A Calorimetric Study of Thermally Induced Conformational Transitions of Ribonuclease A and Certain of Its Derivatives*

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ABSTRACT: Conformational transitions of macromolecules are characterized by varying degrees of cooperativity, the upper limit of which corresponds to the purely two-state process A $\rightleftarrows$ B. A very useful criterion for two-state behavior is afforded by calorimetric measurements: the value for the enthalpy change per mole evaluated by application of the van't Hoff equation, ΔH_{vH}, under a given set of conditions is equal to the calorimetric value, ΔH_{cal}, observed under the same conditions if the transition is of the two-state type; otherwise the inequality $\Delta H_{vH} < \Delta H_{cal}$ holds. The equilibrium data required for the estimation of ΔH_{vH} are best obtained from the calorimetric observations themselves. The thermal transition undergone by bovine pancreatic ribonuclease A has been studied in a highly sensitive differential scanning calorimeter over the pH range 0.4–7.8 at a protein concentration of 0.5% by weight, and at pH 2.8 over a concentration range of 0.1–2.7%. It is concluded on the basis of these measurements, and by comparison with results reported by others, that the transition meets the requirements for a two-state process at pH values below about 2, but that as the pH is raised above this value deviations from the simple two-state situation become increasingly evident. The possible significance of these deviations is discussed.

The thermal transitions of the ribonuclease derivatives ribonuclease S, ribonuclease S′, and S protein at pH 7 are found to deviate widely from the full cooperativity characteristic of a two-state process.

Macromolecules of biological importance are generally characterized by a degree of rigidness of three-dimensional structure which far exceeds that usually observed with synthetic polymers. This arises from the existence of strong intramolecular forces exhibiting much cooperation, one manifestation of which is the relatively sharp thermally induced conformational transitions which have been observed with many biopolymers. The upper limit of cooperativity in a transition is exhibited in a simple two-state (Lumry *et al.*, 1966) transition

$$A \rightleftarrows B \qquad (1)$$

where the cooperative unit coincides with the entire molecule.[1] In some cases of transitions in macromolecules, such as the helix–coil transition of DNA or synthetic polynucleotides, although the transition is sharp enough to be reminiscent of a phase transition, it is quite clear that the average cooperative unit is far smaller than the entire molecule. In other cases, involving smaller molecules such as certain globular proteins, it is less clear how the average cooperative unit compares in size with the hydrodynamic molecule.

If α represents the degree of conversion from the initial state into the final state in the two-state process (reaction 1), the equilibrium constant is $K = \alpha/(1 - \alpha)$. The van't Hoff equation expressing the variation of K with temperature can be written in the form

$$\frac{d\alpha}{dT} = \alpha(1 - \alpha)\frac{\Delta H_{vH}}{RT^2} \qquad (2)$$

where ΔH_{vH} is the standard enthalpy change in reaction 1. In general, ΔH_{vH} may be a function of temperature and therefore of α. In such cases the change in the apparent heat capacity of the solute, ΔC_p, accompanying the transition is different from zero. If the slope of the α *vs.* T plot, frequently called the transition curve or the melting curve, is evaluated at the temperature of half-conversion, T_m

$$\left(\frac{d\alpha}{dT}\right)_{T = T_m} = \frac{\Delta H_{vH}(T_m)}{4RT_m^2} \qquad (3)$$

If the *calorimetrically observed* heat of reaction 1 is Δh cal g^{-1}, and the molecular weight of A and B is M, we have for the two-state system $\Delta H_{vH} = M\Delta h$.[2]

In more complex cases, involving any number of thermodynamically stable states intermediate between the initial and final states, it is still possible to derive from experimental data a measure of the fractional conversion, α,

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[1] The cooperative unit may, of course, include extensive regions which are completely inert so far as the process under consideration is concerned. For example, in the case of a simple keto–enol isomerization, $RCOCH_3 \rightleftarrows RC(OH)=CH_2$, the cooperative unit is the entire molecule regardless of the size of the inert group R.

[2] Since the enthalpy of a single molecular species is in general only slightly dependent on concentration, it is permissible in the present discussion to compare directly enthalpy values determined in dilute solutions with those pertaining to the standard state. The approximation involved here is probably no more serious than that resulting from the usual assumption of unit activity coefficients.

between the initial and final states, although in such systems it is to be expected that different types of experimental observations may lead to different measures of the fractional conversion (Lumry *et al.*, 1966). Since we are concerned here primarily with enthalpy changes, we shall restrict attention to measures of α derived from calorimetric measurements as described below. Such values of α can be utilized in eq 3 to evaluate ΔH_{vH}. However in the case of any macromolecular system, a van't Hoff ΔH is essentially meaningless since additional nonthermodynamic data are always required to permit specification of the size of the "mole," the unit of material to which the van't Hoff ΔH refers. Such nonthermodynamic data can be supplied by calorimetric observations.

It seems probable that any departure from the simple two-state situation will result in a broadening of the transition curve. Although various types of transition other than the two state would give symmetrical transition curves, only the two-state case leads to a value of ΔH_{vH} which is independent of α (provided, of course, that $\Delta C_p = 0$). However, constancy of ΔH_{vH} over the transition is not in general a criterion which is readily applicable to actual experimental curves because of the difficulty of accurately evaluating the slopes of the curves for values of α significantly different from 0.5. A very general criterion for the two-state case is afforded by comparison of the calorimetric and van't Hoff enthalpy values. The broadening of the transition curve referred to above would lead to a reduced ΔH_{vH}, so that in general $\Delta H_{vH} \leq M\Delta h$, with the equality holding only for the two-state case.

The inequality $\Delta H_{vH} < M\Delta h$ resulting from the existence of significantly populated states between the initial and final states may be considered in terms of two hypothetical cases. Equation 4 represents a transition which involves a group of intermediate states in a required sequence of occurrence, with each intermediate transition being characterized by the equilibrium constant, K, and the temperature-independent enthalpy change, ΔH

$$A_1 \underset{\Delta H}{\overset{K}{\rightleftarrows}} A_2 \underset{\Delta H}{\overset{K}{\rightleftarrows}} \cdots \underset{\Delta H}{\overset{K}{\rightleftarrows}} A_n \qquad (4)$$

Such a transition would give a symmetrical transition curve with the van't Hoff enthalpy value at T_m, $\Delta H_{vH}(T_m)$, given by

$$\Delta H_{vH}(T_m) = 4RT_m^2\left(\frac{d\alpha}{dT}\right)_{T = T_m} = \frac{n+1}{3}\Delta H = \frac{n+1}{3(n-1)}M\Delta h \qquad (5)$$

A closely related situation is afforded by molecules which are made up of n identical and independent cooperative units each one of which undergoes transition in a two-state manner with intrinsic equilibrium constant, K, and enthalpy change ΔH. In this case the transition curve is identical with that of a two-state process with $\Delta H_{vH}(T_m) = \Delta H$. The former of these two cases might be considered to be appropriate for an idealized representation of a conformational transition in a globular protein, and the latter for the helix–coil transition in a polynucleotide or a polypeptide. According to the statistical treatments of Zimm and Bragg (1959) and Appliquist (1963) for helix–coil transitions, the cooperativity parameter, σ, is given by the expression

$$\sigma^{1/2} = m\Delta h/\Delta H_{vH} \qquad (6)$$

where m is the residue weight of the monomeric species. The calorimetric criterion for a two-state process, and the calorimetric method for the estimation of the size of the cooperative unit in a conformational transition, are important because equipment is now available (Ackermann, 1958; Ackermann and Rüterjans, 1964; Cruickshank *et al.*, 1968; Privalov *et al.*, 1964; Gill and Beck, 1965; Karasz and O'Reilly, 1966; Danforth *et al.*, 1967; Clem *et al.*, 1969; Jackson and Brandts, 1970) for the accurate determination of the excess heat content of a polymer solution resulting from a thermally induced transition. If the total enthalpy change in the transition is independent of temperature, α is simply the ratio of the enthalpy change due to the fraction of the transition completed at temperature, T, to the enthalpy change corresponding to complete reaction. However, if the apparent heat capacity of the polymeric solute is different after the transition than before, the interpretation of the calorimetric data is somewhat more complex, and indeed is subject to some unavoidable ambiguity. It should be emphasized that entirely analogous difficulties arise in the evaluation of α from observations of any transition-sensitive property of the solute.

