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Interaction of Acetic Acid with Poly-L-glutamic Acid and Serum Albumin*

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With the Technical Assistance of Mr. Robert O. Coombs

ABSTRACT: The combined application of electrophoresis, ultracentrifugation, optical rotatory dispersion, and circular dichroism to the interaction of acetic acid with poly-L-glutamic acid and bovine serum albumin has provided fresh insight into the mechanisms underlying the effect of acetate and other carboxylic acid buffers on the electrophoretic behavior of proteins in acidic media, Thus, for example, the characteristic increase in the net positive charge on the protein molecule due to interaction with the undissociated buffer acid is now understood in terms of binding of the acid to side-chain carboxyl groups with concomitant increase in their p*K*. Moreover, binding need not be cooperative for resolution of bimodal reaction boundaries to occur, since the forementioned mode of interaction generates coupled gradients of acid concentration and pH in the electrophoresis column. Binding of acetic acid favors the helical conformation of poly-L-glutamic acid and causes subtle alteration in the tertiary structure of bovine serum albumin.

Our interest in the interaction of acetic acid with proteins stems from observations on the nonenantiographic, bimodal electrophoretic patterns displayed by a variety of highly purified proteins in acidic media containing acetate or other carboxylic acid buffers (Cann and Phelps, 1957, 1959; Cann, 1958–1961). Experimental evidence has been advanced to support interpretation of the patterns in terms of reversible complexing of the protein with undissociated buffer acid, with concomitant increase in the net positive charge on the protein. Although complex formation does not change the frictional coefficient of the protein molecule significantly, it does cause subtle structural alterations. A theoretical basis for this interpretation was provided (Cann and Goad, 1965) by a theory of electrophoresis of reversibly interacting systems of the type

$$P + nHA \rightleftharpoons P(HA)_n \qquad (1)$$

where P represents a protein molecule or other macromolecular ion in solution and $P(HA)_n$ its complex formed by binding *n* moles of a small, uncharged constituent, HA, of the solvent medium, *e.g.*, undissociated buffer acid. It is assumed that P and $P(HA)_n$ possess different electrophoretic mobilities and that equilibrium is established instantaneously. These computations account for the essential features of the electrophoretic behavior of proteins in acidic media containing varying concentrations of carboxylic acid buffer. Resolution of the patterns into two peaks occurs because of changes in the concentration of HA accompanying reequilibration during differential transport of P and $P(HA)_n$ and maintenance of the resulting concentration gradients of the electrically neutral molecule. Thus, the two peaks correspond to different equilibrium compositions and not to separated P and $P(HA)_n$. Subsequently, Bull and Breese (1967a) demonstrated by equilibrium dialysis that ovalbumin binds undissociated acetic acid and its normal short-chain homologs. It remains to elucidate the mechanism of interaction. The questions posed are: "What are the binding sites on the protein?" "How does binding increase the positive charge on the protein molecule?" "What is the nature and mechanism of the conformational changes wrought by acetic acid?" Answers to these questions have been sought by the combined application of electrophoresis, ultracentrifugation, optical rotatory dispersion, circular dichroism, and measurement of biological activity to the interaction of acetic acid and its homologs with poly-L-glutamic acid, bovine

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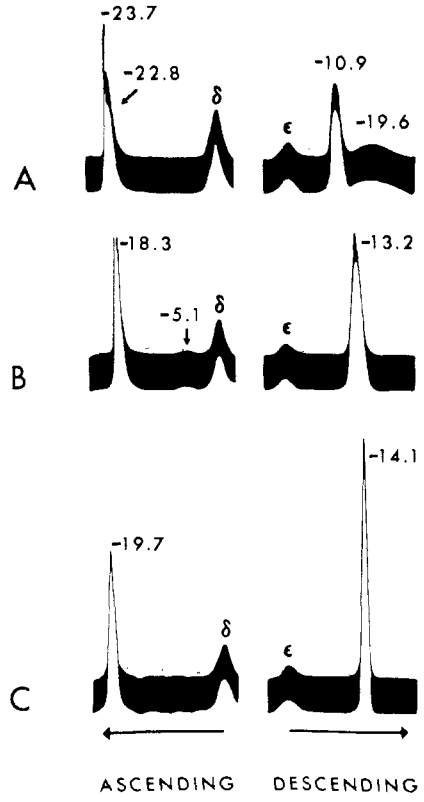

