Relaxation Spectra of Ribonuclease. IV. The Interaction of Ribonuclease with Cytidine 2′:3′-Cyclic Phosphate[1]

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Abstract: The interaction of cytidine 2′:3′-cyclic phosphate with ribonuclease has been investigated with a stopped-flow, temperature-jump technique. Two distinct relaxation processes have been observed. One relaxation process is characterized by a relaxation time which is independent of concentration at high substrate concentrations and is independent of pH over the range 5.5–7. This relaxation process was studied both at 25 and 15° in H_2O and at 15° in D_2O and is due to an isomerization of the enzyme–substrate complex. The second relaxation process is characterized by a relaxation time which is concentration dependent and is due to the binding of the cyclic phosphate to the enzyme. This process was observed only at pH 6.2, 15°. An extension of the work on the interaction of cytidine 3′-phosphate with ribonuclease is also reported. A detailed formal mechanism for the hydrolysis of cytidine 2′:3′-cyclic phosphate to cytidine 3′-phosphate by ribonuclease is presented which can account for all relaxation, steady-state, and equilibrium data in the pH region 5–8. The most important features of this mechanism are the existence of five different states of the enzyme–substrate complex, the presence of at least three ionizable groups at the active site having approximate pK values of 5, 6, and 6.7, and the existence of parallel reaction paths.

Previous papers in this series have dealt with relaxation studies of ribonuclease and its interaction with cytidine 2′:3′-cyclic phosphate, and various competitive inhibitors of ribonuclease action.[2-4] Relaxation studies show that the native enzyme undergoes an isomerization that is characterized by a relaxation time, τ_1, which is of the order of magnitude of milliseconds. The interaction of ribonuclease with cytidine 3′-phosphate is characterized by three relaxation times; one, τ_2, is related to the binding of the nucleotide to the enzyme; τ_3 and τ_4 are characteristic of isomerizations of the enzyme–nucleotide complex.

The relaxation studies were carried out using an equilibrium temperature-jump method[5] which precluded the study of the interaction of model substrates with ribonuclease since the equilibrium between substrates and products is such that the predominant species in solution are cytidine 3′-phosphate and its complexes with ribonuclease.[6] With the development of the stopped-flow, temperature-jump method,[7] studies of the interaction of cytidine 2′:3′-cyclic phosphate and cytidylyl-3′:5′-cytidine with ribonuclease were undertaken. The stopped-flow, temperature-jump method permits the rapid mixing of enzyme with substrates and the application of a temperature perturbation as fast as 16 msec after mixing. This is rapid enough to permit the relaxation spectra of the interaction of the enzyme with substrates to be studied before an appreciable amount of the product of hydrolysis, cytidine 3′-phosphate, is accumulated. This paper presents the results obtained with the system ribonuclease–cytidine 2′:3′-cyclic phosphate and a further extension of the work on the ribonuclease–cytidine 3′-phosphate system. An accompanying paper presents the results obtained with cytidylyl-3′:5′-cytidine.[8]

Experimental Section

Ribonuclease A (phosphate and salt free) was obtained from Worthington Biochemical Corp., lot no. RAF 6084 and RAF 6091, and from Sigma Chemical Co., lot no. 65B-8590. The enzyme was essentially free of aggregrates.[9,10] Cytidine 2′:3′-cyclic phosphate and cytidine 3′-phosphate were prepared as previously described.[2,4] Solutions were prepared by dissolving the solid material (either ribonuclease, cytidine 2′:3′-cyclic phosphate, or cytidine 3′-phosphate) in an 0.15 M KNO_3 solution which had been prepared from freshly boiled distilled water. The concentrations were determined spectrophotometrically as previously described.[2,6] A colorimetric pH indicator (phenol red or chlor phenol red) was added so the final concentration of indicator was about 3×10^{-5} M, and the pH was adjusted with KOH and HNO_3. For studies in D_2O the solutions were prepared as before.[3]

In the stopped-flow, temperature-jump experiments the reactants were placed in the reservoirs and brought to thermal equilibrium at the desired temperature. Stopped-flow experiments were first performed to determine the time course of the over-all reaction. Since the apparatus uses absorption spectrophotometry in the visible region of the spectrum as a means of detecting concentration changes, the enzyme system was coupled with a colorimetric pH indicator. By this means the pH of the solution was determined as a function of time. During the initial portion of the reaction, the first 10%, the change in pH was directly proportional to the extent of reaction. To determine the relaxation spectra of ribonuclease–cytidine 2′:3′-cyclic phosphate, the temperature perturbation was usually applied within the first 1 to 2% of the extent of reaction, where the initial pH had not changed detectably and the amount of product was negligible. The details of the apparatus and the procedure for evaluation of the relaxation times have been described elsewhere.[4,7]

The Michaelis contstants were determined spectrophotometrically[11,12] on a Beckman DK-2A ratio recording spectrophotometer. Care was taken to keep the slit-width constant during a given series of measurements by variation of the sensitivity. The substrate and enzyme were dissolved in 0.05 M Tris–acetate buffer with ionic strength adjusted with KNO_3 to 0.15 M. The substrate

(1) This work was supported by a grant from the National Institutes of Health (GM 13292).

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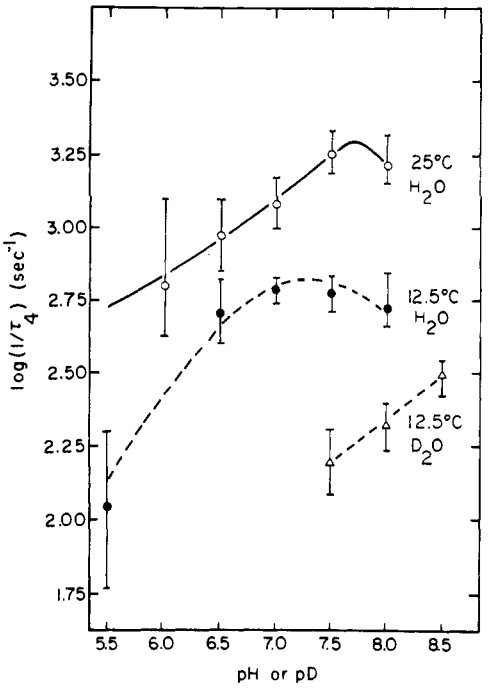


Figure 1. Variation of $1/\tau_4$ with pH (or pD); the solid line is calculated from the mechanism of Figure 5 according to eq 8. The dashed lines have no theoretical significance.

