Cooperative Interaction of Aliphatic Acids with Ribonuclease A*

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ABSTRACT: A variety of biophysical methods have been brought to bear on the mechanism of the reversible interaction of undissociated acetic acid and its short-chain homologs with ribonuclease A. The results of these measurements are synthesized within the context of the three-dimensional structure of the protein. The conclusion reached is that the interaction is a cooperative phenomenon associated with a change in tertiary structure without substantial alteration in secondary structure. Binding of acid to side-chain carboxyl groups on the protein, presumably *via* hydrogen-bond formation, evidently upsets the balance of forces which determines native conformation, hydrophobic bonding of the hydrocarbon moiety of the acid to nonpolar groups in the protein providing the ultimate driving force for the change in conformation. The change in conformation is characterized by (1) exposure of buried tyrosine residues, at least some of which retain a measure of constraint; and (2) relief of the constraint on other of the tyrosine residues in the protein.

The electrophoretic behavior of a variety of proteins in acidic media containing acetate or other carboxylic acid buffers is predicted (Cann and Goad, 1965) by a theory of electrophoresis of reversibly interacting systems of the type

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

where P represents a protein molecule or other macromolecular ion in solution and $P(HA)_n$ its complex formed by binding of 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. Resolution of bimodal reaction boundaries 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 $P(HA)_n$. Although the theory assumes a cooperative interaction, binding of acetic acid to carboxyl groups on the macromolecule evidently need not be cooperative for resolution to occur since such an interaction generates coupled gradients of acetic acid and pH along the electrophoresis column (Cann, 1971). On the other hand, there is little conceptual difficulty in evoking a cooperative interaction when binding causes a change in macromolecular conformation as, for example, in the case of bovine serum albumin (Cann, 1971). In fact, it has now been demonstrated that the reversible interaction of acetic acid and its short-chain homologs with ribonuclease A is a cooperative phenomenon associated with a change in tertiary structure of the protein.

Materials and Methods

Phosphate-free, lyophilized ribonuclease A was obtained from Nutritional Biochemicals Corp. Its sedimentation pattern was normal, and there was no evidence of aggregation. Its near-ultraviolet circular dichroism spectrum was in good agreement with that reported by Pflumm and Beychok (1969a,b), and its far-ultraviolet circular dichroism spectrum was in excellent agreement with that reported by Schellman and Lowe (1968) but 14–25% less intense than reported by Pflumm and Beychok (1969a).

Eastman's valeric and dichloroacetic acids were purified by vacuum distillation; Eastman's *p*-dioxane gave a negative test for peroxides; methanol was Spectrograde; analytical reagent grade urea was recrystallized from 70% ethanol; *N*-acetyltyrosinamide was Mann's CP grade; 2′:3′-cyclic cytidylate sodium and yeast RNA were obtained from Schwarz and Mann, respectively; other chemicals were analytical reagent grade.

Stock solutions of RNase in water were analyzed spectrophotometrically in a thermostated Beckman DK-2 instrument. Other absorption spectra were recorded on a Cary 14 spectrophotometer. The author thanks Dr. Julius Gordon for his kind permission to use this instrument.

Circular dichroism and optical rotatory dispersion spectra were recorded on a Cary Model 60 spectropolarimeter with a Model 6001 circular dichroism attachment, fitted with thermostatable cell holders. Except where indicated, measurements were made at 27.0°. Slits were programmed to yield a 15-Å bandwidth at each wavelength. Concentrations and path lengths were dictated by the absorbance of the acetate buffer; with RNase in 0.2 M NaAc,[1] for example: near-ultraviolet circular dichroism, about 0.9 mg of RNase/ml in a 1-cm cell, although 1.8 mg/ml gave the same results; far-ultraviolet circular dichroism, 2.6 mg/ml in a 0.01-cm cell; optical rotatory dispersion, 4 mg/ml in a 0.01-cm cell. Reduced mean residue rotations, [M′], (deg cm²)/dmole, and ellipticities, $[\theta]_{mrw}$, (deg cm²)/dmole, were calculated in the usual fashion using a value of 110 for the mean residue weight.

Enzymatic activity was measured by three different methods. The Kunitz spectrophotometric assay (Klee, 1966) was made in a Beckman DU spectrophotometer, thermostated at 37 ±

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[1] Abbreviations used are: Ac, acetate; Pr, propionate; Bu, butyrate.

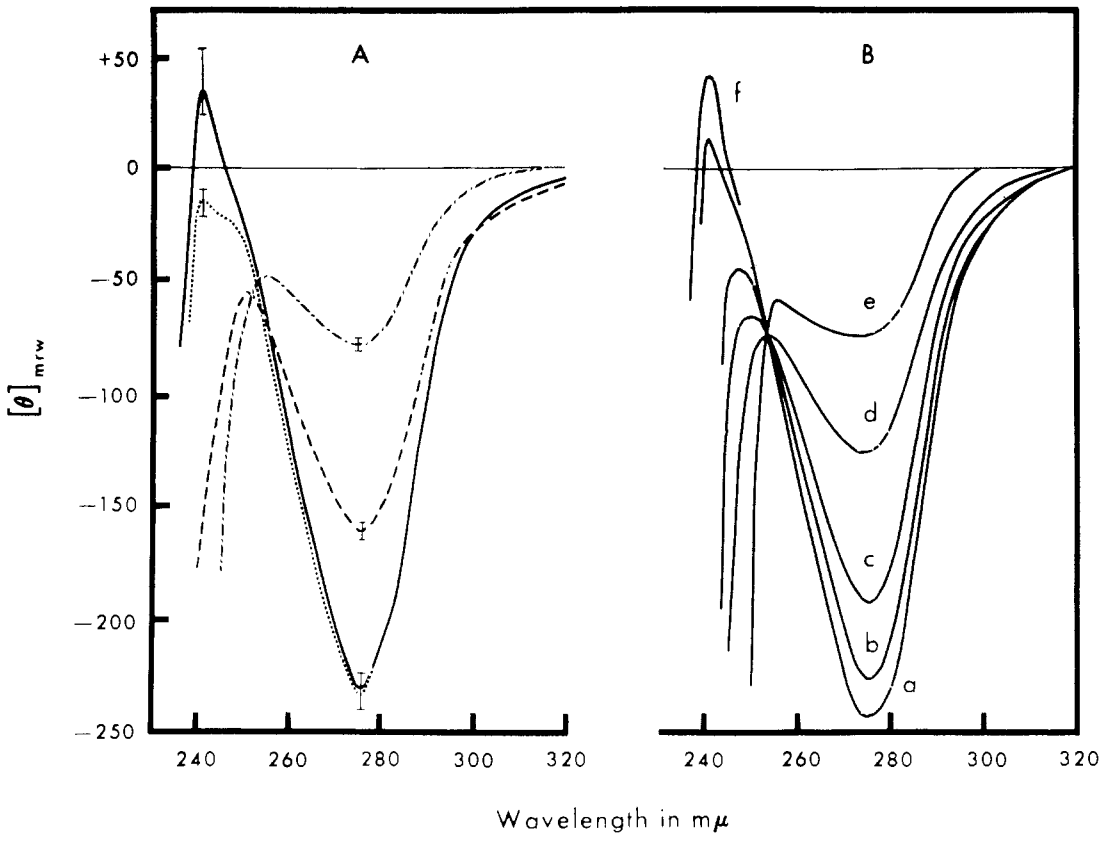

FIGURE 1: Effect of acetate buffer on the near ultraviolet circular dichroism spectrum of RNase: A, ——, 0.02 M NaCl–HCl, pH 2.90; · · · · ·, 0.02 M NaAc–1.31 M HAc, pH 2.85; ---, 2 M urea in 0.2 M NaCl–HCl, pH 3.09; - · -, 2 M urea in 0.02 M NaAc–1.31 M HAc, pH 3.09; average of five or two determinations in absence or presence of urea; error bars designate range of observations. B, effect of increasing buffer concentration at ionic strength 0.2 and pH 3.05; a, 0.1 M NaAc–2.51 M HAc–0.1 M NaCl; b, 0.13 M NaAc–3.27 M HAc–0.07 M NaCl; c, 0.15 M NaAc–3.77 M HAc–0.05 M NaCl; d, 0.17 M NaAc–4.27 M HAc–0.03 M NaCl; e, 0.2 M NaAc–5.02 M HAc; f, 0.2 M NaCl–HCl.

