the molar concentration of buffer acid; $\bar{u}_P$, the constituent mobility of the protein; u_P, the mobility of the uncomplexed protein and $u_{P(\text{HAc})_n}$, the mobility of the protein–acid complex. As previously[2] described, the mean mobility of the descending boundary can be taken as equal to $\bar{u}_P$; the mobility of the sharp fast-moving rising peak, as $u_{P(\text{HAc})_n}$; and the mean mobility of the broad slow-moving rising peak, as u_P. The value of n is chosen so as to make K constant. The computations summarized in Table I indicate that the electrophoretic behavior of insulin is interpretable over a sevenfold range of acetic acid concentration in terms of the binding of a single mole of undissociated acid to the protein.

Discussion

One of the striking features of the interaction of proteins with undissociated acetic acid is that major changes in electrophoretic properties can be mediated by the binding of a small number of acetic acid molecules—2 or 3 in the case of BSA and only 1 with insulin.[8] This suggests a highly specific type of interaction. It has been proposed[4] that complexing of the protein molecule with undissociated acid produces a subtle conformational change which increases the net positive electrical charge on the protein but does not change its frictional coefficient significantly.[9] The essential correctness of this interpretation is indicated by recent extra-electrophoretic measurements made in the laboratory of Dr. Joseph F. Foster. These workers found (personal communication from Dr. Foster) that there is

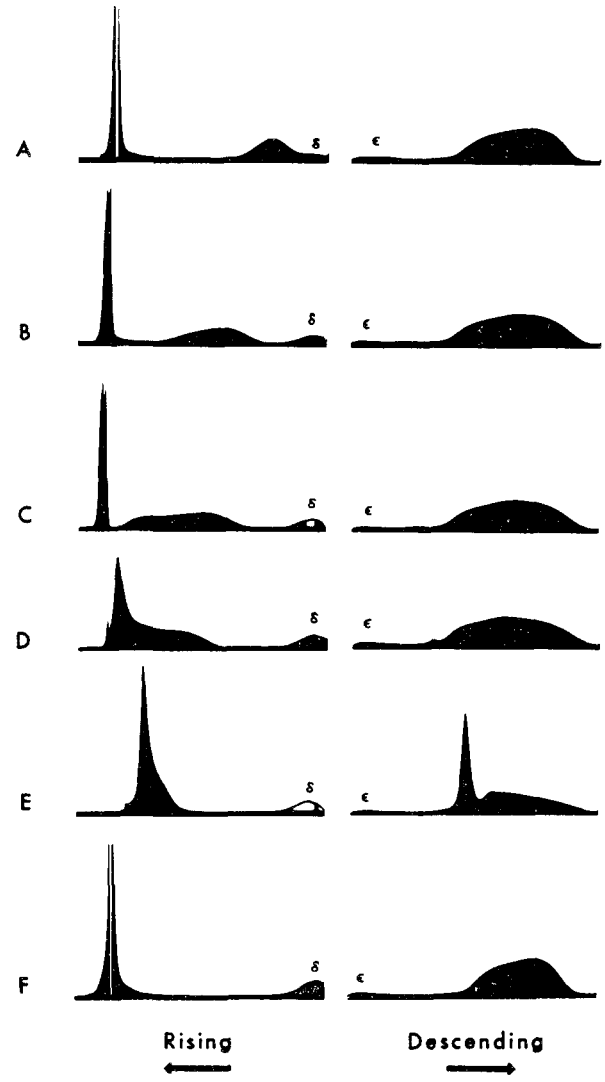

Fig. 1.—Electrophoretic patterns of 1% insulin at *p*H 4.00 ± 0.01: A, 0.02 *M* NaAc–0.1 *M* HAc; B, 0.004 *M* HAc–0.02 *M* HAc–0.016 *M* NaCl; C, 0.003 *M* NaAc–0.015 *M* HAc–0.17 *M* NaCl; D, 0.002 *M* NaAc–0.01 *M* HAc–0.018 *M* NaCl; E, 0.001 *M* NaAc–0.005 *M* HAc–0.019 *M* NaCl; F, 0.02 *M* NaCl–HCl.

(6) H. Svensson, *Acta Chem. Scand.*, **2**, 841 (1948).

(7) E. B. Dismukes and R. A. Alberty, *J. Am. Chem. Soc.*, **76**, 191 (1954).

(8) The protein may bind many moles of acetic acid; but the binding of one or a few moles at critical sites on the protein molecule produces the observed changes in electrophoretic behavior.

(9) The sedimentation constant of ovalbumin is the same within experimental error in media of low and of high acetic acid concentration, *p*H 4.