FIGURE 1: Moving-boundary electrophoretic patterns of poly-L-glutamic acid (PGA): (A) 0.005 M NaAc–0.00218 M HAc–0.035 M NaCl; pH of dialyzed PGA solution, 4.85; mean descending mobility computed from the first moment of the reaction boundary, −14.5. (B) 0.04 M NaAc–0.017 M HAc (pH 4.87); (C) 0.005 M NaAc–0.00213 M HAc–0.000496 M Na_2HPO_4–0.0335 M NaH_2PO_4 (pH 4.82).

serum albumin, and ribonuclease. The results for the first two macromolecules are the subject of this paper. Those for ribonuclease are described in the companion paper (Cann, 1971).

Materials and Methods

Poly-L-glutamic acid sodium was Mann's grade M.A. The electrophoretic and sedimentation experiments were made with material of mol wt 75,000 and the optical rotatory dispersion measurements with material of mol wt 175,000. Both preparations showed virtually the same electrophoretic behavior in acetate containing media.

All experiments on bovine serum albumin (BSA)[1] were made with "fatty acid free" protein. Fatty acid free BSA prepared from Armour's bovine plasma albumin (lot no. D71209) by the charcoal treatment method of Chen (1967) was used for all experiments except those on urea denaturation. For purposes of comparison, the latter experiments were made with the same sample of solvent-extracted protein (Goodman, 1957) used in previous experiments on the inhibition of peptic digestion of BSA by caprylic acid (Klapper and Cann, 1964). Within experimental error, both preparations showed the same circular dichroism spectrum in 0.02 M NaCl–HCl (pH 2.9). Throughout the text fatty acid free BSA is referred to simply as BSA.

Reagent grade chemicals were used except for urea, which was Schwarz–Mann Ultra Pure.

[1] Abbreviation used is: BSA, bovine serum albumin.

Electrophoresis was carried out on 1% poly-L-glutamic acid at field strengths of 4.5–6.5 V cm^{-1} for 40–60 min in a Perkin-Elmer Tiselius apparatus, fitted with a current-regulating power supply. The polypeptide solutions were equilibrated against media containing acetate buffer (NaAc–HAc) by dialysis with stirring for about 36 hr according to the regime: 13 hr at 4°, 9 hr at room temperature followed by 13 hr at 4° with many changes of dialysate. Electrophoretic mobilities ($10^5 \times \mu$ cm^2 sec^{-1} V^{-1}) computed using the conductance of the dialyzed solutions are shown above or beside the corresponding peaks in the electrophoretic patterns displayed in Figure 1. The precision of the mobility measurements is approximately ±3%.

Optical rotatory dispersion and circular dichroism spectra were recorded on a Cary Model 60 spectropolarimeter with a Model 6001 circular dichroism attachment, the sample compartment being maintained at 27°. Slits were programmed to yield a 15-Å bandwidth at each wavelength. Concentrations and path lengths were dictated by the absorbance of acetate buffer: optical rotatory dispersion of poly-L-glutamic acid, 1.5 mg/ml in a 0.1-cm cell; optical rotatory dispersion of BSA, 3 mg/ml in a 1-cm cell over the spectral range 600–260 mμ and 0.18 mg/ml in a 0.1-cm cell in the region of 233 mμ; circular dichroism of BSA, 2 mg/ml in a 1-cm cell for the near-ultraviolet region, and 3 mg/ml in a 0.01-cm cell for the far-ultraviolet region. Reduced mean residue rotations, [M′], (deg cm^2)/dmole, and ellipticities, $[\theta]_{mrw}$, (deg cm^2)/dmole, were calculated in the usual fashion. A value of 114 was used as the mean residue weight of BSA. Optical rotatory dispersion spectra for calculation of the Moffitt–Yang a_0 and b_0 parameters over the pH range 5.6–2.9 were recorded from 600 to 260 mμ. The statistical analysis of Sogami *et al.* (1963) indicates that the most probable value of λ_0 for BSA is 218 ± 2 mμ in the pH range, 9.5–3.55; at lower pH the most probable value is 211 mμ. We compromised on the conventional value of 212 mμ since our primary concern is with relative values of the parameters in acetate buffer as opposed to NaCl.

Results

Poly-L-glutamic Acid. Previously, we advanced electrophoretic and chemical evidence inplicating the carboxyl groups on a protein molecule in its interactions with acetic and formic acid (Cann and Phelps, 1959). Accordingly, poly-L-glutamic acid was chosen as a model for investigating the interaction of acetic acid with macromolecular ions. The results of electrophoretic, optical rotatory dispersion, and ultracentrifugal experiments follow.

