Relaxation Spectra of Ribonuclease. III. Further Investigation of the Interaction of Ribonuclease and Cytidine 3′-Phosphate[1]

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Kinetic studies of the interaction of ribonuclease and cytidine 3′-phosphate have been carried out using the temperature-jump method. Three distinct relaxation processes have been observed which are related to the binding of cytidine 3′-phosphate to ribonuclease. The characteristic relaxation times of these processes have been studied as a function of pH, temperature, and concentrations. A quantitative correlation of all of the available data concerning this interaction can be made if the pH-dependent isomerization of ribonuclease is taken into account. One of the relaxation processes can be associated with the initial complex formation between cytidine 3′-phosphate and ribonuclease. The second-order rate constant for the association reaction is very large ($k \sim 5 \times 10^8$–5×10^9 M^{-1} $sec.^{-1}$) and the initial binding process is associated with an unusually large and positive standard entropy change. The pH dependence of the kinetic constants has been interpreted in terms of three ionizable groups at the active site of the free enzyme with pK values of approximately 5, 6, and 6.7 at 25°. These groups are probably a carboxyl and two imidazole side chains, respectively. The other two relaxation processes are associated with isomerizations of the enzyme–cytidine 3′-phosphate complex. One of these relaxation processes is also observed with ribonuclease–cytidine 2′-phosphate and ribonuclease–pyrophosphate complexes; accordingly, it is probably an isomerization involving a catalytically inactive form of the enzyme–product complex. The large deuterium isotope effect observed on the rate of this isomerization of the ribonuclease–cytidine 3′-phosphate complex at both 25 and 12.5° indicates that a proton transfer is probably involved. One of the relaxation effects is observed only with the ribonuclease–cytidine 3′-phosphate complex and is probably associated with the step prior to the rate-determining step for the formation of cytidine 2′,3′-cyclic phosphate from cytidine 3′-phosphate. The results are interpreted in terms of elementary mechanistic steps and a speculative mechanism which accounts for the hydrolysis of RNA as well as of cyclic phosphates is presented.

Introduction

The first paper in this series[3] presented the results of a kinetic investigation of the interaction of ribonuclease and cytidine 3′-phosphate utilizing the temperature-jump method. Two different relaxation processes were observed: one process was associated with the formation of an enzyme–cytidine 3′-phosphate complex, while the second relaxation effect was associated with an intramolecular transformation of the complex formed. From the pH dependence of the measured second-order rate constant for complex formation, a possible mechanism for the binding of enzyme and cytidine 3′-phosphate was proposed. It was also noted that a quantitative agreement between the binding constants obtained from the temperature-jump study and those obtained from steady-state kinetics[4] was lacking, although the constants obtained by the latter method were not available at all pH values of interest and, moreover, are not necessarily equilibrium constants. The over-all binding constants as measured by difference spectra and Sephadex dialysis have now been determined over a range of pH.[5] A comparison of these results with those from the temperature-jump study discloses a difference in the binding constants which is far outside of experimental error. An additional factor which must be taken into account is that ribonuclease A can exist in two distinct isomeric conformations in the pH range 5 to 7; the kinetics of this process are reported in detail in an accompanying paper.[6]

In this paper, an explanation for the discrepancy between results obtained by the temperature-jump method and by equilibrium methods will be presented; new rate constants have been calculated which differ somewhat from those previously published.[3] The study of the interaction of ribonuclease and cytidine 3′-phosphate has also been extended to another (lower) temperature, and the temperature dependence of all of the relaxation processes has been measured. In addition, a new relaxation process has been observed at the lower temperature and its pH dependence has been determined. A mechanistic interpretation of the results is presented which correlates all of the available data. From the results obtained, some inferences can be made about the amino acid residues around the active site of the enzyme and about the over-all mechanism of the enzymatic reaction.

Experimental Section

Materials and methods were the same as previously described.[3] The pH indicators employed at 12.5° were phenol red in the pH range 7.0–8.0 and chlor phenol red in the pH range 6.0–6.5. The range of enzyme (E_0) and cytidine 3′-phosphate (P_0) used at 12.5° are given in Table I. Some experiments were also carried out in which cytidine 3′-phosphate was replaced by either inorganic pyrophosphate or cytidine 2′-phosphate (prepared as described in an accompanying paper[6]).

(1) This work was supported by a grant from the National Institutes of Health (GM 07803).

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(3) R. E. Cathou and G. G. Hammes, *J. Am. Chem. Soc.*, 86, 3240 (1964).

(4) D. G. Herries, A. P. Mathias, and B. R. Rabin, *Biochem. J.*, 85, 127 (1962).

(5) G. G. Hammes and P. R. Schimmel, *J. Am. Chem. Soc.*, 87, 4665 (1965).

(6) T. C. French and G. G. Hammes, *ibid.*, 87, 4669 (1965).