0.01°, coupled through a Beckman No. 5800 energy-recording adapter to a Honeywell strip-chart recorder of per cent transmission. The author thanks Dr. Oscar K. Reiss for his kind permission to use this equipment. Assay mixtures contained 326 μg of RNA and 4.84 μg of RNase per ml. Production of acid-soluble oligonucleotides was assayed by the procedure of Klee and Richards (1957) except that the digestion mixtures, containing 4.55 mg of RNA and varying amounts of RNase per ml, were incubated for 25 min at 37°. Hydrolysis of 2′:3′-cyclic cytidylate (Fruchter and Crestfield, 1965a) was allowed to proceed for 25 min at 37° in mixtures containing 2.73 mg of substrate and varying amounts of RNase per ml. The reaction was stopped by chilling in an ice bath. The resulting 3′-cytidylate ($R_F \simeq 0.2$) was separated from unhydrolyzed substrate ($R_F \simeq 0.4$) by ascending chromatography of 20–50 μl of reaction mixture on No. 4 Whatman chromatography paper with isopropyl alcohol–ammonia–water (70:5:25, v/v) (Brown *et al.*, 1952). Reaction mixtures and parallel controls containing no enzyme were chromatographed side by side. More than 98% of the control migrated as substrate. The zone of 3′-cytidylate was located with an ultraviolet lamp and excised, a corresponding piece being excised from the control. The cytidylate was eluted with 1 ml of 10^{-2} M HCl. The micrograms of 3′-cytidylate formed in the enzymatic reaction was calculated from the absorbance of the eluate at 279 mμ after subtraction of the control value.

Electrophoresis was carried out on 1% RNase at field strengths of about 6.8 V cm^{-1} for about 70 min in a Perkin-Elmer Tiselius apparatus fitted with a current-regulating power supply. Apparent ascending electrophoretic mobilities ($10^5 \times \mu$ cm^2 sec^{-1} V^{-1}) computed using the conductance of the dialyzed protein solutions are shown above or beside the corresponding peaks in the electrophoretic patterns.

Results

Preliminary electrophoresis and circular dichroism measurements on RNase indicated that interaction with acetic acid causes changes in macromolecular conformation. Subsequently, appeal was made to a variety of biophysical methods in order to gain insight into the mechanism of the interaction and the nature of the forces involved. The results of these experiments are described below.

Near-Ultraviolet Circular Dichroism (Spectral Region, 320–236 mμ). The effect of acetate buffer on the near-ultraviolet circular dichroism spectrum of RNase has been examined under a variety of conditions of pH, buffer concentration, ionic strength, temperature, etc. Let us first consider the results obtained at pH 2.9 and ionic strength 0.02 (Figure 1A). Comparison of the spectra shown in 0.02 M NaAc buffer and 0.02 M NaCl–HCl reveals that, whereas acetate buffer has no effect on the strength and shape of the negative band centered at 276 mμ, it virtually abolishes the weak, positive band at 240 mμ. This effect is reversed by dilution at constant pH. In a typical experiment, a solution containing 1.84 mg of RNase/ml of 0.02 M NaAc buffer was diluted 10-fold with 0.02 M NaCl–HCl (pH 3.06) to give a final pH of 2.9 and the circular dichroism spectrum of the diluted solution recorded using a 5-

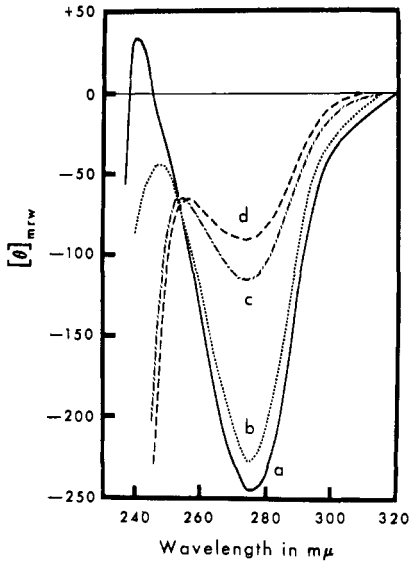

FIGURE 2: Comparative effect of short-chain aliphatic acids on the near-ultraviolet circular dichroism spectrum of RNase at pH 3.05 and ionic strength 0.2: (a) 0.2 M NaCl–HCl, (b) 0.13 M NaAc–3.27 M HAc–0.07 M NaCl, (c) 0.034 M NaPr–2.19 M HPr–0.166 M NaCl, and (d) 0.023 M NaBu–1.28 M HBu–0.177 M NaCl.

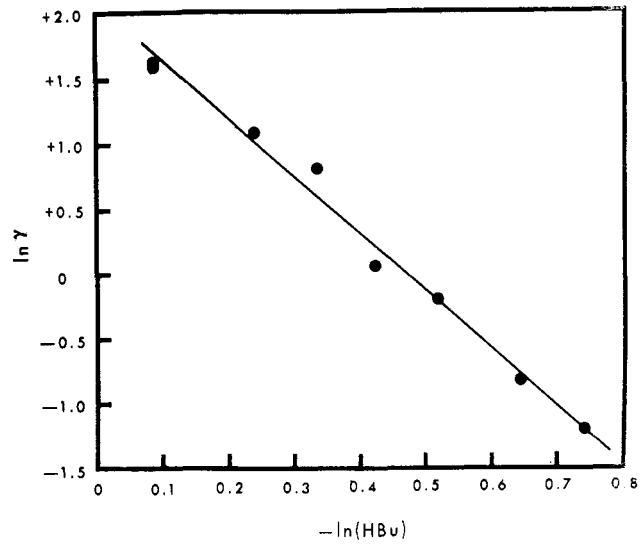

FIGURE 3: Plot of the logarithm of γ, as defined by eq 3, against the logarithm of the butyric acid concentration in 0.2 ionic strength media at pH 3.06. The straight line is the least-squared one.

cm cell. The spectrum of the diluted solution was the same as shown by RNase never exposed to acetate buffer. The question naturally arises as to whether the effect of acetate buffer on the 240-mμ band is not due merely to solvent perturbation of the corresponding chromophore(s) or to a vicinal effect resulting from the bound acetate. These possibilities seem to be eliminated by the observation that acetate buffer destabilizes RNase to denaturation by 2 M urea at pH values less than about pH 3.6 (Figure 1A).

The effect of acetate on the circular dichroism of RNase is quite sensitive to ionic strength and buffer concentration. Thus, while the spectrum is unaffected by 0.02 M NaAc–0.03 M NaCl (pH 3.05), the 240-mμ band is abolished by 0.05 M NaAc buffer. At ionic strength 0.2 the acetate buffer concentration must be increased to more than 0.1 M to affect the same change (Figure 1B).