Electrophoresis. Electrophoresis of poly-L-glutamic acid was carried out at pH 5 and ionic strength 0.04. These conditions were chosen because (1) poly-L-glutamic acid is known to be helical at said pH and ionic strength (Nagasawa and Holtzer, 1964). (2) It has the same value of [M′] at 233 mμ and the same circular dichroism spectrum over the temperature range 10–27° in 0.04 M NaAc buffer as in 0.04 NaCl at pH 5. (3) Ultracentrifugal analyses at 0.3° showed that the polypeptide is not aggregated in these media. The electrophoretic behavior of poly-L-glutamic acid in media containing varying concentrations of acetate buffer at constant pH and ionic strength (maintained with NaCl) is virtually indistinguishable from that shown by proteins. Thus, the electrophoretic patterns at low acetate concentration (Figure 1A) are bimodal and markedly nonenantiographic. Whereas in the descending pattern the fast-moving peak is broad and the slow-moving one sharp, in the ascending pattern the fast-moving peak is hypersharp

and the slow one appears as an intense shoulder. Increasing the buffer concentration results in progressive and characteristic changes, notably in the growth of the slow-moving peak (*i.e.*, the more positive peak) at the expense of the fast one. At the same time a small, quite slow-moving peak develops in the ascending pattern; but this peak is often obliterated by mild convective disturbances. At sufficiently high acetate concentration (Figure 1B) the descending pattern shows a single symmetrical peak with a mobility intermediate in value between those of the two peaks exhibited at low acetate concentration; the ascending pattern, a major peak along with the forementioned, small slow-moving peak.

Fractionation has provided unambiguous evidence that the two peaks shown at low and intermediate acetate concentrations constitute a reaction boundary and are not indicative of inherent heterogeneity. These experiments were made with 3% poly-L-glutamic acid in 0.02 M NaAc–0.00853 M HAc–0.02 M NaCl (pH 5.03) conditions which provide optimum resolution of the two descending peaks. The polypeptide disappearing across the slower peak was removed from the Tiselius cell and reequilibrated against the buffer by dialysis prior to electrophoretic analysis. The electrophoretic patterns of the fraction were the same as those of the unfractionated control and showed two peaks.

The question naturally arises as to whether or not the macromolecular interaction which generates the reaction boundary is not simply a helix–coil transition induced by pH changes produced in the Tiselius cell by the electrophoretic process *per se*. This possibility seems to be eliminated by the following observations. (1) The pH of the solution in the region between the δ boundary and the ascending reaction boundary is only 0.1 pH unit greater than the underlying, dialyzed protein solution. (2) The electrophoretic patterns in 0.002 M NaAc–0.00303 M HAc–0.038 M NaCl (pH 4.5) are essentially the same as at pH 5, and the change in pH across the ascending reaction boundary in the former case is only 0.13 pH unit. Although interpretation of the patterns at pH 4.5 is complicated by aggregation of the polypeptide, pH changes of this magnitude are by no means sufficient to shift the system into the region of the helix–coil transition (Nagasawa and Holtzer, 1964). (3) The electrophoretic patterns in unbuffered 0.04 M NaCl at pH 5 are quite featureless. The ascending pattern shows a single hypersharp peak, the descending pattern a very broad boundary which is skewed forward. The descending boundary simply spread without resolving into peaks upon prolonged electrophoresis with back compensation. If resolution in acetate buffer were merely a reflection of helix–coil transition linked to pH gradients, excellent resolution should have occurred in the unbuffered solution.

Thus, we see that the electrophoretic behavior of poly-L-glutamic acid in acetate-containing media is that predicted for an interaction of the type given by reaction eq 1 as elaborated for a negatively charged macromolecular ion whose mobility decreases upon binding of undissociated buffer acid (Cann and Goad, 1968; see particularly their Figures 9 and 10). But, it is difficult to visualize a cooperative interaction for conditions of pH and ionic strength under which acetate-induced conformational changes evidently do not occur. This seeming paradox is resolved by hypothesizing noncooperative (statistical) binding of undissociated acid to the side-chain carboxyl groups of the polypeptide, presumably through hydrogen-bond formation, according to the pH-dependent reaction scheme

$$CO_2^- + H^+ \rightleftharpoons CO_2H \overset{CH_3CO_2H}{\rightleftharpoons} CO_2H\cdots HO_2CCH_3 \qquad (2)$$