TABLE I

EQUILIBRIUM CONSTANTS FOR BINDING OF UNDISSOCIATED ACETIC ACID TO INSULIN[a] AT 0°

Buffer[b]	$\bar{\nu}_P$	u_P[c] (cm.^{2} sec.$^{-1}$ volt^{-1} × 10^5)	$u_{P(HAc)}$[d]	K $n = 1$ (liter mole^{-1})
0.01 *M* NaAc–0.05 *M* HAc, *p*H 4.00	8.43	3.81	9.49	87.2
.02 *M* NaAc–0.1 *M* HAc, *p*H 3.98	7.64	1.98	8.23	95.9
.01 *M* NaAc–0.05 *M* HAc–0.01 *M* NaCl, *p*H 3.99	7.61	2.77	8.40	123
.008 *M* NaAc–0.04 *M* HAc–0.012 *M* NaCl, *p*H 4.00	7.62	2.99	8.54	126
.006 *M* NaAc–0.03 *M* HAc–0.014 *M* NaCl, *p*H 4.00	7.55	3.46	8.58	132
.004 *M* NaAc–0.02 *M* HAc–0.016 *M* NaCl, *p*H 4.00	7.48	4.50	8.42	158
.003 *M* NaAc–0.015 *M* HAc–0.017 *M* NaCl, *p*H 3.99	7.42	5.66	8.65	95.4
			Average	117 ± 21

[a] Protein concentration, 1%. [b] Ac designates acetate. [c] The first and the last two entries in column corrected for the measured change in conductance across the boundary; other entries corrected for estimated change in conductance. [d] All entries corrected for measured change in conductance across the boundary.

a small but significant difference in the change in optical rotation of BSA with *p*H in acetate buffer as compared to NaCl–HCl.

The difference in net charge between the uncomplexed protein and the protein–acid complex presumably resides in differences in the pK's of several carboxyl groups between the two conformations. The pK's could conceivably increase concomitantly with a conformational change *via* several mechanisms: rupture of hydrogen bonds between carboxylate groups and other side chain groups on the protein, *e.g.* carboxyl groups and tyrosyl residues; rupture of intramolecular ion-pair bonds involving carboxylate groups; changes in electrostatic interaction between charged groups; burying a carboxyl group in the hydrophobic interior of the molecule, etc. The fact that a wide variety of proteins (serum albumins, ovalbumin, conalbumin, γ-globulin, insulin and oxidized but not native ribonuclease) show the same general behavior in carboxylic acid buffers suggests a specific mechanism dependent upon some structural feature common to many globular proteins. The idea that macromolecular structural parameters are important in determining the effect of carboxylic acids on electrophoretic behavior has been pointed out previously[5] in connection with experiments on chemically modified proteins.

It is important to note that these ideas are consistent with the acid–base titration curves of the various proteins. As is generally the case, the electrophoretic net charges, ν, computed[10] from $\bar{\nu}_P$, u_P and even $u_{P(HAc)_n}$ are less than the titration charges at *p*H 4.0 and 2.7 for BSA, ovalbumin and insulin. Of greater significance for this discussion, the electrophoretic net charge of the protein–acid complex is only 3 to 10 protonic charges greater than that of the uncomplex protein. Inspection of the titration curves indicates that this difference in charge readily can be accounted for on the basis of differences in pK's of several carboxyl groups. Finally, the reaction under consideration is strongly *p*H dependent.[5] Tanford[11] has shown recently that if a protein molecule can exist in two conformations and if the equilibrium distribution between these depends on *p*H, then a necessary consequence of the laws of thermodynamics is that at least one titratable group has a different pK in the two conformations.

(10) For insulin in 0.02 *M* acetate buffer, *p*H 3.98: $\bar{\nu}_P = 10$, $\nu_P = 2.6$, $\nu_{P(HAc)} = 11$ and titration charge, about 19. In 0.003 *M* NaAc–0.15 *M* HAc–0.017 *M* NaCl, *p*H 3.99: $\bar{\nu}_P = 9.8$, $\nu_P = 7.5$ and $\nu_{P(HAc)} = 11$. For BSA in 0.02 *M* acetate buffer at *p*H 4.00: $\bar{\nu}_P = 12$, $\nu_P = 3$, $\nu_{P(HAc)_{2.5}} = 13$ and titration charge, about 26. For ovalbumin in 0.04 *M* formate buffer (NaFo–HFo), *p*H 2.69: $\bar{\nu}_P = 14$, $\nu_P = 14$, $\nu_{P(HFo)_n} = 19$ and titration charge, about 30. In the latter case, the rising boundary velocities were not corrected for conductance changes across the boundaries which accounts in large part for $\bar{\nu}_P = \nu_P$.

(11) C. Tanford, *J. Am. Chem. Soc.*, **83**, 1628 (1961).