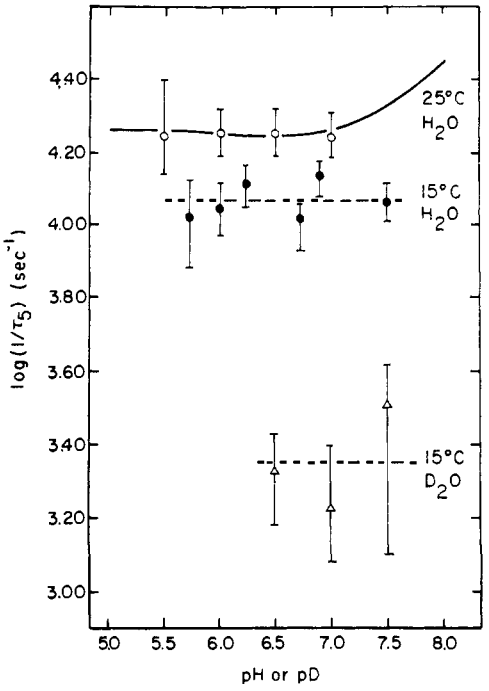


Figure 2. Variation of $1/\tau_5$ with pH (or pD); the solid line is calculated from the mechanism of Figure 5 according to eq 3 at high substrate concentrations. The dashed lines have no theoretical significance.

(3 ml) was pipetted into the blank and the reaction cuvettes and allowed to reach thermal equilibrium. Then 10 μl of stock enzyme solution ($\sim 1 \times 10^{-4}$ *M*) was added to the reaction cell and the change in transmittance at 286 mμ was observed. The transmittance rather than the absorbance was measured, because small concentration changes are easier to detect in this manner with the Beckman DK-2A spectrophotometer.

Results and Interpretation of Data

In the previous studies of the interaction of cytidine 3′-phosphate with ribonuclease,[4] one relaxation time,

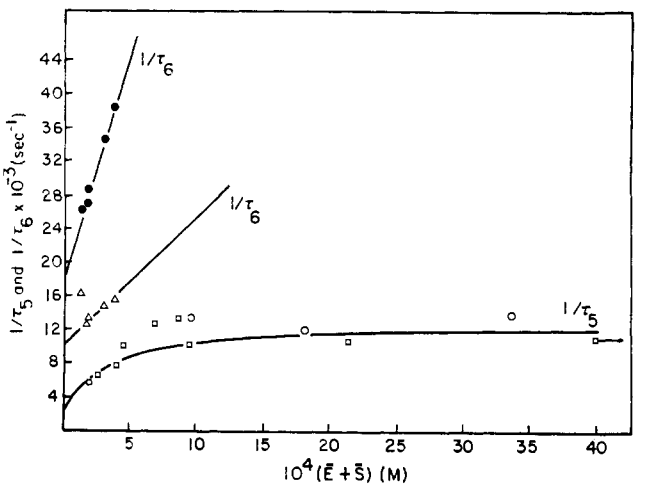


Figure 3. Variation of $1/\tau_5$ and $1/\tau_6$ with ([Ē] + [S̄]) at pH 6.0 and 6.2 at 15°. The symbols used are: $1/\tau_5$ pH 6.0, □; $1/\tau_5$ pH 6.2, ○; $1/\tau_6$ pH 6.2, ●; and △ (see text for details). The solid line for $1/\tau_5$ was calculated from eq 3 using $k_{-6}/k_6 = 3 \times 10^{-4}$ *M*, $k_5 = 1.1 \times 10^4$ sec^{-1}, and $k_{-5} = 2 \times 10^8$ sec^{-1}.

τ_4, could only be studied quantitatively at 12.5° over the pH range 6.5–8. By use of higher nucleotide concentrations and by improvement of the signal-to-noise ratio of the temperature-jump apparatus, measurement of this relaxation time could be extended to lower pH values, to higher temperature, and to D_2O. Figure 1 shows τ_4 as a function of pH (or pD) at 25 and 12.5°. The previous studies[4] showed that $1/\tau_4$ is independent of the concentration of nucleotides and enzyme and is characteristic of an isomerization of the enzyme–nucleotide complex.

The Michaelis constant, K_S, for cytidine 2′:3′-cyclic phosphate was determined at several different pH values and temperatures. The experimental results are shown in Table I. The values for K_S are the average

Table I. Michaelis Constants for the Cytidine 2′:3′-Cyclic Phosphate–Ribonuclease Reaction[a]

Temp, °C	10^4K_S, *M* at pH 5.0	5.5	5.9	6.0	6.2
25	2.6	4.4	...	11.0	...
15	...	...	1.5	1.9	2.1

[a] 0.05 *M* Tris–acetate buffer, $\mu = 0.15$ *M* (KNO_3).

values from plots of $1/v$ *vs.* $1/S$ and of S/v *vs.* S where v is the initial velocity and S is the initial substrate concentration. The best straight line through the experimental points was determined by the method of least squares.

Evidence has been obtained for two relaxation effects when cytidine 2′:3′-cyclic phosphate interacts with ribonuclease. One, designated τ_5, is independent of enzyme and substrate concentrations at high substrate concentrations and has been studied as a function of concentration, pH, temperature, and solvent. Figure 2 shows $1/\tau_5$ as a function of pH (or pD) at 25 and 15°.

At two pH values a concentration-dependent relaxation time was observed. The data at pH 6 and 6.2 are shown in Figure 3. In the low substrate concentration region, the relaxation time depends markedly on concentration. The amplitudes of the relaxation times in the low concentration region are quite small and

therefore the relaxation spectrum is difficult to analyze. An additional complication is that the effects are superimposed on the isomerization process, characterized by τ_1, which, in this pH region, has a time constant of about 600 μsec. Also, the time constants for the relaxation processes are close to the time constant characterizing the temperature perturbation. In fact, depending on how the data are analyzed, at pH 6.2 two sets of values were obtained. The triangles in Figure 3 were evaluated from the photographs of the relaxation effects by drawing the best straight line as the base line and using only that portion of the curve which is present after heating is complete (50 μsec). The solid circles were calculated using a base line which has the curvature of the average value of τ_1 and correcting for the heating time constant. The absolute value of the two sets differs by about a factor of two but within each set the precision of the measurements, as determined by the deviations of several observations at each concentration, was such that the concentration dependence is significant. At pH 6.0 the two methods for evaluation of the photographs did not give significantly different results, and the values obtained are shown in Figure 3.