The spectra obtained at ionic strength 0.2 and varying concentrations of acetate buffer (Figure 1B) reveal that, when the concentration of buffer is increased beyond that required to abolish the 240-mμ band, the band centered at 276 mμ becomes affected. As the concentration is increased, the band progressively decreases in intensity with an isosellipticity point until at the highest concentration attainable it is only 30% as intense as in 0.2 M NaCl–HCl. These effects are completely reversed by a 12.5-fold dilution at constant pH with 0.2 M NaCl–HCl. Nor is the effect of increasing NaAc buffer concentration due to the compensatory decrease in NaCl concentration required to maintain constant ionic strength. Thus, essentially the same results were obtained when the buffer concentration was increased from 0.05 to 0.2 M in the absence of NaCl. The only striking difference between the two sets of experiments is the loss of the isosellipticity point when the ionic strength is allowed to vary along with the buffer concentration. Furthermore, the effect of acetate buffer is attributable to interaction of the protein with undissociated acetic acid rather than with acetate anion. Thus, substitution of sodium dichloracetate for 0.1 M NaCl in a supporting medium containing 0.1 M NaAc buffer (spectrum a of Figure 1B) was without effect on the spectrum.

Short-chain homologs of acetic acid produce the same qualitative changes in the circular dichroism of RNase as described above for acetic acid. Moreover, as illustrated in Figure 2 they are much more effective than acetic acid, the order of increasing effectiveness being acetic < propionic < butyric; and like acetic acid, each shows an isosellipticity point. Indeed, all sets of spectra obtained at constant ionic strength for diverse solvent compositions, different pH values, and temperature exhibit isosellipticity points. This fact permits quantitation of the interaction in terms of two macromolecular species.

For an interaction of the type given by reaction eq 1, the cooperativeness, n, and equilibrium constant, K, can be evaluated by application of the law of mass action in the form

$$\ln \gamma = \ln K + n \ln [\mathrm{HA}] \tag{2}$$

$$\gamma = \frac{[\theta]_0 - [\theta]}{[\theta] - [\theta]_c} \tag{3}$$

where $[\theta]$ is the ellipticity at 276 mμ; and the subscripts 0 and c designate its value in 0.2 M NaCl–HCl and after complete conversion of the protein into its modified conformation, respectively. A concentration of acetic acid sufficiently high to drive the reaction to completion at 27° was not attainable at ionic strength 0.2, but such a concentration was readily realized with either propionic or butyric acid. Accordingly, the value of $[\theta]_c$ for acetic acid was taken to be that shown by its homologs. At elevated temperature $[\theta]_c$ could be realized with acetic acid. In all cases examined, the plot of ln γ *vs.* ln [HA] was approximately linear[2] for extents of reaction ranging from about 20 to 90% (Figure 3). The values of n and K presented in Tables I–III were obtained from the slope and inter-

[2] It is conceivable that this analysis is complicated by possible dimerization of aliphatic acids in aqueous solution (Schrier *et al.*, 1964), although the existence of such dimers is controversial (Jencks, 1969, pp 324–327).

TABLE I: Cooperativenesses and Equilibrium Constants for Interaction of Aliphatic Acids with Ribonuclease A (27.0°, Ionic Strength 0.2).

Aliphatic Acid	No. of Determinations	n[a]	K[b] (l. mole^{-1})^n
		In Water	
Acetic, pH 3.05	5	10.8 ± 0.62	$(3.42 \times 10^{-7})e^{\pm 0.815}$
Acetic, pH 2.55	4	7.1 ± 0.16	$(3.34 \times 10^{-4})e^{\pm 0.181}$
Propionic, pH 3.04	4	8.7 ± 0.51	$(2.83 \times 10^{-3})e^{\pm 0.374}$
Butyric, pH 3.06	5	8.1 ± 0.54	$(6.93 \times 10^{-1})e^{\pm 0.080}$
		In 20% Ethanol, pH 3.05[c]	
Acetic	6	5.0 ± 0.35	$(3.77 \times 10^{-2})e^{\pm 0.263}$
Propionic	8	3.5 ± 0.25	$(1.30)e^{\pm 0.057}$
Butyric	8	4.4 ± 0.19	$(8.03)e^{\pm 0.086}$
Valeric	6	4.1 ± 0.20	$(42.5)e^{\pm 0.202}$

[a] Mean plus or minus standard deviation. [b] Mean value in parentheses multiplied by antilogarithm of standard deviation of the natural logarithm of the mean. [c] The near-ultraviolet circular dichroism spectrum of RNase (0.2 M NaCl–HCl, pH 3.05, at 27°) is the same in 20% ethanol as in water.

TABLE II: Secondary Isotope Effect on the Interaction of Acetic Acid with Ribonuclease A (27.0°, pH (or pD) 3.05, Ionic Strength 0.2).[a]

Acid[b]	n	K (l. mole^{-1})^n	$(K_H/K_D)^{1/n}$
		In H_2O	
Acetic	10.8	3.42×10^{-7}	1.17
d_3-Acetic	11	6.0×10^{-8}	
		In D_2O	
d_1-Acetic	5.8	4.6×10^{-4}	1.10
d_4-Acetic	5.8	2.8×10^{-4}	

[a] Near-ultraviolet circular dichroism spectrum of ribonuclease A in D_2O (0.2 M NaCl–DCl, pD 3.05) was the same within experimental error, as in H_2O. [b] Five determinations on acetic acid in H_2O; two determinations in all of the other cases.

cept of the best linear fit to the data as given by the method of least squares.

Referring to Table I, it will be noted that the value of n for acetic acid at 27° is about 11. This is a gratifying result since there are 11 side-chain carboxyl groups in RNase (Tanford and Hauenstein, 1956), and it is concluded in the preceding paper (Cann, 1971) that acetic acid interacts with proteins by binding to carboxyl groups presumably *via* hydrogen-bond formation. This view is consistent with the large secondary isotope effect revealed by the results presented in Table II.

TABLE III: Effect of Temperature on the Cooperativeness and Equilibrium Constant for Interaction of Acetic Acid with Ribonuclease A (pH 3.05, Ionic Strength 0.2).[a]

Temp (°C)	No. of Determinations	n[b]	K[c] (l. mole^{-1})^n
20.0	2	11	4.8×10^{-8}
23.5	2	12	1.1×10^{-8}
27.0	5	10.8 ± 0.62	$(3.42 \times 10^{-7})e^{\pm 0.815}$
32.0	6	8.0 ± 0.40	$(5.37 \times 10^{-5})e^{\pm 0.512}$
37.0	7	6.1 ± 0.63	$(3.09 \times 10^{-3})e^{\pm 1.095}$

[a] Although in 0.2 M NaCl–HCl the intensity of the 240-mμ circular dichroism band decreased markedly with increasing temperature, the intensity of the 276-mμ band was within experimental error, independent of temperatures from 10 to 37°. [b] At 27° and above, mean plus or minus standard deviation. [c] At 27° and above, mean in parentheses multiplied by antilogarithm of standard deviation of the natural logarithm of the mean.

Deuterated acids like DCO_2H and d_5-phenol are weaker acids than their protium analogs (Jencks, 1969, p 255) and would be expected to form weaker hydrogen bonds.[3]

The value of n for acetic acid decreases by 30–50% and the value of K increases many orders of magnitude when the pH is lowered from 3.05 to 2.55 or when the solvent is changed from H_2O to 20% ethanol (Table I) or to D_2O (Table II). These results are attributed to electrostatic destabilization of the protein due to increased net positive charge, since we note that the pK of its carboxyl groups will be increased on going from H_2O to 20% ethanol (Cohn and Edsall, 1943) or to D_2O (Jencks, 1969, p 276) and that the midpoint temperature (T_m) of the thermal transition of RNase decreases with decreasing pH (Hermans and Scheraga, 1961). Other factors may also contribute; for example, weakening of hydrophobic bonds in the protein molecule by 20% ethanol (Gerlsma, 1968).