In this paper we report the results of a detailed calorimetric study of the thermally induced transition which bovine pancreatic ribonuclease A undergoes in solutions of acid and neutral pH. This transition appears to be quantitatively reversible in solutions of low pH but becomes incompletely reversible when T_m is raised by raising the pH of the solution into the neutral range. In an earlier paper (Danforth *et al.*, 1967) we reported calorimetric measurements of this transition at pH 2.8 and rather high protein concentration (1.5% by weight).

Brandts and Hunt (1967) have made a careful spectrophotometric study of the transition of ribonuclease at low values of the pH, utilizing the decrease in absorbance at 287 mμ which accompanies the transition and interpreting their data on the basis of the two-state hypothesis. Since recent improvements in the differential scanning calorimeter previously described (Danforth *et al.*, 1967) afford the possibility of extending our calorimetric observations down nearly to the level of protein concentration (0.03–0.04%) employed in the spectrophotometric work, it was important to undertake a reexamination of the transition in order to obtain calorimetric data which would be directly comparable with the spectrophotometric results. In addition, the effect of pH on the transition and the transition behavior of certain derivatives of ribonuclease have been investigated.

Materials and Methods

Materials. Bovine pancreatic ribonuclease A was separated by chromatography (Hirs *et al.*, 1953) from material supplied by Light and Co., England. It was found that indistinguishable calorimetric results were obtained using ribonuclease A purchased from Worthington Biochemical Co., N. J. In some cases, the protein was adjusted to pH 6 and heated to 60° for a few minutes to break up aggregates before loading

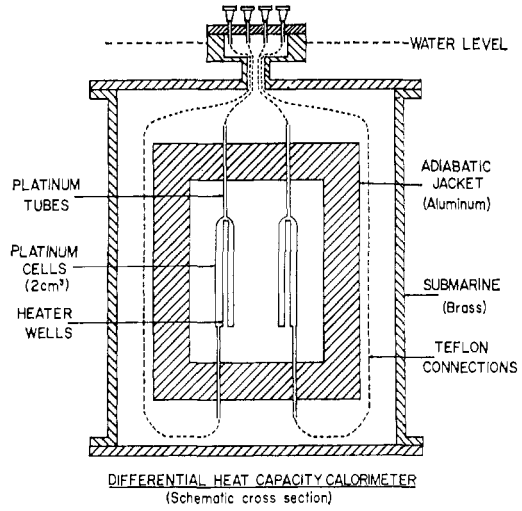

FIGURE 1: Schematic cross section of the differential heat capacity calorimeter, showing the disposition of platinum calorimeter cells within the adiabatic jacket.

into the calorimeter, but since no effect of this treatment could be detected, it was omitted in most of the experiments.

Ribonuclease S, in which the peptide bond between Ala-20 and Ser-21 had been hydrolyzed by treatment with subtilisin, was prepared from Light and Co. ribonuclease by the method of Richards and Vithayathil (1959). Complete details of this procedure and of the purification of the product are given by Hearn (1969).

Ribonuclease S contains the so-called S peptide and S protein. These components were separated by gel filtration on Sephadex G-75 with 5% formic acid as eluent (Hearn, 1969).

Ribonuclease S′ is the designation given to the active enzyme obtained by the interaction of S peptide and S protein. This product appears to be indistinguishable from ribonuclease S, but until this is proven to be the case, most appropriately by X-ray structure analysis, the special designation will be retained.

The concentrations of ribonuclease A and ribonuclease S were determined by spectrophotometry at 277.5 mμ in neutral 0.2 M phosphate buffer, using ϵ 9.8×10^3 M^{-1} cm^{-1}. The concentration of the S protein was also determined spectrophotometrically taking the absorbance of a 0.1% (by weight) solution at 280 mμ to be 0.784 (Sherwood and Potts, 1965).

All chemicals employed were analytical grade commercial products.

Methods. The transition calorimeter previously described (Danforth *et al.*, 1967) was modified in several important respects with very significantly improved performance.

(1) The platinum calorimeter cells were changed to the form shown in Figure 1, and are permanently supported within the adiabatic jacket by means of the platinum filling tubes gold soldered to them. The copper cylinders formerly serving to contain the calorimeter cells were dispensed with, and the junctions of the measuring thermel (10 junctions on each cell) and of the adiabatic thermel (1 junction on each cell associated with two junctions immersed in the adiabatic jacket) are mounted directly on the cells using 0.001 in mylar film for insulation. Two 250-ohm manganin heaters are contained in the reentrant tube in each cell, as in the earlier design. Teflon tubing, as indicated by the dashed lines in Figure 1, leads from the platinum tubes to syringe sockets at the top of the outer brass submarine container. The Teflon tubes are of such a diameter as to give a tight push fit over the platinum filling tubes. For filling, solution is introduced slowly from a syringe through the connection leading to the bottom of the cell until excess appears at the other syringe socket; a second syringe containing some of the same solution is then inserted in this socket, and entrapped air removed by rapidly pulling up on the plunger of this syringe to reduce the pressure on the solution and simultaneously to cause solution to flow through the system to sweep out any thus enlarged air bubbles. It is important to have the cell and solution at a low temperature (~5°) during filling so that boiling of the solution will not occur. Each cell holds about 2 ml; a total of 3–4 ml of solution is required for successful application of this filling technique. The effective volume of each cell is evaluated from measurements of its heat capacity when empty and when filled with water, using the known specific heat and density of water. Appropriate corrections, which are small, are made for the expansion of the solutions during the observation of a transition, the assumption being made that the solutions have the same coefficient of expansion as water. (2) It was mentioned in our earlier paper (Danforth *et al.*, 1967) that continual readjustment of the high-resistance balancing shunt across one of the calorimeter heaters was required during a heating experiment to maintain accurate thermal balance of the two calorimeter cells when both cells were filled with water or the same solution. This operation has now been automated, and thereby rendered more effective, by use of a Model FGE 5110 Data-Trak.[3] (3) A tubing connection, not shown in Figure 1, is included by which dry N_2 can be introduced into the space around the calorimeter cells. It has been found that considerably improved reproducibility is obtained by careful exclusion of moist air, particularly when the calorimeter cells are cooled between runs.