Table I. Range of Enzyme (E_0) and Cytidine 3′-Phosphate (P_0) Concentrations Employed at 12.5°

pH	(E_0), M	(P_0), M
6.0	1.10×10^{-4}	4.5×10^{-5}–3.86×10^{-4}
6.5	1.18×10^{-4}	9.7×10^{-5}–3.59×10^{-3}
7.0	1.15×10^{-4}	9.8×10^{-5}–5.78×10^{-3}
7.5	1.65×10^{-4}	1.05×10^{-4}–5.71×10^{-3}
8.0	1.67×10^{-4}	1.95×10^{-4}–6.14×10^{-3}

Results and Treatment of Data

Before embarking on a detailed analysis of the results obtained, a general description of the behavior observed in this and previous studies will be presented.[3,6] Four different relaxation processes associated with different relaxation times, τ_i, are observed: τ_1 characterizes an isomerization of ribonuclease and is independent of enzyme concentration but is dependent on pH[6]; τ_2 is a function of enzyme and cytidine 3′-phosphate concentrations and of pH[3]; τ_3 is independent of cytidine 3′-phosphate and enzyme concentrations, but is dependent on pH; τ_4 is also independent of cytidine 3′-phosphate concentration but is dependent on pH. All processes are temperature dependent and, in fact, τ_4 could only be measured at 12.5°, although evidence for the occurrence of this relaxation process could be discerned at 25°. In general, the amplitude of all of the relaxation effects was greater at 12.5° than at 25°.

We will now consider in some detail the formation and dissociation of the initial complex formed between cytidine 3′-phosphate and ribonuclease. This process is characterized by τ_2; a possible mechanism is

$$E + P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EP \qquad (1)$$

where E represents ribonuclease and P, cytidine 3′-phosphate. For this mechanism[7]

$$1/\tau_2 = k_1[(E) + (P)] + k_{-1} \qquad (2)$$

Since the over-all binding constant is now known,[5] the equilibrium concentrations, (E) and (P), can be readily calculated, and k_1 and k_{-1} can be determined from a plot of $1/\tau_2$ *vs.* $[(E) + (P)]$. The ratio of the rate constants should then be equal to the binding constant. In point of fact, the ratio k_1/k_{-1} determined by this method is always greater than the equilibrium binding constant, the deviation being greatest at low pH values. In order to explain this discrepancy, we must carefully analyze what each experiment actually measures. The equilibrium binding constant, K_{AP}, can be represented as

$$K_{AP} = \frac{\sum_i (EP_i)}{\sum_j (E_j) \sum_k (P_k)} \qquad (3)$$

where the sums extend over all species present in solution. On the other hand, in the temperature-jump experiment, the equilibrium constant determined is

$$K_{TJ} = \frac{(EP_1)}{\sum_i^{r.e.} (E_i) \sum_j^{r.e.} (P_j)} \qquad (4)$$

(7) M. Eigen and L. de Maeyer in "Technique of Organic Chemistry," Vol. VIII, Part II, S. L. Friess, E. S. Lewis, and A. Weissberger, Ed., Interscience Publishers, Inc., New York, N. Y., 1963, p. 895.

where now the sums are over all species which equilibrate rapidly (r.e.) compared to τ_2, and EP_1 represents all species equilibrating rapidly with the initial complex formed. Note that if $\sum_i^{r.e.}(E_i) = \sum_j (E_j)$, then $K_{AP} \geq K_{TJ}$. However, the observed result is $K_{TJ} \geq K_{AP}$. All of the data can be made consistent if it is recalled that ribonuclease can isomerize and that the associated relaxation time, τ_1, is much greater than τ_2. Since deviations between K_{TJ} and K_{AP} are greatest at low pH values, the most reasonable assumption to make is that the isomeric form which is stable at low pH values is not able to bind cytidine 3′-phosphate. The mechanism in eq. 1 can then be written as

$$\sum_i^{r.e.}(E_i) + \sum_j^{r.e.}(P_j) \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} EP_1 \qquad (5)$$

$$k_{12} \uparrow \quad \downarrow k_{21}$$

$$E'$$

and since $\tau_1 >> \tau_2$

$$1/\tau_2 = k_{23}\left[\left(\sum_i^{r.e.}(E_i) + \sum_j^{r.e.}(P_j)\right)\right] + k_{32} \qquad (6)$$

Since $(E')/\sum_i^{r.e.}(E_i) = k_{21}'/k_{12} = K_{12}'$ is known as a function of pH and temperature,[6] a new constant K_{AP}' can be calculated as

$$K_{AP}' = \frac{\sum_i (EP_i)}{\sum_j^{r.e.}(E_j) \sum_k^{r.e.}(P_k)} \qquad (7)$$

and a plot of $1/\tau_2$ *vs.* $[\sum_i^{r.e.}(E_i) + \sum_j (P_j)]$ can be constructed. The resultant values of k_{23} and k_{32} determined from the slope and intercept of the plot then determine the binding constant K_{TJ} $(=k_{23}/k_{32})$ and furthermore, from eq. 4 and 7, K_{TJ} must be less than or equal to K_{AP}'. (All forms of P are known to equilibrate rapidly.)

