SPECTROSCOPIC EVIDENCE FOR COMPLEXING OF ACETIC ACID WITH BOVINE SERUM ALBUMIN, GRAMICIDIN, AND DIMETHYLFORMAMIDE

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ABSTRACT Acetic acid has a major effect on the absorption spectra of bovine serum albumin, gramicidin, and dimethylformamide in the region, 255 to 200 mμ. Increasing the concentration of acetic acid causes progressively decreasing absorbency accompanied by a large and progressively increasing red shift of the absorption maximum. The decrease in absorbency is interpreted in terms of a reversible complexing of acetic acid with these molecules and the red shift in terms of a non-specific solvent effect.

INTRODUCTION

The electrophoretic behavior of a variety of proteins in acidic media containing acetate buffer or other carboxylic acid buffers is explicable in terms of the reversible binding of undissociated buffer acid by the protein with a concomitant macromolecular conformational change which increases the net positive electrical charge on the protein but does not change its frictional coefficient significantly (1-4). Equilibrium constants for the binding of undissociated acetic acid (HAc) by bovine serum albumin (BSA) (3) or insulin (4) and of a series of aliphatic acids by ovalbumin (3) have been computed from electrophoretic mobilities. These data are consistent with the idea that the acid binds to the protein through formation of a single heterologous hydrogen bond with the carboxyl group of the acid serving as the donor and a protein grouping as the acceptor. This communication presents ultraviolet light absorption studies which furnish independent evidence for the reversible binding of undissociated HAc by BSA, gramicidin, and *N,N*-dimethylformamide (DMF).

MATERIALS AND METHODS

The BSA was Armour's crystalline bovine plasma albumin Lot No. V68802; gramicidin,

Mann's Lot No. B3726; and DMF, Eastman spectrograde Lot No. 9A. The following solvents were used without further purification: Matheson Coleman and Bell spectrograde 2,2,4-trimethylpentane; a freshly opened bottle of Matheson Coleman and Bell spectroquality reagent *p*-dioxane; United Western Laboratories sulfuric acid reagent, assay slightly greater than 99 per cent but less than 100 per cent H_2SO_4; U. S. Industrial Chemicals Co. absolute, pure ethanol U.S.P.-N.F. reagent quality which had the same optical properties as a sample of specially prepared optical ethanol kindly furnished by Dr. Wilhelm R. Frisell. The HAc was Mallinckrodt analytical reagent 99.7 per cent glacial acetic acid.

Ultraviolet absorption spectra were measured at room temperature on a Beckman DU spectrophotometer with photomultiplier attachment. Some of the measurements on gramicidin were made with the Beckman DK-2 recording spectrophotometer. Matched quartz absorption cells of 1 cm path length were used, the solvent serving as the blank. In experiments using salt solutions, acetate buffer (NaAc-HAc) or ethanol-HAc mixtures, the mixed solvent served as the blank. All spectra are plotted in terms of absorbency, $A = \log_{10} I_0/I$, *versus* wave length, λ, in millimicrons, mμ.

Although HAc absorbs in the spectral region studied (wave length of maximum absorption in ethanol, 210 mμ; in 0.1 M NaCl, 203.5 mμ), the value, about 34, of its molar extinction coefficient is much smaller than the values for BSA, gramicidin, and DMF, about 2×10^6, 6×10^5, and 7×10^5, respectively. Consequently, changes in light absorption of the solution due to changes in HAc concentration resulting from the reaction with the solute are negligible. Thus, the spectra shown in the various figures are interpretable solely in terms of solute absorption. A similar argument applies to NaAc.

RESULTS

BSA and Gramicidin. Previously (5), small differences were noted in the 280 mμ absorption band of BSA in 0.1 M NaAc-HAc as compared to 0.002 M NaAc-HAc + 0.09 M NaCl, pH 4. We can now report a major effect of acetate buffer on absorption in the wave length range 255 to 200 mμ. In this spectral region (Fig. 1), BSA in 0.1 M NaCl shows the same skewed absorption band with a maximum at 202.5 mμ for pH values ranging from 7.9 to 4.0. However, changing the solvent from 0.1 M NaCl to 0.1 M NaAc, pH 7.6, causes a striking diminution in absorbency accompanied by a strong shift of maximum absorption towards longer wave length (red shift). These spectral changes are accentuated by changing the solvent to 0.1 M NaAc-0.4 M HAc, pH 4.0. The spectral effects of acetate buffer are reversible as shown by an experiment in which a solution of 4×10^{-3} per cent BSA in acetate buffer was dialyzed for 24 hrs. against many changes of 0.1 M NaCl in order to replace the acetate buffer with NaCl. The dialyzed solution gave the same spectrum, within experimental error, as BSA never exposed to acetate buffer.

