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Structural Studies of Ribonuclease. XI. Kinetics of Denaturation[1,2]

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The kinetics of thermal denaturation of ribonuclease A were studied in the pH range 0.9–3.26 at an ionic strength of 0.16 by a spectrophotometric stopped-flow technique, which measured the change in tyrosyl absorption at 287 mμ. The rate data were complex but could be resolved into two steps representable by the empirical equation: $D_{287} = D_0^1 e^{-\lambda_1 t} + D_0^2 e^{-\lambda_2 t}$; λ_1 and λ_2 were found to be essentially independent of concentration in the range 0.27–1.8 mg./ml. Equilibrium data were also obtained spectrophotometrically in the same pH region where it had previously been shown that the curves are skewed and give nonlinear van't Hoff plots. However, it is now possible to resolve each of the transition curves into two symmetrical transitions which have linear van't Hoff plots. Examination of the relative magnitudes of the changes in absorption involved in the two transitions and in the two kinetic steps led to adoption of a mechanism consisting of two independent parallel and reversible steps, each involving a tyrosyl residue. Adoption of this mechanism and the combined use of the rate and equilibrium data allowed evaluation of all the rate constants. An interpretation of these rate constants is presented.

Introduction

In a previous paper of this series[4] the reversible change of conformation in ribonuclease with varying pH and temperature was studied by means of ultraviolet difference spectra and optical rotation measurements. Using a two-state theory to represent the conformation change, the pH-dependence of the transition temperature T_{tr} and ΔF°_{obsd} and the value of ΔH°_{obsd} were obtained for this equilibrium. Above T_{tr} it was found that ΔH°_{obsd} increases with increasing temperature, suggesting that more than one denatured form participates in the equilibrium at higher temperatures. A theory was developed to account for the pH dependence of T_{tr} and ΔF°_{obsd} in terms of local interactions and general electrostatic effects based on four different possible models; by applying the theory at temperatures up to T_{tr} it was possible to rule out only two of the models.

In this paper the kinetics of the thermal denaturation of ribonuclease have been studied by a spectrophotometric stopped-flow technique in an attempt to gain more information about the mechanism of unfolding and the nature of the forces which stabilize the native conformation. The kinetics are found to be complex but separable into two parallel reversible reactions, each involving a tyrosyl residue. The reversible thermal transition has also been studied by ultraviolet difference spectra and resolved into two independent transitions each of which yields a ΔH° which is constant over the temperature range of the transition. By combining the rate and equilibrium data, a mechanism is formulated which provides information about the various forms in equilibrium with each other and for which all the rate constants are calculable. The temperature dependences of the rate constants for denaturation for both reactions do not follow the Arrhenius law; this behavior is accounted for qualitatively, using recent theories on the kinetics of helix–coil transitions.[5,6] The pH dependence of these rate constants is also discussed qualitatively in terms of hydrophobic and side-chain hydrogen bonding. Finally, the results of this investigation are related to work on ribonuclease by other investigators.

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(2) Presented before the Division of Biological Chemistry at the 145th National Meeting of the American Chemical Society, New York, N. Y., September, 1963.

(3) U. S. Public Health Service Predoctoral Fellow, 1959–1963.

(4) J. Hermans, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, **83**, 3283, 3293 (1961).

(5) P. J. Flory, *J. Polymer Sci.*, **49**, 105 (1961).

(6) M. Saunders and P. D. Ross, *Biochem. Biophys. Res. Commun.*, **3**, 314 (1960).

Experimental

Materials.—Ribonuclease (lots P20B-094 and R20B-094) was obtained as the crystalline material (five times recrystallized) from Sigma Chemical Co. The ribonuclease A fraction was prepared by chromatography of the crystalline protein on unsieved Amberlite IRC-50, XE-64 resin in 0.2 *M* sodium phosphate buffer, pH 6.47,[7] using a 7.5 × 60 cm. column, and was deionized by passage through the mixed bed ion exchanger, MB-1 (Rohm and Haas Co.), using a 5.0 × 40 cm. column. (The material was then lyophilized and stored at 1°). Ribonuclease concentrations were determined by measuring the optical density of a suitably diluted aliquot in 0.01 *M*, pH 7 phosphate buffer at 278 mμ using an extinction coefficient of 0.738 cm.²/mg.[4] All other materials were reagent grade.

pH Measurements.—The pH's were measured on the A scale of a Beckman GS pH meter using Beckman buffers as standards.

Spectrophotometric Stopped-Flow Apparatus.—The kinetics experiments were carried out with a spectrophotometric stopped-flow apparatus[8] which consisted of a lucite mixing chamber, with quartz windows, mounted on a metal stand on a Carl Zeiss PMQ II spectrophotometer. The signal from the photomultiplier of the spectrophotometer was fed directly to a Brush Model RD 561500 high gain d.c. amplifier which amplified the signal and in turn fed it to a Brush Model 232100 oscillograph where it was recorded on chart paper.

The lucite mixing chamber was thermostated by circulating water from a model F Haake circulating pump through channels which had been drilled into it. The temperature of the mixing chamber was calibrated against the temperature of the external bath by inserting a thermocouple into the mixing chamber. Once this calibration was carefully obtained the temperature inside the mixing chamber was known by reading the temperature of the external bath and using the calibration curve. The temperature inside the mixing chamber was known accurately to within a few tenths of a degree and was constant to within five hundredths of a degree. The procedure used was to allow the solutions of ribonuclease and acid to equilibrate thermally in their respective reservoirs for 0.5 hr. before reaction. Each reaction was then carried out by mixing equal volumes of ribonuclease and acid. The size of the reservoirs allowed three runs in succession before another half-hour equilibration period was necessary.

The signal in millivolts, which was recorded as a function of time on the oscillograph chart, was directly proportional to changes in percentage transmission. After each kinetic run had gone to completion, which in all cases represented 100% transmission, a cell was inserted into the light path which cut down the percentage transmission by a known amount. Thus, a given change in millivolts was convertible into a known change in percentage transmission; the graph paper was separately calibrated for each kinetic run. The data on percentage transmission were then easily converted to optical density units. Under each condition of temperature and pH at least five individual runs were used to obtain an average value for the rate constants.

Difference Spectra.—The ultraviolet difference spectra were obtained with a Cary Model 14 recording spectrophotometer. Glass-stoppered 1-cm. silica cells were used to contain the solutions and were thermostated by being inserted in a brass block through which water was circulated from an external bath by a model F Haake circulating pump. As in the mixing chamber in the stopped-flow apparatus a calibration was obtained of the

(7) C. H. W. Hirs, S. Moore, and W. H. Stein, *J. Biol. Chem.*, **235**, 633 (1960).

(8) B. Chance in "Physical Methods of Organic Chemistry," S. L. Friess and A. Weissberger, Ed., Interscience Publishers, Inc., New York, N. Y., 1953, p. 690.