The role of hydrophobic forces in the interaction of RNase with aliphatic acids is underscored by the striking dependence of K on the length of the hydrocarbon moiety of the acid both in H_2O and in 20% ethanol (Table I). As shown in Figure 4, the logarithm of K is approximately a linear function of the number of carbon atoms, N, in the hydrocarbon moiety; and the dependence of ln K on N is weaker in 20% ethanol than in H_2O. The values of

$$\frac{\partial \Delta F^\circ}{\partial N} = -RT\frac{\partial \ln K^{1/n}}{\partial N} \quad (4)$$

calculated from these data are -400 ± 9 cal mole^{-1} (carbon atom)$^{-1}$ in H_2O and -300 ± 58 cal mole^{-1} (carbon atom)$^{-1}$ in 20% ethanol. These values are of the order expected for formation of a hydrophobic bond, *e.g.*, -520 cal mole^{-1} at

[3] The polarizability of d_5-phenol and deuterated molecules in general is slightly less than for their protium analogs (Ingold *et al.*, 1936; De Bruyne and Smyth, 1935); and thus, they would interact less strongly with nonpolar groups in the protein molecule. This would be a small effect, however.

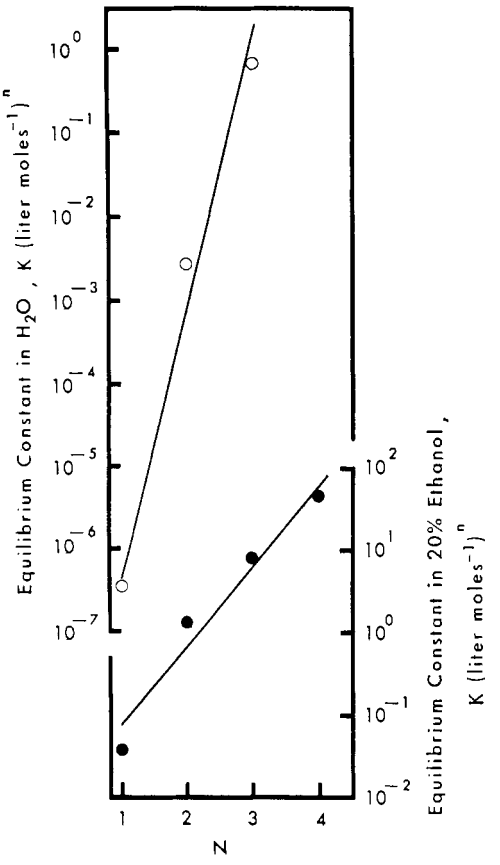

FIGURE 4: Semilogarithmic plot of the equilibrium constant, K, for interaction of normal short-chain aliphatic acids with RNase against the number of carbon atoms, N, in their hydrocarbon moieties.

27° for an alanine–alanine bond (Nemethy and Scheraga, 1962). The lower value in 20% ethanol relative to H_2O is also understandable, since ethanol should weaken hydrophobic interactions between the hydrocarbon moiety of the acid and groups in the protein.

The temperature dependence of the interaction has been examined in the case of acetic acid (Table III). A striking feature of the results is the decrease in the value of n upon raising the temperature from 27 to 37°, which dictates that the Van't Hoff equation be applied to the dependence of $K^{1/n}$ on temperature. Since the plot of ln $K^{1/n}$ against $1/T$ is nonlinear, a least-squares procedure was used to fit $-RT \ln K^{1/n}$ to a general quadratic function of T. The values of $\Delta H°$ and $\Delta S°$ derived therefrom are positive and increase linearly with increasing temperature (Figure 5). The magnitude of $n\Delta H°$ and $n\Delta S°$ is characteristic of protein reactions associated with conformational change; in the temperature range 27–37°, $n\Delta H° \simeq 65$ kcal (mole protein)$^{-1}$ and $n\Delta S° \simeq 190$ eu.

Far-Ultraviolet Circular Dichroism (Spectral Region, about 240–195 mμ). As described above 0.02 M NaAc buffer (pH 2.9) abolishes the 240-mμ band in the near-ultraviolet circular dichroism spectrum of RNase. In contrast, the far-ultraviolet circular dichroism spectrum in 0.02 M NaAc buffer is identical with the spectrum in 0.02 M NaCl–HCl. When the concentration of buffer is increased to 0.2 M NaAc (pH 3.05) however, there is an unusual change in the spectrum (relative to 0.2 M NaCl–HCl) characterized by enhanced intensity in the spectral region between about 240 and 227 mμ and diminished intensity between 227 and 205 mμ (compare curves a and b in Figure 6). The spectra displayed in Figure 6 were obtained at 27°; essentially the same results were obtained at 37° for 0.15 M NaAc–0.05 M NaCl which gives about the same extent of reaction, 90%, as 0.2 M NaAc at the lower temperature, 95%.

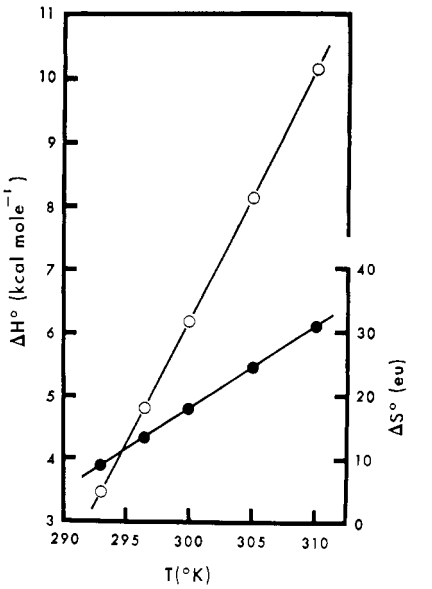

FIGURE 5: Plots of $\Delta H°$ and $\Delta S°$ for the reaction, $1/n\text{P} + \text{HAc} \rightleftharpoons 1/n\text{P}'(\text{HAc})_n$ against the absolute temperature, T. $\Delta C_p = 390$ cal deg^{-1} mole^{-1}.

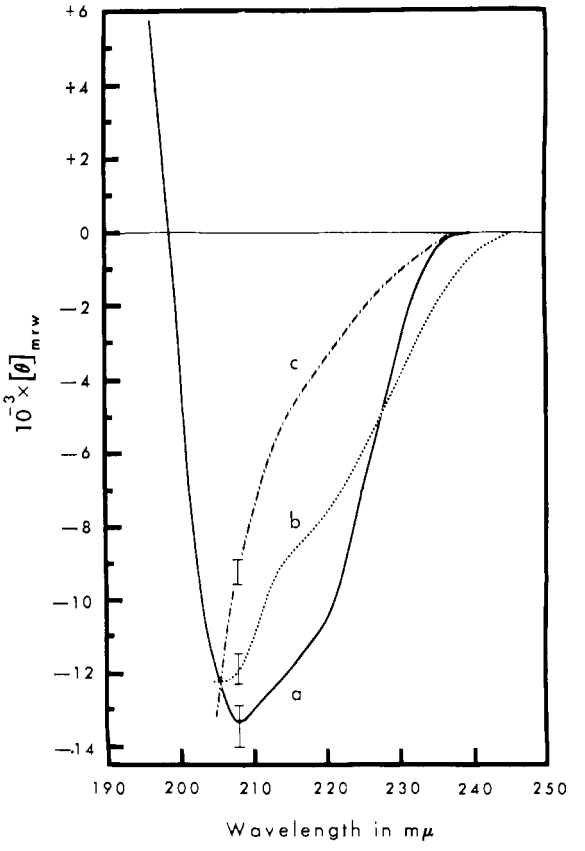

FIGURE 6: Effect of acetic acid or urea on the four ultraviolet circular dichroism spectrum of RNase: (a) 0.2 M NaCl–HCl (pH 3.05), (b) 0.2 M NaAc–5.02 M HAc (pH 3.05), and (c) 8 M urea (pH 8.1). a and b are averages of three determinations; c, average of two determinations. Error bars designate range of observations.

