[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY, ITHACA, NEW YORK]

Structural Studies of Ribonuclease. V. Reversible Change of Configuration[1-3]

BY JAN HERMANS, JR., AND HAROLD A. SCHERAGA

RECEIVED OCTOBER 7, 1960

The reversible change of configuration in ribonuclease with varying *p*H and temperature was studied with ultraviolet difference spectra and optical rotation measurements. The increase in $-[\alpha]$ (indicating loss of helical content) and the "blue shift" of the ultraviolet absorption spectrum (indicating changes in the environment of the tyrosyl groups) parallel each other in the denaturation. Equilibrium data were obtained for the configurational change. These include: the *p*H-dependence of T_{tr} and $\Delta F^0_{obsd.}$, and the value of $\Delta H^0_{obsd.}$ (equal to 51 kcal./mole below T_{tr}, and independent of *p*H). Above T_{tr}, $\Delta H^0_{obsd.}$ increases, suggesting that more than one denatured form participates in the equilibrium above T_{tr}. A theory was developed to account for the *p*H-dependence of T_{tr} and $\Delta F^0_{obsd.}$ in terms of local interactions and general electrostatic effects, based on four different possible models. Applying the theory at temperatures up to T_{tr}, it was possible to rule out two of the models. A distinction between the other two models was not possible. As a result, it is concluded that the native molecule contains one (or possibly two) carboxyl groups with pK^H of 2.5. The intrinsic pK becomes normal (4.6) upon denaturation, this group (or groups) making an important contribution to the *p*H-dependence of T_{tr} and $\Delta F^0_{obsd.}$ for denaturation. Finally, examples are cited to illustrate the importance of the configuration of ribonuclease in determining its reactivity toward various reagents.

Introduction

In a previous paper of this series[5] a three-dimensional model was proposed to account for the available data on the primary, secondary and tertiary structure of ribonuclease. Part of the evidence for this model was obtained from studies of the effect of *p*H and temperature on the reversible change of configuration of ribonuclease in aqueous solution. The present paper considers the details of this configurational change which had been discovered by Harrington and Schellman.[6]

The existence of a configurational change in the ribonuclease molecule was inferred from the observation that the specific optical rotation and specific viscosity of ribonuclease solutions undergo reversible changes with temperature.[6,7] It has also been shown[6,8,9] that the optical density of ribonuclease solutions decreases in the region near 287 mμ at room temperature when the *p*H is lowered from 7 to 1. This has been attributed to a change in the environment of tyrosyl groups. The data reported here show that the changes in optical rotation and in optical density, which occur when the temperature or the *p*H of the solutions is varied, parallel each other.[10] A theoretical treatment of the *p*H-dependence of this reversible configurational change provides information about the forms in equilibrium with each other.

Experimental

Materials.—Armour ribonuclease (lot 381–059) was used without further treatment. The concentration of stock solutions of ribonuclease was determined by measuring the optical density of a suitably diluted aliquot in 0.01 *M*, *p*H 7 phosphate buffer at 278 mμ. An extinction coefficient of 0.715 cm.²/mg., obtained with the aid of a dry-weight determination, was used to compute the concentration.[11]

Glycine (lot 3245) was obtained from Mann Laboratories. All other materials were reagent grade.

***p*H Measurements.**—The *p*H's were measured on the A scale of a Beckman GS *p*H-meter using a Beckman 290–32 glass electrode and a 3.15 *N* KCl calomel electrode. Beckman *p*H 7 buffer and 0.05 *M* KH phthalate and 0.01 *M* sodium borate, prepared according to Bates,[13] were used as *p*H standards.

Difference Spectra.—Ultraviolet difference spectra were obtained with a Beckman DU spectrophotometer equipped with a photomultiplier attachment and thermospacers.[14,15] Glass-stoppered 1 cm. silica cells were used for the solutions. About 30 min. were allowed for thermal equilibrium at each temperature before carrying out spectrophotometric measurements.

The concentration of ribonuclease was 1.90 mg./ml. At low *p*H the desired ionic strength was obtained with KCl and HCl. For *p*H-values above 2 the solutions were buffered with 0.01 *M* citrate, formate, glycine, acetate, carbonate or phosphate, depending on the *p*H, and the remainder of the ionic strength was obtained with KCl.

The difference in optical density, ΔD, between two solutions was measured as a function of wave length, λ. The optical density difference, ΔD_λ, at which ΔD exhibits a maximum, then was studied as a function of *p*H and temperature. In addition, a parameter y may be defined by the equation

$$y = \Delta D_\lambda / (\Delta D_\lambda)_{max} \qquad (1)$$

where $(\Delta D_\lambda)_{max}$ is the largest value of ΔD_λ (*i.e.*, − 0.25) obtained by varying the *p*H and temperature of both solutions. Values of ΔD have also been converted to molar extinction coefficients, $\Delta\epsilon$, expressed in liters/mole/cm., using the known concentration and molecular weight.

Limited access was had to a Cary recording spectrophotometer for a few experiments.[16] With this instrument it is possible to make measurements at high optical density with much greater precision than is obtained with the Beckman DU spectrophotometer. The absorption cell containing the solution was thermostatted with a heating jacket through which water was circulated. The cell containing the solvent was maintained at room temperature. Thus, the dependence of the optical density of the protein solution on temperature at any given wave length could be determined.

(1) This investigation was supported by research grant E-1473 from the National Institute of Allergy and Infectious Diseases, of the National Institutes of Health, U. S. Public Health Service, and by research grant G-6461 from the National Science Foundation.

(2) Presented before the Division of Biological Chemistry at the 134th meeting of the American Chemical Society, Chicago, Illinois, September, 1958.

(3) A summary of this work has been reported.[4]

(4) H. A. Scheraga, C. Y. Cha, J. Hermans, Jr., and C. L. Schildkraut, "Amino Acids, Proteins and Cancer Biochemistry," Academic Press, Inc., New York, N. Y., 1960, p. 31.

(5) H. A. Scheraga, *J. Am. Chem. Soc.*, **82**, 3847 (1960).

(6) W. F. Harrington and J. A. Schellman, *Compt. rend. trav. Lab. Carlsberg, Ser. Chim.*, **30**, 21 (1956).

(7) C. Tanford and R. E. Weber, private communication.

(8) H. A. Scheraga, *Biochem. et Biophys. Acta*, **23**, 196 (1957).

(9) M. Sela and C. B. Anfinsen, *ibid.*, **24**, 229 (1957).

(10) The changes in specific viscosity and optical rotation also parallel each other.[7]

(11) A value of 0.71 cm.²/mg. was obtained by N. Pirzadeh and B. Bonnelycke, as quoted in ref. 12.

(12) P. J. Keller, E. Cohen and H. Neurath, *J. Biol. Chem.*, **233**, 344 (1958).

(13) R. G. Bates, "Electrometric *p*H Determinations," John Wiley and Sons, Inc., New York, N. Y., 1954, pp. 118–119.

(14) J. Hermans, Jr., J. W. Donovan and H. A. Scheraga, *J. Biol. Chem.*, **235**, 91 (1960).

(15) M. Laskowski, Jr., S. J. Leach and H. A. Scheraga, *J. Am. Chem. Soc.*, **82**, 571 (1960).

(16) We are indebted to Profs. P. L. Hartman and R. L. Sproull of the Physics Department for making this instrument available to us.

Optical Rotation.—Optical rotation data were obtained at 436 mμ with a Rudolph Model 80Q high precision polarimeter equipped with a quartz monochromator and oscillating polarizer. The polarimeter tube had a length of 20 cm. and a diameter of 2 mm. Water from a thermostat was run through a jacket around the tube and then through the tube housing. The solutions used were the same as were studied spectrophotometrically.

Results

Effect of Temperature on Difference Spectra.—Previous experiments[8] on the ultraviolet difference spectrum of ribonuclease were carried out at approximately 25° and 0.1 *M* ionic strength where a limiting value of about −800 was obtained for $\Delta\epsilon_{287}$ at low *p*H. In the experiments reported here a similar limiting value of $\Delta\epsilon_{287}$ was obtained; however, it was found to be dependent on the temperature. In studying the temperature dependence of the difference spectrum it was first necessary to establish that Beer's law holds at elevated temperatures, as had been shown previously[8] at 25°. For this purpose pairs of ribonuclease solutions, at *p*H 1.2 and 6.5 (measured at 45°) and ionic strength 0.08 *M*, were compared against each other at 45°. The optical density difference, ΔD, of the solution of low *p*H with respect to the reference solution at high *p*H (at the same protein concentration) was measured as a function of wave length. The results are shown in Fig. 1, the three curves corresponding to protein concentrations of 1.90, 1.27 and 0.63 mg./ml., respectively, the former being the concentration used for all subsequent spectrophotometric and polarimetric measurements. The values of $\Delta D_{287}/c$, c being the concentration in mg./ml., are 0.127, 0.125 and 0.127 cm.^{3}/mg., showing that Beer's law holds at this temperature.

