in the vicinity of Me1 was obscure because Me1 possesses the largest thermal parameters in the molecule. The positive regions have been ascribed to hydrogen atoms and vary in peak height from 0.4 to 1.2 e $A^{-3}$, and their bond distances range from 0.9 to 1.1 A. The orientation of the hydrogen atoms of Me2 is suggestive in that they are oriented so that one hydrogen is directed toward the 3,4 double bond. In this way, Me2 can achieve a staggered configuration with respect to T7 (see Figure 6a).

**Acknowledgment.** We wish to thank Professor W. von E. Doering for bringing this problem to our attention and for providing the *p*-bromophenacyl derivative.

This research has been supported by the National Institutes of Health, Public Health Service, and by Lederle Laboratories Division, American Cyanamid Company. One of us (R. E. D.) is indebted to the Division of General Medical Sciences, Public Health Service, for Public Health Service Fellowship No. 5-Fl-GM-14, 844-03.

# Structural Studies of Ribonuclease. XXV. Enthalpy Changes Accompanying Acid Denaturation[1]

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**Abstract:** An enthalpy titration curve (relative apparent heat content *vs.* pH) has been obtained for ribonuclease in 0.1 *M* KCl between pH 2.2 and 11.5 at 25°. A theoretical curve is able to fit the experimental data. From measurements of the heat change accompanying acidification from pH 9.3 to 2.2 at several temperatures ranging from 10 to 55°, the enthalpy of unfolding of ribonuclease at pH 2.2 and 45° was found to be 109 ± 5 kcal/mole. This result is discussed in relation to previous estimates of the heat of denaturation obtained from heat capacity measurements (70 ± 1 kcal/mole) and from calculations with the van't Hoff equation (114 ± 7 kcal/mole).

Much attention has recently been paid to the conformation of ribonuclease and its change under various conditions. In particular, the thermal transition has been studied by optical rotation and ultraviolet difference spectra measurements, and thermodynamic parameters for the denaturation process have been deduced by assuming models for the reaction.[3-7] The enthalpy of denaturation has also been obtained by calorimetric measurements[8] of the heat capacities of aqueous solutions of ribonuclease at a series of temperatures. Since there are discrepancies in the values of the enthalpy of denaturation obtained by these methods, it was decided to make a direct calorimetric measurement of the enthalpy of denaturation rather than obtain it from the heat capacities.

For this purpose, the heat change accompanying the acidification of ribonuclease solutions (*i.e.*, when the pH is lowered from 9.3 to 2.2) was measured at several temperatures ranging from 10 to 55°. The enthalpy change accompanying the denaturation of ribonuclease was obtained in a manner similar to that employed by Hermans and Rialdi[9] for myoglobin and by Bunville, *et al.*, for DNA.[10]

## Experimental Section

**Materials.** Sigma Chemical Co. five-times crystallized ribonuclease (Lot 114B-1510) was chromatographed (to obtain ribonuclease A) and deionized, as described by Rupley and Scheraga.[11] A sample of the purified, lyophilized protein was found to be free of ribonuclease B by rechromatography on IRC-50 resin using an analytical column. The presence of a small amount of aggregate (less than 1%) was ignored. The lyophilized material was stored in an evacuated desiccator over $P_2O_5$. Concentrations were determined by weighing out the hydrated powder, which contained 7% moisture based on an extinction coefficient[6] of 0.738 $cm^2$/mg at 278 mμ. Deionized distilled water was used, and all other chemicals were reagent grade.

**pH Measurements.** pH measurements were made at 25° with a Radiometer TTT 1a titrator, using Fisher Scientific Co. buffers for standardization.

**Calorimetry.** Calorimetric measurements were carried out with an adiabatic solution calorimeter modeled after the one described by Benjamin.[12]

In a typical experiment, 20–40 mg of ribonuclease was weighed into the sample cell with 3.0 ml (weighed to ±0.1 mg) of either 0.10 or 0.15 *M* KCl. The solution was brought to thermal equilibrium within about 45 min in the calorimeter vessel containing both the cell and about 40 ml (weighed to ±0.1 mg) of salt solution (containing the desired amount of either HCl or KOH) to be mixed with the protein solution. The cell was broken, and the heat of mixing was measured. The resulting solution of about 43 ml contained about 0.5–1.0 mg/ml of ribonuclease, the same con-

(1) This work was supported by a research grant (AI-01473) from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, U. S. Public Health Service, and by a research grant (GB-4766) from the National Science Foundation.