The above analysis is in accord with all available data; values of k_{23}, k_{32}, K_{TJ}, and K_{AP}' at 12.5 and 25° are summarized in Table II. Since K_{AP} and k_{12}/k_{21}' were not measured at exactly these temperatures, the necessary values of these constants were obtained by an extrapolation at constant pH of the logarithm of the equilibrium constants *vs.* the reciprocal temperature. Also the value of K_{AP} at pH 7.5 was obtained by extrapolation of the measured values to this pH. The maximum error in the measured relaxation times is about ±15%, and the estimated errors in k_{23} and k_{32} are ±25%.

The values for k_{23} obtained in this study differ from the previously reported values[3] by less than 25% in the pH range 5.0–7.0; at pH 7.5 the new value of k_{23} is 35% higher than the old value. The greatest effect is on the values of k_{32}; in the pH range 6.5–7.5 the difference is less than about 20% while in the pH range 5.0–6.0, the difference is 35–45%. The net effect on k_{32} has been to abolish any observable pH dependence of k_{32} over the entire pH range studied. The new values are to be preferred since they take into account results not previously available. Finally, it should be pointed out that it has been assumed in this treat-

Table II. Summary of Kinetic Parameters[a]

pH	k_{23}, M^{-1} sec.$^{-1}$	k_{32}, sec.$^{-1}$	K_{TJ}, M^{-1}	K_{AP}', M^{-1}	K_{AP}'/K_{TJ}
		12.5°			
6.0	5.2×10^7	4.4×10^3	1.2×10^4	7.7×10^4	6.5
6.5	2.1×10^7	3.9×10^3	5.3×10^3	2.8×10^4	5.3
7.0	4.5×10^6	4.0×10^3	1.1×10^3	6.6×10^3	6.0
7.5	1.7×10^6	4.2×10^3	4.0×10^2	2.2×10^3	5.5
		25°			
5.0	1.1×10^8	5.3×10^3	2.1×10^4	4.7×10^4	2.2
5.5	1.9×10^8	6.7×10^3	2.8×10^4	7.0×10^4	2.5
6.0	8.7×10^7	6.0×10^3	1.5×10^4	2.2×10^4	1.5
6.5	5.2×10^7	6.4×10^3	8.1×10^3	7.9×10^3	1.0
7.0	1.0×10^7	6.0×10^3	1.7×10^3	2.2×10^3	1.3
7.5	2.8×10^6	6.5×10^3	4.3×10^2	4.7×10^2	1.1

[a] 0.1 M KNO_3.

ment of the data that no coupling exists between the processes characterized by τ_1 and τ_2. An exact calculation of the relaxation spectrum has been made and the errors caused by this assumption have been calculated. At 25° the error arising from coupling has been calculated to be less than 10% in the pH range 5.5–7.5, which is less than the experimental error; at pH 5.0, the maximum error (*i.e.*, for the longest value of τ_2) is 23%. At 12.5° the error involved is less than 10% in the pH range 6.0–7.5.

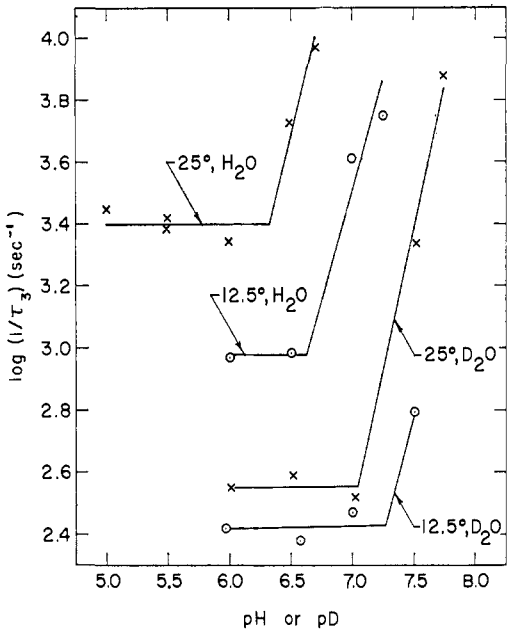

Figure 1. Variation of the relaxation time, τ_3, associated with the chemical relaxation effect observed in solutions containing ribonuclease and cytidine 3′-phosphate, with pH or pD in H_2O and D_2O. The lines have no theoretical significance.

The ratio K_{AP}'/K_{TJ} is equal to $1 + \sum_{i=1}(EP_i)/(EP_1)$. Therefore, this ratio is a measure of the amount of slowly equilibrating isomeric forms (*i.e.*, slow compared to τ_2) of the initially formed complex which is present; it is equal to 1 if all of the isomers equilibrate rapidly compared to τ_2 with the initial complex. Values of K_{AP}'/K_{TJ} are included in Table II. Deviations of this ratio from unity should not be considered significant unless the deviation is greater than about 0.5.

Values of τ_3 have been determined at 12.5 and 25° in both H_2O and D_2O. The results are summarized as a function of pH in Figure 1. In all cases no dependence of the relaxation time on ribonuclease or cytidine 3′-phosphate concentrations could be discerned, although the dependence on enzyme concentration was tested only at 25° in H_2O.

The relaxation process characterized by τ_4 could only be studied quantitatively at 12.5° and the results obtained are shown in Figure 2. This relaxation time was independent of cytidine 3′-phosphate concentration; it could not be detected when D_2O was the solvent.