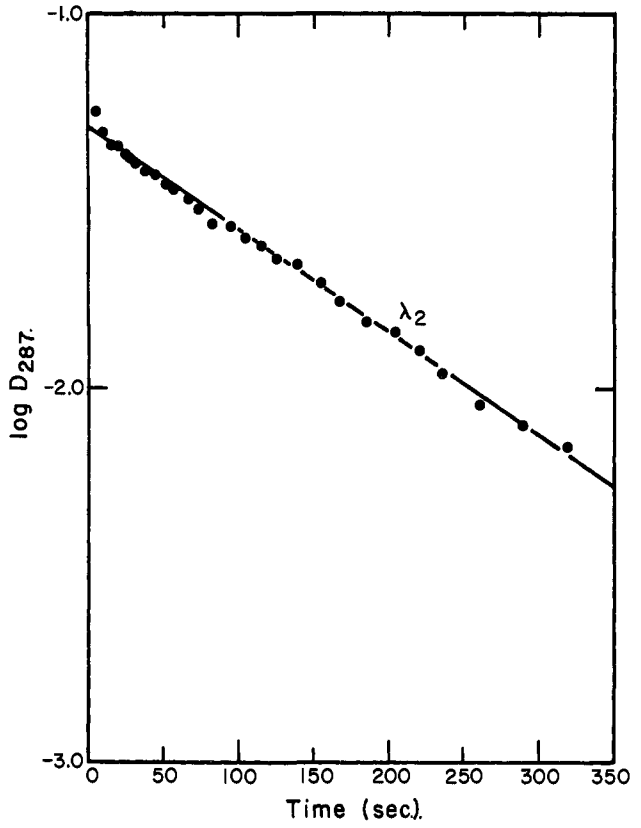

Figure 1a.

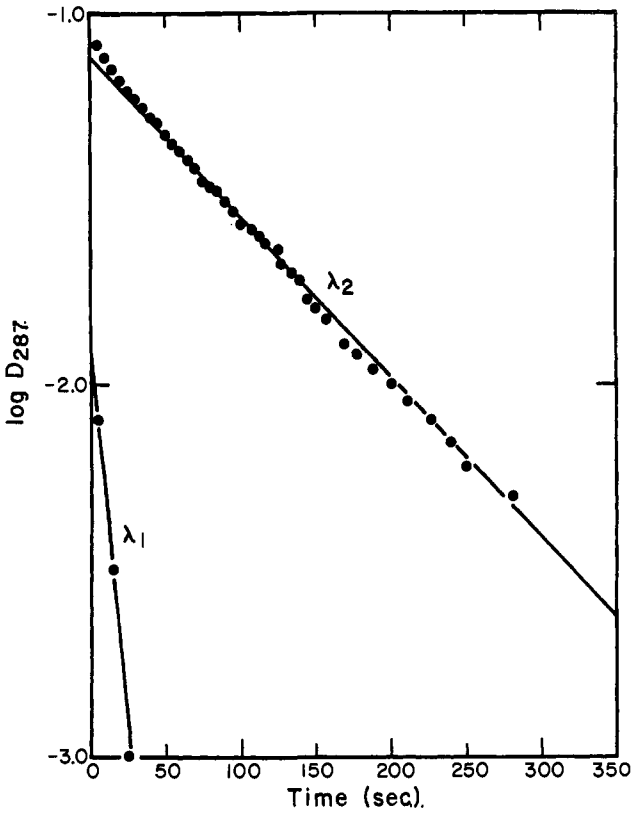

Figure 1b.

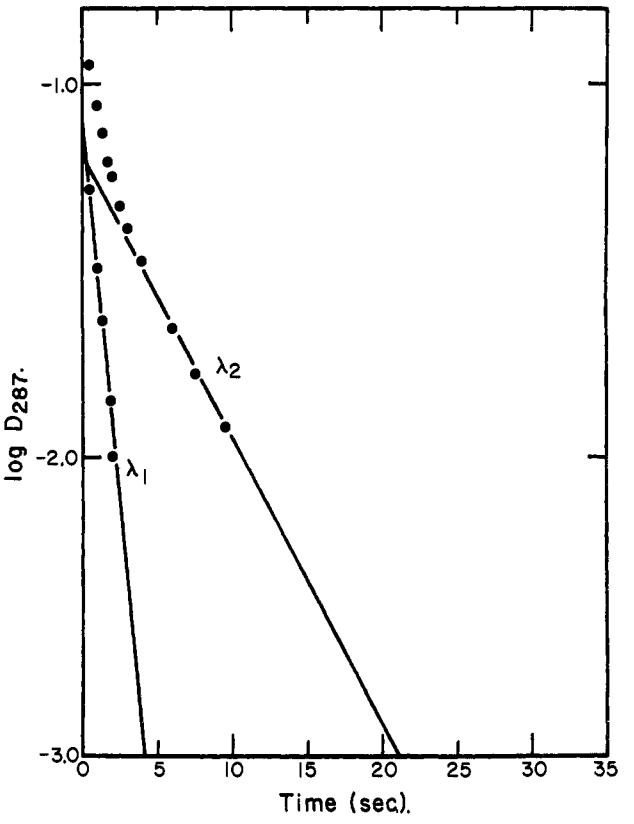

Figure 1c.

Fig. 1.—The kinetics of denaturation of ribonuclease A at pH 0.9, ionic strength 0.16, and protein concentration 0.95 mg./ml.: a, 20°; b, 25°; c, 40°. Figure 1a shows that at 20° the reaction follows apparent first-order kinetics with apparent first-order rate constant λ_2; 1b and 1c show that at higher temperatures the reaction is complex but can be divided into two steps with apparent first-order rate constants λ_1 and λ_2; 1c shows that at 40° the faster step has a larger change in absorption than the slower step.

temperature of the solutions in the cells against the temperature of the external bath, so that the temperature was always known by using the external bath temperature and the calibration curve. The reference solution was always the same as the solution being studied except that it was maintained at 5°. It should be noted this is a different reference solution from the one used by Hermans and Scheraga.[4] It was found that, by using this reference, the peak wave length shifted from about 284 to 287 mμ as the temperature of the solution under study was raised. Thus, by using ΔD_{peak} instead of ΔD_{287}, all the transition curves had a tail which extended to lower temperatures than did those of Hermans and Scheraga.[4] However, the difference between the data of Hermans and Scheraga[4] and those in this paper is only slight and would make no material difference in either their conclusions or ours.