The simplest formal mechanism consistent with the results obtained with ribonuclease–cytidine 2′:3′-cyclic phosphate is

$$\begin{array}{ccc} & E' & \\ & \uparrow\downarrow & \\ k_1 & & k_{-1} \\ & E & \end{array} + S \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} ES_1 \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} ES_2 \qquad (1)$$

If the binding step is rapid relative to the other steps and if the reaction $ES_1 \rightleftharpoons ES_2$ is rapid relative to the isomerization of the free enzyme, the relaxation times characterizing the mechanism are

$$1/\tau_1 = k_{-1} + k_1 \frac{k_6[\bar{E}](k_5 + k_{-5}) + k_{-5}k_{-6}}{k_6([\bar{E}] + [\bar{S}])(k_5 + k_{-5}) + k_{-5}k_{-6}} \qquad (2)$$

$$1/\tau_5 = k_{-5} + k_5 \frac{k_6([\bar{E}] + [\bar{S}])}{k_6([\bar{E}] + [\bar{S}]) + k_{-6}} \qquad (3)$$

$$1/\tau_6 = k_6([\bar{E}] + [\bar{S}]) + k_{-6} \qquad (4)$$

where $[\bar{E}]$ and $[\bar{S}]$ are the equilibrium concentrations of the enzyme species E and cytidine 2′:3′-cyclic phosphate, respectively. The equilibrium concentrations in this case are those at a given time, t, after initiation of the reaction where the decomposition of ES_2 is slow compared to the rate of equilibration of all of the steps in eq 1. In most cases t is about 16 msec. The above equations are valid if the relaxation of the three reactions is not significantly kinetically coupled. A more exact evaluation of the relaxation spectrum indicates that this approximation is adequate for the system and results under consideration. The expression for $1/\tau_6$ indicates that a plot of $1/\tau_6$ *vs.* $([\bar{E}] + [\bar{S}])$ is linear and allows the evaluation of k_6 and k_{-6}. The equilibrium concentrations, $[\bar{E}]$ and $[\bar{S}]$, were calculated from the Michaelis constants and the known equilibrium constant for the isomerization of the free enzyme.[3] The implicit assumption made here is that the Michaelis constant is a true equilibrium constant. This is a good assumption since the rates of the binding step and the conversion between ES_1 and ES_2 are about three orders of magnitude larger than the maximum turnover number of the enzyme–cytidine 2′:3′-cyclic phosphate complex.

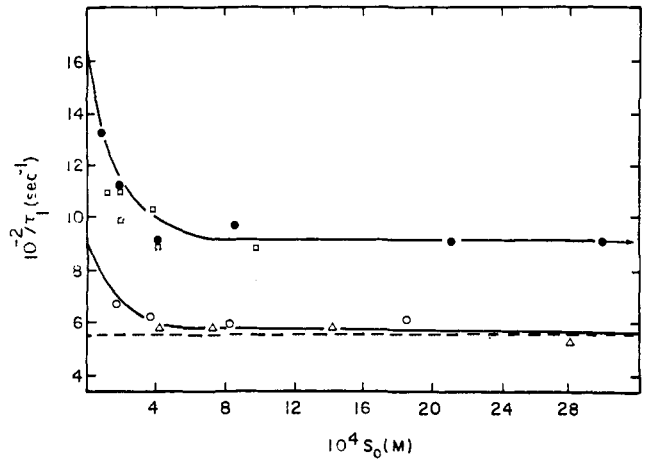


Figure 4. Variation of $1/\tau_1$ with S_0 at several pH values, 15°: pH 6.0, ●; pH 6.2, □; pH 6.7, △; pH 6.9, ○. The dashed line is the value of k_{-1} obtained from ref 3. The solid lines have no theoretical significance.

This means that to a very good approximation, E′, E, S, ES_1, and ES_2 are in equilibrium with respect to one another. Since only 1 or 2% of the substrate has decomposed to product, the assumption has also been made that the concentrations of product and enzyme–product complexes are negligible.

As mentioned above, the evaluation of τ_6 gives two sets of experimental values. The rate constants obtained are $k_6 = 2$ or 5×10^7 M^{-1} sec^{-1} and $k_{-6} = 1$ or 2×10^4 sec^{-1}. The experimental error is so large that these results can only be regarded as fixing the values of the rate constants within a factor of 2 or 3.

The concentration dependence observed at pH 6.0 (see Figure 3) can be explained by use of eq 3. At low substrate concentrations, the relaxation time would be concentration dependent due to the factor multiplying k_5. At high substrate concentrations k_{-6} can be neglected compared to $k_6([\bar{E}] + [\bar{S}])$, and the factor multiplying k_5 becomes unity; thus $1/\tau_5$ becomes concentration independent as the concentration of substrate is raised. This is the behavior observed in Figure 3. The theoretical curve in Figure 3 is calculated using the following parameters: $k_{-6}/k_6 = 3 \times 10^{-4}$ M, $k_5 = 1.1 \times 10^4$ sec^{-1}, and $k_{-5} = 2 \times 10^3$ sec^{-1}. Actually both at pH 6.0 and 6.2, in the regions where $([\bar{E}] + [\bar{S}])$ is less than 10^{-3} M, both τ_5 and τ_6 are probably present, but since the relaxation effects are so small, the precision of the method does not allow one to distinguish between a single relaxation time or two closely spaced relaxation times. What is being measured is probably a weighted average of the two relaxation processes, with τ_5 being predominant at pH 6.0 and τ_6 at pH 6.2. In all cases, the reactions in eq 1 are assumed to be not significantly kinetically coupled: more exact calculations indicate this approximation is valid within experimental error.

The isomerization of free ribonuclease, characterized by τ_1, could be observed in the presence of quite high concentrations of cytidine 2′:3′-cyclic phosphate, though the amplitude decreased and eventually the effect disappeared. The relaxation time became longer as the concentration of substrate increased and leveled off at the higher concentrations (see Figure 4). The relaxation effect was still visible when the enzyme was

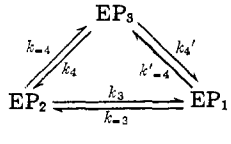
95% saturated with cytidine 2′:3′-cyclic phosphate. This concentration dependence of τ_1 is qualitatively consistent with the behavior predicted by eq 2. However, eq 2 predicts that $1/\tau_1$ should approach k_{-1} at high substrate concentrations; this occurs at pH values near 7, but at pH values near 6, $1/\tau_1$ levels off at a value which is about 70% greater than k_{-1}. The influence of cytidine 3′-phosphate and cytidylyl-3′:5′-cytidine[8] on the behavior of $1/\tau_1$ is distinctly different than that of cytidine 2′:3′-cyclic phosphate. When cytidine 3′-phosphate is added to the enzyme τ_1 does become longer (by about 70%), but no plateau region is reached and τ_1 disappears when the enzyme is 26% saturated. With the dinucleoside phosphate, τ_1 becomes 45% longer and disappears when the enzyme is between 57 and 73% saturated. Since τ_1 is observed even when ribonuclease is 95% saturated with cytidine 2′:3′-cyclic phosphate it appears likely that the enzyme–substrate complex can isomerize to a slight extent in a similar manner to the free enzyme and that the value of $1/\tau_1$ in the plateau region is characteristic of the isomerization of the enzyme–substrate complex.