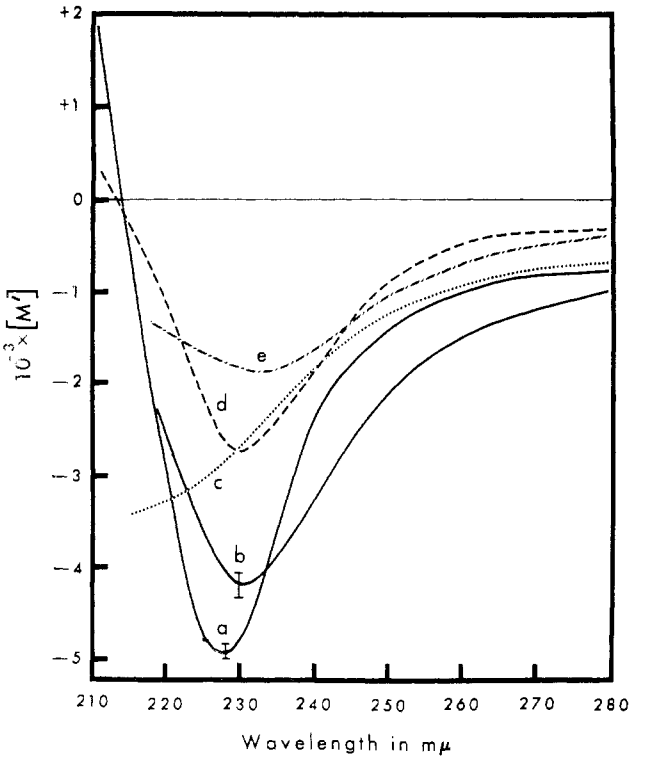

FIGURE 7: Effect of acetic acid and denaturing agents on the optical rotatory dispersion of RNase: (a) 0.2 M NaCl–HCl (pH 3.05) (average of two determinations), (b) 0.2 M NaAc–5.02 M HAc (pH 3.06) (average of two determinations), (c) 8 M urea at pH 7.9, (d) 30% *p*-dioxane containing 0.2 M NaCl–HCl (pH 3.04), and (e) 0.2 M NaCl–HCl (pH 3.04) at 72°. Error bars designate range of observations.

Optical Rotatory Dispersion (Spectral Region, 280 to about 210 mμ). Within a small experimental error, 0.02 M NaAc buffer (pH 2.9) has no effect on either the depth or shape of the 227-mμ trough of the far-ultraviolet negative Cotton effect shown by RNase. Experiments in 2 M urea analogous to those shown in Figure 1A for circular dichroism confirm that 0.02 M NaAc buffer destabilizes the protein to denaturation relative to 0.02 M NaCl–HCl.

In contrast to low acetate concentration, 0.2 M NaAc buffer (pH 3.05) does have a small effect on the 227-mμ trough (Figure 7). The somewhat broadened trough is shifted red by 3–4 mμ, and its depth is decreased by about 15%. These measurements were made at 27°. At 37° the trough in both chloride and acetate (0.15 M NaAc–0.05 M NaCl) is shallower by about 15% than at the lower temperature, but within experimental error the difference between the two spectra is the same.

Ultracentrifugation. The sedimentation coefficient of 0.49% RNase in 0.2 M NaCl–HCl (pH 3.07) has the value, $s_{20,w} = 1.82$ S. When 0.2 M NaAc buffer of the same pH is substituted for NaCl–HCl, the sedimentation coefficient decreases in value to $s_{20,w} = 1.45$ S.

Electrophoresis. Although the effect of acetate buffer on the electrophoretic patterns of RNase at pH 4 is rather weak (Cann and Phelps, 1959), the effect is quite strong at pH 3.7 (Figure 8A). This finding is consistent with circular dichroism measurements at these pH values. Thus, while carboxylic acid buffers have no significant effect on the near-ultraviolet spectrum at pH 4, they do affect the spectrum at pH 3.7. Whereas acetate and propionate buffers (ionic strength 0.15, 1.31 M acid) cause a graded and possible significant decrease in the

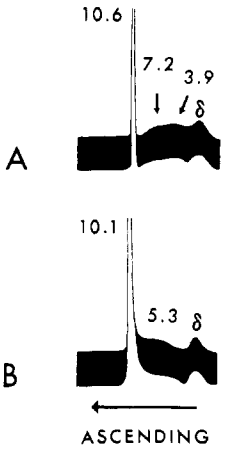

FIGURE 8: Ascending moving-boundary electrophoretic patterns of RNase: (A) 0.005 M NaAc–0.0458 M HAc–0.035 M NaCl, pH of dialyzed protein solution, 3.68; (B) 0.0036 M NaBu–0.0458 M HBu–0.0364 M NaCl (pH 3.66).

intensity of the 240-mμ band, butyric acid not only abolishes the 240-mμ band but decreases the intensity of the 276-mμ band by 50%. Accordingly, one might expect acetate and butyrate buffers to affect the electrophoretic patterns differently at pH 3.7. In particular, the more strongly interacting butyrate buffer should favor fast peaks over slow ones relative to acetate buffer (Cann and Phelps, 1959). Comparison of the ascending reaction boundaries displayed in Figure 8 shows that this is, in fact, the case.

Activity Measurements. The enzymatic activity of RNase has been measured at pH 3, 3.7, and 4.0 in solutions containing varying concentrations of acetate buffer at constant ionic strength and pH by three different methods: the Kunitz spectrophotometric assay; production of acid-soluble oligonucleotides from RNA; and the hydrolysis of 2′:3′-cyclic cytidylate using paper chromatography to separate product from substrate. The results of these measurements are summarized in Figure 9. It is evident that appropriate concentrations of acetate buffer inhibit enzymatic activity, and that there is a nice correspondence between the effect of acetate buffer on the activity of RNase and on its near-ultraviolet circular dichroism spectrum. Consider, for example, the results of the Kunitz spectrophotometric assay at pH 3 and ionic strength 0.2 (curve a, Figure 9A). Increasing the concentration of acetate buffer has little effect on activity below about 0.13 M, which is about the concentration at which acetate abolishes the 240-mμ circular dichroism band at said ionic strength (Figure 1B). Further increase in concentration causes a progressive decrease in activity which parallels the effect of acetate buffer on the intensity of the 276-mμ band (Figure 1B). Likewise, at ionic strength 0.05, increasing the buffer concentration from 0.02 to 0.05 M (curve b of Figure 9A) causes a 60% decrease in activity which correlates with abolition of the 240-mμ band. These findings are substantiated by the results of the other two assay methods (Figure 9B,C). Indeed, hydrolysis of 2′:3′-cyclic cytidylate has revealed that 0.2 M acetate buffer causes significant inhibition of enzymatic activity even at pH 3.7 and 4.0 (see legend to Figure 9), which is consistent with the forementioned effects of buffer acids on the circular dichroism at pH 3.7 and on the electrophoretic behavior at pH 3.7 and 4.0.