Similar behavior is shown by gramicidin in ethanol-HAc solutions (Fig. 2). In the absence of HAc, the absorption spectrum of gramicidin in ethanol shows three major bands: a trimodal band in the region 310 to 250 mμ, a band at 222 mμ, and one at 204 mμ. Whereas changing the solvent from ethanol to ethanol-HAc

has little, if any, effect on the 310 to 250 mμ absorption, the other two bands are profoundly altered. As the concentration of HAc is increased, absorbency in the 240 to 200 mμ region decreases continuously and the wave length of maximum absorption shifts progressively into the red. The reversibility of both the hypochromic effect and the red shift was shown by an experiment in which a 5×10^{-3} per cent solution of gramicidin in ethanol–0.314 M HAc was diluted fivefold with ethanol. The spectrum of the diluted solution was, within experimental error, the

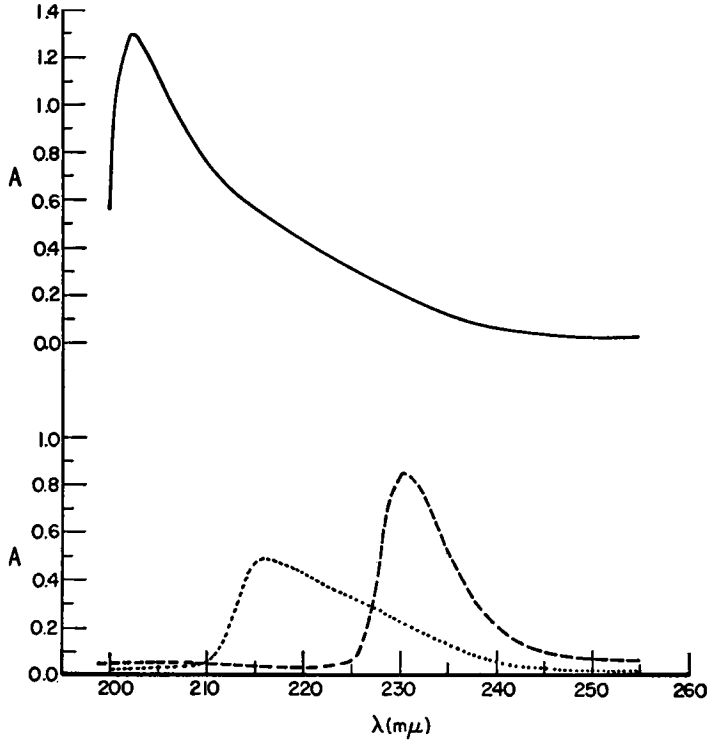

FIGURE 1 Far ultraviolet absorption spectrum of BSA: ——, 4×10^{-3} per cent protein in 0.1 M NaCl, pH 4.0, 5.6, or 7.9; . . . . . , 4×10^{-3} per cent protein in 0.1 M NaAc, pH 7.6; - - -, 2×10^{-3} per cent protein in 0.1 M NaAc-0.4 M HAc, pH 4.0. The maximum absorbency of 4×10^{-3} per cent protein in 0.1 M NaAc-0.4 M HAc, pH 4.0, is 0.19.

same as that of a control solution containing 0.063 M HAc and 10^{-3} per cent gramicidin never exposed to a higher acetic acid concentration. The possibility that the spectral effects of HAc might be due to acetate ions rather than undissociated acid molecules was eliminated by comparing the spectrum of gramicidin in ethanol-0.120 M HAc–0.062 M NaAc with spectra obtained in ethanol–acetic acid without added NaAc. If acetate ions were responsible for the spectral effects, the added

NaAc should have had an effect at least an order of magnitude greater than HAc alone. Actually, it was only about one-fourth as effective as the equivalent concentration of HAc.