The geometry of the apparatus is such that a major portion of the heat transfer from the calorimeter heaters is by means of convection. We have ascertained that no significant artifacts arise from convection by showing that the results obtained at the normal heating rate of 18°/hr are accurately duplicated when the heating rate is reduced to 4.5°/hr.

The primary data for each transition experiment consisted in values of the total energy fed back to the calorimeter in which the transition was taking place and the temperature at intervals of 1 min. From these data and the known concentration of protein in each experiment, curves of the type illustrated in Figure 2 (left side) were constructed. From these curves, transition curves similar to the one shown in Figure 2 (right side), giving α as a function of temperature, are readily constructed either numerically as outlined in our previous publication (Danforth *et al.*, 1967) or graphically as follows. The instrument is adjusted in the low-temperature region, where it is assumed no transition is going on, to give a horizontal base line. The essentially temperature-independent slope of the heat curve in the high-temperature

[3] R. I. Controls, Minneapolis, Minn. 55424.

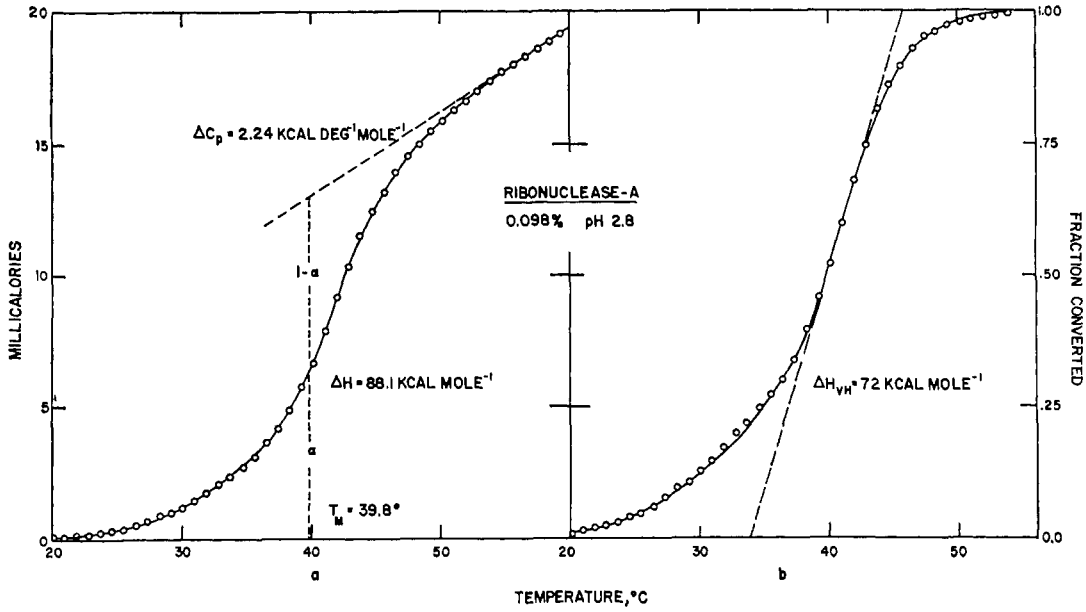

FIGURE 2: (a) A typical plot of feedback energy, ΔQ, *vs.* temperature during the transition of ribonuclease A. Protein concentration, 0.098% by weight, 0.2 N glycine buffer (pH 2.8). (b) Transition curve evaluated graphically as explained in the text.

region indicates that the *apparent* heat capacity of the solute increases during the transition. A vertical line at any specified temperature between the horizontal low-temperature base line and the high-temperature base line is divided by the experimental curve into segments which are taken to be proportional to α and $1 - \alpha$. This procedure is based on two assumptions in addition to the assumption mentioned above that a low-temperature range is available in which no transition takes place: (1) The increase in the apparent heat capacity of the solute is assumed to be strictly proportional to the extent of the conversion between the initial and the final states, regardless of how many intermediate states may be involved, and is not otherwise dependent on temperature. (2) The enthalpy increase observed at any temperature is assumed to be equal to the total transition enthalpy at that temperature multiplied by α. As indicated above, equivalent assumptions are involved in the interpretation of the variation with temerature of any transition-related property. The van't Hoff value for the enthalpy change at T_m, $\Delta H_{vH}(T_m)$, is calculated by eq 3 from a graphically estimated value of $(d\alpha/dT)_{T=T_m}$.

It is important to add that other assumptions, leading to conclusions different from those presented below, can equally well be adopted. For example, in their interpretation of the data from a calorimetric study of the thermal transition of chymotrypsinogen, Jackson and Brandts (1970) assumed that a gradually increasing rate of absorption of heat below the main transition, which is also evident in our data for ribonuclease, is to be attributed to a temperature-dependent heat capacity for the native state of the protein. The numerical values for various transition parameters presented in the section on Results were obtained on the basis of the assumptions outlined in the preceeding paragraph. Examples of transition parameters obtained on the basis of other possible sets of assumptions will be given in the Discussion.

It is evident that if protons are liberated or taken up by the protein during the transition and the solution is well buffered there will be a contribution to the observed heat of transition resulting from protonation or deprotonation of the buffer. If there is no other interaction with the buffer species, a similar contribution will be included in the van't Hoff enthalpy value, in such a manner as to have no influence on the ratio of the van't Hoff value to the calorimetric value. All the enthalpy values reported here, both van't Hoff and calorimetric, have been corrected for buffer ionization heats, the change in proton binding by the protein being estimated from the variation of T_m with pH as described later. The heat of the first ionization of glycine was taken to be 600 cal mole^{-1} at T_m (Izatt and Christensen, 1968). The heat of

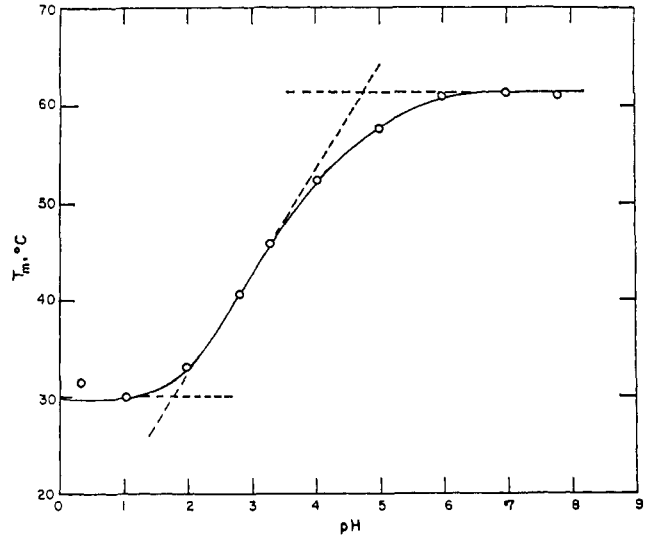

FIGURE 3: The variation with pH of the temperature of half-completion, T_m, of the thermal transition of ribonuclease A. Protein concentration, approximately 0.5% by weight.