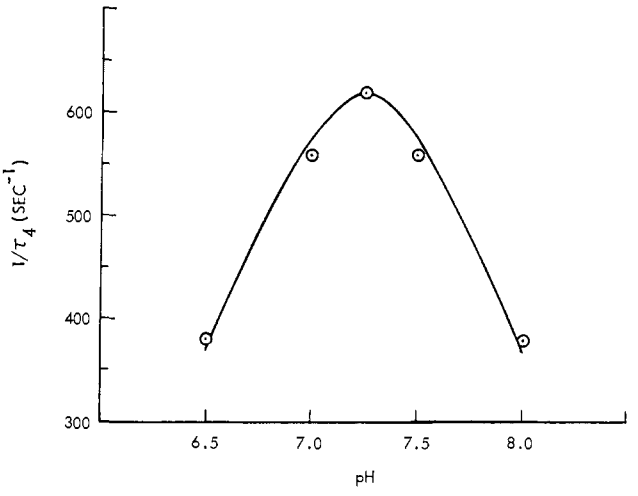

Figure 2. Variation of the chemical relaxation time, τ_4, with pH at 12.5°. The solid line is a theoretical curve calculated according to eq. 14 as described in the text.

A chemical relaxation effect with a relaxation time having a pH dependence and a lack of dependence on enzyme and inhibitor concentrations similar to that of τ_3 was also observed in solutions containing 1.0–2.0 $\times 10^{-4}$ M ribonuclease and 0.5–1.0 $\times 10^{-3}$ M cytidine 2′-phosphate at 12.5°; values of $1/\tau_3$ for this system are shown in Figure 3 as a function of pH.

A chemical relaxation effect was also observed in solutions containing enzyme and 0.4–1.2 $\times 10^{-3}$ M inorganic pyrophosphate at 25° in the pH range 5.0–6.6[8]; it was not possible to extend this study to

(8) These experiments with pyrophosphate were done by Dr. Thayer C. French.

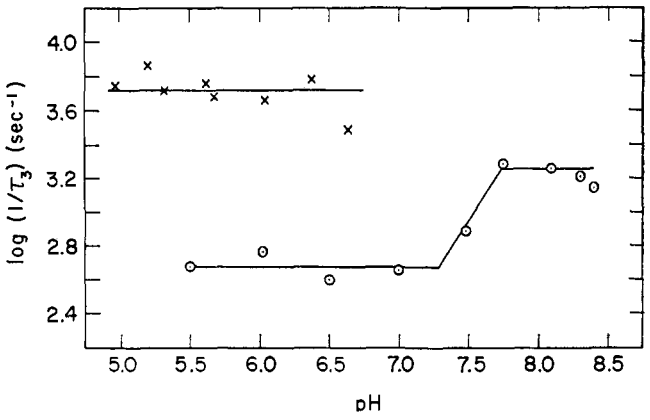

Figure 3. Variation with pH of the relaxation time, τ_3, associated with the chemical relaxation effects observed in solutions containing ribonuclease and cytidine 2′-phosphate at 12.5°, and inorganic pyrophosphate at 25°, respectively: O, cytidine 2′-phosphate; ×, inorganic pyrophosphate. The solid lines have no theoretical significance.

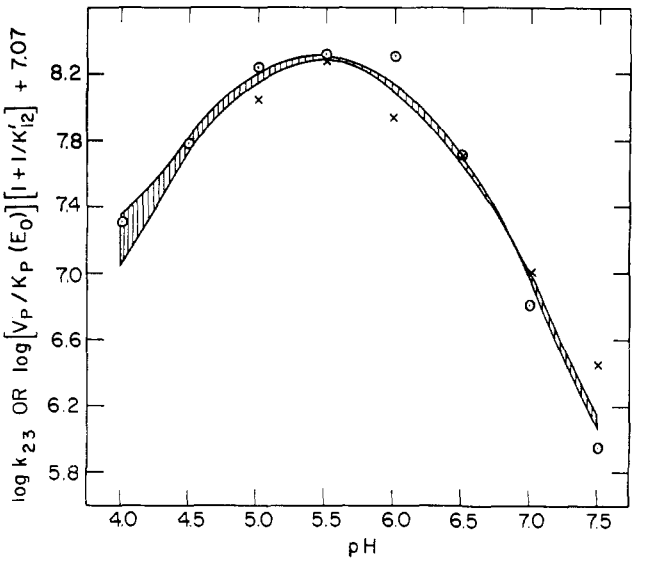

Figure 4. Variation of k_{23} and $V_P[1 + 1/K_{12}']/K_P(E_0)$ with pH at 25°. The points are experimentally determined values: ×, k_{23}; O, $V_P[1 + 1/K_{12}']/K_P(E_0)$. The solid lines enclosing the vertically striped area denote the limits of the theoretical curves calculated according to eq. 8–11 in the text.

higher pH values as the concentration of pyrophosphate necessary to saturate the enzyme was so large that buffering prevented the detection of the small change in hydrogen ion concentration accompanying the relaxation process. In the pH range studied, the relaxation time of this effect is constant within experimental error and is probably analogous to the processes described by τ_3 for the previously discussed cases. Values of $1/\tau_3$ are shown in Figure 3. A relaxation effect with a relaxation time analogous to τ_4 was not observed in the systems containing pyrophosphate and cytidine 2′-phosphate.