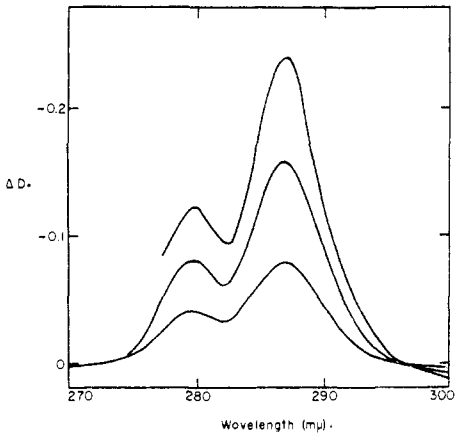

Fig. 1.—Optical densities of solutions of ribonuclease at *p*H 1.2 measured against solutions of the same concentration at *p*H 6.5 at 45° in 1 cm. cells: ionic strength 0.08 *M*. The concentrations are 1.90, 1.27 and 0.63 mg./ml. for the top, middle and bottom curve, respectively. The *p*H 6.5 solutions were the reference of zero optical density.

In order to carry out measurements of the temperature-dependence of the difference spectra with the Beckman spectrophotometer, it was necessary to show that the optical density of the reference solution is independent of temperature. For this purpose the following experiments were carried out with a Cary spectrophotometer. The optical densities of ribonuclease solutions (*p*H 0.9 and *p*H 7, respectively) were each measured against *water* at a series of temperatures. Whereas such experiments cannot be carried out with sufficient precision with the Beckman spectrophotometer at the high optical densities of the protein solutions used here, it is possible to do so with the Cary instrument. It was found that the optical density of the *p*H 7 solution at 287 mμ is independent of temperature up to 43° and decreases above this temperature; the optical density of the *p*H 0.9 solution at 287 mμ decreases with the temperature from 25 to 40°[17] and becomes independent of temperature above 40°. Therefore, in subsequent experiments with the Beckman spectrophotometer, a *p*H 7 solution was used as the reference below 43° and a *p*H 0.9 solution as the reference above this temperature.

The variation of ΔD_{287} with temperature at a series of *p*H values, at an ionic strength of 0.16 *M*, is shown in Fig. 2. These data were obtained with the Beckman instrument in the following manner. A solution at any given *p*H was measured against a reference solution (*p*H 6.8 or *p*H 0.9, as discussed above) at a series of temperatures, both solutions being at the same temperature. Even though the *p*H 0.9 solution was the reference above 43°, all the data above 43° have been calculated with respect to a hypothetical reference by subtracting the value of ΔD_{287} (with respect to the *p*H 0.9 solution) from −0.244, the latter being the value of ΔD_{287} for the solution of *p*H 0.9 with respect to the *p*H 6.8 reference at 43°. In this manner, all the data of Fig. 2 could be plotted on a comparable scale. The vertical line at 43° in Fig. 2 divides the two regions where the *p*H 6.8 and *p*H 0.9 solutions, respectively, were used as the reference.

Reversibility.—Since we shall later discuss the data of Fig. 2 in terms of a theory of reversible denaturation, it is necessary to determine whether the processes giving rise to these curves are reversible. We shall regard such processes as reversible if the values of ΔD_{287} obtained by cooling the solu-

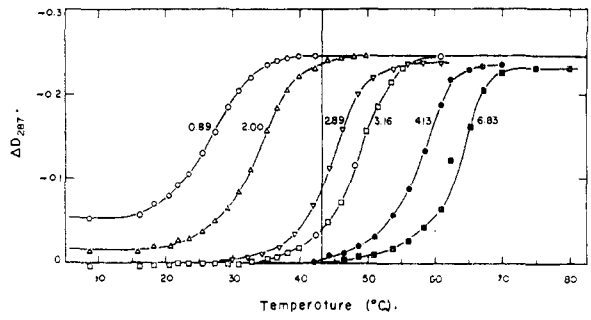

Fig. 2.—Optical densities of solutions at 1.90 mg./ml., ionic strength 0.16 *M*, as a function of temperature, when compared with a reference solution of zero optical density at *p*H 6.83 and low temperature (<43°). The *p*H's of the solutions are indicated. The actual measurement requires the use of the *p*H 6.83 solution as the reference to the left of the vertical line at 43°, and the use of the *p*H 0.9 solution as the reference to the right of this line, as explained in the text.

(17) The behavior below 25° was also investigated but with the Beckman rather than with the Cary spectrophotometer (see below).

tions agree with those obtained on the heating part of the cycle.

Ribonuclease can be completely, irreversibly denatured if a 1.90 mg./ml. solution at *p*H 7 is heated at 95° for 20 minutes. Such a preparation is enzymatically inactive. Its absorption spectrum, *at all temperatures between 0 and 80°*, is the same as that of a reversibly denatured protein *at 80°*.[18] Similarly, the optical rotation of reversibly and irreversibly denatured ribonuclease are the same *at high temperature*.[19,20] Furthermore, the same spectrum is observed when ribonuclease is irreversibly denatured at low *p*H (say *p*H 0.9, 45 min. at 100°) and also when such low *p*H material is brought to *p*H 6.5. Since ribonuclease is *reversibly* denatured at *p*H 0.9 and 45°, by the above criterion, if kept under these conditions for only 10 min. it is seen that the irreversibly unfolded molecules are indistinguishable from the reversibly unfolded ones by means of spectrophotometric (and also optical rotation[20]) measurements. This property makes it possible to determine the extent of irreversible denaturation and, at the same time, provides an estimate of the error made by incorrectly treating the data of Fig. 2 as if the irreversibly denatured material were reversibly denatured.

The test of reversibility of the curves of Fig. 2 was made at both extremes of the *p*H range, *viz.*, *p*H 0.9 and 6.8 between the temperatures 15 and 80°. If two solutions (one at *p*H 0.9 and the other at *p*H 6.8) are heated from 15 to 43° and then cooled to 15°, the 15° value of ΔD should be re-attained at the end of the cycle if the processes are reversible. In actual practice it is found that the new value of ΔD at 15° depends on the length of time that the solutions were maintained at 43°, *i.e.*, there is an augmentation in $-\Delta D$ at 15° which increases with the time during which the solutions were at 43°. Approximately 30 min. are required to attain thermal equilibrium at each temperature. In this minimum period of 30 min. at 43° less than 5% of the material has become *irreversibly* denatured, as indicated by the slight increase in $-\Delta D$ at 15°. Comparison with solutions of *p*H 0.9 and 6.8, which had not been heated, showed that the entire increase in $-\Delta D$ upon returning the heated solutions to 15° is attributable to the presence of irreversibly denatured material in the *p*H 0.9 solution, *i.e.*, there is no denaturation (reversible or irreversible) in the *p*H 6.8 solution kept at 43° for 30 min.[21]

(18) If the solution is brought to 80° rapidly, the spectrum measured, and the solution rapidly cooled, the denaturation is reversible. However, if the protein is kept for a long period at the elevated temperature, then it becomes irreversibly denatured.

(19) J. Hermans, Jr., and H. A. Scheraga, unpublished results.

(20) J. Hermans, Jr., and H. A. Scheraga, *Biochim. et Biophys. Acta*, **36**, 534 (1959).

(21) The criterion for reversibility stated at the beginning of this section requires superposition of the curves found on the cooling and those found on the heating part of the cycle. Our reversibility measurements concern only the initial and final values at the low temperature end of the cycle; *i.e.*, we did not perform measurements at intermediate temperatures. For these intermediate temperatures we refer to the results obtained by Harrington and Schellman,[6] who found a very slight hysteresis, but also an increase in the low-temperature value of $-[\alpha]_D$ after completion of the cycle. The slight degree of irreversible denaturation, which corresponds to this increase, accounts for the hysteresis. If the cycle were carried out rapidly, no denaturation would occur and hysteresis would presumably be absent.

It has been observed that no irreversible denaturation occurs if the maximum temperature in the heating part of the cycle does not exceed the transition temperature,[22] T_{tr}. Between the transition temperature (27°) at *p*H 0.9 and 43°, a measurable amount of irreversibly denatured material appears, the maximum amount being less than 5%, as indicated above, if the heating time does not exceed 30 min.