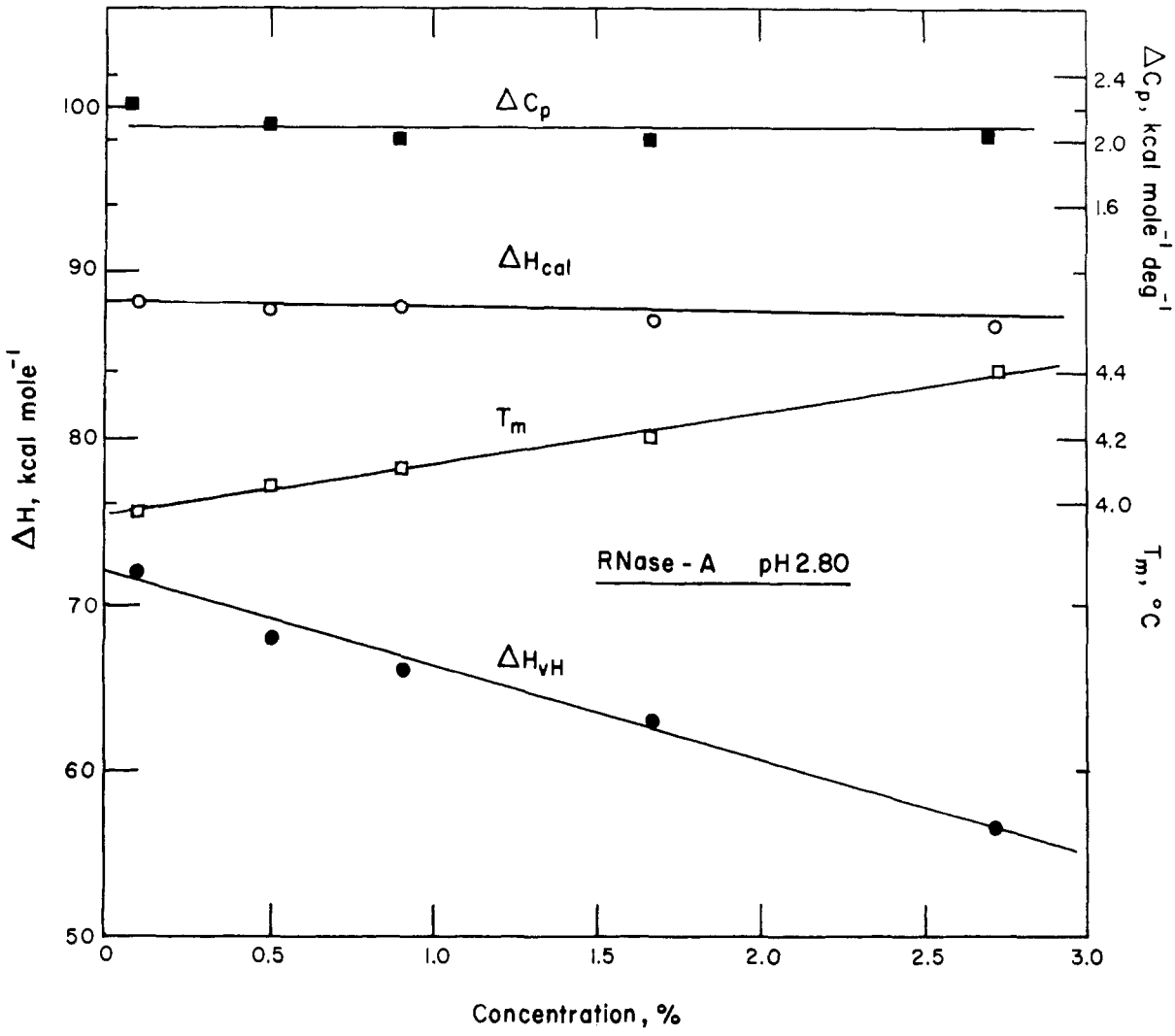

FIGURE 4: The dependence on protein concentration of the parameters for the thermal transition of ribonuclease A in 0.2 N glycine buffer (pH 2.8).

ionization of acetic acid, approximately −90 cal mole^{-1}, is so small as to be completely negligible.

Results

Variation of T_m with pH. Values for T_m estimated from transition curves constructed as described above are shown as a function of pH in Figure 3. The transition experiments were carried out at a protein concentration of approximately 0.5%. It has been frequently noted, most recently by Alberty (1969) and by Ptitsyn and Birshtein (1969), that the change in proton binding in the transition can be evaluated from the slope of a curve such as the one in Figure 3 by means of the equation

$$\frac{\partial T_m}{\partial \mathrm{pH}} = \frac{2.303 \Delta n R T_m^2}{\Delta H} \qquad (7)$$

where $\Delta n = n_{\mathrm{final}} - n_{\mathrm{initial}}$ is the difference in proton binding between the final and initial states. The maximum proton change is seen to occur at pH 3, where $\Delta n = 2.2$ protons absorbed per molecule of ribonuclease. Although this expression is strictly correct only for a two-state process, its application in the present case to the estimation of corrections for buffer ionization heats does not lead to significant error because the corrections are small.

Variation of the Enthalpy of Transition with Protein Concentration. Experiments were performed in 0.2 N glycine buffer (pH 2.80) at protein concentrations in the range 0.1–2.7% by weight, with the results summarized in Table I. The values of T_m in the second column were read from the transition curves, and the values of $\Delta H_{cal}(T_m)$ were read from the heat curves at T_m. As seen in Figure 4, there is a small trend of T_m and $\Delta H_{vH}(T_m)$ with protein concentration, the origin of which is not known, whereas $\Delta H_{cal}(T_m)$ and ΔC_p, the final increase in the apparent heat capacity of the solute, are practically independent of protein concentration. It is evident that there is a significant discrepancy between the calorimetric and van't Hoff values of the enthalpy which persists even on extrapolation to infinite dilution. At very low concentration the van't Hoff value is 82% of the calorimetric value.

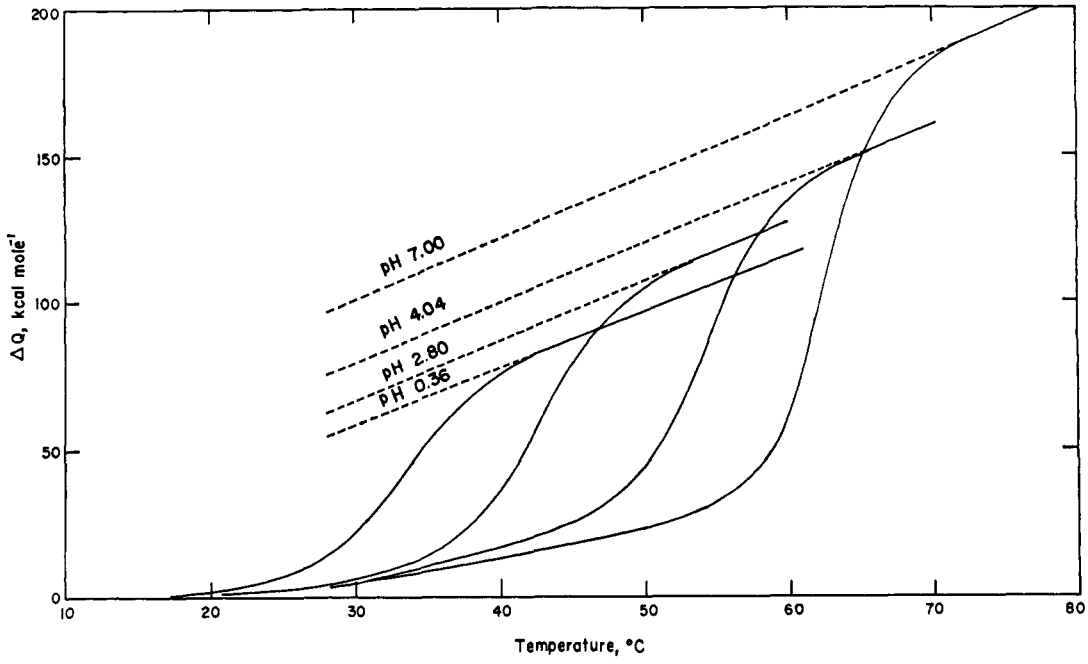

FIGURE 5: A selection of calorimetric curves illustrating the effect of pH on the shape of the transition curve. Protein concentration, approximately 0.5% by weight. Experimental conditions at various pH values are given in Table III.