The possibility that the effect of acetate buffer on activity might be due to poisoning of the enzyme by trace metallic contaminants was eliminated by experiments in which the

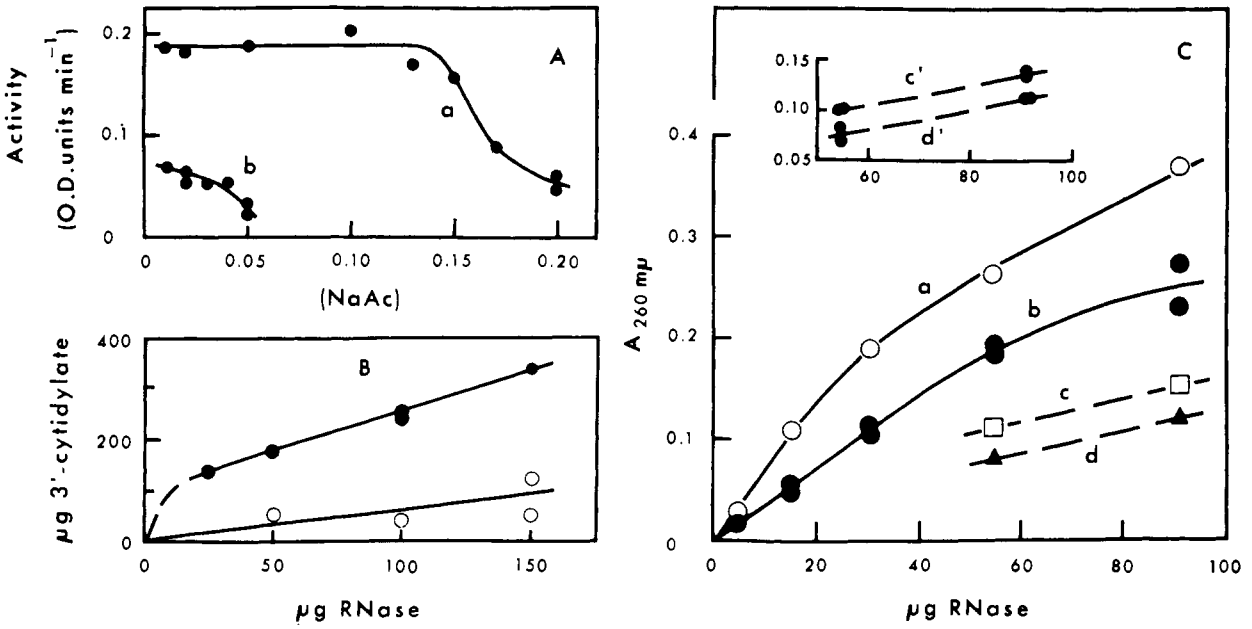

FIGURE 9: Effect of acetate buffer concentration on the activity of RNase at pH 3.05 and constant ionic strength: (A) Kunitz spectrophotometric assay; plot of activity (initial rate of change in OD) *vs.* molar concentration of NaAc at ionic strength 0.2 (curve a) and ionic strength 0.05 (curve b). (B) Hydrolysis of 2′:3′-cyclic cytidylate; plot of micrograms of 3′-cytidylate produced under standard conditions against micrograms of RNase in reaction mixture; (●) 0.05 M NaAc–1.26 M HAc–0.15 M NaCl; (○) 0.2 M NaAc–5.02 M HAc; the nonlinear relationship at high acetate concentration is undoubtedly due to product inhibition. (C) Production of acid-soluble oligonucleotides; plot of absorbance of reaction products at 260 mμ against micrograms of RNase in digestion mixture; curve a, 0.05 M NaAc–1.26 M HAc–0.15 M NaCl; curve b, 0.2 M NaAc–5.02 M HAc; curve c, 0.02 M NaAc–0.744 M HAc–0.03 M NaCl; curve d, 0.05 M NaAc–1.86 M HAc; curves c′ and d′ same as c and d but with a different lot of substrate. Hydrolysis of 2′:3′-cyclic cytidylate gave the following results at pH 3.66 (3 mg of substrate and 100 μg of RNase per ml): in 0.05 M NaAc–0.425 M HAc–0.15 M NaCl, 620 ± 10 μg of 3′-cytidylate; in 0.2 M NaAc–1.70 M HAc, 470 ± 6 μg. At pH 4.02 (1 mg of substrate and 25 μg of RNase per ml): in 0.01 M NaAc–0.387 M HAc–0.19 M NaCl, 214 ± 8 μg; in 0.2 M NaAc–0.760 M HAc, 160 ± 9 μg.

acetic acid was vacuum redistilled or the reaction mixtures contained 10^{-3} M EDTA.

Comparison to Denaturation. Interaction of acetic acid with RNase and urea denaturation of the protein share certain characteristic features. Thus, for example, the near-ultraviolet circular dichroism spectrum of RNase in 0.2 M NaAc–5.02 M HAc (pH 3.05) (curve e of Figure 1B) is quite similar to its spectrum in 8 M urea[4] at pH 8.2. Also, 0.2 M NaAc–5.02 M HAc produces a two-peaked, difference absorption spectrum (Figure 10A) characteristic of tyrosine spectral shifts and quantitatively similar to that produced by 8 M urea (Sela and Anfinsen, 1957; Bigelow and Geschwind, 1960). In 8 M urea all six tyrosine groups of RNase ionize normally (Blumenfeld and Levy, 1958), and it is generally held that denaturation exposes the three "buried" groups in the native protein (Tanford and Hauenstein, 1956) to the solvent. Accordingly, the difference in molar extinction coefficient at 287 mμ was corrected for solvent perturbation[5] of six tyrosine groups (Donovan, 1969). The corrected values are -3.07×10^3 for 0.2 M NaAc buffer and -2.92×10^3 for 8 M urea. There are two possible interpretations of this result. The simplest one is that interaction of acetic acid with RNase exposes the three "buried" tyrosine residues to the solvent. An alternative interpretation is that acetic acid enhances tyrosyl-charge or tyrosyl hydrogen-bonding interactions which in turn causes a blue shift that would be in addition to the blue shift attending exposure of buried tyrosine residues; this would give an apparently higher number of exposed tyrosine residues.

Although interaction with acetic acid and urea denaturation share the foregoing features, it cannot be concluded that they cause the same changes in macromolecular conformation. Comparison of the far-ultraviolet circular dichroism of RNase in 0.2 M NaAc buffer (pH 3.05) and in 8 M urea (Figure 6, curves b and c, respectively) and the optical rotatory dispersion in the two solvents (Figure 7, curves b and c) reveals that acetic acid is no where near as disruptive of structure as urea. The same comparative conclusion holds for interaction with acetic acid and heat denaturation (Figure 7, curves b and d).

The question remains whether the conformational change wrought by aliphatic acids is mediated by binding of acid molecules to the protein or is merely due to a change in water structure. The latter possibility seems to be eliminated by experiments with *p*-dioxane in solutions containing 0.2 M NaCl–HCl (pH 3.05). The results can be summarized as follows. (1) Although 30% (v/v) dioxane in water produces the same change in the near-ultraviolet circular dichroism of RNase as does 0.2 M NaAc buffer (about 30% HAc at pH 3.05), curve e in Figure 1B, it causes a much greater change in optical rotatory dispersion (compare curves b and e in Figure 7); (2) 20% dioxane decreases the intensity of the 276-mμ circular dichroism band by only 12%. In contrast, only half as much butyric acid, which is an isomer of dioxane,

[4] At 27° $[\theta]_{276} = -74°$ in 0.2 M NaAc (pH 3.05) and $-90°$ in 8 M urea (pH 8.2); the latter represents about a 60% reduction in intensity over that of the native protein. Pflumm and Beychok (1969a) report about a 40% reduction in intensity at pH 8.0, presumably, at room temperature. They also report that $[\theta]_{276}$ in 8 M urea decreases with increasing pH.