Results

Kinetic Form.—It has previously been shown that the stability of ribonuclease A is dependent on pH in the pH region 0.9–7.0, the stability being greatest at pH 7.0 and decreasing at lower pH's.[4] Thus, for example, at pH 7.0 and 40° the molecules are in the native conformation, while at pH 0.9 at this temperature they are reversibly unfolded. By suddenly shocking the pH of a solution of ribonuclease at 40° from pH 7.0 to 0.9, the system is put into a thermodynamically unstable state and will immediately begin to denature to restore thermodynamic equilibrium. In Fig. 1a, 1b, and 1c are shown examples of the rate data for this reaction at 20, 25, and 40°, respectively, all at pH 0.9, ionic strength 0.16, and a protein concentration of 0.95 mg./ml. The data were obtained using the spectrophotometric stopped-flow technique which measured the time course of the decrease in optical density at 287 mμ. In each experiment unbuffered ribonuclease A at pH 7.0 was rapidly mixed with the proper amount of acid to shift the pH to 0.9. In each of the figures the data are plotted in the form log D_{287} *vs.* time; D_{287} is here directly proportional to the concentration of native material. It can be seen in Fig. 1a that, with the pos-

TABLE I

pH AND TEMPERATURE DEPENDENCE OF λ_1 ($\lambda_1 \times 10^2$ SEC.$^{-1}$) SHOWN WITH THE AVERAGE DEVIATION FROM THE MEAN

pH	25°	30°	35°	40°	45°	50°	55°
0.9	7.7 ± 0.04	24.1 ± 2.2	49.4 ± 5.5	121 ± 12	140 ± 14	125 ± 12	117 ± 9
1.2	6.69 ± 0.41	26 ± 3	46 ± 2	70 ± 6	93 ± 3	122 ± 13	147 ± 3
2.0		28.3 ± 1.4	32.8 ± 2.2	52 ± 2	80.4 ± 2.5	80.5 ± 2.9	72.1 ± 1.6
2.5			40.6 ± 0.6	44.6 ± 1.3	75.4 ± 8.3	74.7 ± 6.5	88.8 ± 5.6
2.9				93.3 ± 10.4	99.2 ± 9.6	126 ± 7	120 ± 8
3.3				76.1 ± 8.6	119 ± 7	130 ± 6	123 ± 6

TABLE II

pH AND TEMPERATURE DEPENDENCE OF λ_2 ($\lambda_2 \times 10^2$ SEC.$^{-1}$) SHOWN WITH THE AVERAGE DEVIATION FROM THE MEAN

pH	20°	25°	30°	35°	40°	45°	50°	55°
0.9	0.64 ± 0.05	0.94 ± 0.03	2.35 ± 0.57	6.98 ± 0.15	20.7 ± 0.3	33.6 ± 2.3	32.2 ± 2.5	35.0 ± 2.4
1.2		1.07 ± 0.04	2.45 ± .07	6.36 ± .22	16.4 ± 2.6	25.8 ± 1.3	26.4 ± 1.1	40 ± 1
2.0			1.70 ± .07	3.33 ± .03	11.0 ± 0.3	23.6 ± 0.9	23.9 ± 0.5	21.5 ± 0.8
2.5				3.83 ± .13	6.37 ± .16	20.0 ± 1.7	20.0 ± 2.5	20.0 ± 1.5
2.9					8.31 ± .05	13.1 ± 0.4	27.1 ± 2.2	26.3 ± 1.2
3.3					20.5 ± 1.2	27.7 ± 1.5	26.8 ± 0.8	37.1 ± 1.3

sible exception of the first two points, at 20° the kinetics appear to be first order over the entire range of the reaction with apparent first-order rate constant λ_2. At 25° and 40° the kinetics are complex, the first-order type plot giving a curve similar to the kind obtained from two parallel first-order reactions with rate constants λ_1 and λ_2.[9] It should also be noted that at 40° the faster step shows a greater change in D_{287} than does the slower step. Since the thermal denaturation of ribonuclease is known to be reversible,[4] the constants λ_1 and λ_2 cannot be simple first-order rate constants but must be functions of various elementary rate constants, some for reactions in the direction of denaturation and some for reactions in the direction of renaturation, the exact functional relationship depending on the mechanism. Although the data in Fig. 1b and 1c were separated to obtain λ_1 and λ_2 in a manner analogous to that of two parallel first-order reactions,[9] this is not meant to imply at this point, in the absence of independent confirmatory evidence, that this is the actual mechanism, since various other types of mechanism (*i.e.*, sequential mechanisms) can yield similar results when plotted in the form used in Fig. 1b and 1c. This point will be considered further in the Discussion section. At this stage all that can be said is that the time course of the decrease in optical density can be represented by an empirical equation of the type

$$D_{287} = D_0^1 e^{-\lambda_1 t} + D_0^2 e^{-\lambda_2 t} \qquad (1)$$

where t is time, $D_0^1 + D_0^2$ is the value of D_{287} at zero time, and λ_1 and λ_2 are two parameters obtained from Fig. 1a and 1b in the manner previously described.

Concentration Dependence.—To determine whether the parameters λ_1 and λ_2 were dependent on the concentration of ribonuclease, a series of kinetics experiments was carried out at 0.27, 0.54, 0.95, and 1.81 mg./ml. of protein. These experiments were all run at 35°, ionic strength 0.24, and pH 0.9. Although there seemed to be a slight tendency for the values of λ_1 and λ_2 to increase with decreasing concentration, the change was such that plots of log λ_1 and log λ_2 *vs.* log (concentration) yielded slopes of −0.23 and −0.17, respectively. It is known that ribonuclease A aggregates during lyophilization[10] and this mild concentration dependence could be due to a small amount of dimerization, the dimers denaturing at a different rate from the monomers. However, it is also known that heating to 60° reverses the dimerization[10] and the protein will then remain monomeric upon cooling. Rate experiments were carried out on solutions which were heated to 60° and then cooled and no change was found in the kinetics; thus it does not seem as though aggregation could be the cause of the mild concentration dependence. Since it will be shown later that, under the conditions of these experiments, λ_1 and λ_2 do reduce to simple first-order rate constants, they would be first order to within ±0.2 unit.

It should also be mentioned that it is not known what effect concentration may have on the thermal transition curve of ribonuclease. If it were slightly concentration dependent this would cause a corresponding concentration dependence in the rate experiments. The dependence of λ_1 and λ_2 on concentration is so slight that, for the purposes to which the data are put, it will be considered negligible.

Dependence on pH and Temperature.—Experiments similar to those shown in Fig. 1a, 1b, and 1c were conducted at a series of pH's from 0.9 to 3.36 and at a series of temperatures at each pH. The values of λ_1 and λ_2 obtained are summarized in Tables I and II. Each value of λ_1 and λ_2 shown in these tables is an average value based on at least five individual experiments of the kind shown in Fig. 1a, 1b, and 1c. Included with each value in the tables is the average deviation from the mean value. It is to be noted that the experiments at higher pH's have been carried out only at the higher temperatures. This is because at these pH's the protein is more stable and doesn't begin to unfold until higher temperatures are reached. It should also be mentioned that, since the thermal unfolding is a reversible process, the kinetics experiments at lower temperatures followed the reaction to a point of equilibrium only part of the way through the thermal transition. Only at the higher temperatures has the equilibrium been shifted completely to the unfolded form; therefore the rate data are for complete unfolding only at higher temperatures.