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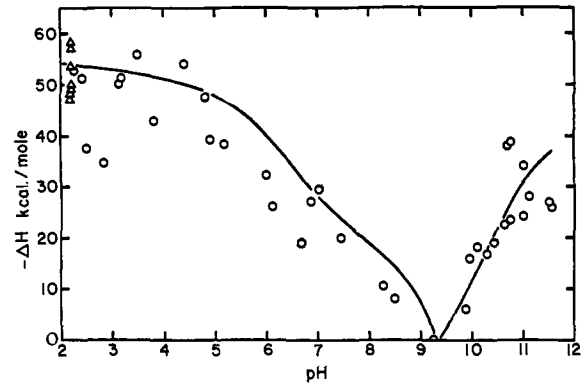


Figure 1. Enthalpy changes which accompany the addition of HCl (below pH 9.3) or KOH (above pH 9.3) to ribonuclease at pH 9.3 dissolved in 0.15 *M* KCl at 25°. The curve is a theoretical one: O, 0.15 *M* KCl, 25°; △, 0.10 *M* KCl, at various temperatures between 10 and 25°.

centration as used for previous equilibrium studies.[4,6] The observed heat changes ranged up to 0.180 cal (corresponding to a temperature change of about 0.0025°), which is considerably in excess of the heat change observed in a blank experiment in which the same two solvents were mixed in the absence of protein; the latter exothermic heat change, amounting to 0.048 ± 0.005 cal, was subtracted from that observed with the protein solution.

Only 1 hr was required for the complete measurement, the solution being at chemical and thermal equilibrium within about 5 min after mixing the reactants in the calorimeter. The experiments were conducted in the temperature range of 10–55°. In this temperature range, the heat of dilution of ribonuclease *at constant pH and temperature* in 0.15 *M* HCl–KCl solution at pH 2.2 and in 0.15 *M* sodium phosphate buffer at pH 4.6 and 7.5 was found to be within the limits of error (±0.005 cal) of breaking the sample cell.

The calorimeter is also equipped to measure the heat capacity of the solutions, which (for dilute solutions) is essentially the same as those of the solvents, within our experimental error of ±0.1% in the measurement of heat capacity.

The observed enthalpy changes were converted to a molar basis, using a molecular weight of 13,680 g/mole and a moisture content of the lyophilized solid of 7%.

## Results

**Proton Binding.** Proton binding (and dissociation) experiments were carried out at 25° by addition of HCl or KOH, respectively, in 0.15 *M* KCl, to a solution of ribonuclease at pH 9.3 in 0.15 *M* KCl. The latter pH is somewhat lower than the isoionic pH[13] of 9.6 because of the presence of a small amount of dissolved $CO_2$. The heat liberated is shown as a function of pH in Figure 1. The solid line represents the theoretical curve computed in the manner described in the Discussion section. Since the protein is in its native conformation at 25°, on the acid side of the isoionic point, these data are heats of ionization for the native protein at this temperature.

**Acid Denaturation.** Similar experiments were carried out at several temperatures by bringing a ribonuclease solution from pH 9.3 in 0.1 *M* KCl to pH 2.2 in 0.1 *M* KCl.[14] These results are shown in Figure 2, from which it can be seen that heat is liberated at low temperatures and absorbed at high temperatures, the rapid

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

(14) The pH of 2.2 was measured at 25° after the experiment. However, slight deviations from pH 2.2 in the temperature range of 10 to 55° should not affect the measured value of $\Delta H$ significantly, since the only group ionizing at this pH is the carboxyl group, whose heat of ionization is small.

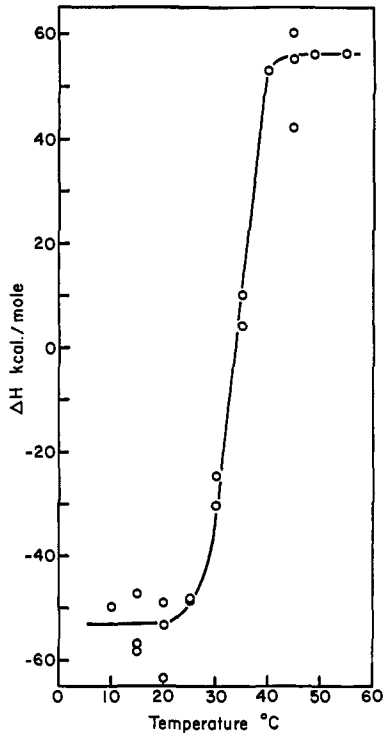


Figure 2. Temperature dependence of the enthalpy change which accompanies the addition of HCl to solutions of ribonuclease at pH 9.3 in 0.1 *M* KCl so that the final pH measured at 25° is pH 2.2. The curve is drawn through the experimental points.

change in $\Delta H$ occurring in the range 25–45° where ribonuclease is known to undergo a transition at pH 2.2. In fact, the previously reported transition temperature[4] of 35° is in good agreement with the data of Figure 2.