Effect of pH on the Transition Parameters. The data obtained in experiments performed at various values of the pH, with ribonuclease A present in about 0.5% concentration, are given in Table II, and the transition parameters derived from these data are listed in Table III. The variation of T_m with pH has been discussed above. It is seen that $\Delta H_{vH}(T_m)$ and ΔC_p are essentially independent of the pH, whereas $\Delta H_{cal}(T_m)$ varies strongly with pH even when corrected to a constant temperature using the observed values of ΔC_p. At very low values of the pH there is good agreement between the calorimetric and van't Hoff values of the transition enthalpy, but the difference between these quantities becomes increasingly large as the pH is raised. Inspection of Figure 5, in which a selection of experimental curves is presented, shows that the increasing discrepancy between the calorimetric and van't Hoff enthalpies is paralleled by increasing asymmetry of the curves.

At low pH the transition appears to be completely reversible, as judged by the fact that a second experiment performed with a single filling of the calorimeter gives results indistinguishable from these obtained in the first heating. At pH values above 4.0 the transition becomes detectably irreversible. This is illustrated in Figure 6, in which the experimental curves for two heatings of a 0.54% solution at pH 6.2 are given. Presumably the irreversible part of the denaturation is a time- and temperature-dependent phenomenon at any given pH. Although the thermodynamic parameters given for the transitions at the higher pH values may be seriously in error because of the irreversibility, it is unlikely that the excess enthalpies given in Table II are much affected by irreversibility at temperatures below 55–60°.

Transition Parameters for Derivatives of Ribonuclease A. Transition experiments with ribonuclease S, ribonuclease S′, and the S protein, all in unbuffered solutions at pH 7 in 0.2 N NaCl solution at a concentration of approximately 0.5%, are summarized in Table IV and Figure 7. As is the case with ribonuclease A, the transitions of its derivatives are not fully reversible at pH 7. The results for ribonuclease S and ribonuclease S′ are the same within experimental uncertainty; the curve for the latter material is omitted from Figure 7.

One can conclude from Figure 3 that there is no change in protonation accompanying the transition of ribonuclease A at pH 7. Direct measurements (Hearn, 1969) of the pH changes accompanying the transitions of the ribonuclease derivatives at a concentration of about 1% together with back-titrations of the substances at a temperature corresponding to full unfolding, indicated *liberation* of 1.2–1.3 protons/molecule. Because of buffering by the protein, the pH decreases amounted to approximately 0.6 unit. These decreases would tend to increase slightly the values for $\Delta H_{vH}(T_m)$ estimated from the transition curves.

TABLE I: Conformational Transition of Ribonuclease A in 0.2 N Glycine Buffer at pH 2.80.

Protein Concn (% by Wt)	T_m (°C)	ΔH_{cal} (T_m) (kcal mole^{-1})	ΔH_{vH} (T_m) (kcal mole^{-1})	ΔC_p (kcal deg^{-1} mole^{-1})
0.098	39.8	88.1	72.0	2.24
0.495	40.6	87.7	68.0	2.11
0.900	41.0	87.8	66.0	1.99
1.66	42.0	87.1	63.0	1.99
2.72	44.0	86.6	56.0	2.03
	Av	87.5	Av	2.07
	Av dev	±0.5	Av dev	±0.07

TABLE II: Excess Enthalpies of the Solute in Solutions of Ribonuclease A Relative to the Solutions at 0–5° at Various Values of the pH.

Temp (°C)	ΔQ (kcal mole^{-1}) at pH									
	0.36	1.05	2.02	2.80	3.28	4.04	5.00	6.23	7.00	7.80
15.0	0.45	0.50								
16.5	0.65	0.96	0.20	0.42						
18.0	1.15	1.81	0.40	0.65	0.42	0.51	0.43			0.75
19.5	1.70	2.91	0.65	0.90	0.73	0.68	0.60	0.50	0.32	1.35
21.0	2.89	4.10	1.03	1.05	0.98	0.89	0.90	0.62	0.65	1.93
22.5	3.96	5.95	1.55	1.43	1.30	1.03	1.15	0.85	0.88	2.81
24.0	5.82	8.51	2.21	2.10	1.92	1.67	1.53	1.08	1.38	3.37
25.5	7.94	11.6	3.94	2.85	2.35	2.08	1.95	1.88	1.96	3.94
27.0	11.3	15.9	6.31	3.52	2.93	3.02	2.69	2.51	2.78	4.90
28.5	15.8	22.5	10.1	4.82	4.02	3.85	3.56	3.22	3.66	6.05
30.0	22.2	30.4	14.9	6.38	5.35	5.02	4.95	4.14	4.91	7.22
31.5	30.5	40.5	22.2	8.14	6.90	6.37	6.01	5.20	6.12	8.50
33.0	40.2	49.4	31.9	10.3	8.32	7.92	7.78	6.21	7.50	10.1
34.5	50.0	57.1	42.9	13.4	10.2	9.56	9.40	7.45	8.75	11.6
36.0	58.6	64.1	54.3	17.0	12.1	11.2	11.2	8.66	9.86	13.3
37.5	65.8	70.0	63.1	22.6	13.4	12.9	12.7	9.91	11.2	14.5
39.0	71.9	75.2	70.5	30.2	17.2	15.3	14.5	11.2	12.6	15.8
40.5	77.0	79.6	76.3	39.4	21.5	17.7	16.5	12.6	14.6	17.1
42.0	81.1	83.5	81.2	51.6	26.6	20.1	19.0	14.1	15.8	18.6
43.5	83.9	86.5	83.9	66.0	34.6	22.9	20.9	15.7	17.0	20.4
45.0	86.8	89.6	86.8	78.3	45.4	25.6	23.9	17.6	18.5	22.1
46.5	89.8	92.6	92.3	88.1	59.3	29.1	26.8	19.5	19.9	24.0
48.0	92.7	95.7	95.2	96.1	76.4	34.2	30.2	21.4	21.2	26.1
49.5	95.5	98.6	98.1	102.3	91.0	41.1	33.1	23.6	22.8	28.5
51.0	98.4	101.8	101.1	109.9	103.1	50.4	36.6	26.2	24.8	30.8
52.5	101.2	104.5	104.0	111.4	111.3	64.0	41.2	28.1	27.1	33.5
54.0	104.1	107.6	107.0	114.8	118.4	80.8	47.2	31.2	30.1	37.0
55.5	107.0		109.9	117.8	123.6	99.9	55.4	36.0	33.4	41.4
57.0	110.0		112.6	121.0	127.7	114.5	66.7	42.4	39.0	47.1
58.5	112.9		115.5	124.1	130.9	126.2	84.0	52.4	47.9	55.9
60.0	115.9		118.5	127.2	134.0	134.2	102.9	65.6	63.8	70.4
61.5			121.5		137.0	140.0	121.6	86.5	87.3	93.1
63.0					140.1	145.1	136.7	109.0	116.5	117.0
64.5					143.1	149.4	147.6	129.6	141.1	140.6
66.0					146.1	152.5	156.2	145.2	159.0	156.6
67.5						155.6	163.5	155.4	169.9	168.4
69.0						158.8	169.4	162.8	177.8	176.7
70.5							174.6	168.9	183.7	183.8
72.0							179.7	174.2	188.2	189.8
73.5							184.1	178.2	191.4	195.3
75.0							189.0	182.1	194.5	200.2
76.5							192.3	185.1	197.6	204.6
78.0							195.8	188.2	200.7	209.0
79.5							199.2	191.3		212.4
81.0							202.6			215.4
82.5										218.4
84.0										221.4