It is known that, even at very low temperatures, there is a difference in absorption between ribonuclease A at pH 7 and pH 0.9, which has been attributed to the titration of one or more carboxyl groups which interact, perhaps by side chain hydrogen bonding, with one or more tyrosyl groups.[4] We have attempted to study the rate of this process by shocking the pH from 7.0 to 0.9 at low temperature but found that the rate was too fast to measure with our instrument; the existence of such a rapid reaction is consistent with an ionization process. Thus, in the experiments shown in Fig. 1a, 1b, and 1c, this change in absorption takes place rapidly with the mixing and is not included in the time course

(9) A. A. Frost and R. G. Pearson in "Kinetics and Mechanism," John Wiley and Sons, Inc., New York, N. Y., 1953, pp. 149–151.

(10) A. M. Crestfield, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **238**, 618 (1963).

of the decrease in optical density shown in the figures. At higher pH's this low temperature difference in absorption disappears.

Experiments at 235 mμ.—A few experiments were also carried out in a similar manner at 235 mμ. At this wave length the change in absorption is due to the peptide group and thus might be expected to follow different kinetics representing changes in the molecule as a whole rather than more local changes around specific groups such as tyrosyl. The results of these experiments are shown in Table III. By comparing these results with those in Tables I and II under the same conditions of temperature and pH it can be seen that the values of λ_1 and λ_2 are smaller at 235 mμ than at 287 mμ with the exception of λ_2 at pH 2.5 and 40°. The form of the rate curves at 235 mμ is the same as at 287 mμ but the rates are generally slower.

TABLE IIIa

VALUES OF $\lambda_1 \times 10^2$ SEC.$^{-1}$ MEASURED AT 235 mμ

pH	35°	40°
0.9	22.9 ± 1.7	60.1 ± 1.1
2.5		36.8 ± 1.7

TABLE IIIb

VALUES OF $\lambda_2 \times 10^2$ SEC.$^{-1}$ MEASURED AT 235 mμ

pH	35°	40°
0.9	5.20 ± 0.18	10.9 ± 0.4
2.5		7.53 ± 0.14

Equilibrium Transition Curves.—Because of the complexity of the kinetics it was felt that additional information would be necessary before any mechanism for the denaturation could be proposed. Since previous investigation of the thermal transition in ribonuclease had shown that application of a two-state theory gave nonlinear van't Hoff plots,[4] it was felt that further examination of these transition curves would be useful. Figure 2 shows the effect of temperature on the optical density of ribonuclease A at pH 0.9, ionic strength 0.16, and a protein concentration of 0.95 mg./ml. The reference solution used in this experiment was the same solution maintained at 5°. The solid line is drawn through the experimental points. These results differ slightly from those reported previously[4] in that they show a long tail extending down into the low temperature region. It was discovered in the course of carrying out these experiments that the spectral peak shifted from about 284 mμ at 5° to 287 mμ at 20° as the solution temperature was raised, the peak then remaining at 287 mμ at all the higher temperatures. The wave length of the spectral peak was used at each temperature in Fig. 2 rather than the value 287 mμ, as was used previously.[4] This explains the low temperature tail in these results. Since correlation of degrees of unfolding with spectral changes is of necessity done on an empirical basis it is difficult to decide which of the two references is superior. However, the differences between the two sets of results are very small and make very little quantitative and no qualitative differences in any of the conclusions. It should also be noted that the different reference solutions used here account for the absence from Fig. 2 of the usual low temperature difference spectrum which has been attributed to carboxyl group ionization,[4] since in this case both the reference solution and the solution under study are at pH 0.9.

The problem of nonlinear van't Hoff plots has been solved by resolving the transition in Fig. 2 into two-component transitions which are shown by the dotted lines. This resolution was made by plotting the first derivative of the transition curve in Fig. 2 to obtain a

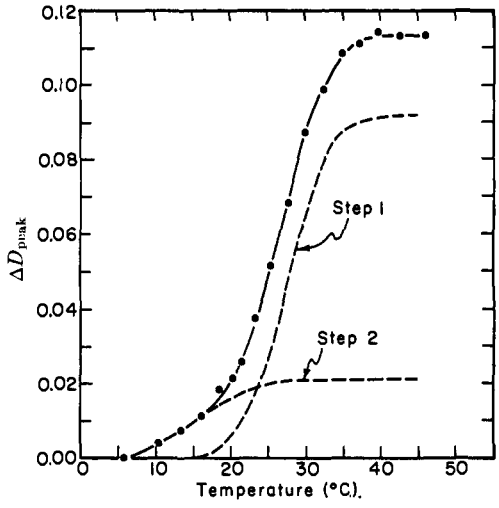

Fig. 2.—The effect of temperature on the optical density of ribonuclease A at pH 0.9, ionic strength 0.16, and protein concentration 0.95 mg./ml. The reference solution was maintained at a temperature of 5°. The solid line is drawn through the experimental points. The dotted lines labeled steps 1 and 2 demonstrate resolution of the experimental data into two composite transition curves. Step 1 corresponds to the fast step in Fig. 1 with apparent rate constant λ_1 and step 2 with the slow step in Fig. 1 with apparent rate constant λ_2.

curve having two overlapping peaks. By separating the two peaks and then integrating them separately, a first approximation of the resolution into two transitions was obtained. The two curves shown in Fig. 2 were then obtained by small trial and error adjustments which smoothed out the curves and improved their symmetry. The two basic requirements for the resolution were first that the two components had to be symmetrical so that their van't Hoff plots would be linear and second that the final resolution be unique. Various attempts were made to find other resolutions which would yield two symmetrical curves whose sum would yield the original curve, but no other combination was successful. Thus we are convinced that the resolution shown in Fig. 2 is unique.