Such an interaction would decrease the negative charge on the macromolecule at constant pH. Moreover, reequilibration during differential transport of the several polypeptide–acetic acid complexes containing different numbers of acetic acid molecules would be expected to produce coupled gradients of acetic acid and pH along the electrophoresis cell, in which event a noncooperative interaction might be expected to ape a cooperative one. A test of this hypothesis is to replace the NaCl in a supporting medium containing low concentration of acetate buffer with an indifferent buffer such as phosphate to uncouple the gradients of acetic acid and pH. The composition of the phosphate buffer must be such as to maintain the pH and ionic strength. If our hypothesis is correct, the electrophoretic patterns in the mixed buffer will show a single peak; and the electrophoretic mobility of the descending peak will be equal to the mean mobility of the descending reaction boundary shown in the medium containing only the acetate buffer. Comparison of the patterns displayed in Figure 1C to those in Figure 1A reveals that this is, in fact, the case.

The value of the intrinsic association constant between acetic acid and poly-L-glutamic acid was estimated from the mean descending mobilities presented in Figure 1A,B for low and high acetate concentrations and the potentiometric titration curve of the polypeptide in 0.04 M KCl. The calculation was made as follows. (1) The net electrophoretic charge at high acetate concentration was taken to be the product of the titration charge at the pH of the dialyzed polypeptide solution at low acetate concentration and the ratio of mobility at high to that at low acetate concentration. (2) The difference between the titration charge at the pH of the dialyzed polypeptide solution at high acetate concentration and the net electrophoretic charge was taken to be the moles of acetic acid bound per mole of polypeptide, $\bar{r}$. (3) The intrinsic association constant, K, was calculated to the first approximation by use of the equation

$$\bar{r} = \frac{nK[\mathrm{HAc}]}{1 + K[\mathrm{HAc}]} \qquad (3)$$

assuming the total number of binding sites, n, equal to the degree of polymerization of the polypeptide. (4) A rapidly converging iterative procedure was then applied by calculating the moles of acetic acid bound at low acetate concentration and subtracting this quantity from the titration charge at low acetate concentration before carrying through the foregoing steps once again, etc. The value of the association constant computed in this manner[2] is $2 \pm 0.6\ M^{-1}$.

Optical Rotatory Dispersion. The conclusion reached above is that interaction of acetic acid with poly-L-glutamic acid decreases the negative electrical charge on the macromolecular ion. In that case, acetate buffer should favor the helical form of the polyglutamate in the pH region of the helix–coil transition. This idea was confirmed by optical rotatory dispersion measurements, the depth of the 233-mμ trough of the far-ultraviolet negative Cotton effect being taken as a measure of helical content. The results displayed in Figure 2 demonstrate that the polypeptide is, in fact, more helical in 0.2 M

[2] A critique of previous estimates of the association constant made by application of the weak electrolyte moving-boundary theory to protein patterns (Cann, 1960, 1961) is given in footnote 7 of Cann and Goad (1965). Although it is inappropriate to use the apparent mobilities of the peaks in a reaction boundary to calculate equilibrium constants, the mean mobility of the descending reaction boundary can be used for this purpose since it is the average mobility of the various macromolecular species in the underlying equilibrium mixture.

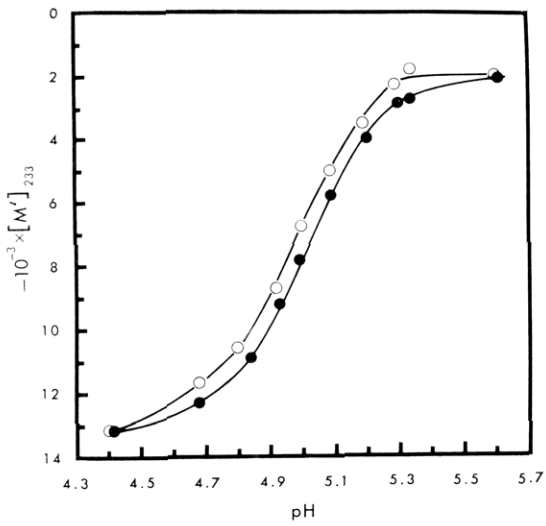

FIGURE 2: Comparison of the helix–coil transition of poly-L-glutamic acid in 0.2 M NaCl–HCl (○) with the transition in 0.2 M NaAc buffer (●).