Discussion

The simplest explanation of the observed pH dependence is that ribonuclease and cytidine 3′-phosphate can exist in several different states of ionization depending upon the pH and that some of these states cannot take part in the reaction. The equilibrium between these different states must be adjusted rapidly compared to τ_2 if they are to have an effect upon k_{23}. Independent experiments show that the protolytic reactions occurring with the enzyme and with cytidine 3′-phosphate are in fact adjusted rapidly compared to τ_2.

Obviously the pH dependence of k_{23} is determined only by ionizable groups on free cytidine 3′-phosphate and free ribonuclease. The steady-state parameter, $V_P(1 + 1/K_{12}')/K_P(E_0)$, where V_P is the maximum velocity for the formation of cytidine 2′,3′-cyclic phosphate, K_P is the Michaelis constant of cytidine 3′-phosphate, (E_0) is the total enzyme concentration, and $(1 + 1/K_{12}')$ corrects for the fact that only one of the isomeric forms of ribonuclease is active, should have the same pH dependence as k_{23}.[9] Although the quantity $V_P(1 + 1/K_{12}')/K_P(E_0)$ cannot be directly measured, it has been recently calculated from thermodynamic and kinetic data,[10] and a plot of k_{23} and $V_P(1 + 1/K_{12}')/K_P(E_0)$ *vs.* pH at 25° is shown in Figure 4. It can be seen that the pH dependence of the two parameters is the same within experimental error. Before this pH dependence can be interpreted in terms of ionizable groups on the enzyme, some assumption must be made concerning the form (or forms) of cytidine 3′-phosphate which binds to the enzyme. We will consider four cases which cover the most likely (and simplest!) possibilities: (1) only the monoanion binds; (2) only the dianion binds; (3) both mono- and dianions bind to the same form of the enzyme with equal values of k_{23}; and (4) both mono- and dianions bind to the enzyme with equal values of k_{23}, but the dianion binds to a form of the enzyme with one more proton than in the case of the monoanion. With these assumptions, it was necessary to assume the existence of two, two, three, and two ionizable groups, respectively, at the active site of ribonuclease in order to fit the experimental data. The formal equations which describe the data in these four cases are (*cf.* ref. 3 for the details of how such equations can be derived)

Case 1

$$k_{23} = \frac{k_{23\,max}}{[1 + (H^+)/K_a + K_b/(H^+)][1 + (H^+)/K_1 + K_2/(H^+)]} \quad (8)$$

Case 2

$$k_{23} = \frac{k_{23\,max}}{[1 + K_a/(H^+) + K_aK_b/(H^+)^2][1 + (H^+)/K_2 + (H^+)^2/K_1K_2]} \quad (9)$$

Case 3

$$k_{23} = \frac{k_{23\,max}}{1 + (H^+)/K_a + K_b/(H)^+ + K_bK_c/(H^+)^2} \quad (10)$$

Case 4

$$k_{23} = \frac{k_{23\,max}[1 + K_2/K_A]}{1 + (H^+)/K_a + K_b/(H^+)} \quad (11)$$

(9) L. Peller and R. A. Alberty, *J. Am. Chem. Soc.*, 81, 5907 (1959).
(10) J. T. Bahr, R. E. Cathou, and G. G. Hammes, *J. Biol. Chem.*, 240, 3372 (1965).

where $k_{23\ max}$ is the pH-independent value of the second-order rate constant, K_a, K_b, and K_c are ionization constants of groups on the enzyme, K_1 is the ionization constant associated with the cytidine 3′-phosphate ring nitrogen, and K_2 is the ionization constant associated with the secondary phosphate group. A summary of the parameters associated with these mechanisms and the various ionizable species involved is given in Table III. The locus of points calculated with eq. 8–11 and Table III for the 25° data are included in Figure 4. All of the mechanisms fit the data equally well; a better fit to the data can be obtained by assuming more complex mechanisms, but the data do not warrant this. This analysis is considerably more detailed than previously given for similar data,[3] but a considerably greater amount of information is now available. Since the data at 12.5° are more sparse and steady-state kinetic parameters are not available at this temperature, the results obtained at this temperature are not as reliable. All of the mechanisms involve ionizable groups on the enzyme having pK values of about 5 and 6.7 (at 25°). One mechanism also involves a group with a pK of about 6. Since a group with a pK of 6.1 has already been implicated at the active site,[6] a reasonable postulate is that at least three ionizable groups are located in the vicinity of the active site characterized by pK values of about 5, 6, and 6.7.

