tions that did not also produce formylmethylflavin or the isoalloxazine analog. The aldehyde group at the 3′- or 4′-position appears to be relatively stable to air. We suggest that the introduction of a hydroxyl group at the 2′-position would make the 3′-ketone unstable in the environment of the isoalloxazine nucleus. The cleavage of the side chain could have occurred before the cyclization, but we cannot propose a satisfactory mechanism. It is unlikely that hydrogen abstraction at the 2′-position would lead to the aldehyde when the group attached to the 2′-position is not hydrogen. Most oxidations of α-hydroxycarbonyl groups indicate that the hydroxy fragment converts to an aldehyde[37] for a secondary hydroxy function.

A variation of this mechanism has been proposed for VI.[25] Air oxidation of the reduced isoalloxazine nucleus induces a break of the N–O bond at the N-10 position. The resulting alkoxyl radical could abstract a hydrogen from the media (*i.e.*, hydrogen peroxide or side-chain fragments). As in the case of formylmethylflavin, a bimolecular-reaction mechanism provides an appealing alternative. However, unlike the other situation, the cyclic intermediate has an oxidation potential different from the 1,10-dihydro derivative. Therefore, we feel that VI differs from the other isoalloxazines by the nature of the hydrogen-abstraction process, and it does not follow a bimolecular path.

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Relaxation Spectra of Ribonuclease. VI. The Interaction of Ribonuclease with Uridine 3′-Monophosphate[1]

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Abstract: Kinetic studies of the interaction of ribonuclease with uridine 3′-monophosphate were performed using the temperature-jump method. Changes in both ultraviolet absorption and pH were measured. A partial reinvestigation of the relaxation spectra of ribonuclease–cytidine 3′-monophosphate binding was also carried out. For both nucleotides two relaxation processes are observed which can be attributed to an initial association–dissociation of the enzyme and nucleotide followed by an isomerization of the enzyme–nucleotide complex. These two processes were studied at 25° in the pH range 4.5–7.5. The interaction of ribonuclease with both uridine and cytidine 3′-nucleotides has qualitatively similar relaxation characteristics as a function of pH. However, the dissociation rate constant for uridine 3′-monophosphate binding is approximately two times that for cytidine 3′-monophosphate. A minimal mechanism consistent with all of the data involves three ionizing groups on ribonuclease and two isomeric states of the ribonuclease–3′-nucleotide complex. One of the ionizing groups on ribonuclease is postulated not to interact directly with the 3′-nucleotides, but instead reflects a change in conformation associated with the isomerization of the free enzyme *and* the enzyme–product complex. These results can be correlated with the known three-dimensional structure of the enzyme.

Previous papers in this series have included relaxation studies on the interaction of ribonuclease with cytidine 3′-monophosphate,[3,4] cytidine 2′:3′-cyclic phosphate,[5] and cytidylyl 3′:5′-cytidine.[6] Since the apparent association constants of ribonuclease with cytidine 3′-monophosphate are greater than those with uridine 3′-monophosphate,[7] a comparison of the relaxation spectra of ribonuclease with these nucleotides is of interest. During the course of the current investigation, which utilized an improved temperature-jump apparatus having a substantially greater signal-to-noise ratio, some discrepancies were found with previous data; therefore a partial reinvestigation of the ribonuclease–cytidine 3′-monophosphate interaction was undertaken. The effects designated τ_2[3,4] and τ_3,[3,4] which were previously observed by measurement of pH changes with a pH indicator, were corroborated in the present study by direct observation of the change in the ultraviolet difference absorbance which accompanies ribonuclease–nucleotide binding.[7] The results indicate that two relaxation processes are associated with ribonuclease–nucleotide binding: one is associated with an initial association–dissociation reaction and the other with an isomerization of the enzyme–product complex. The pH dependence of the relaxation times was determined, and a mechanism is discussed which attempts to correlate these results with the known chemical and structural features of ribonuclease.

(1) This work was supported by a grant from the National Institutes of Health (GM 13292).
(2) National Institutes of Health Postdoctoral Fellow 1966–1968.
(3) R. E. Cathou and G. G. Hammes, *J. Am. Chem. Soc.*, **86**, 3240 (1964).
(4) R. E. Cathou and G. G. Hammes, *ibid.*, **87**, 464 (1965).
(5) J. E. Erman and G. G. Hammes, *ibid.*, **88**, 5607 (1966).
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Experimental Section

Bovine pancreatic ribonuclease A was obtained as a phosphate-free lyophilized powder from Worthington Biochemicals Corp. and was used without further purification. The concentration of

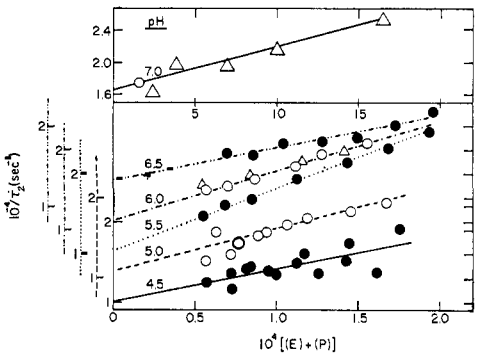

Figure 1. Variation of $1/\tau_2$ with [(E) + (P)] at different pH values. The circles represent experiments where absorbancy changes at 255 mμ were measured; the triangles represent experiments employing pH indicators: phenol red at pH 7.0 and chlorophenol red at pH 6.0. The ordinate has been shifted for each pH for clarity.

ribonuclease was determined as described previously.[7] Nucleotides were prepared and analyzed as described previously.[7] All other reagents were either reagent grade or the best grade available commercially.