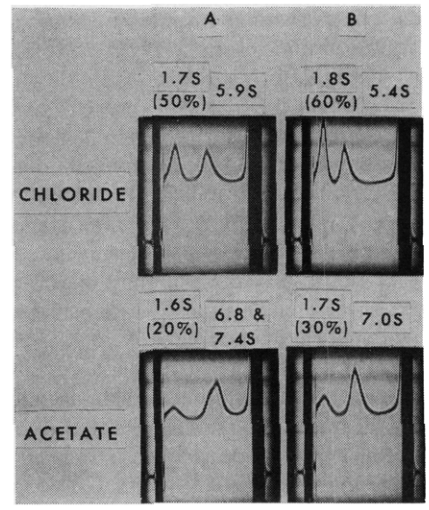

FIGURE 3: Comparison of the sedimentation patterns of poly-L-glutamic acid (PGA) at 10° in NaCl and in NaAc (pH 4.19): A, upper figure: 0.04 M NaCl, pH adjusted with HCl; lower figure: 0.04 M NaAc, pH adjusted with HAc. B, upper figure: 0.1 M NaCl, pH adjusted with HCl; lower figure: 0.1 M NaAc, pH adjusted with HAc. Sedimentation coefficients shown above corresponding boundaries. The percentage of the slower sedimenting boundary corrected for radial dilution given in parentheses. Concentration of PGA, 0.96%; time of sedimentation, 95 min in A and 79 min in B; 60,000 rpm; bar angle, 35°.

NaAc buffer than in 0.2 M NaCl–HCl, *i.e.*, the midpoint of the transition is shifted alkaline in acetate buffer. The magnitude of the alkaline shift indicates an intrinsic association constant of 0.4 M^{-1} between acetic acid and the polypeptide. Quite a good fit to the data in acetate buffer is obtained simply by (1) equating the number of moles of acid bound (as computed from eq 3 using $K = 0.4\ M^{-1}$ and assuming n equal to the total number of side-chain carboxyl groups on the polypeptide) with the change in charge on the macromolecule induced by acetic acid binding; (2) subtracting this change in charge from the titration charge in 0.2 M KCl to give the net charge in acetate buffer; and (3) assigning to $[M']_{233}$ that value which in 0.2 M NaCl corresponds to a titration charge equal to the net charge in acetate buffer. When this calculation is refined by taking into account the dependence of the degree of dissociation of the carboxyl groups upon charge, the fit is excellent. When comparing the value, 0.4 M^{-1}, of the association constant deduced in this manner with the value, 2 ± 0.6 M^{-1}, obtained from electrophoretic mobilities of the helical form of the polypeptide, it must be borne in mind that the value from the helix–coil transition is an average of the constants for the helical and coil conformations.

Ultracentrifugation. As illustrated in Figure 3, acetate buffer promotes the aggregation of poly-L-glutamic acid at pH 4.2. (It is worthwhile reiterating that the polypeptide is not aggregated in either NaCl or NaAc solutions at the higher pH values of 4.8–5.0 used in the electrophoretic experiments, *e.g.*, Figure 1B; aggregation does not ensue until the pH is lowered to pH 4.5.) The possibility that enhancement of aggregation by acetate buffer at pH 4.2 might be caused by metals or other contaminants in the acetic acid or sodium acetate was eliminated by an experiment in which the acetic acid was redistilled and the polypeptide solution contained 10^{-3} M EDTA. The sedimentation pattern was the same, within epxerimental error, as that obtained with reagent grade acetic acid and no added chelating agent. Thus, the enhanced aggregation is evidently another consequence of the decrease in negative charge on the macromolecular ion attending acetic acid binding. (Note that an increase in ionic strength at constant pH causes an increase in the charge on poly-L-glutamic acid and a decrease in the extent of aggregation.) Conversely, the results of the electrophoretic, optical rotatory dispersion, and ultracentrifugal measurements when taken together leave little, if any, doubt that interaction of acetic acid with poly-L-glutamic acid decreases the negative charge on the polypeptide.