In order to determine whether the heat of protonation is temperature dependent, the following experiment was performed. A 3.0-ml aliquot of a stock solution (containing 14.2 mg of aspartic acid and 22.4 mg of histidine in 50 ml of 0.1 *M* KCl at pH 8.5), simulating the number and kinds of groups of ribonuclease which are protonated when the pH 9.3 solution is adjusted to pH 2.2, was brought to pH 2.2 with 40 ml of dilute HCl in 0.1 *M* KCl at 15 and 45°, respectively. The enthalpy change at 15 *and* 45° was 0.076 ± 0.002 cal. Thus, the heat of protonation of the amino acid solution was independent of temperature at 15 and 45°, within the limits of experimental error.

**Effect of Prior Irreversible Denaturation.** A sample of ribonuclease [which was denatured (largely irreversibly[4]) by maintaining it at 95° at pH 0.9 for 45 min, deionized by passing it through a mixed-bed resin column, and lyophilized] was adjusted from pH 9.3 to pH 2.2 at both 15 and 45°, in the same manner as the native protein. The enthalpy changes were −39 ± 1 and −21 ± 2 kcal/mole at 15 and 45°, respectively, compared to −53 and +56 kcal/mole for the native molecule at the same temperatures.

**Effect of Prior Oxidation.** A sample of oxidized ribonuclease (prepared by the procedure of Hirs,[15] and kindly supplied by Dr. J. P. Riehm of this laboratory) was subjected to the same pH change from 9.3 to 2.2. The enthalpy changes were −9 and +29 kcal/mole at 15 and 45°, respectively. Only one deter-

(15) C. H. W. Hirs, *J. Biol. Chem.*, **219**, 611 (1956).

mination was made at each temperature because of an insufficient supply of the oxidized protein.

## Discussion

**Protonation in Absence of Change of Conformation.** Consider first the data at low temperature (10–25°). In this temperature range, ribonuclease does not undergo any change of conformation[4] between pH 2.2 and 9.3. Hence, the observed values of $\Delta H$ at any temperature in this range must correspond to the heat of protonation of the native molecule, in going from pH 9.3 to 2.2, *i.e.*

$$\Delta H_{ion} = H_{2.2} - H_{9.3} \quad (1)$$

where $H_{2.2}$ and $H_{9.3}$ are the relative apparent heat contents of the protein at the two pH values. Since $\Delta H_{ion}$ is essentially independent of temperature, in the range 10–25°, $H_{2.2}$ and $H_{9.3}$ must vary with temperature in the same manner.

For the mixture of amino acids which simulates some of the ionizing groups of ribonuclease

$$\Delta H'_{ion} = H'_{2.2} - H'_{9.3} \quad (2)$$

where the primes indicate that the heat contents pertain to the mixture of amino acids. Since $\Delta H'_{ion}$ is independent of temperature in the range 15–45°, we shall assume that $\Delta H_{ion}$, for the protein in the absence of a change of conformation, is also independent of temperature in the range 25–45° (as well as 10–25°). Any enthalpy changes in excess of $\Delta H_{ion}$ will therefore be attributed (see below) to a change in conformation, $\Delta H_{den}$. The latter quantity will include any enthalpy changes due to the fact that the electrostatic factor, $w$, will not be the same for the native and denatured proteins.[16]

In the absence of a change of conformation we may account for the data of Figure 1 in the same manner as was done by Hermans and Rialdi for myoglobin.[9] The value of $\Delta H_{ion}$ at any pH, at a given temperature, is given by

$$\Delta H_{ion} = \Sigma \alpha_i \Delta H_i \quad (3)$$

where $\Delta H_i$ is the heat of protonation of the $i$th kind of group and $\alpha_i$ is the fraction of that kind of group which has been protonated in going from pH 9.3 to any given pH. The values of $\alpha_i$ are related to the intrinsic ionization constant $K_i°$ for the given kind of ionizing group by the equation[17]

$$\frac{\alpha_i}{1 - \alpha_i} = K_i° e^{2wZ} / a_{H^+} \quad (4)$$

where the electrostatic factor, $w$, depends on the size and shape of the protein molecule and on the ionic strength, $Z$ is the net charge on the molecule, and $a_{H^+}$ is the hydrogen ion activity. The values of $K_i°$ have been determined for ribonuclease.[13] An empirical eighth degree polynomial was obtained for the dependence of $Z$ on pH, using the data of Hauenstein[18,19] at ionic strength 0.15. Values of $w$ ranging from 0.061 to 0.122 were used, but the computed curve of $\Delta H_{ion}$ *vs.* pH was not sensitive to this variation (within 5% in $\Delta H_{ion}$) since the enthalpy of ionization of the carboxyl group is so small when $Z$ is large (and $Z$ is small when the $\alpha$-amino group becomes protonated). A value of 0.061 was used for the calculation of the curve shown in Figure 1.