Discussion

Comparison with Previous Results. Beck *et al.* (1965) studied the transition of ribonuclease A at pH 2.8 by essentially the same method as employed in the present work. The solvent used was 0.15 M KCl and 0.01 M phthalate buffer, and the protein concentration was 1.4–2.7% by weight. These authors reported values of 70 ± 1 kcal mole^{-1} for $\Delta H_{cal}(T_m)$, 52 kcal mole^{-1} for $\Delta H_{vH}(T_m)$, and 0.66 kcal deg^{-1} mole^{-1} for ΔC_p. All these values are significantly

TABLE III: Transition of Ribonuclease A at Various Values of the pH.

pH	Solvent	T_m (°C)	ΔH_{cal} (T_m) (kcal mole^{-1})	ΔH_{vH} (T_m) (kcal mole^{-1})	ΔH_{cal} (25°)[a] (kcal mole^{-1})	ΔC_p (kcal deg^{-1} mole^{-1})
0.36	HCl	31.5	61.2	62	48.7	1.90
1.05	0.1 N NaCl, HCl	29.9	59.0	59	49.1	2.02
2.02	0.2 N glycine buffer	31.2	65.6	67	53.3	1.98
2.80	0.2 N glycine buffer	40.6	87.7	68	54.8	2.11
3.28	0.2 N glycine buffer	45.8	105.0	67	63.4	2.00
4.04	0.2 N acetate buffer	52.3	126.0	64	69.2	2.08
5.00	0.2 N acetate buffer	57.8	151.0	60	77.2	2.25
6.23	0.2 N acetate buffer	60.8	155.5	66	81.2	2.05
7.00	0.2 N NaCl	61.3	168.0	(88)	92.9	2.07
7.80	0.2 N NaCl	61.2	178.0	72	105.5	2.00
				Av 65		Av 2.05
				Av dev ±3		Av dev ±0.07

[a] $\Delta H_{cal}(25°) = \Delta H_{cal}(T_m) - \Delta C_p(T_m - 25)$.

lower than those found by us. One contribution, amounting to 3–4 kcal mole^{-1}, to the discrepancy in $\Delta H_{cal}(T_m)$, arises from the fact that the heat of ionization of phthalic acid at T_m is −1.0 kcal mole^{-1} (Izatt and Christensen, 1968) whereas that for glycine is about +0.6 kcal mole^{-1}, and that 2.2 protons are absorbed per molecule of protein during the transition. Beck *et al.* used a heating rate in their experiments less than one-tenth as large as ours, so that their material was exposed to elevated temperatures for much longer periods. The data in Table I of their publication indicate a decrease in the enthalpy of transition on successive heatings of a single solution. The relatively small difference between the value reported for $\Delta H_{vH}(T_m)$ and that found in the present work is probably due to inadequate buffering in the experiments performed by Beck *et al.* At a protein concentration of 2.7% about 0.0044 M proton would be absorbed by the protein, whereas the concentration of protonated phthalic acid present was about 0.005 M. Since an increase in pH raises the transition temperature, inadequate buffering would have the effect of broadening the experimental transition curve. We cannot account for the discrepancy in ΔC_p.

The work of Beck *et al.* (1965) has been carried further by Reeg (1969), who studied the concentration dependence of the transition enthalpy employing a much higher heating rate than used by Beck *et al.* Reeg concluded that at pH 2.8 and protein concentrations below 0.5%, $\Delta H_{cal}(T_m) = \Delta H_{vH}(T_m)$, so that under these conditions the transition appears to be two state in character. His value for $\Delta H_{cal}(T_m)$, 85.1 kcal mole^{-1} at low concentration is in reasonable agreement with ours, but his value for $\Delta H_{vH}(T_m)$ is considerably higher than ours. He also found $\Delta C_p \approx 1.5$ kcal deg^{-1} mole^{-1}. The source of these discrepancies is unknown.

Kresheck and Scheraga (1966) determined calorimetrically the enthalpy changes attendant on altering the pH of a ribonuclease A solution from pH 9.3 to 2.2 in the temperature range 10–55°. The heat liberated at temperatures below 20°, where the protein remains in its native state throughout the pH range used, was attributed to the heats of protonation of the various titratable groups in the protein and was assumed to remain constant over the entire temperature range. On this basis, a value of 109 ± 5 kcal mole^{-1}, which falls within the range of calorimetric values listed in Table II, was estimated for the enthalpy of denaturation. Since no evidence of the large ΔC_p for the unfolding process was observed in these experiments, and the overall heat of proton addition was assumed to be temperature independent, Kresheck and Scheraga (1966) believed the enthalpy of the denaturation process to be independent of temperature.

Discussion of earlier estimates of the enthalpy change in the transition of ribonuclease A, based on the van't Hoff equation, are given in the papers by Beck *et al.* (1965) and Kresheck and Scheraga (1966). Ginsburg and Carroll (1965) followed the transition at pH 2.1 in solutions of low ionic strength by observations of absorbance, viscosity, and optical rotation. They found nearly identical transition curves for all methods of observation, which lead to T_m and $\Delta H_{vH}(T_m)$ values in reasonable agreement with those reported in Table III. Ginsburg and Carroll found an effect of sulfate ions, and to a lesser extent phosphate ions, in stabilizing the protein. Thus with Cl^- as the only anion present the

TABLE IV: Transition of Ribonuclease A and Various of Its Derivatives in 0.2 N NaCl at pH 7.0.

Substance	T_m (°C)	ΔH_{cal} (T_m) (kcal mole^{-1})	ΔH_{vH} (T_m) (kcal mole^{-1})	ΔH_{cal} (25°) (kcal mole^{-1})	ΔC_p (kcal deg^{-1} mole^{-1})
Ribonuclease A	61.3	168	97	92.9	2.07
Ribonuclease S	47.7	107	62	59.8	2.08
Ribonuclease S′[a]	47.1	111	63	64.0	2.08
S protein	37.6	55	30	37.1	1.42

[a] S protein:S peptide = 1:1.25.