DMF. Interpretation of the effect of HAc on the spectra of BSA and gramicidin clearly requires a knowledge of the behavior of a model compound such as DMF. Background information was furnished by a preliminary investigation of solvent-induced frequency shifts in the absorption spectrum of DMF. The results

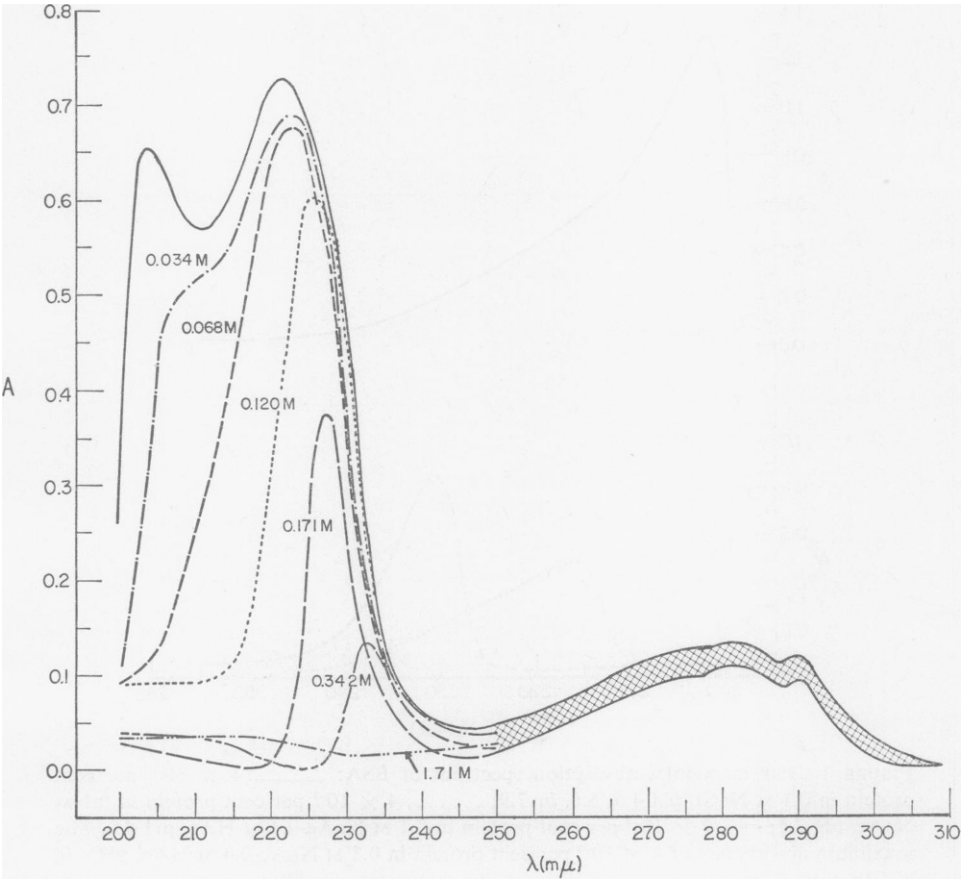

FIGURE 2 Absorption spectra of gramicidin in ethanol (_____) and ethanol-HAc solutions (___ . ___, ___ ___, ..., and ___ . . ___). Concentration of HAc shown beside corresponding spectrum; 1 mg gramicidin per 100 ml solution. In the cross-hatched region the various spectra differ little, if at all, from one another.

are presented in Figs. 3 and 4. It was found that the absorption maximum of DMF shifts progressively towards shorter wave length (blue shift) as the solvent is changed from 2,2,4-trimethylpentane to ethanol to water, which is also the order of