[5] It is assumed that the spectral perturbation produced by HAc is the same as NaAc. Some justification for this assumption is that urea and guanidium chloride produce the same perturbation (Donovan, 1969).

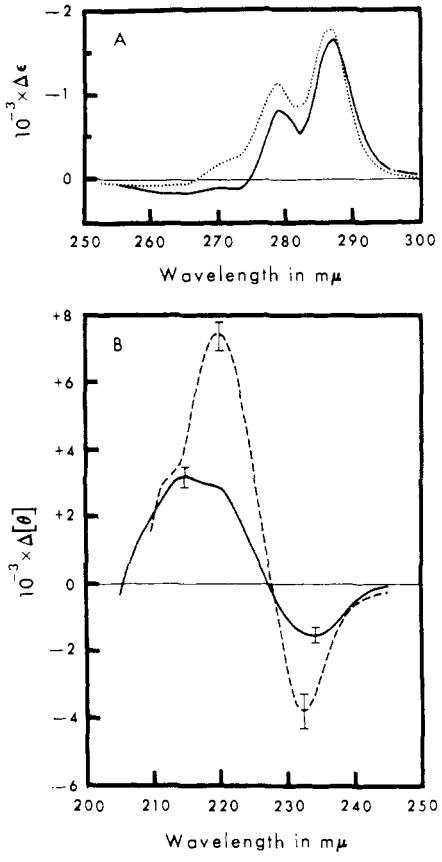

FIGURE 10: Comparison of the difference spectra of RNase and *N*-acetyltyrosinamide produced by 0.2 M NaAc–5.02 M HAc (pH 3.05) referred to 0.2 M NaCl–HCl in the first instance and methanol in the second: A, —, difference absorption spectrum of RNase, plot of molar extinction coefficient, $\Delta\epsilon$, *vs.* wavelength; · · · · ·, difference absorption spectrum for 3 moles of *N*-acetyltyrosinamide, 3 × $\Delta\epsilon$ plotted on same scale as $\Delta\epsilon$ for RNase. B, difference circular dichroism spectrum of RNase, —, and *N*-acetyltyrosinamide, ---; error bars designate mean deviations. The difference circular dichroism spectrum of RNase is the difference between curves a and b of Figure 6. Note that the difference circular dichroism spectrum of *N*-acetyltyrosinamide is not normalized to the tyrosine concentration found in the protein; normalization to three buried tyrosine residues is effected by multiplying the values of $\Delta[\theta]$ by 3/124, where 124 is the total number of amino acid residues in the protein.

decreases the intensity by 72% at the same ionic strength and pH. This result shows that the carboxyl group is essential for the interaction. (3) Near-ultraviolet circular dichroism spectra obtained at different concentrations of dioxane do not exhibit an isosellipticity point. In addition we note that (1) 20% HAc (ionic strength 0.2, pH 3) abolishes the 240-mμ circular dichroic band and decreases the intensity of the 276-mμ band significantly (curve b in Figure 1B), but 20% ethanol has no effect on the spectrum at pH 3; (2) only 5% HAc (ionic strength 0.02, pH 3.2) is sufficient to destabilize RNase to denaturation by 2 M urea; and (3) as little as 0.5% HAc (ionic strength 0.2, pH 5) promotes helix formation in poly-L-glutamic acid (Cann, 1971).

Measurements on N-Acetyltyrosinamide. Finally, it seemed pertinent to examine the spectral properties of the model compound, *N*-acetyltyrosinamide, in particular its difference spectra produced by 0.2 M NaAc buffer (pH 3.05), referred to methanol. In Figure 10A, the difference absorption spectrum for 3 moles of the model compound is plotted on the same scale as the molar difference spectrum of RNase produced in this case by the buffer referred to 0.2 M NaCl–HCl. The value of 3 × $\Delta\epsilon$ at 287 mμ for the model compound is seen to be -1.80×10^3. This is to be compared to the value, -2.35×10^3, for the molar extinction of RNase corrected for solvent perturbation of three tyrosine residues, *i.e.*, those accessible to the solvent in the native protein. Since the corrected value for RNase is the difference produced by the transfer of the three buried tyrosine residues into acetate buffer, it would appear that methanol makes a fairly good approximation to the environment of the buried residues.

The circular dischroism spectrum of *N*-acetyltyrosinamide has a prominent positive band at 226 mμ which has been attributed to the aromatic moiety of this compound (Beychok and Fasman, 1964; Pflumm and Beychok, 1969a). Recently, Ettinger and Timasheff (1971) reported that change in medium from aqueous to increasingly methanolic results in a red shift of this band without changes in intensity. We can confirm this observation and add that the red shift is accompanied by a 10% decrease in intensity when the solvent is changed from acetate buffer to absolute methanol. The difference circular dichroism spectrum produced by acetate buffer relative to methanol is presented in Figure 10B.

Discussion

We interpret our findings to mean that binding of undissociated acetic acid and its short-chain hemologs to RNase causes reversible changes in the tertiary structure of the protein without substantial alteration in secondary structure. Consider, for example, the effect of acetic acid on the 240-mμ circular dichroism band, which is known to be a most sensitive indicator of native conformation (Beychok, 1966; Pflumm and Beychok, 1969b). At low ionic strengths, relatively low concentrations of acid abolish the band but do not produce detectable changes in the 276-mμ band, the far-ultraviolet circular dichroism or the 227-mμ trough of the far-ultraviolet Cotton effect. Abolition of the 240-mμ band is accompanied by destablization of the protein to urea denaturation and a large decrease in enzymatic activity.

High concentrations of acetic acid cause more extensive alterations in structure characterized by exposure of buried tyrosine residues; a moderate increase in frictional coefficient corresponding possibly to a doubling of the effective volume of the protein molecule; large positive values of $n\Delta H^\circ$ and $n\Delta S^\circ$; and loss of enzymatic activity. It is clear, however, that acetic acid is much less disruptive of structure than either urea or heat. In fact, the small effect of acetic acid on the 227-mμ optical rotatory dispersion trough indicates that there is little change in the amounts of α helix and β structure in the protein. The most plausible explanation of these results is that binding of acetic acid causes the compact protein molecule to swell with dislocation of its N-terminal segment. The N-terminal segment comprises the 25 amino acid residues before the first S–S cross-link and contains the His-12 residue, which along with His-119 and Lys-41, is in the active site of the enzyme (Crestfield *et al.*, 1963a,b; Hirs, 1962; Marfey *et al.*, 1965; Wyckoff and coworkers, 1967). Thus, even a small dislocation of the right sort could result in loss of enzymatic activity. Support for this explanation is provided by the experiments of Crestfield and coworkers (Crestfield *et al.*, 1962; Fruchter and Crestfield, 1965a,b) on the structure of the active RNase dimer formed when RNase is lyophilized from 50% acetic acid and the product is dissolved in phosphate buffer. The dimer has two active sites, each containing a His-12 resi-

due from one monomer and a His-119 residue from the other. Evidently in 50% acetic acid the N-terminal segment (the first 20 residues of which are now known to be bonded to the surface of the hydrophobic core of the protein (Wyckoff *et al.*, 1967)) dissociates from the surface. Upon cooling the solution to −80° and lyophilizing, some of the dissociated segments are assumed to become bound to adjacent molecules in a manner such as to preserve the specific conformation required for enzymatic activity. In passing, Crestfield *et al.* (1962) note that the S-peptide can be separated from RNase S-protein by Sephadex filtration with 50% acetic acid as eluent.