Experiments of this kind were then carried out in the pH range 0.9–3.26 and in each case it was found possible to resolve the transition curve into two symmetrical transition curves with linear van't Hoff plots. Also, in each case it was found that the resolution consisted of a larger and a smaller transition which have been numbered steps 1 and 2, respectively, in Fig. 2. Tables IV and V summarize the values of the thermodynamic parameters obtained from these experiments. It is to be noted that the two transitions show different pH de-

TABLE IV

pH DEPENDENCE OF THE THERMODYNAMIC PARAMETERS FOR TRANSITION 1

	pH					
	0.9	1.2	2.0	2.5	2.9	3.3
ΔH_1, kcal./mole	63.6	57.2	74.7	78.7	79.2	105.2
ΔS_1, e.u.	211	190	277	254	251	328
$T_{1\ trans}$, °C.	27.5	28.0	32.9	37.0	42.5	48.0

TABLE V

pH DEPENDENCE OF THE THERMODYNAMIC PARAMETERS FOR TRANSITION 2

	pH					
	0.9	1.2	2.0	2.5	2.9	3.3
ΔH_2, kcal/mole	44.9	53.1	48.5	32.8	26.5	21.1
ΔS_2, e.u.	155	182	164	109	88	70
$T_{2\ trans}$, °C.	15.7	18.7	23.3	27.0	26.5	29.5

pendences, the larger transition (*i.e.*, step 1) displaying increasing enthalpy and entropy changes with increasing pH and the smaller one (*i.e.*, step 2) showing decreasing enthalpy and entropy changes with increasing pH. The values of the enthalpy are considered to be accurate to within 3 kcal./mole and the entropy to within ±10 e.u.

Discussion

Kinetic Model.—In the previous section it has been shown that the reaction appears to proceed by a two-step mechanism and that the transition curves could be resolved into two symmetrical component transition curves which yielded linear van't Hoff plots. Although the actual mechanism could be rather complicated and still be representable empirically by eq. 1, we have chosen to discuss only two of the simplest alternative types as shown by eq. 2 and 3.

$$N \underset{k_1^b}{\overset{k_1^f}{\rightleftharpoons}} D_1 \underset{k_2^b}{\overset{k_2^f}{\rightleftharpoons}} D_2 \qquad (2)$$

$$\left.\begin{aligned} N_1 &\underset{k_1^b}{\overset{k_1^f}{\rightleftharpoons}} D_1 \\ N_2 &\underset{k_2^b}{\overset{k_2^f}{\rightleftharpoons}} D_2 \end{aligned}\right\} \qquad (3)$$

Equation 2 represents a mechanism in which the native protein N partially denatures to an intermediate D_1 which then further denatures to produce the final product D_2. This mechanism would be consistent with the form of the kinetics which we have obtained and also with the resolution of the transition curves into two components. Equation 3 is a mechanism consisting of two parallel reversible reactions. We do not mean to imply by eq. 3 that there are two kinds of native protein N_1 and N_2 each of which denatures, but rather that two parts of the native molecule, one part symbolized by N_1 and the other by N_2, denature reversibly and independently. This mechanism would also be cosistent with the form of our kinetic data and the resolution of the transition curves. In general, it would be difficult in a situation such as this to make a choice between these two mechanisms, but in this particular case it can be done. In Fig. 2 it can be seen that, as the temperature is raised, unfolding occurs first by transition 2 which is the smaller of the two transitions in terms of the ultimate change in absorption. This step is then followed at higher temperatures by transition 1 which ultimately has the larger change in absorption. Thus, if the reaction occurred by the mechanism of eq. 2 transition 2 would represent conversion of N to D_1 and transition 1 conversion of D_1 to D_2. However, by referring to Fig. 1a, 1b, and 1c it can be seen that the faster step ultimately shows the greater change in absorption and does not even proceed appreciably at 20° where the transition is just beginning to occur in Fig. 2. Thus, trying to use eq. 2 leads one to the conclusion that the first step has the smaller change in absorption (from the equilibrium data) and that it has the larger change in absorption (from the kinetic data). This difficulty is avoided by choosing eq. 3. In Fig. 2 the smaller transition would represent the step N_2 to D_2 and the larger transition N_1 to D_1, the latter having a higher transition temperature than the former. In the kinetics experiments of Fig. 1a, 1b, and 1c the fast step with apparent first-order rate constant λ_1 corresponds to the step N_1 to D_1 and the slower step with rate constant λ_2 corresponds to the step N_2 to D_2. Thus, we have here a case where, although N_1 is thermodynamically more stable against thermal denaturation than N_2, it reacts at a faster rate once denaturation begins. We therefore choose the mechanism of eq. 3 in preference to that of eq. 2. Adoption of this mechanism leads us to believe that there are two regions of the ribonuclease molecule which denature essentially independently, each at their own rate and with their own thermodynamic parameters which are those shown in Tables IV and V. It is also known that treatment with urea is accompanied by a further change in absorption over that shown in Fig. 2,[11,12] so that there must still be an additional hard core of structure which is not disrupted at all by the thermal transition.

Adoption of the mechanism of eq. 3 allows calculation of the rate constants k_1^f, k_2^f, k_1^b, and k_2^b by eq. 4, 5, 6, and 7.

$$k_1^f/k_1^b = K_1 \qquad (4)$$

$$k_1^f + k_1^b = \lambda_1 \qquad (5)$$

$$k_2^f/k_2^b = K_2 \qquad (6)$$

$$k_2^f + k_2^b = \lambda_2 \qquad (7)$$

In these equations K_1 and K_2 are equilibrium constants of transitions 1 and 2 such as those shown in Fig. 2 and λ_1 and λ_2 are the parameters obtained from the kinetic experiments such as those shown in Fig. 1a, 1b, and 1c. It can be seen from eq. 4–7 that, at temperatures well above the transition temperatures for transitions 1 and 2 where K_1 and K_2 are much larger than 1, $k_1^f \approx \lambda_1$ and $k_2^f \approx \lambda_2$, while at lower temperatures k_1^b and k_2^b will not be negligible.

Since the most important element in the previous discussion, which led to the selection of the mechanism of eq. 3, concerned the relative magnitudes of the changes in absorption involved in the two steps, it seems appropriate at this point to discuss briefly the possible sources of error and the reliability of obtaining D_0^1 and D_0^2 of eq. 1 from the kinetic data. In Fig. 1a, 1b, and 1c one could in principle obtain these values simply by looking at the extrapolation of the two straight lines to time zero. In practice, however, it turns out that these values showed a large amount of scatter from run to run, thus making the error involved in taking an average value too large to be practical. One cause of this scatter is the fact that, in originally obtaining the data from the chart paper of the oscillograph, zero time was assumed to coincide exactly with a scale division of the paper rather than at the initial point of deflection of the pen. This introduces no error in the λ values and was convenient for obtaining the data from the paper. However, this probably introduces only a small error and the rest of the scatter must be assumed to be random experimental error. It can easily be seen that small differences in λ_1 can introduce larger differences in D_0^1 because of the steepness of the slope. Thus, any attempt to compare Fig. 1a, 1b, and 1c with Fig. 2 using D_0^1 and D_0^2 quantitatively is not possible. However, since the argument presented in favor of eq. 3 was only a qualitative one, it seems to be well supported by all our data since a careful inspection of all the runs at all the pH's showed that at the higher temperatures D_0^1 became greater than D_0^2 even though precise values of these parameters could not be obtained.