Discussion

The interactions of cytidine 3′-phosphate and of cytidine 2′:3′-cyclic phosphate with ribonuclease can be studied independently. This is a consequence of the rapid binding of the small molecules to the enzyme and subsequent rapid interconversions of each species separated by the relatively slow rate-determining step. When the cyclic phosphate binds to the enzyme, for example, a rapid intramolecular conversion, characterized by τ_5, occurs; this indicates at least two intermediate species of bound cytidine 2′:3′-cyclic phosphate exist. The two intermediates and the free enzyme and substrate can be considered to be in equilibrium since the turnover number for the ribonuclease-catalyzed hydrolysis of cytidine 2′:3′-cyclic phosphate[12] is at least three orders of magnitude slower than the interconversion and binding steps.[11] With this equilibrium approximation, the relaxation and steady-state data for the cyclic phosphate can be obtained without being influenced by cytidine 3′-phosphate, and *vice versa*. The interaction of cytidine 3′-phosphate and cytidine 2′:3′-cyclic phosphate are considered in detail separately and then a formal mechanism for the hydrolysis of the cyclic phosphate to cytidine 3′-phosphate which is sufficient to explain all the relaxation, steady-state, and equilibrium data in the pH range 5–8 at 25° is presented.

The interaction of cytidine 3′-phosphate with ribonuclease is characterized by three relaxation times: τ_2, τ_3, and τ_4.[4] The dependence of the relaxation times on pH, temperature, concentration, and solvent have been investigated. The number of relaxation times indicates a minimum of three enzyme–cytidine 3′-phosphate complexes must exist. With neglect of various possible ionization states which are in rapid equilibrium, two ways in which the three complexes can be arranged are linearly as in eq 5 or cyclically as in eq 6. No way

$$EP_3 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} EP_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP_1 \qquad (5)$$



(6)

of differentiating between the two schemes with the available experimental data exists, but the linear sequence is used since it is the simpler of the two. The scheme in eq 5 gives the following expressions for the relaxation times at high substrate concentrations

$$1/\tau_3 = k_3 + k_{-3} \qquad (7)$$

$$1/\tau_4 = k_4 + k_{-4}\left(\frac{k_{-3}}{k_3 + k_{-3}}\right) \qquad (8)$$

Here the assumption has been made that $1/\tau_3 \gg 1/\tau_4$ which is consistent with the experimental results. Nine possible ways of including the binding step and the rate-determining step with the linear arrangement of the intermediates exist. Cytidine 3′-phosphate may bind to the enzyme to form either EP_1, EP_2, or EP_3 initially, and for each of these cases EP_1, EP_2, or EP_3 may react in the rate-determining step. Since the interaction of ribonuclease with all substrates[4,8] and many inhibitors[4] is associated with a relaxation time similar to τ_3, that is, the pH dependence and solvent D_2O effect are similar, and since τ_4 was only observed with cytidine 3′-phosphate, it was assumed that the process related to τ_3 immediately follows the binding step. Presently no evidence is available for or against this hypothesis. The complexes are arbitrarily assumed to occur in the mechanism sequentially in the order EP_1, EP_2, and EP_3, although a cyclic mechanism is certainly possible; however such changes would not affect the qualitative features of the mechanism.

An additional consideration is necessary with cytidine 3′-phosphate. This molecule exists predominantly in two ionized forms in the pH region investigated. The secondary phosphate group has a pK value of 5.9 at 25°[6] so cytidine 3′-phosphate can exist as a monoanion or a dianion (cytidine 3′-phosphate has two other ionizable groups, the primary phosphate group with a pK of about 1 and the ring nitrogen on the base which has a pK of 4.3[6]). The possibilities exist that only the monoanion binds to the enzyme, only the dianion binds, or that both species bind either at the same or different rates. The monoanion almost certainly binds because cytidine 2′:3′-cyclic phosphate, dinucleoside phosphates, and ribonucleic acid cannot exist in the dianionic form and all three bind to the enzyme. The question is whether or not the dianion of cytidine 3′-phosphate binds in addition to the monoanion. Since the active site is positively charged in the pH region of strongest binding, it would seem that the dianion would bind more strongly then the monoanion if electrostatic forces play an important role in the binding. However, no convincing experimental data exist upon which to base any conclusions concerning dianion binding. Since it considerably simplifies the mechanism and doesn't change any of the *qualitative* mechanistic features, the dianion binding is assumed to be negligible.

Regardless of which of the several possible mechanisms is considered, linear or cyclic sequence of intermediates, monoanion binding, or both mono- and dianion binding, two features are common to all mechanisms. At least three ionizable groups on the enzyme must be postulated to explain the pH dependence of all experimental quantities. In the free enzyme these groups have apparent pK values of about