Table III. Mechanistic Parameters for the Formation of the Cytidine 3′-Phosphate–Ribonuclease Complex

Case	Substrate species[a]	Enzyme species[b]	Enzyme pK values	$k_{23\ max}$, M^{-1} sec.$^{-1}$
		25°		
1	PH^-	EH_2, *EH*, E	5.0, 6.7	4.2×10^8
2	P^{2-}	*EH_2*, EH, E	4.8, 6.8	4.5×10^9
3	P^{2-}, PH^- equal rates	EH_3, *EH_2*, EH, E	5.0, 5.9, 6.7	3.7×10^8
4	P^{2-}, PH^- equal rates	*EH_2*, EH, E EH_2, *EH*, E	5.0, 6.7	3.7×10^8
		12.5°		
1	PH^-	EH_2, *EH*, E	5.0, 7.1	1.3×10^8
2	P^{2-}	*EH_2*, EH, E	4.8, 7.1	1.7×10^9
3	P^{2-}, PH^- equal rates	EH_3, *EH_2*, EH, E	5.2, 6.0, 7.5	8.5×10^7
4	P^{2-}, PH^- equal rates	*EH_2*, EH, E EH_2, *EH*, E	5.0, 7.1	1.0×10^8

[a] Only the substrate species which bind are listed. [b] The enzyme species which bind are italicized.

If it is desired to have the same ionization state of the free enzyme for the forward and reverse directions of the enzymatic reaction, only mechanisms 1 and 4, as written, are possible; this follows directly from a consideration of the pH dependence of the equilibrium constant. However, mechanisms 2 and 3 can be made to conform to this restriction by the simple addition of rapidly equilibrating protolytic equilibria. This consideration, therefore, does not rule out any of the mechanisms.

The values of $k_{23\ max}$ are not greatly affected by slight alterations in the pK values so that some significance can be attached to the activation parameters associated with it. The activation parameters for $k_{23\ max}$ and k_{32} (which is independent of pH) are summarized in Table IV. Included are values of the standard enthalpy ($\Delta H°$) and entropy ($\Delta S°$) changes associated with the binding process. The possible experimental error in these parameters is large and they are at best reliable to about ±6 kcal./mole or ±20 e.u. Regardless of the mechanism chosen, $k_{23\ max}$ is close to or equal to the value expected for a diffusion-controlled reaction[11]; however, the rather large values of $\Delta H°$* suggest the process may not be a simple diffusion-controlled reaction. In all cases the standard entropy change is unusually large and positive for a simple association reaction and this could imply an over-all loosening of the enzyme accompanying binding in accordance with the idea of "entropy compensation."[12]

Table IV. Activation and Thermodynamic Parameters at 25°

Case[a]	$\Delta H°$*, kcal./mole	$\Delta S°$*, e.u.	Log (k_{23max}/k_{32})	$\Delta H°$, kcal./mole	$\Delta S°$, e.u.
k_{23}					
1	15	31	4.83	11	58
2	13	28	5.86	9	55
3	19	45	4.78	15	72
4	17	36	4.78	13	63
k_{32}	4	−27			

[a] The cases are the same as in Table III.

The temperature dependence of the pK values is also worth noting. In all cases, the group or groups with lower pK values, *i.e.*, 4.8–5.9, have the same pK values at 25 and 12.5°. On the other hand, the group with a pK of 6.7–6.8 has a pronounced temperature dependence; the shift in the pK value going from 25 to 12.5° corresponds to a standard enthalpy of ionization of about 10 to 20 kcal./mole. The ionization constant of a carboxyl group has little or no temperature dependence[13] while that of a normal imidazole group is about 8 kcal./mole.[13]

The groups at the active site with pK values of about 5 and 6.7 are probably a carboxyl group and an imidazole, respectively. An identification of the remaining group is more difficult; the lack of temperature dependence of the ionization constant would make it appear to be a carboxyl group. However, the pK value (6) is abnormally high for a carboxyl group; moreover, the group would probably have to be in a quite negatively charged environment to have the observed pK value. As ribonuclease is a basic protein and contains *positive* charge clusters,[14] this is highly unlikely. On the other hand, an imidazole group would have a lower pK in a positively charged environment. It is more likely, therefore, that the group is an imidazole which has an abnormal temperature coefficient It is also possible that experimental error has obscured any observable temperature dependence of the ionization constant. It must be borne in mind, however, that these arguments are based on circumstantial evidence, and that the identity of this group cannot

(11) R. A. Alberty and G. G. Hammes, *J. Phys. Chem.*, **62**, 154 (1958).
(12) G. G. Hammes, *Nature*, **204**, 342 (1964).
(13) R. B. Martin, "Introduction to Biophysical Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1964, p. 65.
(14) G. I. Loeb and H. A. Saroff, *Biochemistry*, **3**, 1819 (1964).

be ascertained with any great certainty at present. In view of other evidence, the two histidine residues involved are almost certainly numbers 12 and 119 in the amino acid sequence.[15,16] The exact location of the carboxyl group in the amino acid sequence cannot be reasonably guessed at this time.