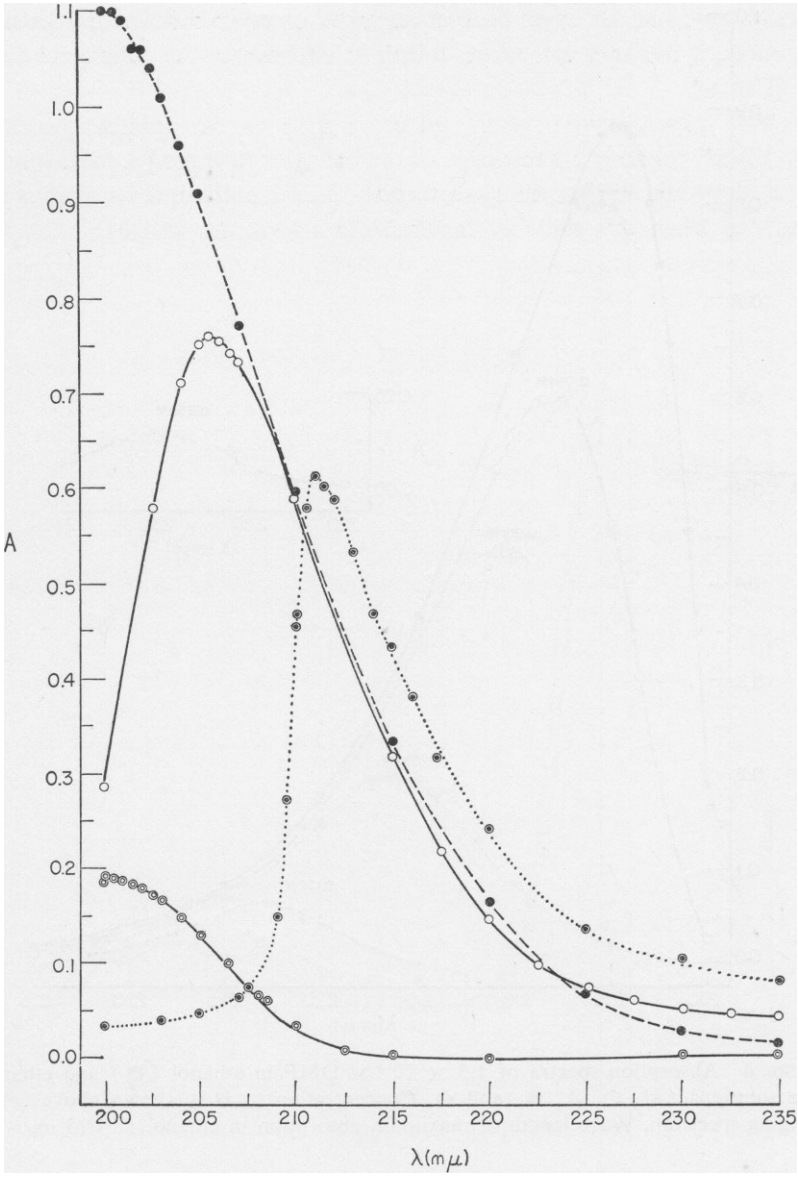

FIGURE 3 Absorption spectra of 1.3×10^{-4} M DMF in various solvents: 2,2,4-trimethylpentane, ○; water, ●; 99 to 100 per cent H_2SO_4, ⊙; *p*-dioxane, ◉. Wave length of maximum absorption in 2,2,4-trimethylpentane, 205.5 mμ; *p*-dioxane, 211 mμ.

increasing hydrogen-bonding tendency of these solvents. Changing the solvent from 2,2,4-trimethylpentane to H_2SO_4 likewise causes a blue shift which correlates with the fact that DMF is monoprotonated in 100 per cent H_2SO_4 (6). On the other