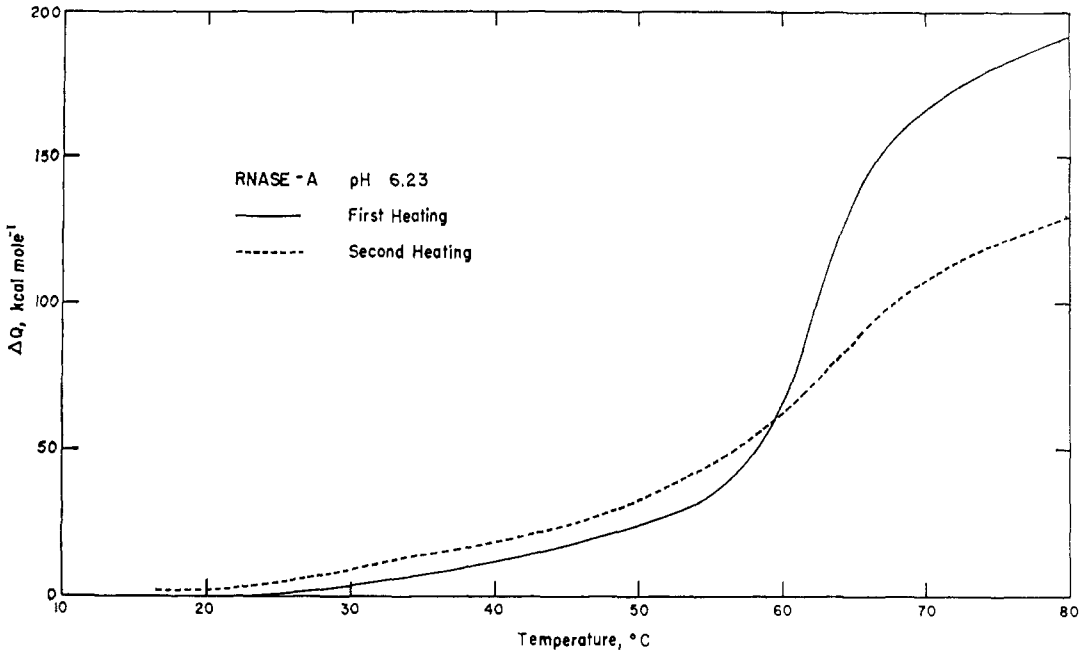

FIGURE 6: Calorimetric curves showing the lack of reversibility of the transition of ribonuclease A at high pH. Protein concentration, 0.5% by weight; 0.2 N NaCl (pH 6.23), solid curve, first heating; dashed curve, second heating.

values for T_m and $\Delta H_{vH}(T_m)$ were, respectively, 29° and 46 kcal mole^{-1}; these were changed to 32° and 57 kcal mole^{-1} in the presence of $H_2PO_4^-$–H_3PO_4, and to 43° and 69 kcal mole^{-1} in the presence of SO_4^{2-}–HSO_4^-. It is probable that our samples of ribonuclease A, which were not as rigorously deionized as those of Ginsburg and Carroll, contained sufficient phosphate to give a small degree of stabilization.

Brandts and Hunt (1967) have reported a very careful spectrophotometric study of the transition in the pH range 1.13–3.15 at protein concentrations of 0.03–0.04%, and a thermodynamic analyses of the resulting data in terms of the two-state hypothesis. The values they obtained for ΔH_{vH} at 30° are in satisfactory agreement with our calorimetric ΔH_{vH} values at the same temperature up to pH 2.5, but at higher values of the pH the calorimetric values decrease with increasing pH much more rapidly than the spectrophotometric values. This discrepancy may result, to an unknown extent, from the dependence of the calorimetric ΔH_{vH} on protein concentration shown in Figure 4. Brandts and Hunt (1967) report an average value for ΔC_p of 2.01 ± 0.03 kcal deg^{-1} mole^{-1}, independent of pH, in excellent agreement with the directly observed calorimetric value of 2.05 ± 0.07 kcal deg^{-1} mole^{-1}.

TABLE V: Transition Parameters for Ribonuclease A (Temperature-Dependent Heat Capacity for Native State).

pH	T_m (°C)	ΔH_{cal} (T_m) (kcal mole^{-1})	ΔH_{vH} (T_m) (kcal mole^{-1})	ΔC_p (T_m) (kcal deg^{-1} mole^{-1})
3.28	47.0	86	88	0.86
4.04	53.8	96	88	0.79
5.00	59.5	117	88	0.83

Alternative Interpretations of the Calorimetric Data. Jackson and Brandts (1970) have very recently reported a calorimetric investigation of the thermally induced transition of chymotrypsinogen. They observed an increasing rate of absorption of heat with increasing temperature below the main transition range, similar to that we found with ribonuclease. They attributed this heat absorption to a temperature-dependent apparent heat capacity for the native state of the protein, and found that this assumption led to close agreement between $\Delta H_{cal}(T_m)$ and $\Delta H_{vH}(T_m)$ in the pH range (1.95–3.02) included in their study.

A similar interpretation has been applied to a selection of our data. The equation

$$\Delta Q = -8.0 + 0.11t + 0.011t^2 \qquad (8)$$

where t is the centigrade temperature, fits the data at pH 7.00 reasonably well between 20 and 55°. This corresponds to an apparent heat capacity increasing linearly with temperature with a slope of 0.0016 cal deg^{-2} g^{-1}. If eq 8 is used as base line in place of the horizontal base line employed in deriving the quantities listed in Table III, the results given in Table V are obtained. It is seen that this treatment leads to values for $\Delta H_{vH}(T_m)$ and $\Delta H_{cal}(T_m)$ which are essentially in agreement at pH 3.28 and 4.04, but not at pH 5.00.

A different interpretation of the data can be based on the assumption that there are two transitions. Again it is found (see Table VI) that the apparent adherence to the requirements for a two-state transition, in this case for both the lower and upper transitions, can be extended to about pH 4. Calculations with several different sets of assumed parameters for the lower transition at pH 5, including nonvanishing values for the change in apparent heat capacity, made it