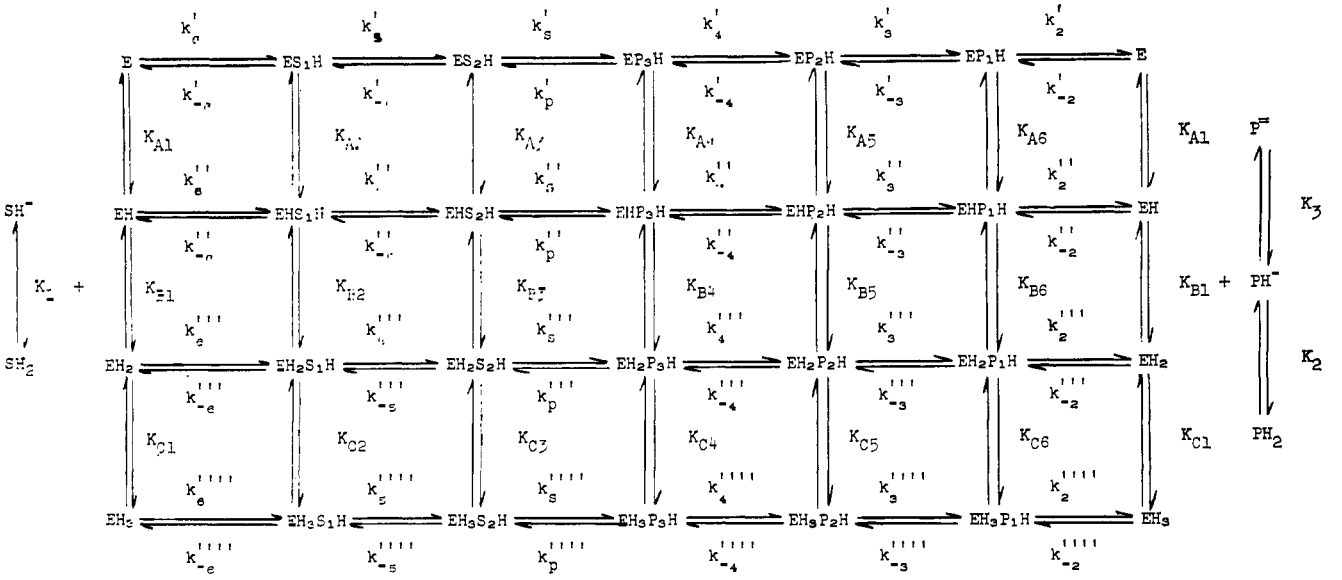


Figure 5. Formal mechanism for the hydrolysis of cytidine 2′:3′-cyclic phosphate to cytidine 3′-phosphate by ribonuclease. SH_2 and SH^- are the neutral species of monoanion of cytidine 2′:3′-cyclic phosphate. PH_2, PH^-, and P^{2-} are the neutral, monoanion, and dianion species of cytidine 3′-phosphate. E, EH, EH_2, and EH_3 are the various protonated forms of the free enzyme. The remaining species are the various protonated forms of the enzyme–substrate and enzyme–product complexes. Some protons are omitted for clarity. The values for the macroscopic ionization constants and pH-independent rate constants are given in Tables II and III.

5, 6 and 6.7 at 25°. These values were necessary to explain the pH dependence of the second-order rate constant for the binding of cytidine 3′-phosphate to ribonuclease.[4] Secondly, regardless of the mechanism, parallel paths must be postulated (more than one ionized form of a species must react) in order to simultaneously explain the apparent dissociation constant of the enzyme–cytidine 3′-phosphate complex and the pH dependence of the relaxation times. By use of these two postulates, all of the mechanisms which have been discussed can qualitatively fit the relaxation, steady-state, and equilibrium data for the binding of cytidine 3′-phosphate to ribonuclease. Note that the implicit assumption has been made that all complexes are mechanistically significant; presently this cannot be either proved or disproved.

The results obtained on the interaction of cytidine 2′:3′-cyclic phosphate with ribonuclease do not give much help in determining a mechanism for the hydrolysis since the pH dependence of the second-order rate constant could not be determined. If it is assumed that the cyclic phosphate binds to the same ionized forms of the enzyme as cytidine 3′-phosphate with the same relative rate constants, and that the dissociation step is pH independent analogous to results obtained with cytidine 3′-phosphate, then no mechanism for the interaction of cyclic phosphate has been found which is compatible with any of the possibilities used to fit the data for the interaction of ribonuclease and cytidine 3′-phosphate. If the cyclic phosphate binds to the enzyme in a different manner than to cytidine 3′-phosphate, then enough latitude is available to fit the data. Cytidine 2′:3′-cyclic phosphate probably does bind differently than cytidine phosphate. One piece of experimental evidence which supports this conclusion is the pH dependence of K_S and K_P, the Michaelis constants for the two species: K_S goes through a minimum at about pH 5.0[12] and K_P goes through a minimum at pH 5.5.[13] (See Figure 7). Additional evidence is the different effects of cytidine 2′:3′-cyclic phosphate and cytidine 3′-phosphate on the ribonuclease isomerization, characterized by τ_1, discussed above.

The mechanism which was chosen to fit the experimental data in the pH region 5–8 is shown in Figure 5. Only the monoanion of the substrate and product bind to the enzyme. The isomerization of the enzyme is not shown, but was explicitly taken into account when fitting the data to this mechanism. Provision is not made for the possibility that the ES_1 species can isomerize since if this occurs, it occurs only to a small extent. All of the protolytic equilibria are assumed to be buffered by other ionizable groups on the enzyme (*cf.* ref 2 for a discussion of this point). The apparent macroscopic ionization constants are shown in Table II and the pH-independent rate constants in Table III. These constants were obtained by a trial and error procedure utilizing all available kinetics and equilibrium data.

Table II. Enzyme, Substrate, and Enzyme–Substrate Ionization Constants at 25° (See Figure 5)

Ionization constant	i = 1	2	3	4	5	6
pK_{Ai}	6.7	7.4	8.0	8.0	6.7	7.7
pK_{Bi}	6.0	7.1	7.1	7.4	7.4	6.0
pK_{Ci}	5.0	6.0	6.3	4.0	5.0	4.0
pK_i[81]	3.9	4.3	5.9			

Macroscopic ionization constants were utilized to simplify the calculations. If the macroscopic ionization constants are well separated, they approach the values of the microscopic constants. In the mechanism in Figure 5 all of the pK values are 0.6 or more apart except for two cases. In the ES_1 species two ionization constants are reversed; that is, the species with three protons has a smaller ionization constant than the

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

Table III. pH-Independent Rate Constants at 25° (See Figure 5)

i	2	−2	3	−3	4	−4	s sec^{-1}	p	5	−5	6	−6
Rate constant	10^{-8} sec^{-1}	10^{-7} M^{-1} sec^{-1}	10^{-8} sec^{-1}	10^{-8} sec^{-1}	10^{-8} sec^{-1}	10^{-2} sec^{-1}		10^{8} sec^{-1}	10^{-3} sec^{-1}	10^{-3} sec^{-1}	10^{-7} M^{-1} sec^{-1}	10^{-4} sec^{-1}
k_i'	6.2	3.1	45	10	0.32	0.15	~0	~0	8.7	28	0.073	5.3
k_i''	6.2	31	~0	~0	3.2	30	5.3	9.6	8.7	9	0.46	5.3
k_i'''	6.2	31	1.8	1	0.45	4.2	35	3.2	8.7	9	5.8	5.3
k_i''''	6.2	3.1	0.9	5	~0	~0	~0	~0	12	6.4	5.8	0.53

species with two protons. This reversal of ionization constants seems reasonable since $1/\tau_3$ rises much more steeply with increasing pH than would be predicted from a normal ionization curve.[4] The behavior is similar to that observed in the ionization of carboxylic acids where the apparent ionization constant becomes larger as the pH is increased due to homologus double hydrogen bonds.[14] The numerical pK values for the enzyme–substrate complexes should not be taken too literally; they are only indications of the pH region in which the groups ionize.