The values of k_1^f, k_1^b, k_2^f, and k_2^b obtained by applying eq. 4–7 are summarized in Tables VI and VII.

Temperature Dependence of Rate Constants.—In Fig. 3 and 4 are shown the Arrhenius plots of log k_1^f and log k_2^f *vs.* $1/T$ at various pH's It is evident from the curvature of these plots that the usual straight line form of the Arrhenius plot does not apply in these cases. Both plots seem to have a different temperature dependence in the high temperature regions than in the low temperature regions.

(11) M. Sela and C. B. Anfinsen, *Biochim. Biophys. Acta*, **24**, 229 (1957).
(12) C. C. Bigelow, *J. Biol. Chem.*, **286**, 1706 (1961).

TABLE VIa

pH AND TEMPERATURE DEPENDENCE OF $k_1^f \times 10^2$ IN SEC.$^{-1}$

pH	20°	25°	30°	35°	40°	45°	50°	55°
0.9		2.12	17.3	49.4	121	140	125	117
1.2		1.96	16.3	43.0	121	140	125	117
2.0			6.63	22.4	50.4	80.4	80.5	72.1
2.5				11.4	33.9	75.4	74.7	88.8
2.9					21.5	72.9	121	120
3.3					0.83	19.4	99.5	120

TABLE VIb

pH AND TEMPERATURE DEPENDENCE OF $k_1^b \times 10^2$ IN SEC.$^{-1}$

pH	20°	25°	30°	35°	40°	45°	50°	55°
0.9		5.58	6.83					
1.2		4.71	9.67	3.07				
2.0			21.6	10.5	1.61			
2.5				29.2	4.20			
2.9					71.8	26.5	4.6	
3.3					75.3	100.0	30.7	3.25

TABLE VIIa

pH AND TEMPERATURE DEPENDENCE OF $k_2^f \times 10^2$ IN SEC.$^{-1}$

pH	20°	25°	30°	35°	40°	45°	50°	55°
0.9	0.48	0.89	2.35	6.98	20.7	33.6	32.2	35.0
1.2		1.01	2.45	6.36	16.4	25.8	26.4	40.0
2.0			1.48	3.25	11.0	23.6	23.9	21.5
2.5				3.68	6.37	20.0	20.0	20.0
2.9					7.31	12.8	27.1	26.3
3.3					14.8	22.7	24.6	37.1

TABLE VIIb

pH AND TEMPERATURE DEPENDENCE OF $k_2^b \times 10^2$ IN SEC.$^{-1}$

pH	20°	25°	30°	35°	40°	45°	50°	55°
0.9	0.15	0.05						
1.2		0.06						
2.0			0.22	0.09				
2.5				0.23				
2.9					0.96	0.28		
3.3					5.69	5.03	2.18	

Two similar theories of the kinetics of helix–coil transformations have been proposed by Flory[5] and by Saunders and Ross.[6] Saunders and Ross have applied the steady-state approximation to a kinetic model while Flory has applied the solution of the gambler's ruin problem of probability theory to a one-dimensional random walk. Ross and Sturtevant have found experimental verification of these theories in their study of the kinetics of formation of the two-stranded poly (A + U) helix.[13] Following Flory's terminology, the mechanism for the helix–coil transition is represented by eq. 8

$$H_n \underset{k_-}{\overset{k_i}{\rightleftarrows}} H_{n-1}C_1 \underset{k_-}{\overset{k_+}{\rightleftarrows}} H_{n-2}C_2 \underset{k_-}{\overset{k_+}{\rightleftarrows}} \cdots \quad H_1C_{n-1} \underset{k_i^*}{\overset{k_+}{\rightleftarrows}} C_n \qquad (8)$$

where $H_{n-i}C_i$ denotes the species comprising a sequence of $n - i$ helical segments adjoining a sequence of i random segments, k_i is the rate constant for initiating helical breaks or uncoiling from the ends, and k_+ is the rate constant for propagation; k_i^* and k_- play similar roles in the direction of helix formation. The rate of unfolding for large n and $r > 1$, derived by Flory, is given in eq. 9.

$$-dC_H/dt = k_i(1 - 1/r)C_H \qquad (9)$$

Here $r = k_+/k_-$ is an equilibrium constant for conversion of a helical segment to a random segment. At temperatures well above the transition temperature $1/r$ becomes zero and the apparent rate constant reduces simply to k_i, the rate-determining step being that of initiation. At lower temperatures as $1/r \rightarrow 1$ the second factor plays a dominant role and the rate falls off rapidly with decreasing temperature as the transition temperature is approached. For a simple helix–coil transition where the initiation step consists of breaking a few peptide hydrogen bonds it would be expected that k_i would have a positive temperature coefficient. The Arrhenius plot would be expected to be linear at high temperature with a positive enthalpy of activation and depart from linearity at lower temperatures toward T_{tr}. For the more complex case of a reversible protein denaturation a term analogous to $(1 - 1/r)$ should play a similar role to that discussed above for a simple helix–coil transition; however, k_i cannot have the same

(13) P. D. Ross and J. M. Sturtevant, *J. Am. Chem. Soc.*, **84**, 4503 (1962).

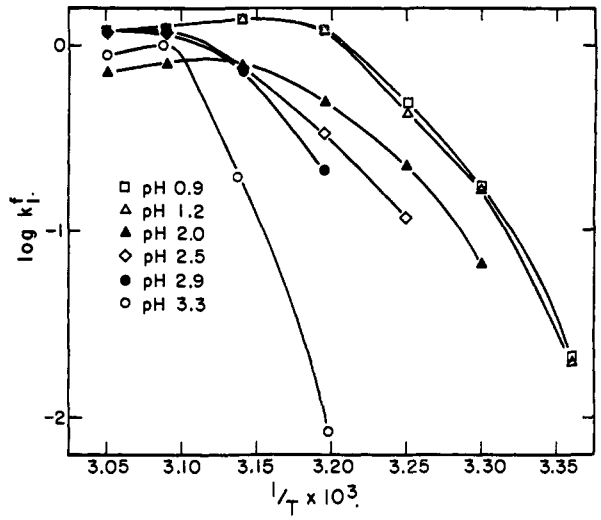

Fig. 3.—The logarithm of k_1^f, the first-order rate constant for step 1, plotted against the reciprocal of the absolute temperature.