The question of reversibility at *p*H 6.8 may be considered in a similar manner, except that now the heating and cooling cycle[23] occurs between 43 and 80°. Upon re-attaining 43°, the increase in $-\Delta D$ is attributed to the irreversibly denatured material, whose concentration can be determined. Thus, the denaturation of a solution at *p*H 6.8 in a heating cycle was carried out, and the spectrum of the *p*H 6.8 solution with respect to a *p*H 0.9 solution was measured. After the measurements at each temperature, the temperature of the solutions was returned to 43° and the difference spectrum again measured; changes in optical density with respect to that found initially were interpreted as indicating the presence of irreversibly denatured material in the *p*H 6.8 solution. By this procedure, the fraction of irreversibly denatured material was found to be insignificant after 30 min. periods at 50 and 55°, respectively. At temperatures above 55°, the per cent. of irreversibly denatured material increased with each measurement at a higher temperature, reaching 35% after the measurement at 80°. The per cent. of irreversibly denatured material increased with the length of time at the elevated temperatures. The values of ΔD were corrected for the presence of irreversibly denatured material, once the concentration of such material was determined. The corrected data yielded a smoother curve of ΔD *vs.* T than did the uncorrected data. The corrected curve showed a value of T_{tr} at *p*H 6.8 which was only 0.3° higher than the uncorrected curve in Fig. 2. Since the time of heating in these reversibility tests was longer than usual, the actual error in the data of Fig. 2 is probably less than 0.3°, which is insignificant as far as the use to which the data of Fig. 2 will be put is concerned.

Having considered the reversibility problem at the extremes of *p*H and temperature, we may regard the transition temperatures at intermediate *p*H's (Fig. 2) as characteristic of the reversible denaturation.

Finally, it may be noted that the curves in Fig. 2 are not symmetrical about T_{tr}. While this skewness will be discussed further below, it is of interest to note here that the smooth curve, obtained by correcting for the presence of irreversibly denatured material, is qualitatively just as unsymmetrical as the uncorrected one at *p*H 6.83 in Fig. 2.

Effect of Temperature on Optical Rotation.—Optical rotation measurements were made on three of the solutions used to obtain the data of Fig. 2.

(22) The transition temperature is defined as the temperature at which half of the material is *reversibly* denatured and is taken as the midpoint of each of the curves of Fig. 2.

(23) If the *p*H 0.9 solution is kept above 43°, the protein slowly becomes irreversibly denatured, without any change in spectrum. Such a solution is, therefore, a useful reference for observing the changes in the spectrum of the *p*H 6.8 solution above 43°.

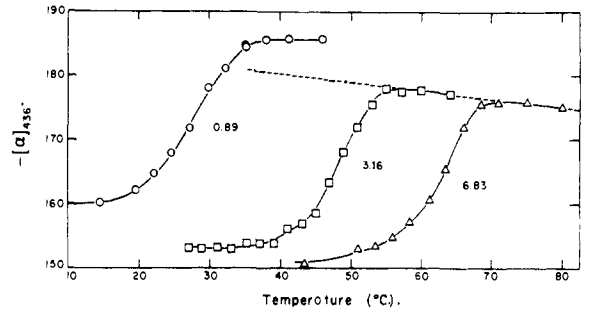

Fig. 3.—Optical rotation of ribonuclease solutions as a function of temperature at three *p*H values. The dotted line is the linear extrapolation of the high-temperature values of $[\alpha]_{436}$ to low temperatures. The values determined by this line are taken to be $[\alpha]_{436}$ of completely unfolded ribonuclease at any given temperature.

The specific rotation data are shown in Fig. 3. In cases where the maximum value of $[\alpha]$ varies with temperature, as it does for the *p*H 3.16 and *p*H 6.83 solutions, the maximum change in $[\alpha]$, *i.e.*, $\Delta[\alpha]_{max}$, is computed[6,7] at each temperature by linearly extrapolating the high temperature values of $[\alpha]$ to the temperature at which $\Delta[\alpha]$ was measured. The justification of this procedure is that the optical rotation of solutions of oxidized ribonuclease, which is in a randomly-coiled form,[6] shows a similar gradual decrease with increasing temperature over an appreciable temperature range. It appears that a single (dotted) line represents the extrapolation at both *p*H's. With the values of $\Delta[\alpha]_{max}$ thus determined, it is possible to compute a value of y, the fraction converted, where y is defined as

$$y = \Delta[\alpha]/\Delta[\alpha]_{max} \qquad (2)$$

where $\Delta[\alpha]$ is the increment at any temperature over the low-temperature limiting value of $[\alpha]$. The values of y from eq. 2 are plotted in Fig. 4, where filled symbols represent optical rotation measurements. Similar values of y, computed from the spectrophotometric data of Fig. 2 according to eq. 1, are represented in Fig. 4 by open symbols. It

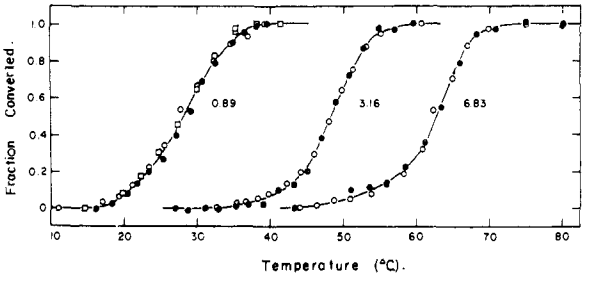

Fig. 4.—Comparison between optical densities and optical rotation. The fraction of the molecules converted to the denatured form as determined by the value of ΔD (open symbols) and by the value of $[\alpha]_{436}$ (closed symbols) are plotted against temperature.

may be noted that the changes in optical density and in optical rotation parallel each other. (Use of equation 1 requires that the values of ΔD_λ and $(\Delta D_\lambda)_{max}$ be measured at the same temperature. However, since $(\Delta D_{287})_{max}$ is essentially independent of temperature, the value of $(\Delta D_{287})_{max}$ at each *p*H could be used to compute the values of y in Fig. 4 at varying temperatures.)

Equilibrium Constants.—It is possible to define an equilibrium constant $K_{obsd.}$ by

$$K_{obsd.} = y/(1 - y) \qquad (3)$$

Values of $K_{obsd.}$ may thus be computed at any *p*H and temperature from the data of Fig. 2. These are plotted as log K *vs.* $1/T$ at each *p*H in Fig. 5.

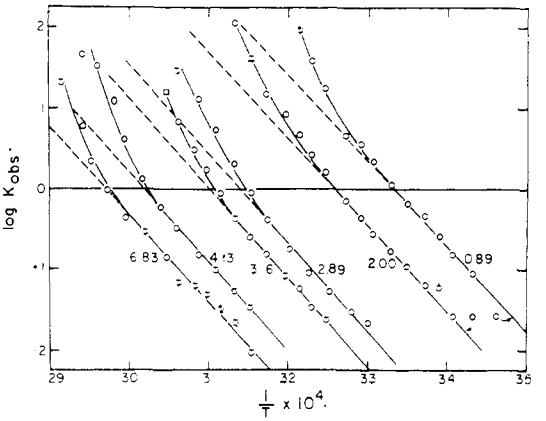

Fig. 5.—The logarithm of the equilibrium constant for the denaturation reaction, determined from the values of ΔD, is plotted against the reciprocal of the absolute temperature. Six parallel straight lines have been drawn to approximate the results at low temperatures (high $1/T$). At high temperatures the results deviate from these lines.

It may be noted that straight lines are obtained at low temperatures (high values of $1/T$) at each *p*H but that curvature appears at high temperatures.

The straight lines of Fig. 5 are parallel with a slope d log $K_{obsd.}$/d$(1/T) = -1.1 \times 10^4$. From this a value of 51 kcal./mole may be computed for the heat of reaction by means of the equation

$$\Delta H^0_{obsd.} = -R \frac{\mathrm{d} \ln K_{obsd.}}{\mathrm{d}(1/T)} \qquad (4)$$

The curvature (arising from the skewness of the curves in Fig. 2) leads to higher values of $\Delta H^0_{obsd.}$ at higher temperatures, as has been reported.[6,7] The curvature may be attributed to the existence of more than one reversibly denatured form of ribonuclease at high temperatures. If this is so, then the various (reversibly and irreversibly) denatured forms have the same specific rotation and ultraviolet absorption. This is not unreasonable in view of the fact, already cited, that the low temperature reversibly and high-temperature irreversibly denatured forms are indistinguishable by both of these properties. It may be noted that the curvature persists even when a correction is made for the presence of *irreversibly* denatured material.