In computing the curve above pH 9.3, $\Delta H_i$ is replaced by $\Delta H_i - 13{,}800$ in order to account for the neutralization of the ionizing proton by added base.[20] A summary of the values of $K_i°$ and $\Delta H_i$ used in this study and, for comparison, those used by Hermans and Rialdi[9] to fit the myoglobin data is given in Table I.

Although chloride ions are bound to ribonuclease,[21,22] no data are available for the heat of such binding. Therefore, this effect is neglected in the calculation of both $Z$ and $\Delta H_i$.

**Table I.** Parameters Used to Calculate the Enthalpy Titration Curve for Ribonuclease and Myoglylobin[9]

| Group[a] | $pK_i°$ Ribonuclease | $pK_i°$ Myoglobin | $\Delta H_i$, kcal/mole Ribonuclease | $\Delta H_i$, kcal/mole Myoglobin |
|---|---|---|---|---|
| Carboxyl | | | | |
| Normal (8) | 4.6 | 4.4 | 0.5 | 1.6 |
| Buried (1) | 2.5 | ... | 0.5 | ... |
| Buried (1) | 3.35 | ... | 0.5 | ... |
| Terminal (1) | 4.6 | 4.4 | 0.5 | 1.6 |
| Imidazole (4) | 6.5 | 6.5–6.6 | 5.5 | 7.1 |
| $\alpha$-Amino (1) | 7.8 | 7.8 | 11 | 11 |
| $\epsilon$-Amino (10) | 10.2 | 10.0 | 11 | 12.7 |
| Phenolic (3) | 10.0 | 10.0 | 6.1 | 6.1 |

[a] The numbers in parentheses indicate the number of each group considered to ionize between pH 2.0 and 11.5 in ribonuclease.

Within these limitations, it can be seen that the theoretical curve of Figure 1 agrees fairly well with the experimental data. This agreement is consistent with the fact that there is no change in conformation between pH 2.2 and 9.3 in the temperature range of 10–25°.

**Heat of Denaturation.** If ribonuclease at 45° is brought from pH 9.3 to pH 2.2, the observed $\Delta H$ is +56 kcal/mole. Since we have found no change in the ultraviolet difference spectrum at pH 9.3 between ribonuclease at 45 and at 25°, we may regard the molecule as being in its native conformation at 45° at pH 9.3. Therefore, the observed $\Delta H$ at 45° is

$$\Delta H_{obsd} = \Delta H_{ion} + \Delta H_{den} \quad (5)$$

where $\Delta H_{ion}$ is the heat of protonation of *native* ribonuclease in going from pH 9.3 to 2.2. Since we assume this to be independent of temperature,[23]

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(17) K. Linderstrøm-Lang, *Compt. Rend. Trav. Lab. Carlsberg*, **15**, No. 7 (1924).

(18) J. D. Hauenstein, Ph.D. Thesis, State University of Iowa, 1955.

(19) Hauenstein[18] dismissed the possibility of ion binding. However, it will be shown below that the theoretical curve of $\Delta H_{ion}$ *vs.* pH is not very sensitive to different values of $w$, and uncertainties in $Z$ (arising from a neglect of chloride binding) have less of an effect on the calculated curve than does $w$.

(20) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," 2nd ed, Reinhold Publishing Corp., New York, N. Y., 1950, pp 492–494.

(21) H. A. Saroff and W. R. Carroll, *J. Biol. Chem.*, **237**, 3384 (1962).

(22) G. I. Loeb and H. A. Saroff, *Biochemistry*, **3**, 1819 (1964).

(23) The temperature dependence of $\Delta H_{ion}$ of the *native* protein may be expressed as (temperature designated in subscripts)

$$(\Delta H_{ion})_{45} = (\Delta H_{ion})_{10} + \int_{10}^{45} \Delta C_p^{native}\, dT$$

where

$$\Delta C_p^{native} = (C_p)_{protonated}^{native} - (C_p)_{unprotonated}^{native}$$

The assumption that $(\Delta H_{ion})_{45} = (\Delta H_{ion})_{10}$ is equivalent to the assumption that $\Delta C_p^{native} = 0$. Besides the fact that the latter is a reasonable assumption, it is verified by the experiment on the mixture of amino acids.