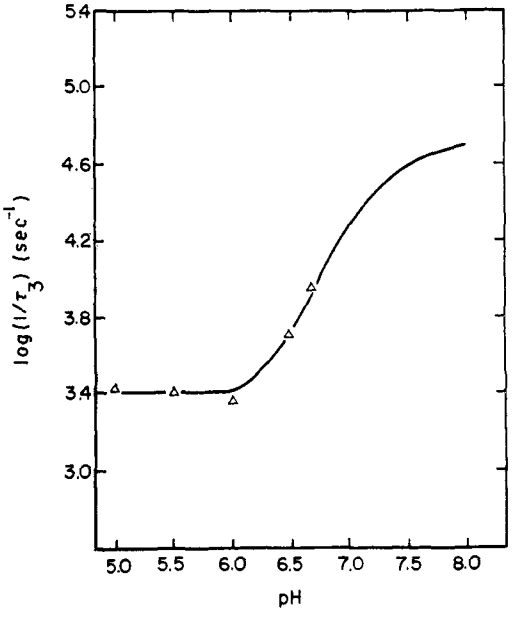


Figure 6. Variation of $1/\tau_3$ with pH at 25°. Experimental values taken from ref 4. The solid line is calculated from the mechanism in Figure 5 according to eq 7.

The expressions for $1/\tau_3$, $1/\tau_4$, $1/\tau_5$, and $1/\tau_6$ have been given in eq 7, 8, 3, and 4, respectively; $1/\tau_2$ is given by

$$1/\tau_2 = k_{-2}([\bar{E}] + [\bar{P}]) + k_2 \tag{9}$$

The steady-state constants are given by

$$K_S = \frac{(1 + K_I)k_{-5}k_{-6}}{(k_5 + k_{-5})k_6} \tag{10}$$

$$\frac{V_S}{[E_0]} = \frac{k_s k_5}{(k_5 + k_{-5})} \tag{11}$$

$$K_P = \frac{(1 + K_I)k_2k_3k_4}{[k_3k_4 + k_{-3}(k_4 + k_{-4})]k_{-2}} \tag{12}$$

(14) H. A. Scheraga, "Protein Structure," Academic Press Inc., New York, N. Y., 1956, p 59.

$$\frac{V_P}{[E_0]} = \frac{k_p k_{-3}k_{-4}}{[k_3k_4 + k_{-3}(k_4 + k_{-4})]} \tag{13}$$

where the rate constants in these equations are the apparent rate constants at a given pH, K_I is the apparent equilibrium constant between the isomers of the free enzyme,[3] V_S and V_P are the maximum velocities for the forward and reverse reactions, respectively, and $[E_0]$ is the total enzyme concentration.

The theoretical curves for $1/\tau_4$ and $1/\tau_5$ as a function of pH at 25° are shown with the experimental data in Figures 1 and 2, respectively. The calculated curve for $1/\tau_3$ and the experimental points are shown in Figure 6. A plot of $1/\tau_2$ *vs.* $([\bar{E}] + [\bar{P}])$ is linear and allows k_{-2} and k_2 to be determined directly. The second-order rate constant, k_{-2}, as a function of pH is shown in ref 3 along with theoretical curves calculated for several mechanisms. The mechanism proposed in this paper fits the experimental points equally well.

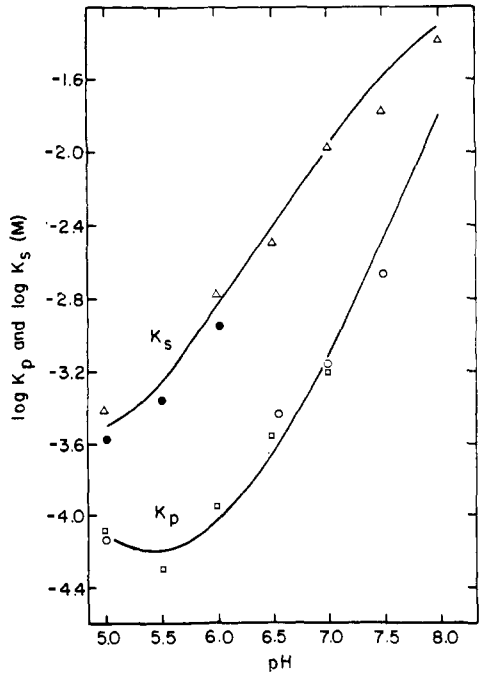


Figure 7. Variation of K_S and K_P with pH, 25°. The solid lines are calculated from the mechanism in Figure 5 according to eq 10 and 12. Symbols used are: K_S from ref 12, △; K_S, this work, ●; K_P from ref 12, ○; K_P from ref 13, □.

Figure 7 shows the theoretical curves for K_P and K_S along with the experimental points. Since K_S depends upon the apparent rate constants, k_6 and k_{-6}, and $1/\tau_6$ was not observed, at 25°, the values of these two rate constants were arbitrarily assigned values at pH 6.0 which were compatible with the values observed at

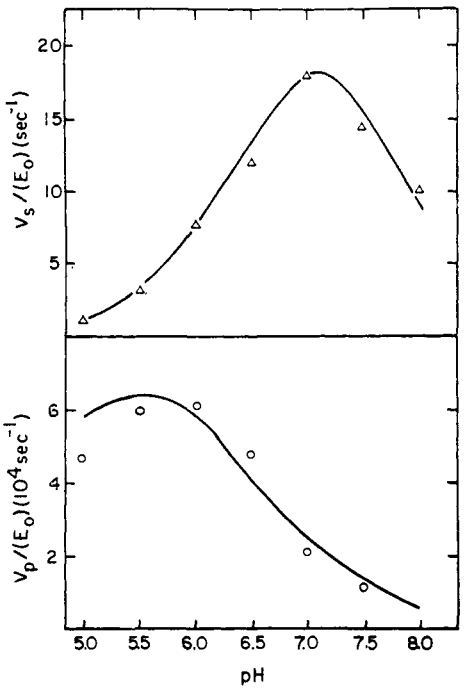


Figure 8. Variation of $V_S/[E_0]$ and $V_P/[E_0]$ with pH 25°. The solid lines are calculated from the mechanism in Figure 5 according to eq 11 and 13. Symbols used are $V_S/[E_0]$ from ref 12, △; $V_P/[E_0]$ calculated from eq 14 (see text), O.