While we have postulated a small number of denatured forms to account for the skewness, Schellman[24] has shown how essentially the same amount of skewness may arise from the existence of a large number of different size helices in a polypeptide chain of given length. As a result of "unraveling" of the ends of the helical portions the

(24) J. A. Schellman, *J. Phys. Chem.*, **62**, 1485 (1958).

transition is broadened[24] on the side where the helices predominate (*i.e.*, below T_{tr}). However, it should be noted at this point that the changes in the ΔD data (which arise from abnormal tyrosyl groups) and in the $[\alpha]$ data (which indicate the "helix content") parallel each other. This parallelism can best be explained by assuming that the disruption of the interaction which is responsible for the abnormality of the (at most three) tyrosyl groups is the result of a change in the folding in a limited part of the molecule, at least in its early stages. This interpretation of the parallelism of the ΔD and $[\alpha]$ data provides some support for our treatment of the initial stage of the transition as a one-step equilibrium.

Since the high temperature forms of reversibly denatured material (and also irreversibly denatured material) do not make a significant contribution to the value of the transition temperature,[25] up to T_{tr}, the values of T_{tr} are not significantly in error, as already pointed out. They are plotted against *p*H in Fig. 6, which also includes the results of some curves not represented in Fig. 2. In the *p*H range covered, T_{tr} appears to be independent of *p*H below *p*H 0.5 and above *p*H 6. According to theory,[26] the values of T_{tr} should decrease in the alkaline range. However, we have not yet investigated the alkaline *p*H range. On the other hand, the decrease of T_{tr} in both the acid and alkaline ranges *has* been observed with ribonuclease *films*.[27]

In Fig. 6, the points represented by the open circles were obtained with solutions each containing one of the carboxylic acid/carboxylate buffers indicated in the Experimental section. The filled circles correspond to 0.01 *M* phosphate buffer and the open triangle to 0.005 *M* phosphate buffer. The higher transition temperature in phosphate buffer indicates that phosphate stabilizes the native ribonuclease molecule, as it also does in 8 *M* urea.[28]

Effect of Ionic Strength.—Since T_{tr} becomes independent of *p*H below *p*H 0.5 and above *p*H 6, it can be seen that the maximum change in T_{tr}, *i.e.*, $(\Delta T_{tr})_{max}$, with *p*H at ionic strength 0.16 is 35.6°. In order to decide whether electrostatic effects play a role in determining the value of $(\Delta T_{tr})_{max}$, this quantity was re-determined at several ionic strengths by means of ultraviolet difference spectra measurements. The results are shown in Table I. No data were obtainable at *p*H 0.7 below 0.16 *M* since the *p*H cannot be lowered sufficiently at lower ionic strength.

The value of T_{tr} at *p*H 0.7 increases considerably with increasing ionic strength. Consequently $(\Delta T_{tr})_{max}$ decreases. Since the protein carries a rather high positive charge at this *p*H, it might appear that the higher ionic strength shields the charges, reducing electrostatic repulsions which could be important in denaturation and which would (in the absence of salt) decrease T_{tr}, *i.e.*, salts appear to stabilize the native protein. In agreement with this notion, the effect of ionic strength is smaller at *p*H 6.5 where the charge on the protein is smaller than at *p*H 0.7. It thus seems that electrostatic forces may contribute to the *p*H-dependence of the stability of the native molecule. Unfortunately, there are some anomalous effects of ionic strength, for which we have no explanation,

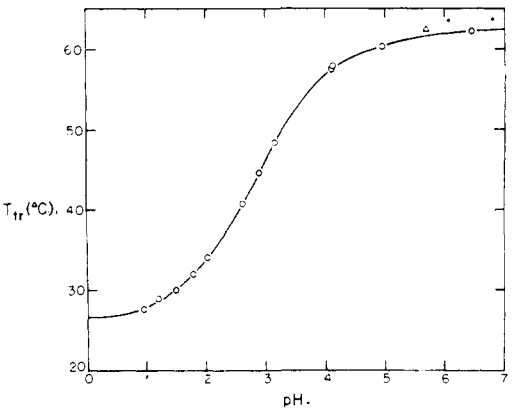

Fig. 6.—The transition temperature, which was determined from the optical density data of Fig. 2 and a number of similar experiments, is plotted against *p*H. The horizontal parts at both high and low *p*H should be noted. Open circles refer to solutions with only HCl and KCl to make up the desired *p*H and ionic strength (0.16 *M*). At higher *p*H's the solutions contained 0.16 *M* KCl and 0.01 *M* of one of several carboxylic acids as a buffer. The filled circles were obtained with solutions containing 0.01 *M* phosphate as the buffer, the triangle, 0.005 *M* phosphate.

TABLE I

TRANSITION TEMPERATURES AT EXTREME *p*H'S AT VARIOUS IONIC STRENGTHS (UNBUFFERED SOLUTIONS)

Ionic strength, *M*	T_{tr}, °C. *p*H 6.5	T_{tr}, °C. *p*H 0.7	$(\Delta T_{tr})_{max}$
0.0	61.9		
.01	62.3		
.10	62.3		
.16	62.2	26.6	35.6
.32	61.7	31.5	30.2
.50	62.7		
1.0	63.7		
1.1	63.9	39.4	24.5
2.0	66.0		
4.0	67.5[a]		

[a] Uncertain due to precipitation at high temperature.

and which, therefore, weaken the above conclusions deduced from the experiments on the effect of ionic strength. The anomalies manifest themselves at high ionic strength in (1) an increase in T_{tr} at *p*H 6.5 above ionic strength 0.5, and (2) in a change in the shape of the ΔD *vs.* T curve.

Discussion

Conversion of T_{tr} Values to Free Energies.—Since the theory in the appendices is presented in terms of free energies, these quantities may be obtained from the experimental data by means of the equation

$$\Delta F^0_{obsd.} = -RT \ln K_{obsd.} \qquad (5)$$

$K_{obsd.}$ is related to the measured optical densities by equations 1 and 3. In principle, $\Delta F^0_{obsd.}$ is then ob-

(25) This conclusion is based on the fact that the experimental data fall on straight lines at all *p*H's in Fig. 5 for $T \leq T_{tr}$.

(26) H. A. Scheraga, *J. Phys. Chem.*, **64**, 1917 (1960).

(27) A. Nakajima and H. A. Scheraga, *J. Am. Chem. Soc.*, **83**, 1575 (1961).

(28) M. Sela, C. B. Anfinsen and W. F. Harrington, *Biochim. et Biophys. Acta*, **26**, 502 (1957).

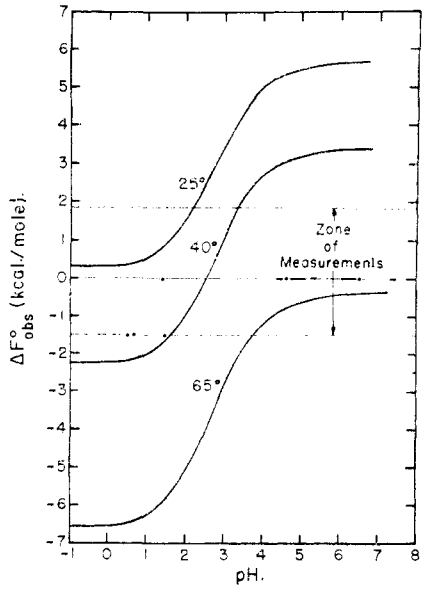

Fig. 7.—Free energies of denaturation at three temperatures as a function of pH, computed from the transition temperatures with the aid of equation 7.

tainable at any pH and temperature from the experimental data. In practice, reliable values of $K_{obsd.}$ are available only in the transition region, *i.e.*, for $0.01 < K_{obsd.} < 100$. Inspection of Fig. 2 reveals that the transition region encompasses only about 20° at each pH, whereas a range of about 40° must be bridged in order to compare measurements at pH 0.9 and pH 6.8. Therefore, it is necessary to extrapolate the values of $K_{obsd.}$ to higher and lower temperatures at each pH.