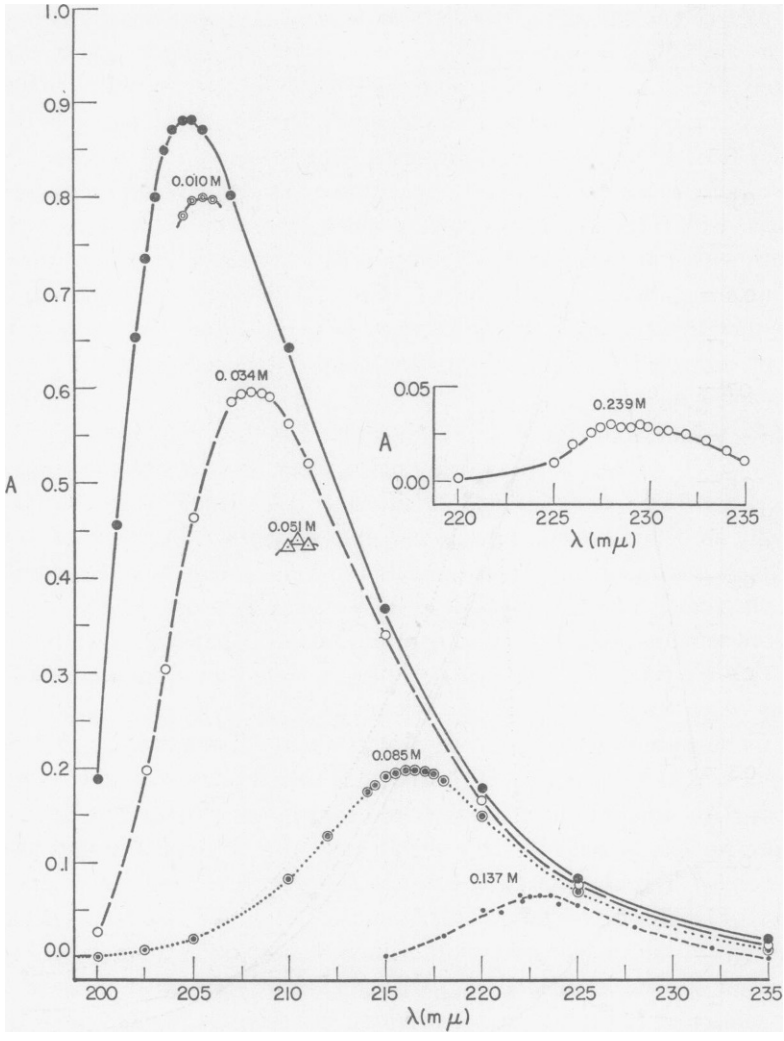

FIGURE 4 Absorption spectra of 1.3×10^{-4} M DMF in ethanol (●) and ethanol-HAc solutions (◎, ○, △, ◉, and •). Concentration of HAc shown above corresponding spectrum. Wave length of maximum absorption in ethanol, 204.75 mμ.

hand, changing the solvent from 2,2,4-trimethylpentane to the non-hydrogen-bonding solvent, dioxane, causes the well known red shift which results from polarization that is induced in the solvent by the transition dipole of the solute and from dipole-dipole forces between solute and solvent (7, 8).

As shown in Fig. 4, the behavior of DMF in ethanol-HAc is similar to that of BSA and gramicidin. Increasing the concentration of HAc in the solvent causes progressively decreasing absorbency accompanied by progressively increasing red

shift. A plot of the maximum absorbency against the wave length of maximum absorption was concave downwards except at low wave lengths where it was approximately linear. In other words, increasing the HAc concentration at the higher concentrations produces a much larger red shift relative to the decrease in maximum absorbency than at the lower concentrations. These observations indicate that HAc exerts two independent effects on the spectrum. The simplest interpretation is that HAc reacts[1] with DMF to form a complex which absorbs in the spectral region, 350 to 200 mμ, with a molar extinction coefficient at least 100-fold smaller than that of DMF. Increasing the concentration of HAc increases the concentration of the complex, thereby decreasing the absorbency of the solution, and at the same time causes a progressive shift of the absorption band of the uncomplexed DMF towards longer wave length.[2]

The HAc-induced red shift is unusually large. The frequency shift of 5270 cm^{-1} induced by 0.239 M HAc is about 3½ times as large as that observed on changing the solvent for DMF from ethanol to dioxane. This result is in contrast to the theoretically predicted solvent-induced red shifts of the 500 mμ band of phenol red (8). In this system glacial HAc is predicted to induce a shift which is only about 10 per cent greater than that of dioxane. However, our measurements were carried out at wave lengths close to a very intense absorption band of HAc beginning at 185 mμ (9). Consequently, the polarizability of HAc should increase rapidly with decreasing wave length in the region where DMF absorbs (10). Since red shifts increase in magnitude with increasing polarizability of the solvent and since the concentration of HAc near a DMF molecule is certainly greater than its bulk concentration, HAc should induce a greater red shift in our system than in the case of visible spectra. The large observed red shift may also be related in part to the fact that HAc shows a weak absorption band at 210 mμ. It is conceivable that exciton interaction of DMF with neighboring HAc molecules should also be considered. The large red shift of the visible absorption of indigo observed in benzene by increasing concentration can be explained in part by exciton interaction between nearest neighbor molecules (11); but exciton theory is not as yet sufficiently well developed for application to our system. Finally, it is important to note that these considerations are consistent with the observation that HAc induces a red shift in the 222 mμ band but not in the 310 to 250 mμ band of gramicidin.

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[1] Simple protonation of DMF seems to be eliminated by the observation that the spectrum of DMF in ethanol-0.1 M HCl is essentially the same as in ethanol containing the appropriate amount of water. The experiments with gramicidin have eliminated acetate ion as the effective agent.