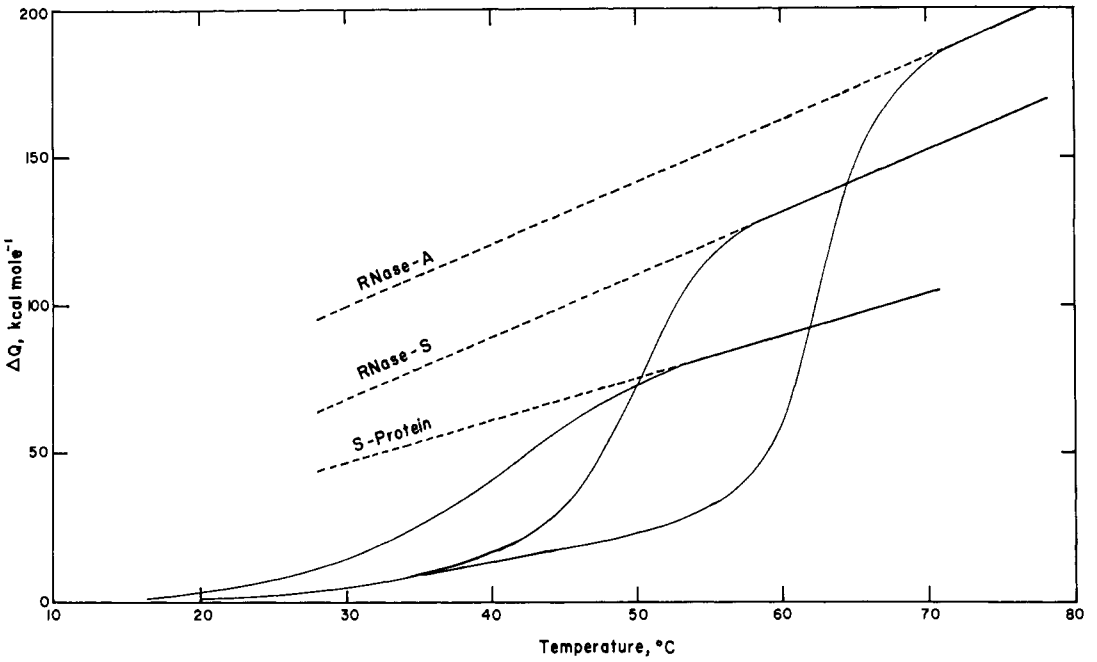

FIGURE 7: Calorimetric transition curves for ribonuclease A, ribonuclease S, and S protein in 0.2 N NaCl at pH 7.00 (room temperature). Protein concentration, approximately 0.5% by weight.

TABLE VI: Transition Parameters for Ribonuclease A (Resolution into Two Transitions).

	Values Assumed for Lower Transition				Values Calculated for Upper Transition			
pH	T_m (°C)	$\Delta H_{cal}(T_m)$ (kcal mole^{-1})	$\Delta H_{vH}(T_m)$ (kcal mole^{-1})	ΔC_p (kcal deg^{-1} mole^{-1})	T_m (°C)	$\Delta H_{cal}(T_m)$ (kcal mole^{-1})	$\Delta H_{vH}(T_m)$ (kcal mole^{-1})	ΔC_p (kcal deg^{-1} mole^{-1})
3.28	38.0	26	26	0	46.8	88	89	2.0
4.04	42.0	32	32	0	53.4	95	87	2.08
5.00	42.7	40	40	0	59.4	114	84	2.25

seem clear that the data at that pH cannot be analyzed in terms of two two-state transitions.

There can be no doubt that the data at higher pH values could be decomposed in a quite arbitrary manner into several successive two-state transitions, since three adjustable parameters (T_m, ΔH, and ΔC_p) are available for each such transition.

Cooperativity of the Transition. At pH values up to 2.0, and perhaps a little higher, the conformational transition of ribonuclease A appears to be fully cooperative, as judged by the equality $\Delta H_{vH}(T_m) = \Delta H_{cal}(T_m)$ evidenced in Table III. At higher values of the pH the situation is less clear. The fact that both alternative interpretations applied in the preceeding section fail to extend the apparent adherence to two-state behavior above pH 4 inclines us to the view that at values of the pH above 2 several intermediate states are indeed the cause of the gradually increasing heat absorption before the main transition sets in. On the other hand, it is difficult to rule out the possibility that this failure is actually the result of irreversibility at higher values of the pH.

Ginsburg and Carroll (1965) found closely agreeing transition curves for ribonuclease A at pH 2.1 whether the extent of the transition was estimated from measurements of absorbance, optical rotation, or viscosity. This finding suggests that at this low pH the transition is quite accurately two state in character.

Brandts and Hunt (1967) found that their extensive spectrophotometric data for the transition at pH values up to 3.15 could be fitted, to within 0.1–0.2% of the total absorbance change, to an equation based on the two-state assumption, and have used this fact as justification for their thermodynamic treatment which is necessarily based on the two-state assumption. It seems probable that their criterion for full cooperativity is not as sensitive as the calorimetric criterion.

Increase in Heat Capacity. An outstanding feature of the calorimetrically observed transition is the continuing absorption of heat after the completion of the transition, which can be considered as being due to a marked increase in the apparent heat capacity of the solute resulting from the unfolding. This large increase, which has been noted by a

number of authors (see the reviews by Tanford, 1968, and Brandts, 1969) for various protein denaturation processes, is generally attributed to increased exposure of hydrophobic groups to the solvent, with concomittant orientation of water molecules in a hydrogen-bonded structure the disruption of which on heating leads to increased heat absorption as compared with ordinary bulk water (Kauzmann, 1959; Privalov, 1968; see, however, Holtzer and Emerson, 1969).

It is interesting that the calorimetric data indicate that the increase in apparent heat capacity remains constant over the entire pH range included in our work only if it is assumed that the heat capacity of the native protein is independent of temperature and of pH (see Table III). The absence of curvature in the calorimetric plots at temperatures above the transition range (see especially the data for pH 0.36 in Table II and Figure 5) shows that the apparent heat capacity of the unfolded protein at each pH is essentially independent of temperature. These two observations are consistent with the view that both the native and the unfolded proteins have apparent heat capacities which are independent of temperature, and thus lend support to the conclusion that at pH values above 2 the transition is not of the simple two-state type. They also suggest that, so far as the increase in exposure of hydrophobic groups is concerned, the denaturation process is identical at pH 0.4 and 7.8, although the free energies and enthalpies at any fixed temperature vary widely over the pH range.

Effect of pH on the Transition. The well-known effect of pH on the T_m for the ribonuclease transition shows that the protein is markedly destabilized by having its net positive charge increased. The data in Table III show a parallel decrease in the enthalpy of transition, calculated to a fixed temperature to remove the effect of the large ΔC_p, as the pH is lowered. Further interpretation of this decrease is complicated by the significant alteration in the shape of the experimental transition curve as the pH is altered.

Transitions of Ribonuclease Derivatives. Hydrolysis of a single peptide bond in ribonuclease A to form ribonuclease S leads to a marked destabilization of the protein, as evidenced by a decrease of 14° in T_m. The enthalpy change accompanying the transition is correspondingly decreased by about 30 kcal mole^{-1} at 25°, roughly the same amount as the decrease in ΔH_{cal} (25°) for ribonuclease A resulting from a similar change in T_m produced by a pH change. Further severe destabilization results from removing the S peptide from the S protein, with again a large decrease in ΔH_{cal} (25°). This time the decrease is considerably larger than that observed with ribonuclease A when a similar change in T_m is produced by lowering the pH, presumably because the heat absorbed on unfolding the S peptide is not included. This is in keeping with the observation that ΔC_p for ribonuclease S is the same as that for ribonuclease A, whereas that for the S protein is 30% lower.

It is clear that none of these transitions at pH 7.0 meets the requirement for a two-state process, that $\Delta H_{vH}(T_m) = \Delta H_{cal}(T_m)$.

Conclusions

The thermally induced conformational transition of ribonuclease A fulfills, within experimental uncertainty, the requirements for a fully cooperative, two-state process at low values of the pH up to approximately pH 2. It appears to be impossible to decide with full confidence, on the basis of the techniques presently available, whether the transition is two state above this pH or is of more complicated character. The calorimetric data presented in this paper indicate that the apparent heat capacity of the denatured (unfolded) protein is probably independent of both temperature and pH. The observed constancy, at all pH values studied, of the overall change in apparent heat capacity of the protein in going from native protein at 5° to denatured protein at elevated temperature then suggests, but does not prove, that the apparent heat capacity of the native protein is also independent of both temperature and pH. If this is actually the case, as we believe it to be, it follows that the transition involves more than two states at values of the pH above 2.

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