Equation 6 is suitable as an extrapolation formula.

$$-RT \ln K_{obsd.} = \Delta F^0_{obsd.} = \Delta H^0_{obsd.} - T\Delta S^0_{obsd.} \quad (6)$$

where $\Delta H^0_{obsd.}$ is the standard heat of denaturation and $\Delta S^0_{obsd.}$ is the standard entropy of denaturation, both of which are independent of T. For an equilibrium between only two forms of molecules, this relation would be expected to hold within experimental error (say ±10%) over a range of fifty degrees. Unfortunately, the plots of log $K_{obsd.}$ *vs.* $1/T$ in Fig. 5 show that equation 6 holds only when log $K_{obsd.} \leq 0$, *i.e.*, when $T \leq T_{tr}$ at any pH. From this it was concluded that, above T_{tr}, the equilibrium is one between native ribonuclease and *more than one* form of reversibly denatured ribonuclease. The theory thus cannot be compared with the experiments unless the following *assumption* is made: *Below T_{tr} there exists an equilibrium between only two forms of molecules.* This one-step equilibrium has a constant standard enthalpy, $\Delta H^0_{obsd.}$ and entropy, $\Delta S^0_{obsd.}$ of reaction, independent of temperature in the range 15 to 70°. As a consequence, we can compute the values of $K_{obsd.}$ and $\Delta F^0_{obsd.}$ in the whole temperature range, using equation 6 (applied to experimental data obtained below T_{tr}). Since $\Delta F^0_{obsd.} = 0$, *i.e.*, $\Delta H^0_{obsd.} - T_{tr}\,\Delta S^0_{obsd.} = 0$ at the transition temperature, eq. 6 becomes

$$\Delta F^0_{obsd.} = \Delta H^0_{obsd.}\left[1 - \frac{T}{T_{tr}}\right] \quad (7)$$

Experimentally $\Delta H^0_{obsd.}$ was found to be independent of pH and equal to 51 ± 5 kcal./mole (Fig. 5; see also Appendix III); for every pH at which T_{tr} was determined we can, therefore, compute $\Delta F^0_{obsd.}$ at any value of T with the aid of equation 7. Curves representing the pH-dependence of $\Delta F^0_{obsd.}$ at three temperatures are shown in Fig. 7. The region of temperature and pH where measurements were made is indicated by the horizontal dashed lines. Actually, only the measurements at temperatures below T_{tr}, *i.e.*, at points for which $\Delta F^0_{obsd.} > 0$, were used to compute $\Delta F^0_{obsd.}$ since only for these points does equation 7 describe the experimental results.

The curves in Fig. 7 have flat portions at low and high pH, which correspond, of course, to the flat portions of the curve of T_{tr} against pH shown in Fig. 6. The difference in $\Delta F^0_{obsd.}$ between these two limits is seen to be equal to 5.4 kcal./mole at 25°.

Models.—In order to compute theoretical curves corresponding to the experimental ones of Fig. 7, it is necessary to introduce a model for the ribonuclease molecule. Four plausible models are described[29] in Appendix II. These were selected on the basis that they can account for the value of the maximum difference in $\Delta F^0_{obsd.}$ (5.4 kcal./mole at 25°) shown in Fig. 7. The models were tested further to see whether they can account[30] for the pH-dependence of $\Delta F^0_{obsd.}$ and of T_{tr}. The pK's and distribution of the groups in the various models[30] are listed in Table II.

The theory discussed in the appendices is essentially that presented previously[26] but modified (in the case of models B, C_1 and C_2) to take account of a possible contribution from a difference in the electrostatic free energy between native and denatured forms.

Theoretical Free Energies.—The calculation of the free energies of protein molecules as a function of pH (for given pK's and w's) is described in Appendix II (equations A20 and A21). The pH-dependent part of $\Delta F^0_{obsd.}$, *viz.*, ΔF^0_H, then was obtained as the difference in free energy between native and denatured molecules for models A, B, C_1 and C_2 and plotted in Fig. 8 at 25°. Since ΔF^0_H approaches zero in the limit of low pH, the ordinate, ΔF^0_H, for the *experimental* data was ob-

(29) The notation used to specify the pK's of the groups involved in these models is as follows: pK^0 is the intrinsic pK of a free group, in the absence of any interactions; pK^E is the pK in the presence of a general electrostatic interaction, but in the absence of local interactions (such as hydrogen bonding, hydrophobic bonding, local electrostatic interaction, etc.), and is given by the equation

$$pK^E = pK^0 - 0.868\,wZ$$

where Z is the net charge on the molecule and w is an electrostatic interaction factor (see eqs. A2 and A3 of Appendix I); pK^H is the pK in the absence of a general electrostatic interaction, but in the presence of local interactions; $pK_{obsd.}$ is the observed value of the pK in the presence of general electrostatic and local interactions (see eq. A4 of Appendix I).

(30) In the next paper in this series these models also will be used to account for: (1) the titration curve of ribonuclease, which is known to show certain abnormalities[31] at low pH, and (2) the difference titration curve between native and denatured ribonuclease molecules. The next paper also will discuss the origin of the abnormality of the carboxyl and tyrosyl groups of native ribonuclease and provide a justification for the pK's of the groups in the four models of Appendix II.

(31) C. Tanford and J. D. Hauenstein, *J. Am. Chem. Soc.*, **78**, 5287 (1956).

TABLE II

DISTRIBUTION OF IONIZING GROUPS IN THE DIFFERENT MODELS

	Model	Carboxyl groups pK^H = 2.50[a]	Carboxyl groups pK^H = 3.65[a]	Carboxyl groups pK^H = 3.75[b]	Carboxyl groups pK^0 = 4.60[b]	Imidazole groups pK^0 = 6.50[b]	w (at ionic strength 0.16)
A	Native	2	..	1	8	4	0.061
	Denatured	..	..	1	10	4	.061
B	Native	1	1	1	8	4	.061
	Denatured	..	1	1	9	4	.047
C_1	Native	..	1	1	9	4	.061
	Denatured	..	1	1	9	4	.034
C_2	Native	1	1	1	8	4	.061
	Denatured	1	1	1	8	4	.034

[a] These groups with abnormal pK's are included on the basis of changes in optical density and optical rotation with pH which are to be discussed in the next paper.[30] [b] These groups have normal[31] pK's.

tained by an arbitrary adjustment of $\Delta F^0_{obsd.}$ to zero at low pH. This adjustment is equivalent to subtracting ΔF^0_{unf} from $\Delta F^0_{obsd.}$ (see eq. A6 of Appendix I). It can be seen from Fig. 8 that the agreement between the experimental data and the theoretical curve for model A is good, for model B fair but for models C_1 and C_2 very poor.[32]

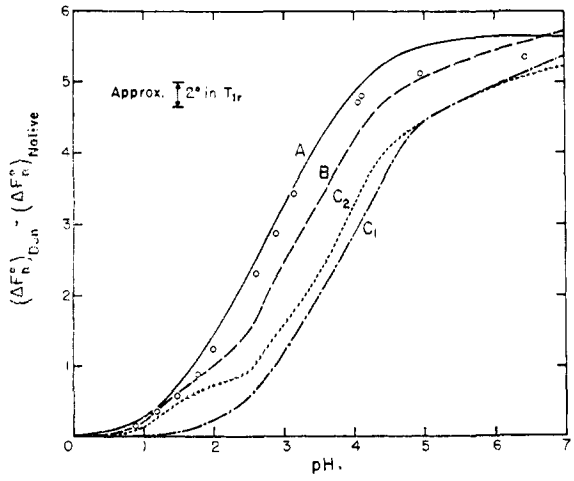

Fig. 8.—The pH-dependent part of the free energy of denaturation in kcal./mole computed for the four models. Curves are theoretical. The open circles are experimental values at T = 300°K. obtained from the transition temperatures (eq. 7). The zero of the ordinate was chosen arbitrarily at the low pH limit of all curves.

If the theoretical ΔF^0_H values are converted[33] to values of T_{tr}, they can be compared with experimental values, as shown for models A and B in Fig. 9. From Fig. 9 it is not possible to make a choice between models A and B, even though Fig. 8 provides a slight preference for model A.

Variation of the Ionic Strength.—An additional distinction between the models is their behavior as the ionic strength is varied. Although the ΔF^0_H values are dependent on w for all models, and thus on ionic strength, this is not true of the maximum value of this function. The maximum value of ΔF^0_H contains only $w_N - w_D$ (see Appendix II, eq. A25), which is zero for model A but not for the other models. Therefore, the difference between the transition temperatures at very low and very high pH should not vary with ionic strength for model A but should for the other models.