Interaction of acetic acid and its homologs with RNase is a cooperative phenomenon, the value of n being interpreted as the number of undissociated acid molecules that must be bound in order for the structural transformation to occur. When considering possible mechanisms of interaction, it is instructive to recall the behavior of RNase in NaCl–HCl at low pH. RNase does not expand in acid solution at 25° and ionic strength 0.25, even when it attains its maximum positive change at pH 1 (Buzzell and Tanford, 1956). Undoubtedly, intramolecular hydrogen bonding and hydrophobic bonding taken together are sufficiently strong at 25° for the molecule to withstand the disruptive tendency of the repulsive electrostatic interaction between the positive charges on it surface. Extensive unfolding does occur, however, when the temperature is raised to only 40° (Tanford, 1961). While hydrophobic bonds become stronger with increasing temperature (up to about 60°), hydrogen bonds become weaker. Evidently, the balance of forces is now unfavorable for maintenance of native conformation. A similar combination of forces is implicated in the interaction of RNase with aliphatic acids.

Hydrogen bonding of acid molecules to side-chain carboxyl groups on the protein (Cann, 1971) has several consequences. First, the increase in pK of the side-chain groups increases the net positive charge on the protein at constant pH. This, in turn, increases the repulsive electrostatic force which tends to disrupt native conformation. Also, the X-ray crystallographic structure of RNase S (Wyckoff and coworkers, 1967) indicates that two of its carboxyl groups participate in either hydrogen bonding or charge–charge interactions with other residues: Asp-38 with Tyr-92, Asp-14 with Tyr-25 and with Arg-33. The Asp-14–Arg-33 interaction helps hold the S-peptide in place. Binding of aliphatic acid to these two carboxyl groups would certainly weaken the structure of the protein. Finally, hydrophobic bonding is of major importance for the interaction. Under certain conditions, *e.g.*, 0.2 M acetate buffer at pH 3, possible changes in water structure may weaken hydrophobic bonding within the protein molecule; but this cannot be the principal effect. We propose that the binding of aliphatic acid renders the surface of the protein, or parts thereof, hydrophobic or at least more so than in the absence of acid, for one can imagine the hydrocarbon moieties of the bound acid molecules as extending outward into the aqueous solvent. Since this would be an energetically unfavorable situation, we visualize a compensatory alteration of conformation such that the hydrocarbon moieties are withdrawn from contact with water and disposed in a nonpolar environment. Thus, binding of aliphatic acid to the protein upsets the balance of forces which determines native conformation, hydrophobic bonding of the hydrocarbon part of the acid with nonpolar groups in the protein molecule providing the ultimate driving force for the conformational change. These ideas are in accord with the observed dependence of the value of n and K on solvent composition, pH, length of the hydrocarbon moiety of the acid, and temperature. With respect to the temperature dependence of n, for example, it is to be expected that increasing the temperature will increase the strength of the hydrophobic bond formed between the hydrocarbon part of the acid and nonpolar groups in the protein. Accordingly, it should require fewer bound acid molecules to affect the change in conformation at higher temperatures.

Turning our attention to the effect of acetic acid on the circular dichroism of RNase, we seek an explanation on the one hand for the drastic reduction in the intensity of the near-ultraviolet band centered at 276 mμ and, on the other hand, for the unusual change in the far-ultraviolet spectrum. The latter is by far the more provocative, since the optical rotatory dispersion measurements speak against any major change in secondary structure. Consequently, an explanation must be sought in terms of perturbation of aromatic residues. In particular, we focus on the buried tyrosine residues which are exposed to the solvent by interaction of the protein with high concentrations of acetic acid.

The difference far-ultraviolet circular dichroism spectrum of RNase produced by 0.2 M NaAc buffer (pH 3) referred to 0.2 M NaCl–HCl is compared in Figure 10 with the difference spectrum of *N*-acetyltyrosinamide produced by acetate buffer referred to methanol. Both spectra show a negative band centered at about 233 mμ; a positive band in the spectral region, 215–220 mμ; and a crossover at 227–228 mμ. Moreover, the ratio of intensities at the extrema is the same in both cases. While these similarities are hardly fortuitous, it is disconcerting that the intensity of the difference spectrum of the protein is more than an order of magnitude greater than expected for transfer of three buried tyrosine residues into the solvent. (See legend to Figure 10 for normalization of the difference spectrum of *N*-acetyltyrosinamide to the tyrosine concentration in the protein.) This seeming paradox can apparently be resolved in terms of the theory of optical rotation and the X-ray crystallographic structure of RNase A and RNase S (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967). In their classical paper on the theory of optical rotation Kauzmann *et al.* (1940) point out that factors which decrease the freedom of rotation of groups about bonds will cause an increase in the first-order contribution to the optical activity. Two of the examples quoted are: (1) for quartz, sodium perchlorate, etc., the optical rotation goes from zero for the melt to exceedingly high values for the crystal; and (2) for tartaric acid, the amorphous acid at room temperature has a specific rotation of +0.76° at 592 mμ and +15.9 at 180° for 588 mμ, while the crystalline acid at room temperature has a specific rotation of −636° for 578 mμ. It is possible therefore that a tyrosine residue locked into relatively rigid position might exhibit a much larger circular dichroism than the freely rotating group in *N*-acetyltyrosinamide. The X-ray crystallographic structure of RNase S reveals that all 6 tyrosine residues are partly constrained. The titratable residues, Tyr-73, -76, and -115, have their hydroxyl ends exposed; Tyr-92 may be hydrogen bonded to Asp-38; Tyr-97 is completely buried and probably hydrogen bonded to Lys-41; and Tyr-25 may be hydrogen bonded to Asp-14 and is largely buried. (Tyr-25 would likely be completely buried in RNase A.) If one accepts the proposition that these residues have an anomalously intense circular dichroism due to hindered rotation and grants the possibility that after the buried residues are exposed to solvent they are still subject to a measure of constraint,[6] it becomes possible to reconcile the intensities of the difference

[6] No implication is meant as to the state of the residues exposed in the urea-denatured protein.

spectra of RNase and *N*-acetyltyrosinamide. Allowance must be made, of course, for the possibility that minor disruption of β structure may occur when RNase swells. In that event, there would be a positive contribution to its difference spectrum in the region of 217 mμ (Pflumm and Beychok, 1969a).

While it seems evident that at least some of the buried tyrosine residues which are exposed to the solvent by acetic acid retain a measure of constraint, the constraint on other of the tyrosine residues in the protein is evidently relieved by the interaction. This conclusion follows from the drastic reduction (about 75%) in the intensity of the near-ultraviolet circular dichroism band centered at 276 mμ. Horwitz *et al.* (1970) have assigned 40–50% of the strength of this band to disulfide bridges, 45–35% to the three exposed tyrosine residues, and 20–15% to the buried tyrosine residues. Pursuant to the argument presented above, the contribution of the tyrosine residues to this band would be greatly diminished by relief of the constraint on some of them. Also, the contribution of the disulfide bridges would be altered by alteration of the protein conformation in their immediate environs.

In conclusion, the results described above and in the companion paper, when taken along with the theory of electrophoresis of interacting systems, provide the understanding required for unambiguous interpretation of the electrophoretic behavior of proteins in media containing acetate or other carboxylic acid buffers. It emerges from these investigations that electrophoresis is one of the most sensitive methods available for detecting and characterizing interactions of proteins with small molecules. Moreover, the interaction of proteins with acetic acid provides a model for specific allosteric interactions. RNase is a particularly good choice for this purpose since its X-ray crystallographic structure provides the fundamental information required for a detailed description of its reactions.

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