$\Delta H_{ion} = -53$ kcal/mole (see Figure 2). In eq 5, $\Delta H_{den}$ is the enthalpy change for the following process, *at 45° and pH 2.2.*

native protonated ribonuclease ⟶ denatured protonated ribonuclease (6)

Substituting the value $\Delta H_{obsd} = +56$ kcal/mole, we obtain $\Delta H_{den} = 109 \pm 5$ kcal/mole.

In order to compare this value with that obtained from the van't Hoff equation,[4,6] it must be recalled that a two-state model was assumed (*two* two-state models being assumed by Scott and Scheraga,[6] since their kinetic data indicated that the transition consists of *two* independent steps) and equilibrium constants were evaluated at a series of temperatures. Since the van't Hoff plots obtained by Scott and Scheraga[6] were linear, the enthalpy changes computed from them correspond to reaction 6 (at pH 2.2), with the $\Delta H$'s being independent of temperature. The sum of the $\Delta H$'s for the two reactions considered by Scott and Scheraga[6] is 114 ± 7 kcal/mole over the pH range of 0.9 to 3.3 (covering about a 30° range of transition temperatures). Hence, within the experimental errors quoted, we may conclude that the calorimetrically measured $\Delta H_{den}$ is in good agreement with that determined by Scott and Scheraga using the van't Hoff calculation. Both of these values are considerably higher than the value of 70 kcal/mole at pH 2.8 obtained by Beck, *et al.*,[8] from heat capacity measurements.

While the value of Scott and Scheraga agrees with that obtained calorimetrically in this work, we cannot consider this a verification because of the uncertainty in the correct value[23] of $\Delta H_{ion}$ at 45°. If this agreement were real, it is still *not* a verification of the assumption made by Scott and Scheraga that ribonuclease unfolds in two two-step processes; even if *many* intermediates were involved, the van't Hoff approximation used by Scott and Scheraga could conceivably give a value close to 114 kcal/mole.

Consider next the discrepancy between our value of 109 kcal/mole at pH 2.2 and the value of 70 kcal/mole obtained by Beck, *et al.*,[8] at pH 2.8. The difference in pH would not seem to be an important factor.[6] Perhaps, the difference in concentration may be one cause, since the experiments of Beck, *et al.*, were made with ribonuclease concentrations 25 times greater than those used here; it is known that the observed heat of transition of poly-γ-benzyl-L-glutamate is lower at higher concentrations.[24] In addition, we found a slow exothermic step at 45° ($\Delta H = -20 \pm 10$ kcal/mole) with a half-life of about 10 min upon acidification of *1%* solutions of ribonuclease; this slow step, which was not observed at the *low* concentrations used in the experiments of Figures 1 and 2, could also contribute to the value of $\Delta H$ obtained by Beck, *et al.* Another factor may be the presence of irreversibly denatured material in the samples of Beck, *et al.*, since ribonuclease becomes progressively irreversibly denatured the longer it stands above the transition temperature[4] (our samples were denatured within 5 min, whereas those of Beck, *et al.*, were kept above the transition temperature for about 10 hr). Also, Beck, *et al.*, heated their solid material to constant weight at 105° before preparing solutions, a procedure which might have caused some denaturation.

**Irreversibly Denatured Ribonuclease and Oxidized Ribonuclease.** Making similar assumptions as in the case of reversible denaturation, we may conclude that the irreversibly denatured material has some residual structure which is unfolded with a *small* absorption of heat. The values observed upon acidification of irreversibly denatured ribonuclease are uncertain to the extent of an unknown degree of aggregation (involving an unknown heat of aggregation).

Oxidized ribonuclease must possess some noncovalent structure and undergo a conformational change upon protonation at 25° with an absorption of about 44 kcal/mole (the difference between −9 and −53 kcal/mole in going from pH 9.3 to 2.2 at 25°). Perhaps, in a similar way, some noncovalent structure may be present in the unprotonated form of *reduced* ribonuclease and may be responsible for the correct disulfide pairing which occurs during the oxidation experiments carried out by Anfinsen and his colleagues;[25,26] recently,[27] a noncovalent interaction, involving tyrosyl residue 115, has been so implicated.

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(25) M. Sela, J. H. White, Jr., and C. B. Anfinsen, *Science*, **125**, 691 (1957).
(26) C. B. Anfinsen, *Brookhaven Symp. Biol.*, **15**, 184 (1962).
(27) M. E. Friedman, R. Goldberger, and H. A. Scheraga, Abstracts, 152nd National Meeting of the American Chemical Society, New York, N. Y., Sept 1966.