Bovine Serum Albumin. The electrophoretic behavior of BSA and its derivatives in media containing varying concentrations of acetate buffer at pH 4 has been examined in detail previously (Phelps and Cann, 1956; Cann and Phelps, 1959; Cann, 1958, 1960). Optical rotatory dispersion and circular dichroism measurements on defatted BSA now provide evidence that binding of acetic acid causes subtle alterations in the tertiary structure of the albumin molecule. Thus, in the pH range 4.6–2.9, 0.02 M sodium acetate buffer causes quite significant changes in the optical rotatory parameters, a_0 and b_0, relative to those in 0.02 M NaCl–HCl (Figure 4A,B) apparently without a corresponding change in helicity as judged by the depth of the 233-mμ trough of the far-ultraviolet negative Cotton effect (Figure 4C).

Although the weak, negative dichroism bands shown by BSA in the region of 260–270 mμ (Velluz and Legrand, 1965) are unaffected by substitution of acetate buffer for NaCl–HCl (pH 2.8–2.9); acetate buffer does cause a small (on the order of 10%) but significant enhancement of the negative, far-ultraviolet bands (compare spectra a and b in Figure 5). The possibility that this enhancement reflects an increase in helical content of the protein seems to be precluded by the insensitivity of $[M']_{233}$ to acetate[3] (Figure 4C), and one must entertain the idea that perturbation of chromophores other than peptide groups is responsible for the enhancement. Such perturbation could be due merely to vicinal effects resulting from bound acetic acid, or it could be due to altered interactions of the

[3] The contribution to $[M']_{233}$ of the changes in ellipticity at 220 and 207 mμ upon substitution of acetate buffer for NaCl–HCl is evidently largely compensated by changes in ellipticity of opposite sense at still lower wavelengths. This possibility could not be explored, however, since it proved impossible to extend our measurements below 205 mμ owing to the high absorbance of acetate buffer.

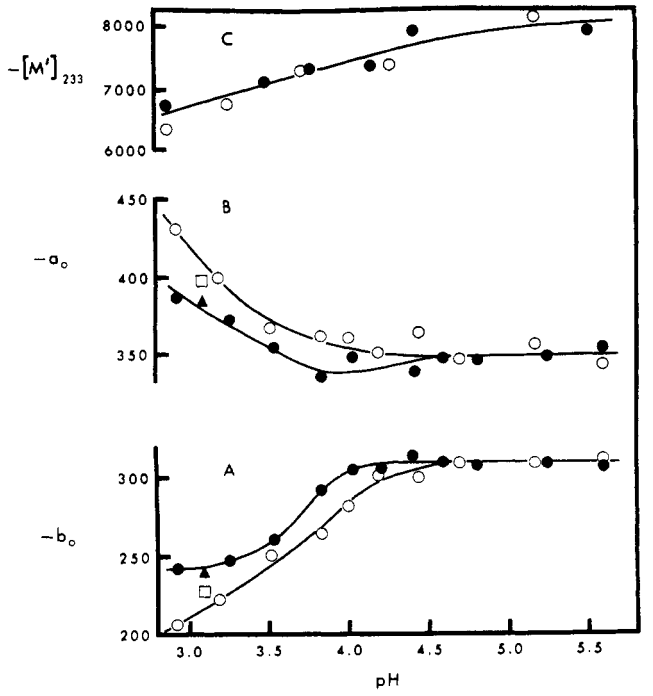

FIGURE 4: The effect of acetate buffer upon the optical rotatory dispersion parameters of fatty acid free BSA. Plots of $-b_0$, $-a_0$, and $-[M']_{233}$ against pH: (○) 0.02 M NaCl–HCl and (●) 0.02 M NaAc buffer. In A and B: (□) 0.005 M NaAc–0.015 M NaCl and (▲) 0.01 M NaAc–0.01 M NaCl.