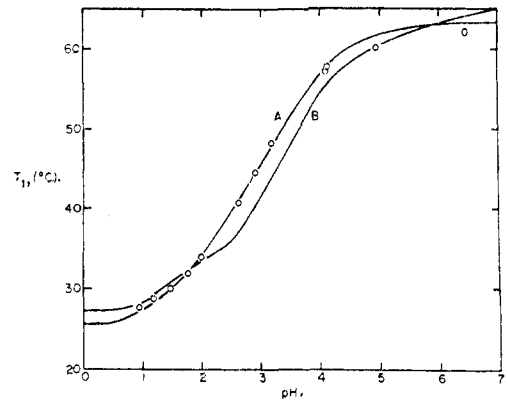

Fig. 9.—Transition temperatures as a function of pH. Points are experimental, curves are theoretical for models A and B.

The data of Table I do indicate a dependence of $(\Delta T_{tr})_{max}$ on ionic strength, which might lead one to prefer model B over model A. However, because of the ionic strength anomalies cited in the Results section, the experiments on the variation of ionic strength are inconclusive as far as the choice between model A and model B is concerned.

Conclusion Regarding Models.—None of the models chosen gives perfect agreement with the experimental results. It is easy to exclude models C_1 and C_2 on the basis of the pH dependence of $\Delta F^0_{obsd.}$ but a choice cannot be made between models A and B. We are thus left with the conclusion[30] that the native molecule contains one, and possibly two, carboxyl groups with pK^H = 2.5. These groups become normal (pK^0 = 4.6) upon denaturation and play an important role in the pH-dependence of $\Delta F^0_{obsd.}$

Role of Denaturation in Ribonuclease Reactivity.—A knowledge of the details of the helix-random coil equilibrium (Figs. 2 and 4) provides a basis for understanding the behavior of ribonuclease in various reactions.

As a first example, we consider the susceptibility of ribonuclease to attack by proteolytic enzymes. If the site of attack must be in an unfolded part of

(32) In making this comparison it should be recalled that our lowest pH-value of T_{tr} (Fig. 6) lies almost but not quite in the pH-independent region. This may affect our choice of value of ΔF^0_{unf} in our adjustment of ΔF^0_H to zero at low pH. Thus, we have obtained $\Delta F^0_{obsd.}$ from T_{tr} (eq. 7) by an extrapolation and, also, with the assumption that $\Delta H^0_{obsd.}$ is constant within ±10%.

(33) This conversion again requires the addition of an arbitrary value of $\Delta F^0_{unf.}$ to ΔF^0_H to obtain $\Delta F^0_{obsd.}$

the molecule, then the nature of the hydrolytic products may differ as the temperature and pH is varied. Under the conditions used by Anfinsen[34] for peptic digestion of ribonuclease (pH 1.8 and 37°), it can be seen from Fig. 2 that ribonuclease is almost completely denatured. Anfinsen found that pepsin split the fourth peptide bond from the C-terminus. However, Ginsburg and Schachman[35] obtained different results at pH 2.1 and 25.5°. From Fig. 2, it can be seen that ribonuclease is only slightly unfolded under these conditions; therefore, it is not surprising that the results of Ginsburg and Schachman differ from those of Anfinsen. The data of Fig. 2 are the basis for planning experiments to use proteolytic enzymes to locate the non-helical regions of native ribonuclease and also the portions of the molecule involved in the reversible change of configuration.[36]

As a second example we consider the reaction of ribonuclease with iodoacetate.[37,38] Apart from other reactive side-chain groups, at least one methionyl residue was found to react at pH 2.8 and 40° and not at pH 5.5 and 40°, even though the reaction between methionine itself and iodoacetate proceeds at a pH-independent rate. Again, from the data of Fig. 2, it appears that this methionyl residue reacts only when the molecule is in the unfolded form. If the specific reactive methionyl residue is identified, it should be possible to decide which portion of the ribonuclease molecule is involved in the reversible transition. The accumulation of more information of this kind will provide details of the secondary and tertiary structure of the native molecule and, eventually, a confirmation or disproval of the proposed model.[5]

Appendix I

Electrostatic Interactions

In this appendix we show that general electrostatic interactions can make a significant contribution to the free energy of denaturation.

The electrostatic free energy of Z charges uniformly distributed on the surface of a spherical macromolecule, which is dissolved in water containing strong electrolyte, is given by[39-43]

$$F^0_{el} = RTwZ^2 \qquad (A1)$$

where

$$w = \frac{N\epsilon^2}{2DRT}\left[\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right] \qquad (A2)$$

where N, R and T have their usual meaning, D is the dielectric constant, ϵ is the electronic charge, κ is the inverse of the radius of the ionic atmosphere and b and a are the hypothetical radii of the molecule and of exclusion, respectively.

As a result of this electrostatic free energy, the degree of ionization, x, of a specific group on a protein will be given by

$$\frac{xH}{1 - x} = K^E = K^0 e^{2wZ} \qquad (A3)$$

where K^0 is the ionization constant in the absence of electrostatic interactions, and H is the hydrogen ion activity. If the group is hydrogen bonded we may write

$$\frac{xH}{1 - x} = K_{obsd.} = K^H e^{2wZ} \qquad (A4)$$

where K^H contains K^0 and also a contribution from the hydrogen bond.[44] These equations may be averaged over all the molecules at any given pH; in particular, for a large number, n, of identical groups with intrinsic ionization constant K^0, eq. A3 becomes

$$\frac{rH}{n - r} = K^E = K^0 e^{2wZ} \qquad (A5)$$

where r is the average number of the n groups which have dissociated protons at the given pH.

The standard free energy of denaturation may be written as[26,45]

$$\Delta F^0_{obsd} = \Delta F^0_{unf} + \Delta F^0_H \qquad (A6)$$

where ΔF^0_{unf} pertains to the backbone hydrogen bonds[46] (and is independent of pH) and ΔF^0_H pertains to pH-dependent interactions such as the general electrostatic effect, local electrostatic effects, side-chain hydrogen bonds, etc. In this appendix we shall, for the moment, disregard all but the general electrostatic contribution to ΔF^0_H and refer to the latter as $(\Delta F^0_H)_{el}$.

According to equation A1

$$(\Delta F^0_H)_{el} = RT(w_D - w_N)Z^2 \qquad (A7)$$

where the subscripts D and N refer to the denatured and native molecules, respectively, at the pH where the net charge is Z on both the native and denatured molecules. It should be noted that Z will be the same for the native and denatured molecules only in certain special cases. Such a situation, which we are considering here, is found in the limits of high and low pH, *i.e.*, in the flat parts of the titration curve where no groups ionize in either the native or denatured molecule. (In Appendix II, eq. A22, we shall take into account differences in Z between the native and denatured molecules in the *intermediate* pH range.) We have observed (Fig. 7) that the pH-dependent part of $\Delta F^0_{obsd.}$ (assumed in this appendix to be $(\Delta F^0_H)_{el}$) is 5.4 kcal./mole larger at pH 6.5 ($Z = 5$)[31] than at pH 0 ($Z = 19$)[31] when $T = 298°$. Therefore, we may write, with the aid of eq. A7

$$(\Delta F^0_H)_{el,\ Z=5} - (\Delta F^0_H)_{el,\ Z=19} = RT(w_D - w_N)(5^2 - 19^2)$$
$$= 200\ (w_N - w_D)\ \text{kcal./mole} \qquad (A8)$$

at $T = 298°$. If we assume, for the moment, that the term in eq. A8 is equal to 5.4, then we obtain

$$w_D = w_N - 5.4/200 \qquad (A9)$$

Since $w_N = 0.061$[31] at 0.15 ionic strength, we obtain $w_D = 0.034$. The value 0.034 is a reasonable one[46] for w_D. Therefore, we have to consider the possibility that the pH-