[2] This interpretation assumes that either the absorption band of DMF represents a single electronic transition or is the sum of two or more bands with the same sensitivity to HAc. It is conceivable, of course, that the spectrum represents the sum of two or more closely situated bands with different sensitivities to HAc in which case quantitative interpretation would seem to be virtually impossible.

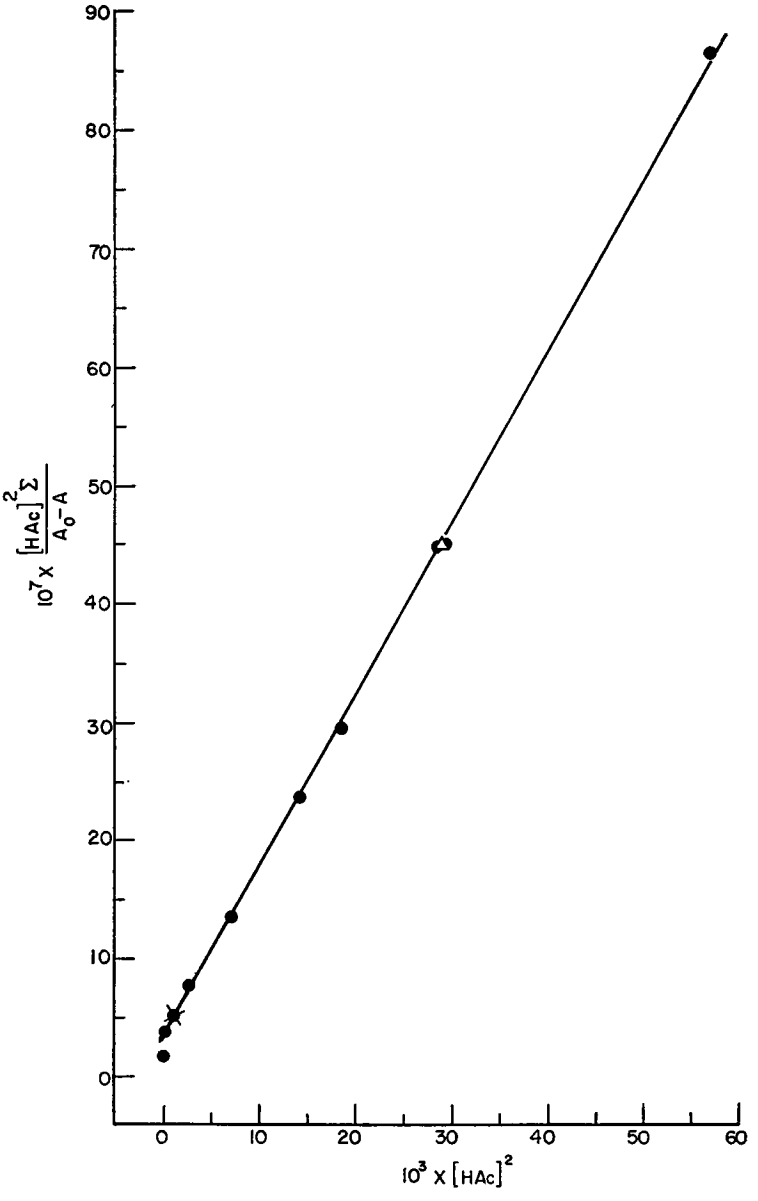

FIGURE 5 Determination of $(\epsilon_0 - \epsilon_1)$ and K for the reaction of DMF with HAc (see Equation 2): ●, $10^4 \times \Sigma = 1.21$ to 1.32; △, $10^4 \times \Sigma = 6.85$ and [HAc] = 0.171M; X, previous solution diluted fivefold with ethanol to demonstrate the reversibility of the reaction.

Granting the above and disregarding the red shift by translating all the spectra to a common wave length of maximum absorption, the decrease in absorbency of DMF solutions with increasing concentration of HAc is interpretable quantitatively in terms of the reaction,

$$\mathrm{DMF} + 2\mathrm{HAc} \rightleftharpoons \mathrm{DMF \cdot 2HAc} \tag{1}$$