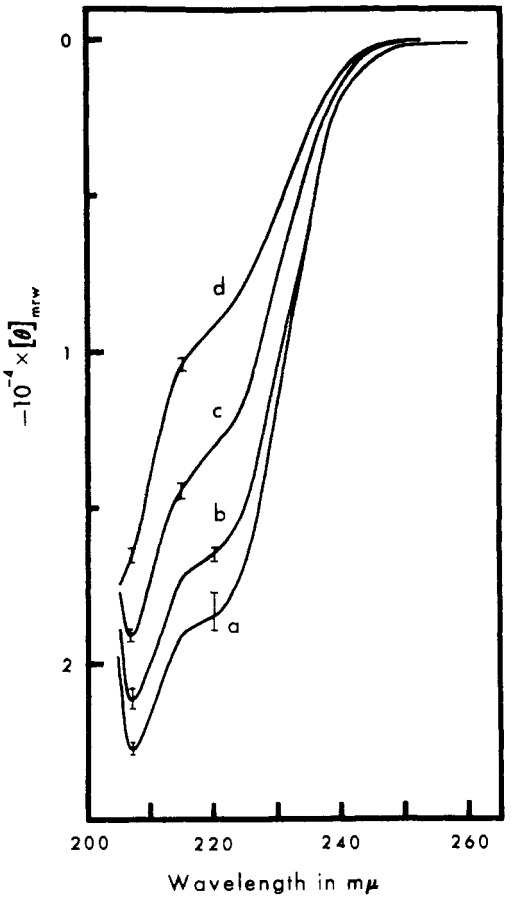

FIGURE 5: The effect of acetate buffer on the accessible portion of the far-ultraviolet circular dichroism spectrum of fatty acid free BSA in the absence and presence of urea: curve a, 0.02 M NaAc–1.31 M HAc (pH 2.88); curve b, 0.02 M NaCl–HCl (pH 2.86); curve c, 2 M urea solution containing 0.02 M NaAc–2.45 M HAc (pH 2.86); curve d, 2 M urea solution containing 0.02 M NaCl–HCl (pH 2.85). Curves a and b are averages of three determinations; curves c and d, averages of two determinations. The error bars designate range of observations. The same results as shown by curve d were also obtained in 2 M urea solution containing either 0.02 M NaCl–0.02 M HCl (pH 2.87) or 0.02 M HCl (pH 2.84).

chromophores with other groups in the protein because of spatial reorientation, *i.e.*, local conformation changes. The first of these possibilities seems to be eliminated. Evidence for the absence of vicinal effects is twofold. First, the near-ultraviolet (260–270 mμ) circular dichroism spectrum is unaffected by acetate buffer; strong vicinal effects would be expected to produce shifts in this spectral region as well as in the far-ultraviolet region. Secondly, acetate buffer inhibits denaturation of BSA by 2 M urea at pH 2.9 as revealed by comparison of spectra c and d in Figure 5. The fact that spectrum c has a negative peak at 207 mμ, while spectrum d has none, indicates that a true conformational change occurs on substitution of acetate buffer for NaCl–HCl. It can be concluded, therefore, that binding of acetic acid causes subtle alterations in the tertiary structure of BSA, in the absence as well as the presence of urea.

Discussion

The foregoing results provide fresh insight into the mechanisms underlying the effect of acetate and other carboxylic acid buffers on the electrophoretic behavior of proteins in acidic media. Thus, the results with poly-L-glutamic acid are in complete accord with the previous conclusion (Cann and Phelps, 1959) that carboxyl groups on the protein play an important role in its interactions with acetic and formic acid. A salient feature of the interaction is that binding of acetic acid increases the net positive charge on the protein molecule (Cann and Phelps, 1957). This is now understood in terms of reaction eq 2 according to which the binding of acetic acid to carboxyl groups increases their pK thereby increasing the net positive charge on the protein at constant pH.[4] This, in turn, explains the influence of pH on the interaction as illustrated graphically for ovalbumin and BSA in Figure 2 of Cann and Phelps (1959). As the pH of the protein solution is progressively lowered from pH 4.5 to 2.7 and the carboxylate groups titrated, the number of sites to which acetic or formic acid can bind with concomitant increase in positive charge decreases. Accordingly, it requires increasingly high concentration of acid to produce the changes in electrophoretic patterns characteristic of the interaction, *e.g.*, growth of fast peaks at the expense of slow ones in the case of proteins acid to their isoelectric pH.

We visualize acetic acid binding to side-chain carboxyl groups of poly-L-glutamic acid and proteins *via* hydrogen-bond formation. The values of the intrinsic association constant derived from electrophoretic mobilities and from the effect of acetic acid on the helix–coil transition of poly-L-glutamic acid are on the order of 1 M^{-1}. This is the order of magnitude expected for hydrogen-bond formation in water (Jencks, 1969). Thus, the value of the equilibrium constant for formation of the intermolecular hydrogen bond in the bifluoride ion is

[4] At sufficiently high concentration of aliphatic acids, ovalbumin evidently binds more undissociated acid than can be accounted for by carboxyl groups alone (Bull and Breese, 1967a). It may be that peptide groups also participate in binding under these conditions.