(34) C. B. Anfinsen, *J. Biol. Chem.*, **221**, 405 (1955).
(35) A. Ginsburg and H. K. Schachman, *ibid.*, **235**, 115 (1960).
(36) J. A. Rupley and H. A. Scheraga, *Biochim. et Biophys. Acta*, **44**, 191 (1960).
(37) H. G. Gundlach, W. H. Stein and S. Moore, *J. Biol. Chem.*, **234**, 1754 (1959).
(38) H. G. Gundlach, S. Moore and W. H. Stein, *ibid.*, **234**, 1761 (1959).
(39) K. Linderstrøm-Lang, *Compt. rend. trav. lab. Carlsberg*, **15**, No. 7 (1924).
(40) R. K. Cannan, *Chem. Revs.*, **30**, 395 (1942).
(41) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).
(42) G. Scatchard, in "Proteins, Amino Acids and Peptides," Ed. by E. J. Cohn and J. T. Edsall, Reinhold Publishing Co., New York, N. Y., 1943, p. 20.
(43) J. T. Edsall, *ibid.*, p. 444.
(44) M. Laskowski, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, **76** 6305 (1954).
(45) J. A. Schellman, *Compt. rend. trav. Lab. Carlsberg, Ser. Chim.*, **29**, 230 (1955).
(46) Obviously, this is a crude calculation depending, among other things, on the validity of the Linderstrøm-Lang model[39] for the native and denatured protein. On the other hand, since the native protein is compact, it is reasonable to expect that $w_D < w_N$, whether or not permeation of the denatured molecule is allowed.[47] Since w is constant over a wide range of pH in denaturing media,[48] we can estimate a lower limit for w_D as 0.018 from experiments in 8 M urea, 0.1 M KCl[49] (the value in our solvent, 0.16 M KCl, being essentially the same because of approximate cancellation of the effects of adding urea and decreasing the KCl concentration). Thus the value 0.034 is reasonable since it lies in the range $0.018 < w_D < 0.061$.
(47) C. Tanford, *J. Phys. Chem.*, **59**, 788 (1955).
(48) C. Y. Cha and H. A. Scheraga, *J. Am. Chem. Soc.*, **82**, 54 (1960).
(49) O. O. Blumenfeld and M. Levy, *Arch. Biochem. Biophys.*, **76**, 97 (1958).

dependent part of $\Delta F^0_{obsd.}$ arises from a general electrostatic effect. Actually, it is shown in the sub-section entitled "Theoretical Free Energies" that the general electrostatic effect is only *part* of ΔF^0_H.

Appendix II

Electrostatic and Local Interactions

In this Appendix we provide the basis for the discussion in the text showing that a model based only on a general electrostatic effect is insufficient to account for the denaturation data. Rather, local interactions, *e.g.*, side-chain hydrogen bonding, must also be included. It must be emphasized that side-chain hydrogen bonding is only one of several possible types of local interaction. Further discussion of the nature of the local interactions will be given in the next paper.[30]

pH-Dependence of Free Energy of Ionization (General Electrostatic Effect; No Configurational Change).—In Appendix I we considered the dependence of ΔF^0_H on *p*H at the extremes of the *p*H range. We now wish to consider the *p*H-dependence of ΔF^0_H in the intermediate range for a molecule whose groups ionize according to equation A3. Therefore, we shall first consider in this section of Appendix II the *p*H dependence of the free energy of ionization of such groups, assuming only a general electrostatic effect and no change in molecular configuration over the *p*H range involved.

Consider a native protein in solution existing as species carrying different numbers of hydrogen ions: AH_n, AH_{n-1}, AH_{n-2}, etc., where the charges have been omitted for simplicity. The *p*H of the solution determines the amount of material which will be present as each of these species. The distribution of the material over the various species will determine the free energy of the protein in solution. This free energy can be split into two parts, a *p*H-independent (F^0_0) and a *p*H-dependent one (F^0_h).

$$F^0 = F^0_0 + \Delta F^0_h \qquad \text{(A10)}$$

At very low *p*H, when the molecules are all present as species AH_n, the *p*H-dependent part, ΔF^0_h, is equal to zero. In going from all AH_n molecules to the mixture of different species, hydrogen ions are liberated. If the average number dissociated per protein molecule in the solution is r, the relation (A11) holds[50]

$$\frac{1}{RT}\frac{\partial \Delta F^0_h}{\partial \ln H} = r \qquad \text{(A11)}$$

It is worth pointing out that this is a general equation, no restricting conditions having been imposed on the molecule in deriving it. An equivalent equation, in terms of a grand partition function, has been given by Harris and Rice.[51]

The theory of electrostatic interaction between charges on a protein molecule[39-43] provides the means of relating r and H. Taking as our model a molecule with n groups each with the same ionization constant K^0 (in the absence of electrostatic interaction) and with charge approaching $Z = Z^0$ in the limit $H \to \infty$, we have from equation A5

$$H = \frac{n - r}{r} K^0 e^{2w(Z^0 - r)} \qquad \text{(A12)}$$

since the number of charges diminishes by one with every ionization, assuming no chloride binding. ΔF^0_h is obtained by integrating equation A11

$$\frac{1}{RT}\Delta F^0_h = \int r \frac{\mathrm{d} \ln H}{\mathrm{d}r} \mathrm{d}r + \text{constant} \qquad \text{(A13)}$$

The derivative d ln H/dr is obtained from eq. A12, and the final result is

$$\frac{1}{RT}\Delta F^0_h = -\{wr^2 - n \ln (n - r) - \text{constant}\} \qquad \text{(A14)}$$

The constant is determined by the normalizing condition that ΔF^0_h is equal to zero at very low *p*H. *i.e.*, when $r = 0$, so that[52]

$$-\frac{1}{RT}\Delta F^0_h = wr^2 + n \ln \frac{n}{n - r} \qquad \text{(A15)}$$

Writing $1 + \frac{r}{n - r}$ for $\frac{n}{n - r}$, and using equations A5 and A12 we have

$$-\frac{1}{RT}\Delta F^0_h = wr^2 + n \ln (1 + K^E/H) = wr^2 + n \ln (1 + K^0 e^{2w(Z^0 - r)}/H) \qquad \text{(A16)}$$

As a special case when $w = 0$, we have

$$-\frac{1}{RT}\Delta F^0_h = n \ln (1 + K^0/H) \qquad \text{(A17)}$$

pH-Dependence of Free Energy of Ionization in the Presence of Hydrogen-Bonding (No Change of Configuration).—If the ionizing groups are hydrogen-bonded, the ionization constants will be equal to $K_{obsd.} = K^H e^{2wZ}$ instead of $K^E = K^0 e^{2wZ}$. Thus equation A16 becomes

$$-\frac{1}{RT}\Delta F^0_h = wr^2 + n \ln (1 + K^H e^{2w(Z^0 - r)}/H) \qquad \text{(A18)}$$

In the special case where $w = 0$, we have

$$-\frac{1}{RT}\Delta F^0_h = n \ln (1 + K^H/H) \qquad \text{(A19)}$$

pH-Dependence of the Transition from Native to Denatured Molecules.—We are now in a position to compute the *p*H-dependence of ΔF^0_H taking into account a general electrostatic effect and side-chain hydrogen bonding.

In the general case, where the groups are not identical, we shall write equation A18 as

$$-\frac{1}{RT}\Delta F^0_h = wr^2 + \sum_i \ln (1 + K_i^H e^{2w(Z^0 - r)}/H) \qquad \text{(A20)}$$

where

$$r = \sum_i x_i \qquad \text{(A21)}$$

and the x_i's are defined by equation A4.

---

(50) This relationship may be proved as follows. Equations 5 and 7, pages 451 and 452, of ref. 43 may be written in our notation as

$$r = \frac{\partial \ln x_n}{\partial \ln H}$$

where x_n is the fraction of all the molecules which is in the form AH_n. The various ionization equilibria may be written as

$$AH_n \rightleftarrows AH_{n-1} + H \rightleftarrows AH_{n-2} + 2H \rightleftarrows \ldots \rightleftarrows AH_{n-i} + iH \rightleftarrows \ldots \rightleftarrows A + nH$$

The equilibrium condition is

$$\mu_n = \mu_{n-1} + \mu_H = \mu_{n-2} + 2\mu_H = \ldots = \mu_{n-i} + i\mu_H = \ldots = \mu_0 + n\mu_H$$

where the μ's are the chemical potentials of the various species and of hydrogen ion. The chemical potential of the solute is given by

$$\mu = \sum_{i=0}^{n} x_{n-i}(\mu_{n-i} + i\mu_H)$$

Therefore, applying the equilibrium condition and writing μ_n for $(\mu_{n-i} + i\mu_H)$

$$\mu = \mu_n \sum_{i=0}^{n} x_{n-i} = \mu_n$$

But, at any *p*H, μ_n is given by

$$\mu_n = \mu_n^0 + RT \ln c_n = \mu_n^0 + RT \ln x_n c_0$$

where c_n is the concentration of species AH_n at the given *p*H, and c_0 is the total protein concentration. At very low *p*H (*i.e.*, $H \to \infty$), $x_n = 1$; at higher *p*H's, x_n is less than unity. Therefore, the change in the molar free energy upon raising the *p*H is given by

$$\Delta F^0_h = (\mu_n)_H - (\mu_n)_{H \to \infty} = RT \ln x_n$$

Differentiating, we obtain

$$\frac{1}{RT}\frac{\partial \Delta F^0_h}{\partial \ln H} = \frac{\partial \ln x_n}{\partial \ln H}$$

Substitution of the first equation in this footnote leads to eq. A11.