pH 6.2, 15°, namely, $k_6 = 2.7 \times 10^7\ M^{-1}$ sec^{-1} and $k_{-6} = 3 \times 10^4$ sec^{-1}. The ionization constants and pH-independent rate constants in the mechanism were then varied until a good fit of the experimental data was achieved. Figure 8 shows the theoretical curves for $V_S/[E_0]$ and $V_P/[E_0]$ along with the experimental points; $V_P/[E_0]$ was not measured experimentally but calculated from the experimental values of the over-all equilibrium constant, K_{eq}, $V_S/[E_0]$, K_P, and K_S according to the equation

$$\frac{V_P}{[E_0]} = \frac{K_P}{K_{eq}K_S}\frac{V_S}{[E_0]} \qquad (14)$$

(The calculated experimental values of $V_P/[E_0]$ shown in Figure 8 differ somewhat from those calculated in ref 5 due to an earlier calculation error and use of more reliable steady-state parameters.) The complete mechanism for the conversion of cytidine 2′:3′-cyclic phosphate to cytidine 3′-phosphate is consistent with the over-all equilibrium constant between product and substrates

$$K_{eq} = \frac{[\text{cytidine 3′-phosphate}]}{[\text{cytidine 2′:3′-cyclic phosphate}]} = \frac{K_P V_S}{V_P K_S} = \frac{k_2k_3k_4k_5k_6}{k_{-2}k_{-3}k_{-4}k_{-5}k_{-6}} \qquad (15)$$

The experimental data agree with the values of K_{eq} for this mechanism well within experimental error. The steady-state constants, eq 10–13, are relatively complex functions of the rate constants and any attempt to use the steady-state constants to determine the ionization constants of the intermediates, in general, would not be successful.

Though the mechanism presented in Figure 5 is not unique, it is one of the simplest that can be found to simultaneously fit all of the relaxation, steady-state, and equilibrium data in the pH region 5 to 8 for the ribonuclease-catalyzed hydrolysis of cytidine 2′:3′-cyclic phosphate to cytidine 3′-phosphate. The proton uptake observed when a nucleotide binds to ribonuclease[15] cannot be completely explained by this mechanism. The calculated maximum proton uptake when cytidine 3′-phosphate binds to ribonuclease qualitatively agrees above pH 5.5 with the experimental data for cytidine 2′-phosphate binding.[15] From pH 5.5 to 4 the mechanism predicts much less proton liberation than is observed. Better agreement in this low pH region can be obtained by reducing pK_{C4}, pK_{C5}, and pK_{C6} to about 3 but then the steady-state data cannot be fit as well. Since the relaxation times were observed only down to pH 5.0, the mechanism is probably not valid below this pH. For example, inclusion of another ionizable group on the enzyme with a pK value below 5 would permit the calculated proton uptake to be in reasonable agreement with the experimental data.

In spite of the apparent complexity of the mechanism of action of ribonuclease and the difficulty in postulating a fairly unique mechanism, several general features appear to be a necessary part of the actual mechanism (whatever it may be). (1) Five different states of the enzyme–substrate complex must exist in the hydrolysis of cytidine 2′:3′-cyclic phosphate to cytidine 3′-phosphate. (2) At least three ionizable groups on the enzyme are associated with the catalytic activity (the pK values are about 5, 6, and 6.7 on the free enzyme). (3) Parallel reaction paths must be postulated. (4) Cytidine 2′:3′-cyclic phosphate interacts with ribonuclease in a different manner than cytidine 3′-phosphate. The results obtained also indicate that the Michaelis constants are equilibrium constants.

Ideally, one would like to transform the formal mechanism of Figure 5 into a chemical mechanism. Unfortunately such a transformation is presently almost entirely speculation. Certainly most of the proposed mechanisms[4,16,17] are insufficient to be consistent with all of the available data. Although the basic mechanisms proposed could certainly be modified, extensive speculation is unwarranted until more structural information about ribonuclease is available. Some possible relationships between structure and mechanism will be briefly mentioned in the accompanying paper.

Appendix

pH Dependence of the Apparent Rate Constants for the Mechanism of Figure 5

$$k_2 = \frac{k_2' + \frac{[H^+]}{K_{A6}}k_2'' + \frac{[H^+]^2}{K_{A6}K_{B6}}k_2'' + \frac{[H^+]^3}{K_{A6}K_{B6}K_{C6}}k_2''''}{\left(1 + \frac{[H^+]}{K_{A6}} + \frac{[H^+]^2}{K_{A6}K_{B6}} + \frac{[H^+]^3}{K_{A6}K_{B6}K_{C6}}\right)}$$

$$k_{-2} = \frac{k_{-2}' + \frac{[H^+]}{K_{A1}}k_{-2}'' + \frac{[H^+]^2}{K_{A1}K_{B1}}k_{-2}''' + \frac{[H^+]^3}{K_{A1}K_{B1}K_{C1}}k_{-2}'''}{\left(1 + \frac{[H^+]}{K_2} + \frac{K_3}{[H^+]}\right)\left(1 + \frac{[H^+]}{K_{A1}} + \frac{[H^+]^2}{K_{A1}K_{B1}} + \frac{[H^+]^3}{K_{A1}K_{B1}K_{C1}}\right)}$$

(15) J. P. Hummel and H. Witzel, *J. Biol. Chem.*, 241, 1023 (1966).
(16) D. Findlay, D. G. Herries, A. P. Mathias, B. R. Rabin, and C. A. Ross, *Biochem. J.*, 85, 152 (1962).
(17) H. Witzel, *Progr. Nucleic Acid Res.*, 2, 221 (1963).