about 4 M^{-1}. The existence of acetic acid dimers in water is controversial (Schrier *et al.*, 1964; Jencks, 1969). If they exist, the association constant of 0.1 M^{-1} indicates that the hydrogen bond is quite weak. On the other hand, factors such as the low dielectric constant of the cavity formed by the macromolecule in water should act to increase the strength of hydrogen bonds between side-chain carboxyl groups and acetic acid molecules. Moreover, hydrophobic bonding between the methyl group of acetic acid and the hydrocarbon moiety of the carboxyl side chain of poly-L-glutamic acid appears possible for a hydrogen-bonded complex (Schrier *et al.*, 1964) and could conceivably contribute to the free energy of binding.

The results with poly-L-glutamic acid also bear on the mechanism of resolution of bimodal reaction boundaries. While theoretical calculations (Cann and Goad, 1965) have established that cooperative interactions of the type shown by reaction eq 1 can generate relatively intense concentration gradients of the small molecule along the electrophoresis column, it is difficult to visualize how a simple, noncooperative interaction could generate gradients sufficiently strong to cause resolution into peaks. It now seems apparent, however, that noncooperative binding of neutral molecules such as undissociated acetic acid to ionizable groups on the macromolecule will generate coupled gradients of acetic acid and pH, and that the electrophoretic behavior of such an interaction will ape cooperative binding. This is most clearly seen by considering the simplest case of a single complex formed in accordance with reaction eq 2. In this case, the mean amount of acetic acid bound per mole of macromolecule will vary as the product of the concentrations of hydrogen ion and acetic acid and, consequently, as the square of the acetic acid concentration. Thus, the equilibrium composition will vary along the electrophoresis column in accordance with the square of the local concentration of unbound acetic acid. In other words, the effective magnitude of the concentration gradients of acetic acid through the reaction boundary is greatly increased.

Acetic acid has been shown to induce conformational changes in macromolecules. Thus, for example, it favors the helical configuration of poly-L-glutamic acid and causes changes in the optical rotatory dispersion parameters (a_0 and b_0) and circular dichroism spectrum of BSA interpretable in terms of subtle alterations in the tertiary structure of the protein. When such conformational changes occur under the same conditions for which electrophoretic behavior indicates interaction with the buffer acid (as is the case with BSA), there is little conceptual difficulty in evoking a cooperative interaction. It can be expected that here too resolution of the reaction boundary will be enhanced by coupled gradients of acid and pH.

The subtle effect of acetate buffer on the tertiary structure of BSA is consistent with its effect on the pH profile of the N–F transition of the protein. Leonard and Foster (1961) found that, in contrast to the profile obtained in 0.02 M NaCl, the profile in 0.02 M NaAc shows evidence for a double transition. The midpoint of the combined transitions in NaAc occurs at a slightly lower pH value than in NaCl as judged by interpolation of the pH profiles presented in their Figures 2 and 3.

Inhibition of urea denaturation of BSA by acetic acid is reminiscent of the inhibition of its peptic digestion by caprylic acid (Klapper and Cann, 1964). No doubt both phenomena share a common mechanism rooted in the conformational adaptability of BSA (Karush, 1950, 1952; Markus and Karush, 1958) whereby binding of acid is associated with a subtle shift in macromolecular conformation to a state refractory to urea denaturation and the action of pepsin. Glazer and Sanger (1963) have presented data suggesting that binding of 2 moles of stearic acid by BSA is associated with a conformational rearrangement resulting in the accessibility of a greater number of tyrosine residues to iodination. It is conceivable that the enhancement of the negative, far-ultraviolet circular dichroism bands of BSA by acetic acid is due to perturbation of aromatic residues, but little is known about the contribution of side-chain residues to this spectral region (Ettinger and Timasheff, 1971).

Finally, the inhibition of urea denaturation of BSA by acetate buffer is in striking contrast to the destabilizing effect of acetate reported for other proteins. Thus, for example, Gibbs (1954) found that the rate of heat denaturation of human serum albumin at pH values less than about pH 4.3 is greater in the presence than in the absence of acetate buffer due to a sizable decrease in the activation energy; and Bull and Breese (1967b) found that acetic acid and its normal short-chain homologs are powerful denaturants of ovalbumin at acid pH values. The question of the effect of acetic acid and its short-chain homologs on the conformation and stability of ribonuclease is examined in depth in the companion paper (Cann, 1971).

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