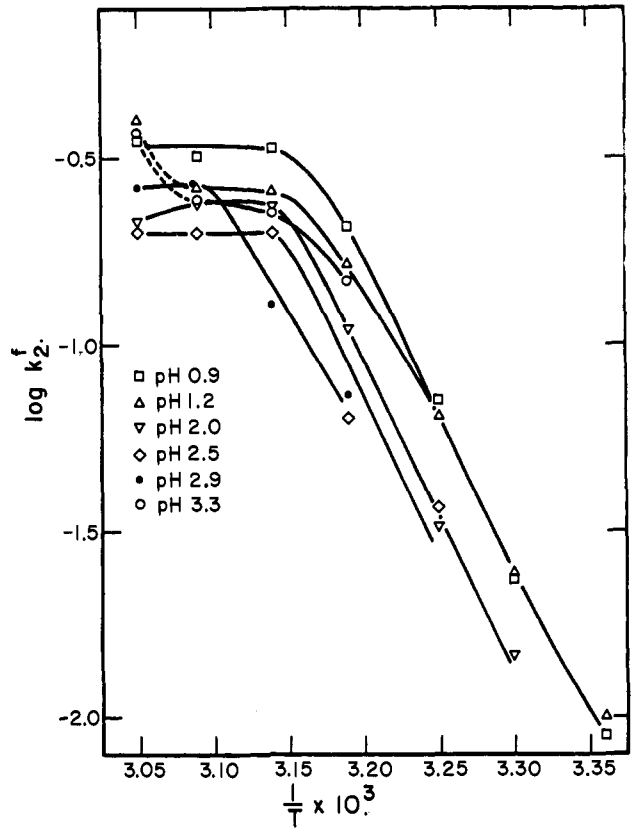

Fig. 4.—The logarithm of k_2^f, the first-order rate constant for step 2, plotted against the reciprocal of the absolute temperature.

simple interpretation. Laskowski and Scheraga have proposed a theory of protein denaturation in which the activation process consists of breaking a critical number of side chain hydrogen bonds, the activated molecules then denaturing by a helix–coil transition.[14] In a footnote in their paper they discuss an alternate formulation in which the species with the side chain hydrogen bonds ruptured are considered to be in an intermediate state rather than an activated state. Recent theoretical work by Némethy and Scheraga on hydrophobic bonding in proteins[15] makes it necessary to consider the intermediate (or activated) state as having been attained by rupture of both hydrogen and hydrophobic bonds. Thus, the rate constant of the initiation step k_i of Flory's theory for a simple helix–coil transition may reflect rupture of hydrophobic bonds and side-chain hydrogen bonds as well as peptide hydrogen bonds when applied to a reversible protein denaturation. This would be reflected in the dependence of k_i on both temperature and pH. The temperature dependence of k_1^f in the high temperature region shown at various pH's in Fig. 3 seems to have a negative coefficient while k_2^f shown in Fig. 4 seems to have a coefficient that is either slightly positive or zero depending on the pH. This is clearly a reflection of the different activation processes for each case, but in the absence of any specific detailed knowledge of these processes it is difficult to attempt a quantitative explanation. The rapid decrease in rate with lower temperature is simply a reflection of the $(1 - 1/r)$ factor in Flory's theory which gives the probability that a given initiation is followed by a succession of steps which ultimately lead to complete denaturation. If one assumes that the activation process involves the rupture of some critical combination of side chain hydrogen bonds and hydrophobic bonds, one might expect the rate constant k_i to either increase or decrease with increasing temperature since hydrogen bonds tend to weaken but hydrophobic bonds strengthen with increasing temperature.[15] Thus, with this modified interpretation of k_i we feel that the data in Fig. 3 and 4 are in qualitative agreement with theory.

Considering the renaturation process from the point of view of Flory's theory leads to an equation similar to eq. 9 with k_i^* replacing k_i and r replacing $1/r$. Thus, at temperatures well below T_{tr} where $r \rightarrow 0$ the apparent rate constant reduces to k_i^*, the rate constant for initiating a helical nucleus. At higher temperatures closer to the transition temperature the factor $(1 - r)$ becomes important and the rate falls off rapidly with increasing temperature as the transition temperature is approached from below. Although not many values of k_1^b and k_2^b have been obtained, it can be seen in Tables VIb and VIIb that at all pH's the values do decrease with increasing temperature as the transition temperatures are approached, as previously shown in Tables IV and V. Thus, on the basis of these data, the renaturation process also seems to follow theory.

pH Dependence of Rate Constants.—Figures 5 and 6 show the pH dependence of k_1^f and k_2^f plotted in the form log k *vs.* pH at various temperatures. It can be seen that both k_1^f and k_2^f show a pH dependence in the pH region 1.0–3.0 which has been attributed to abnormal carboxyl groups which are interacting with the tyrosyl groups, thereby causing the change in absorption. It has been previously suggested that the carboxyl and tyrosyl groups may be hydrogen bonded to each other, the hydrogen bond being surrounded by a hydrophobic environment.[4] Since the mechanism consists of two independent parallel reactions taking place in different regions of the molecule there must be at least two of these tyrosyl–carboxyl group interactions involved, one in each of the two separate regions. In Fig. 5 it can be seen that above pH 3.0, especially at 40 and 45°, the rate falls off very rapidly with pH. This is due to the term $(1 - 1/r)$ discussed previously since the transition temperatures in this pH region are higher than at the lower pH's. This effect is not noticeable in Fig. 6 because, for step 2, the transition temperatures are too low even at the higher pH's.

It should be noticed in Tables VIb and VIIb that the rate constants k_1^b and k_2^b increase with increasing pH, which is just what is expected, since at constant temperature r decreases with increasing pH; thus the term $(1 - r)$ increases. Also the constant k_i^* may itself have some pH dependence.

Results at 235 mμ.—As discussed previously, the experiments at 235 mμ followed the same form as those at 287 mμ but the rates were slower. Since the absorption at 235 mμ is due to the peptide group, it should include contributions from the whole molecule whereas the absorption at 287 mμ is due to changes in the environment of the tyrosyl groups only. The difference in rate between these two kinds of experiments may be attributable to the fact that when the protein denatures the tyrosyl groups are exposed rapidly to the more aqueous environment and the rest of the molecule then unfolds more slowly. This seems reasonable since the tyrosyls are probably involved in interactions such as side chain hydrogen bonds to carboxyl groups which are responsible for maintaining the native conformation. If, as has been suggested, there are tyrosyl and carboxyl groups side chain hydrogen bonded to each other and surrounded by a hydrophobic environment,[4] then what one sees at 287 mμ may be the rupturing of this whole region of interaction and the bringing of the tyrosyl and carboxyl groups from the hydrophobic to an aqueous environment. The experiments at 235 mμ would follow this plus the subsequent further unfolding of the backbone chain by a helix–coil type transition. We do not mean to imply that each method sees a different reaction but merely emphasizes different aspects of the same over-all reaction, the runs at 235 seeing the over-all process and those at 287 emphasizing the key first part of this process. The fact that the mechanism is the same for each method strengthens the conclusion made previously that the unfolding occurs by two parallel independent reactions.