(51) F. E. Harris and S. A. Rice, *J. Phys. Chem.*, **58**, 725 (1954).

(52) This equation is similar to equation 1 of Scatchard's paper[41] but not identical with it. Scatchard's equation refers to the free energy of the species AH_k with only one value of k and must still be averaged over all k's. This averaging has been performed in arriving at equation A12 by making use of equation A3 which has been derived by an averaging procedure.

In the native protein the groups will have ionization constants K_i^H, different from K_i^0, because of the local interactions. In the denatured molecule the local interactions are assumed to be absent, and the dissociation constant is K_i^0, rather than K_i^H. Also, when the general electrostatic effect is included, the native molecule has a w-factor equal to w_N, the denatured one equal to w_D. Thus the pH-dependent part of the free energy change upon going from native to denatured molecules is given by

$$-\frac{1}{RT}\Delta F^0_H = -\frac{1}{RT}[(\Delta F^0_h)_D - (\Delta F^0_h)_N] =$$

$$w_D r_D^2 - w_N r_N^2 + \sum_i \ln \frac{1 + K_i^0 \exp\{2w_D(Z^0 - r_D)\}/H}{1 + K_i^H \exp\{2w_N(Z^0 - r_N)\}/H} \quad (A22)$$

Combining equations A6 and A22 and converting standard free energies to equilibrium constants, we obtain

$$K_{obsd.} = K_{unf} \prod_i \times \frac{[1 + K_i^0 \exp\{2w_D(Z^0 - r_D)\}/H] \exp(w_D r_D^2)}{[1 + K_i^H \exp\{2w_N(Z^0 - r_N)\}/H] \exp(w_N r_N^2)} \quad (A23)$$

where K_{unf} contains all pH-independent contributions. In this paper we are restricting our treatment to the case where $K_i^H \geq K_i^0$ for all groups. In such a case, the maximum variation of ΔF^0_H with pH is the same as the value of ΔF^0_H at very high pH ($H = 0$) (since $\Delta F^0_H = 0$ at low pH by definition). We can write equation A22 as

$$-\frac{1}{RT}(\Delta F^0_H)_{H=0} = w_D n^2 - w_N n^2 + \sum_i \ln \frac{K_i^0 \exp\{2w_D(Z^0 - n)\}}{K_i^H \exp\{2w_N(Z^0 - n)\}} \quad (A24)$$

since $Ke^{2w(Z^0 - r)}/H \gg 1$ and both r_D and r_N are equal to n in this limit (all groups ionized).

This result can be written as

$$-\frac{1}{RT}(\Delta F^0_H)_{H=0} = (w_D - w_N)(2nZ^0 - n^2) + \sum_i \ln \frac{K_i^0}{K_i^H} \quad (A25)$$

These equations are the basis for our discussion of the experimental results on the reversible denaturation of ribonuclease.

Models.—The following models will be considered in our attempt to account for the pH-dependence of ΔF^0_H for ribonuclease at low pH.

1. **Model A.**—Both the native and denatured molecules have the same value of $w = 0.061$ (at 0.16 M ionic strength[31]). Two equivalent carboxyl groups[30] have $pK^H = 2.50$ in the native molecule (*i.e.*, $pK_{obsd.} = 1.5$ at 0.16 M ionic strength and pH 1.5), and $pK^0 = 4.60$ in the denatured molecule. In this model, ΔF^0_H arises solely from local interactions (*e.g.* hydrogen bonds). The basis for the choice of this model is described below and in the next paper of this series.[30]

For this model, the maximum value of ΔF^0_H, according to eq. A25 with $w_D = w_N$, is given by

$$-\frac{1}{RT}\Delta F^0_H = \sum_i \ln \frac{K_i^0}{K_i^H} \quad (A26)$$

If $pK_i^0 = 4.6$ and $pK_i^H = 2.5$ and if there are two terms in the sum of equation A26, then the right hand side of this equation will be equal to 5.8 kcal./mole, which is in good agreement with the observed value of 5.4 kcal./mole. If w_D were made smaller than w_N, the calculated value would increase, giving poorer agreement; on the other hand, $w_D > w_N$ is unlikely (see Appendix I). Therefore, if this model is to be used, w_N must be taken equal to w_D.

2. **Model B.**—Only one carboxyl group[30] has $pK^H = 2.50$ in the native molecule and $pK^0 = 4.60$ in the denatured molecule. According to the above calculation, this group contributes only 2.9 kcal./mole to $\{(\Delta F^0_H)_{pH\,6.5} - (\Delta F^0_H)_{pH0}\}$ and the remainder is obtained from a generalized electrostatic effect (computed in Appendix I, in the absence of hydrogen bonding). If $w_N = 0.061$, then w_D must be 0.047 (see eq. A8) to account for the remainder of the 5.4 kcal./mole (*i.e.*, $w_N - w_D = 0.014$).

3. **Model C.**—The whole contribution to ΔF^0_H is a generalized electrostatic effect. Thus, according to the calculations in Appendix I, the value of 5.4 kcal./mole is obtainable from $w_N = 0.061$ and $w_D = 0.034$.

From equation A25 it can be seen that, in order to account for the maximum change in ΔF^0_H, we need specify only the change in w and those groups which have a different pK (in the absence of a generalized electrostatic interaction) in the two forms. When applying eq. A22 in order to calculate ΔF^0_H in the intermediate pH-range, knowledge of all pK's is needed. Evidence for the basis of the introduction of the abnormal groups into the various models described in Table II is presented in the next paper.[30]

Appendix III

Enthalpy Changes

In computing values of $\Delta F^0_{obsd.}$, the standard free energy of denaturation, as a function of pH from the observed values of the transition temperature at the beginning of the Discussion section, use was made of the experimental fact that $\Delta H^0_{obsd.}$, the standard enthalpy of denaturation, is 51 kcal./mole and independent of pH. It is worth noting that this is only so within an experimental error of $\pm 10\%$, *i.e.*, ± 5 kcal./mole. Since the total change in free energy of unfolding, observed when changing the pH, is only 5.4 kcal./mole, the constancy of $\Delta H^0_{obsd.}$ within experimental error is not unexpected.

However, in our use of $\Delta H^0_{obsd.}$ we have assumed a rather rigorous constancy, and we may ask if this is justified in light of the conclusions drawn from it. Thus, we may ask how much of ΔF^0_H is an enthalpy contribution.

We shall consider the maximum value of ΔF^0_H which is given by eq. A25. The contributions from the generalized electrostatic effects are separated into two terms in eq. A25. Since the pH-dependent part of $\Delta H^0_{obsd.}$, *i.e.*, ΔH^0_H, can be obtained from ΔF^0_H as

$$\Delta H^0_H = \partial\left(\frac{\Delta F^0_H}{T}\right)/\partial(1/T) \quad (A27)$$

we have as a contribution from the generalized electrostatic effect

$$\frac{\Delta H^0_H}{R} = -(2nZ^0 - n^2)\frac{\partial}{\partial(1/T)}(w_D - w_N) \quad (A28)$$

Dividing this by the expression for that part of ΔF^0_H which is due to the generalized electrostatic effect in eq. A25, we obtain

$$\frac{\Delta H^0_H}{\Delta F^0_H} = \frac{\partial(w_D - w_N)/\partial(1/T)}{T(w_D - w_N)} \quad (A29)$$

Using equation A2 for w, the absolute value of the right hand side of eq. A29 is less than $^1/_3$.[53]

The contribution from local interactions is given by the second term of eq. A25. The corresponding enthalpy contribution is

$$\Delta H^0_H = -R\frac{\partial}{\partial(1/T)}\sum \ln(K_i^0/K_i^H) = \sum(\Delta H_i^H - \Delta H_i^0) \quad (A30)$$

For a carboxyl group, ΔH_i^0 is usually zero, and ΔH_i^H is observed to be zero.[30] Therefore, the contribution of a local interaction to the maximum value of ΔH^0_H is zero.

Thus, the absolute value of ΔH^0_H is at most 5.4/3 or 1.8 kcal./mole from the general electrostatic effect, which is within the 5 kcal./mole error in the observed value of $\Delta H^0_{obsd.} = 51$ kcal./mole.

(53) L. Gruen, M. Laskowski, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, **81**, 3891 (1959).