The absorbency, A, can then be related to the acetic acid concentration [HAc], by the equation[3]

$$\frac{[\mathrm{HAc}]^2\Sigma}{A_0 - A} = \frac{1}{(\epsilon_0 - \epsilon_1)K} + \frac{[\mathrm{HAc}]^2}{(\epsilon_0 - \epsilon_1)} \tag{2}$$

where A_0 is the absorbency in the absence of HAc; Σ, total concentration of DMF both free and complexed with HAc; K, the equilibrium constant for Reaction 1; ϵ_0, the molar extinction coefficient of DMF; and ϵ_1, the molar extinction coefficient of the complex, DMF·2HAc. Since the shape of the absorption band changes somewhat with increasing HAc concentration, Equation 2 has been applied only to the change in maximum absorbency with HAc concentration in Fig. 5. The value, 6.92×10^3, thus obtained for $(\epsilon_0 - \epsilon_1)$ is the same within experimental error as the measured value, 6.84×10^3, of ϵ_0. This result supports our hypothesis that the complex formed between DMF and HAc absorbs weakly, if at all, in the spectral region of interest. The plot shown in Fig. 5 also illustrates the reversibility of complexing between DMF and HAc and gives a value of 450 liter^2 mole^{-2} for K.

Complexing of two HAc molecules with a single molecule of DMF is reminiscent of the reaction between HAc and triethylamine in carbon tetrachloride (12). The compound formed at about half-neutralization of the acid is one in which triethylamine has accepted a proton from the acid dimer to form an ion pair salt, the triethylammonium ion forming a hydrogen bond with one of the carboxylate oxygens of the acetate ion–acetic acid hydrogen-bonded moiety. An analogous compound might be formed between DMF and HAc, particularly since DMF is a weak base being protonated on oxygen in H_2SO_4 (6) and HAc is undoubtedly dimerized in ethanol. On the other hand, it is conceivable that two HAc molecules can each form a hydrogen bond to the carboxyl oxygen of a single DMF molecule. The molecules in crystalline formamide are linked in sheets by double hydrogen bonds between two amido N-H and a single carbonyl oxygen (13).

DISCUSSION

The experiments with DMF can be used as a guide for interpreting the effect of HAc on the spectra of BSA and gramicidin. The conclusion seems justified that undissociated HAc and presumably also acetate ion react reversibly with the

[3] Equation 2 was derived from the following expressions: $A = \epsilon_0$ [DMF] + ϵ_1 [DMF·2HAc]; $A_0 = \epsilon_0\Sigma$; Σ = [DMF] + [DMF·2HAc]; and K = [DMF·2HAc]/[DMF] [HAc]^2.

peptide linkages and, possibly, also tyrosine, tryptophan, histidine and other chromophoric residues (14) in the latter molecules thereby suppressing absorption in the spectra region, 255 to 200 mμ. The spectra shown in Fig. 1 indicate that a very large proportion of these groupings in BSA must be reactive at pH 4 which in turn suggests that at very low protein concentrations the BSA molecule unfolds as a result of exposure to acetate buffer at pH 4. This inference is not too surprising since previous experiments (15) have shown that prolonged exposure of BSA at sufficiently low protein concentration (0.2 per cent or less) to acetate buffer at pH 4 results in irreversible changes in electrophoretic properties.

These results support our previous conclusion from electrophoretic experiments (1-4) that a variety of proteins react reversibly with undissociated HAc. (The effect of HAc on electrophoretic properties is completely reversible provided that the protein concentration is 0.4 per cent or greater). As with DMF, complexing of proteins with HAc is weak. Thus, the intrinsic association constants for binding of HAc to BSA and insulin as computed from mobility data have values of 22 and 117 liter mole^{-1}, respectively (3, 4).

The HAc- and NaAc-induced red shifts observed with BSA and gramicidin are presumably due to non-specific solvent effects and, conceivably, also exciton interaction between uncomplexed chromophoric groupings and neighboring HAc molecules or acetate ions.

Finally, studies of the type reported herein have a strong bearing on our understanding of protein-protein interactions essential for life processes. Recent (as yet unpublished) experiments from this laboratory have shown that the binding of caprylic and other long-chain fatty acids by serum albumin has a strong inhibitory effect upon the specific complexing of this protein with pepsin. Apparently, bound fatty acid stabilizes the serum albumin molecule in a configuration which is refractory to the action of pepsin. It remains to be determined whether the specific binding sites and the conformational changes induced thereby are similar for long-chain fatty acids and HAc. However, it has previously been shown that propionic and valeric acid have essentially the same effect on the electrophoretic behavior of proteins in acidic media as HAc (1) and that both caprylic acid and HAc stabilize BSA against isomerization of pH 4 (16).

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