The relaxation times τ_3 and τ_4 are undoubtedly related to isomerizations of the ribonuclease–cytidine 3′-phosphate complex. The fact that τ_4 is observed only at 12.5° is consistent with the large values of K_{AP}'/K_{TJ} (*cf.* Table I) at this temperature. The variation of $1/\tau_3$ with pH is shown in Figure 1. The form of the pH dependence is about the same at 25 and 12.5°, but the curve is shifted up the pH scale at 12.5°. As was pointed out previously,[3] more than one titratable group must be responsible for the observed pH dependence, and because of the large deuterium isotope effect the reaction probably involves a proton transfer. However, one should bear in mind that the large isotope effect could also be caused by alteration of the protein structure in D_2O. Activation parameters have been calculated for values of $1/\tau_3$ at the lower pH values where $1/\tau_3$ is constant and are: $\Delta H^{\circ *} = 12$ kcal./mole and $\Delta S^{\circ *} = -4$ e.u. in H_2O; and $\Delta H^{\circ *} = 4$ kcal./mole and $\Delta S^{\circ *} = -9$ e.u. in D_2O. The low pH limit of $1/\tau_3$ in H_2O at 25° is smaller by a factor of 7 than the corresponding value of $1/\tau_3$ in D_2O, while at 12.5° this factor is only 3.5.

A relaxation effect corresponding to τ_3 has also been observed when cytidine 2′-phosphate and pyrophosphate are bound to ribonuclease, but not when sulfate ion and cytidine are bound. A plausible postulate is that the isomerization characterized by τ_3 involves the formation of an inactive complex in which the OH group on phosphate ends up in the position where the furanosyl-2′-OH group should be relative to the catalytic groups. The enzyme side chains would then be the same ones associated with τ_1; this is consistent with the fact that when cytidine is bound to the enzyme, both τ_1 and τ_3 are absent.[6] A schematic indication of how this might come about is shown in Figure 5. Small ions, *i.e.*, sulfate, could bind at the group with a pK of 6.7 without affecting τ_1. Pyrophosphate and cytidine 2′- and 3′-phosphate could form inactive complexes in which the OH group on the phosphate forms a weak bond with the carboxyl or imidazole group. Needless to say, this model should not be taken too literally.

The pH dependence of $1/\tau_3$ will fit an equation of the form

$$1/\tau_3 = k + \frac{k'}{1 + (H^+)/K_a + (H^+)^2/K_aK_b} \qquad (12)$$

which corresponds to the mechanism

$$EP_1 \underset{k'}{\overset{k}{\rightleftharpoons}} EP_1' \underset{K_a}{\overset{H^+}{\rightleftharpoons}} EP_1'H^+ \underset{K_b}{\overset{H^+}{\rightleftharpoons}} EP_1'H_2^{2+} \qquad (13)$$

where

$$K_a = \frac{(EP_1')(H^+)}{(EP_1'H^+)}$$

and

$$K_b = \frac{(EP_1'H^+)(H^+)}{(EP_1'H_2^{2+})}$$

(15) A. M. Crestfield, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **238**, 2413 (1963).

(16) W. D. Stein and E. A. Barnard, *J. Mol. Biol.*, **1**, 350 (1959).

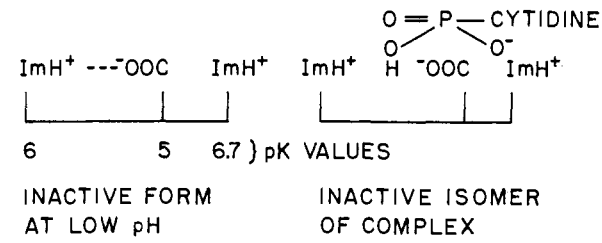

Figure 5. A possible model of the inactive forms of ribonuclease and ribonuclease–inhibitor complexes.

and the protolytic equilibria are assumed to be adjusted rapidly compared to τ_3. Unfortunately $1/\tau_3$ could not be observed over a sufficiently wide pH range to establish the validity of eq. 12. However, this mechanism is sufficient to explain the observed rapid increase of $1/\tau_3$ with increasing pH. It is interesting to note that k, the low pH limit of $1/\tau_3$ and the rate constant for the formation of the inactive complex, is roughly inversely proportional to the binding strength of the anions to ribonuclease.

The experimental values of $1/\tau_4$ at 12.5° were found to fit the bell-shaped curve shown in Figure 2 which can be described by the equation

$$1/\tau_4 = \frac{k_{43}}{1 + (H^+)/K_a + K_b/(H^+)} + k_{34} \qquad (14)$$

where k_{43}, the pH-independent value of $1/\tau_4$, is equal to 9.7×10^2 sec.$^{-1}$ and K_a and K_b, the ionization constants of the enzyme groups involved, have pK values of 6.7 and 7.8, respectively, and k_{34} is negligibly small. A mechanism from which eq. 14 can be derived is

$$\begin{array}{ccc} & EP_2 & \\ & \uparrow\downarrow K_b & \\ EP_1 & \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} & EP_2H \\ & \uparrow\downarrow K_a & \\ & EP_2H_2 & \end{array} \qquad (15)$$

where some of the protons have been omitted for simplicity and the assumption has been made that the protolytic equilibria are adjusted rapidly compared to τ_4. This relaxation process is postulated to be on the main reaction path because it is found only when cytidine 3′-phosphate is the bound substance and its pH dependence is somewhat analogous to that calculated for the maximum velocity of the reverse reaction,[10] except that it is shifted upward on the pH scale (probably because the temperature-jump experiments were carried out at a lower temperature than the steady-state studies). This step could not be the same as that associated with the maximum velocity since $V_P/(E_0)$ is less than 1 sec.$^{-1}$ [10]; accordingly, if $1/\tau_4$ really has the same pH dependence as $V_P/(E_0)$, this step must be just prior to that associated with the rate-determining step for formation of cytidine 2′,3′-phosphate. The rate constant k_{34} must have the same pH dependence as k_{32} and hence is independent of pH. The fact that k_{34} is negligible relative to $1/\tau_4$ simply implies that the concentration of EP_2H is small compared to that of EP_1. The pK values found cannot be identified with particular groups on the enzyme since