Relation to Other Work.—It is known that three of the six tyrosyl residues of ribonuclease are abnormal in their ionization and spectral properties and that some of the carboxyl groups have abnormally low pK values.[16-18] It has been suggested that these abnormalities are due to tyrosyl–carboxyl interactions such as side chain hydrogen bonds and to the presence of neighboring hydrophobic regions.[4] Hermans and Scheraga suggested the existence of one or two tyrosyl–carboxyl side chain hydrogen bonds buried in hydrophobic regions.[4] It has recently been shown that two of the three abnormal tyrosines do not react with iodine and that these two "buried" tyrosines are numbers 25 and 97 in the amino acid sequence.[19,20]

Bigelow has considered some of the changes in tyrosyl absorption under various conditions and found that at low temperature and low pH one tyrosine, which he

(14) M. Laskowski, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, **83**, 266 (1961).

(15) G. Némethy and H. A. Scheraga, *J. Phys. Chem.*, **66**, 1773 (1962).

(16) D. Shugar, *Biochem. J.*, **52**, 142 (1952).

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

(18) C. Tanford, J. D. Hauenstein, and D. G. Rands, *ibid.*, **77**, 6409 (1955).

(19) C. Y. Cha and H. A. Scheraga, *J. Biol. Chem.*, **238**, 2958 (1963). This is paper IX in this series.

(20) C. Y. Cha and H. A. Scheraga, *ibid.*, **238**, 2965 (1963). This is paper X in this series.

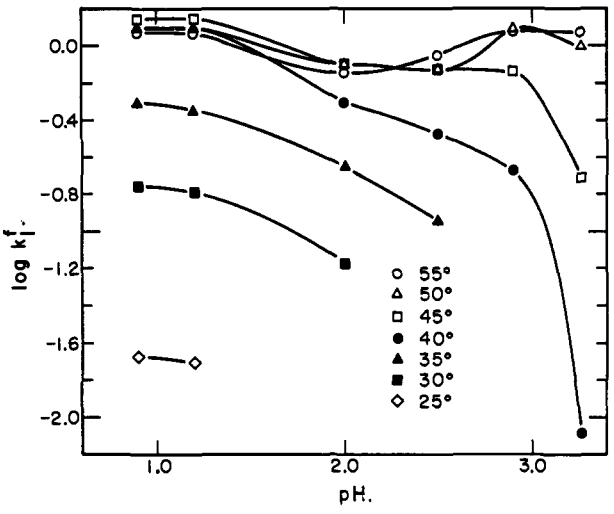

Fig. 5.—The logarithm of k_1^f, the first-order rate constant for step 1, plotted against pH.

called B, was normalized and that at higher temperatures two tyrosyls, called A and B, were normalized. Normalization of A was accompanied by a $\Delta\epsilon_{287}$ of -700 and B by a $\Delta\epsilon_{287}$ of -1000. Stronger treatment such as 8 *M* urea was necessary to normalize all three tyrosines A, B, and C.[21] The work discussed in this paper on the mechanism of the thermal transition in ribonuclease presumably concerns only the two tyrosyls called A and B by Bigelow. Our interpretation of the transition as consisting of two parallel independent transitions taking place in different regions of the molecule seems to be consistent with all of this previous work. Presumably, each of these regions contains an abnormal tyrosine which interacts, possibly through side chain hydrogen bonding with a carboxyl group, the groups being buried in a hydrophobic environment. The disruption of these regions normalizes the tyrosyl and carboxyl groups by rupturing the hydrophobic interactions and the side chain hydrogen bonds and bringing the groups into an aqueous environment, thus accounting for the dependence of the equilibrium and rate data on pH in the region of carboxyl group ionization. These two tyrosines would be those designated A and B by Bigelow and could be tyrosines 25 and 97 which fail to iodinate. The low temperature, low pH difference spectrum seems to involve the ionization of one or more carboxyl groups without any accompanying conformational change in agreement with the interpretation of Hermans and Scheraga,[4] since, as previously discussed, we found this reaction too fast to measure by our spectrophotometric stopped-flow technique.

(21) C. C. Bigelow, *J. Biol. Chem.*, **236**, 1706 (1961).

Recent experiments on the chymotryptic and tryptic hydrolysis of ribonuclease at elevated temperatures indicate that all the residues between 25 and 36 in the amino acid sequence are exposed by carrying out the thermal transition.[22,23] Since tyrosine 25 is also one of the groups which does not iodinate, it is very likely that this region corresponds to one of the regions involved in the mechanism proposed in this paper. It is interesting to note that aspartic acid 83 could be close enough to tyrosine 25 to be hydrogen bonded because they are each next in sequence to a disulfide bridge between Cys 26 and Cys 84. Primary chymotryptic splits also occur

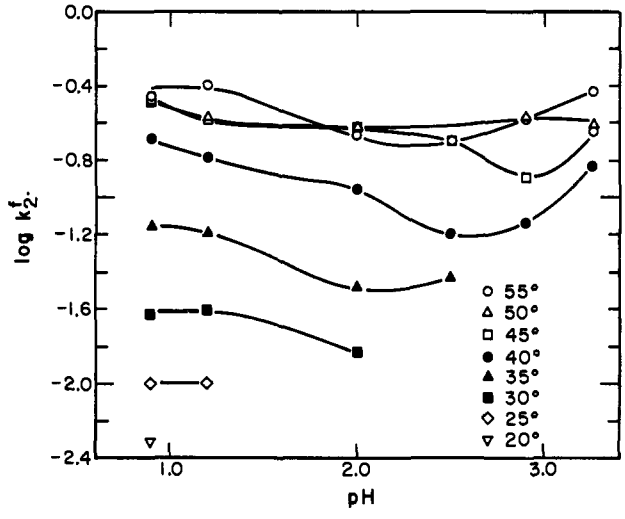

Fig. 6.—The logarithm of k_2^f, the first-order rate constant for step 2, plotted against pH.

at tyr–lys (97–98) and tyr–ser (76–77).[22] Presumably one of these is in the second region in our kinetic mechanism. Although tyr 97 is not iodinated it could be the one tyrosine remaining in the hard core of structure which is only normalized by harsh treatment such as 8 *M* urea and called tyrosine C by Bigelow.[23] Although it is not possible to choose between tyrosines 97 and 76 at this time, experiments are currently under way in this Laboratory to determine which of the carboxyl groups are "buried" and these may yield some clues. It is known that asp 38 is buried,[24] which might favor tyr 97 due to their close proximity to the disulfide bridge between cys 40 and cys 95, but this is only suggestive and further interpretation must await the completion of this work.

(22) J. A. Rupley and H. A. Scheraga, *Biochem.*, **2**, 421 (1963).
(23) T. Ooi, J. A. Rupley, and H. A. Scheraga, *ibid.*, **2**, 432 (1963).
(24) J. Riehm, C. A. Broomfield, and H. A. Scheraga, unpublished results.