$$k_3 = \frac{k_3' + \frac{[H^+]}{K_{A5}}k_3'' + \frac{[H^+]^2}{K_{A5}K_{B5}}k_3''' + \frac{[H^+]^3}{K_{A5}K_{B5}K_{C5}}k_3''''}{\left(1 + \frac{[H^+]}{K_{A5}} + \frac{[H^+]^2}{K_{A5}K_{B5}} + \frac{[H^+]^3}{K_{A5}K_{B5}K_{C5}}\right)}$$

$$k_{-3} = \frac{k_{-3}' + \frac{[H^+]}{K_{A6}}k_{-3}'' + \frac{[H^+]^2}{K_{A6}K_{B6}}k_{-3}''' + \frac{[H^+]^3}{K_{A6}K_{B6}K_{C6}}k_{-3}''''}{\left(1 + \frac{[H^+]}{K_{A6}} + \frac{[H^+]^2}{K_{A6}K_{B6}} + \frac{[H^+]^3}{K_{A6}K_{B6}K_{C6}}\right)}$$

$$k_4 = \frac{k_4' + \frac{[H^+]}{K_{A4}}k_4'' + \frac{[H^+]^2}{K_{A4}K_{B4}}k_4''' + \frac{[H^+]^3}{K_{A4}K_{B4}K_{C4}}k_4''''}{\left(1 + \frac{[H^+]}{K_{A4}} + \frac{[H^+]^2}{K_{A4}K_{B4}} + \frac{[H^+]^3}{K_{A4}K_{B4}K_{C4}}\right)}$$

$$k_{-4} = \frac{k_{-4}' + \frac{[H^+]}{K_{A5}}k_{-4}'' + \frac{[H^+]^2}{K_{A5}K_{B5}}k_{-4}''' + \frac{[H^+]^3}{K_{A5}K_{B5}K_{C5}}k_{-4}''''}{\left(1 + \frac{[H^+]}{K_{A5}} + \frac{[H^+]^2}{K_{A5}K_{B5}} + \frac{[H^+]^3}{K_{A5}K_{B5}K_{C5}}\right)}$$

$$k_s = \frac{k_s' + \frac{[H^+]}{K_{A3}}k_s'' + \frac{[H^+]^2}{K_{A3}K_{B3}}k_s''' + \frac{[H^+]^3}{K_{A3}K_{B3}K_{C3}}k_s''''}{\left(1 + \frac{[H^+]}{K_{A3}} + \frac{[H^+]^2}{K_{A3}K_{B3}} + \frac{[H^+]^3}{K_{A3}K_{B3}K_{C3}}\right)}$$

$$k_p = \frac{k_p' + \frac{[H^+]}{K_{A4}}k_p'' + \frac{[H^+]^2}{K_{A4}K_{B4}}k_p''' + \frac{[H^+]^3}{K_{A4}K_{B4}K_{C4}}k_p''''}{\left(1 + \frac{[H^+]}{K_{A4}} + \frac{[H^+]^2}{K_{A4}K_{B4}} + \frac{[H^+]^3}{K_{A4}K_{B4}K_{C4}}\right)}$$

$$k_5 = \frac{k_5' + \frac{[H^+]}{K_{A2}}k_5'' + \frac{[H^+]^2}{K_{A2}K_{B2}}k_5''' + \frac{[H^+]^3}{K_{A2}K_{B2}K_{C2}}k_5''''}{\left(1 + \frac{[H^+]}{K_{A2}} + \frac{[H^+]^2}{K_{A2}K_{B2}} + \frac{[H^+]^3}{K_{A2}K_{B2}K_{C2}}\right)}$$

$$k_{-5} = \frac{k_{-5}' + \frac{[H^+]}{K_{A3}}k_{-5}'' + \frac{[H^+]^2}{K_{A3}K_{B3}}k_{-5}''' + \frac{[H^+]^3}{K_{A3}K_{B3}K_{C3}}k_{-5}''''}{\left(1 + \frac{[H^+]}{K_{A3}} + \frac{[H^+]^2}{K_{A3}K_{B3}} + \frac{[H^+]^3}{K_{A3}K_{B3}K_{C3}}\right)}$$

$$k_6 = \frac{k_6' + \frac{[H^+]}{K_{A1}}k_6'' + \frac{[H^+]^2}{K_{A1}K_{B1}}k_6''' + \frac{[H^+]^3}{K_{A1}K_{B1}K_{C1}}k_6''''}{\left(1 + \frac{[H^+]}{K_1}\right)\left(1 + \frac{[H^+]}{K_{A1}} + \frac{[H^+]^2}{K_{A1}K_{B1}} + \frac{[H^+]^3}{K_{A1}K_{B1}K_{C1}}\right)}$$

$$k_{-6} = \frac{k_{-6}' + \frac{[H^+]}{K_{A2}}k_{-6}'' + \frac{[H^+]^2}{K_{A2}K_{B2}}k_{-6}''' + \frac{[H^+]^3}{K_{A2}K_{B2}K_{C2}}k_{-6}''''}{\left(1 + \frac{[H^+]}{K_{A2}} + \frac{[H^+]^2}{K_{A2}K_{B2}} + \frac{[H^+]^3}{K_{A2}K_{B2}K_{C2}}\right)}$$

Relaxation Spectra of Ribonuclease. V. The Interaction of Ribonuclease with Cytidylyl-3′:5′-cytidine[1]

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Abstract: The interaction of ribonuclease with a dinucleoside phosphate, cytidylyl-3′:5′-cytidine, has been studied with a stopped-flow, temperature-jump technique. At 15° in the pH range 6–7 two relaxation processes were observed. One relaxation time was independent of concentration and pH and is characteristic of an intramolecular isomerization of the dinucleoside phosphate–enzyme complex. The second relaxation time was dependent on the concentration and pH and is characteristic of the binding of the substrate to the enzyme. These results along with the results of previous studies have been incorporated into a schematic formal mechanism for the hydrolysis of cytidylyl-3′:5′-cytidine to cytidine 3′-phosphate and cytidine.

The ribonuclease-catalyzed hydrolysis of ribonucleic acid proceeds *via* a pyrimidine cyclic phosphate intermediate.[2] This catalytic hydrolysis of ribonucleic acid has been studied to some extent,[3,4] but since the system becomes quite inhomogeneous as ribonucleic acid is hydrolyzed, the data are difficult to interpret. Dinucleoside phosphates have been used to investigate the steady-state kinetics of the formation of the pyrimidine cyclic phosphate intermediate.[5] The second half of the reaction, the hydrolysis of the pyrimidine cyclic phosphate, has been investigated by steady-state techniques with cytidine 2′:3′-cyclic phosphate[6] and uridine 2′:3′-cyclic phosphate[7] as substrates.

The equilibrium temperature-jump method has been used to study the dynamic behavior of ribonuclease[8] and its interaction with cytidine 3′-phosphate, the product of hydrolysis of cytidine 2′:3′-cyclic phosphate, and with various competitive inhibitors of ribonuclease.[9] Since

(1) This work was supported by a grant from the National Institutes of Health (GM 13292).
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