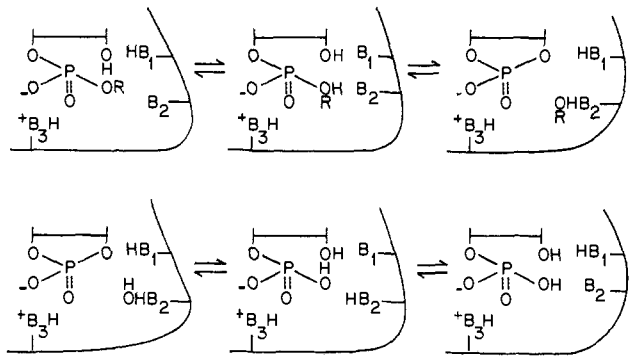

Figure 6. A speculative mechanism for the hydrolysis of RNA and cytidine 2′,3′-cyclic phosphate by ribonuclease.

the pK values would be expected to differ in an unknown manner from those for the same groups on the free enzyme. The temperature dependence of this relaxation time could not be determined as the amplitude of the relaxation effect was too small to be measurable at 25°. This relaxation process was not observed in D_2O, either because structural modifications make the concentration of EP_2H negligible or because $1/\tau_4$ has no isotope effect and is not detectable experimentally since it is obscured by $1/\tau_3$.

In principle, the coupling between the relaxation process should be included in the above analysis; in actual fact the precision of the data does not warrant such a detailed analysis.

At this point, it is worth summarizing the most important points of the proposed interpretation of the data: (1) *at least three ionizable groups are located at the active site; these can be identified with pK values of about 5, 6, and 6.7 (at 25°) and are probably two imidazoles and one carboxyl group;* (2) *the formation of the initial complex is very rapid* ($k \sim 5 \times 10^8$–5×10^9 M^{-1} *sec.*$^{-1}$ *at 25°*) *and is associated with an unusually large positive entropy change;* (3) *the relaxation processes associated with* $1/\tau_3$ *and* $1/\tau_4$ *are isomerizations of the complex formed,* $1/\tau_3$ *possibly being related to the formation and breakdown of an inactive complex, and* $1/\tau_4$ *possibly being related to the step prior to the rate-determining process for the formation of cytidine 2′,3′-cyclic phosphate.*

A formal minimal mechanism for the hydrolysis of cytidine 2′,3′-cyclic phosphate which fits all of the available data is

$$\begin{array}{ccccccc} E + S & \rightleftharpoons & ES & \underset{(V_P/(E_0))}{\overset{(V_s/(E_0))}{\rightleftharpoons}} & EP_2 & \underset{}{\overset{(\tau_4)}{\rightleftharpoons}} & EP_1 \overset{(\tau_2)}{\rightleftharpoons} E + P \\ \uparrow\downarrow (\tau_1) & & & & & & \uparrow\downarrow (\tau_3) \quad \uparrow\downarrow (\tau_1) \\ E' & & & & & & EP_1' \quad E' \end{array} \qquad (16)$$

where S designates cytidine 2′,3′-cyclic phosphate. The protolytic equilibria have been omitted for simplicity and the measured time constants associated with each step are indicated in parentheses. To translate this formal mechanism into meaningful chemistry involves considerable speculation; nevertheless, Figure 6 contains a speculative mechanism which corresponds to $ES \rightleftharpoons EP_2 \rightleftharpoons EP_1$ in eq. 16. Included is an illustration of how this same mechanism would also work for the breakdown of ribonucleic acid to cyclic phosphates. This mechanism has the feature that unlike previously proposed mechanisms, the enzyme begins and ends in the same state of ionization without postulating further equilibria so that the principle of microscopic reversibility is automatically obeyed. Also, the same ionization states of the groups on the enzyme are involved in both the breakdown of RNA to cyclic phosphate and in the subsequent hydrolysis of the cyclic phosphate. This is in agreement with the finding that the pH dependence of these two reactions is quite similar.[17] Because of the speculative nature of the mechanism, no attempt is made to identify B_1, B_2, and B_3 with specific groups on the enzyme.

As with most kinetic studies, many questions remain unanswered. We are presently attempting to study the hydrolysis of cytidine 2′,3′-cyclic phosphate directly with a combination of rapid mixing and relaxation methods and hope ultimately to study the breakdown of RNA.

(17) H. Witzel, *Progr. Nucleic Acid Res.*, 2, 221 (1963).