The temperature-jump apparatus used was similar in design to that described elsewhere.[8,9] The apparatus has a reaction volume of less than 0.2 ml, and an Hanovia xenon–mercury arc lamp, type 901-B-1, was used as a source of ultraviolet radiation. The light was focused into the center of the reaction mixture through a conical lens and was defocused onto the photomultiplier through another conical lens. Exposure of the reaction mixture to the intense radiation of this lamp was minimized since the nucleotides were observed to undergo significant photolysis after exposure times greater than 30 sec. However, quite high signal-to-noise ratios can be obtained with this arrangement. All solutions used in temperature-jump experiments were prepared from freshly boiled distilled and deionized water and were 0.2 *M* in KCl. The relaxation processes associated with the binding of ribonuclease with uridine and cytidine 3′-nucleotides were detected by measurement of absorption changes at 255 and 260 mμ, respectively. Because of the large absorbance of the nucleotides and ribonuclease at these wavelengths, only limited ranges of concentration of the reactants could be investigated using ultraviolet light: the concentration ranges employed are summarized in Table I. In experiments designed to study the isomerization of the ribonuclease–3′-nucleotide complex, the enzyme was brought to at least 85% saturation with nucleotide: the ranges of ribonuclease and nucleotide concentrations employed over the pH range investigated were 0.5–1.02 × 10^{-4} *M* and 1.0–5.93 × 10^{-3} *M*, respectively. In these experiments pH indicators were used to detect pH changes accompanying the relaxation processes. The indicators employed at a concentration of 2 × 10^{-5} *M* were chlorophenol red (pH range, 5.2–6.6) and phenol red (pH range, 6.6–7.5) and the changes in absorbance were observed at 573 and 558 mμ, respectively. In all temperature-jump experiments the reactants were equilibrated at 17.5° and a 7.5° temperature jump was applied. Thus all rate data were obtained at 25°. For each experiment at least eight photographs of the oscilloscope trace were taken and analyzed.

All pH measurements were performed with a Radiometer pHM-26 pH meter; the error in the pH measurement is estimated to be ±0.05 pH units.

Table I. Range of Ribonuclease (E_0) and Uridine 3′-Monophosphate (P_0) Concentrations Employed in Temperature-Jump Experiments

pH	$10^5(E_0)$, *M*	$10^5(P_0)$, *M*
4.5[a]	4.3–5.8	1.8–19.0
5.0[a]	3.9–5.1	1.8–17.5
5.5[a]	3.9–4.6	1.8–21.5
6.0[b]	4.0–4.8	2.7–17.3
6.5[a]	5.6	2.7–19.7
7.0[a]	4.8	13.2
7.0[c]	4.3	7.0–168

[a] Absorbancy changes at 255 mμ used to detect relaxation effects. [b] Both absorbancy changes at 255 mμ and pH changes detected with 2 × 10^{-5} *M* chlorophenol red indicator observed at 573 mμ used to detect relaxation effects. [c] 2 × 10^{-5} *M* phenol red indicator used to detect relaxation effects at 558 mμ.

Results and Treatment of Data

General Consideration of the Relaxation Processes. Previous temperature-jump studies on ribonuclease–nucleotide binding from this laboratory[3-6] have relied on the use of pH indicators to observe the various relaxation processes. This technique precludes studies at pH values lower than about 5 and relaxation effects observed in the pH range 5–6 have small amplitudes which are difficult to analyze. Since the nucleotide absorption spectra are markedly perturbed on binding with ribonuclease,[7] it is possible to measure *directly* absorbance changes to study the relaxation effects of ribonuclease–nucleotide binding. Previously the optics of the apparatus and the unavailability of an intense, stable ultraviolet lamp prevented this. The relaxation processes for ribonuclease–uridine 3′-monophosphate binding were studied at 255 mμ which is the wavelength of the maximum absorbance difference associated with ribonuclease–uridine 3′-monophosphate binding.[7] The amplitude of the effect associated with the initial association–dissociation reaction (see below) observed at 255 mμ was approximately five times that observed using the pH indicator technique under identical conditions; therefore more reliable and precise data can be obtained.

Two relaxation processes were found to be associated with ribonuclease–uridine 3′-monophosphate binding. The faster of these processes has observed relaxation times from 40 to 100 μsec and is dependent on the concentration of the reactants. This effect can be interpreted as representing the initial binding reaction

$$E + P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \qquad (1)$$

where E represents ribonuclease and P represents uridine 3′-monophosphate. For this mechanism[10]

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

where $1/\tau_2$ is the observed reciprocal relaxation time, (E) is the equilibrium concentration of free ribonuclease and (P) is the equilibrium concentration of free uridine 3′-monophosphate. Since the association constants for ribonuclease–uridine 3′-monophosphate binding as a function of pH are known,[7] (E) and (P) can be calculated and values of k_2 and k_{-2} can be obtained from plots of $1/\tau_2$ *vs.* [(E) + (P)]. (This procedure for treating the data may not be strictly correct; however, as will be clear later the assumption of more complex mechanisms does not alter the results very much.) Such plots for different values of pH are illustrated in Figure 1. At each pH at least 60 individual temperature-jump experiments were performed. The values of k_2 and k_{-2} were obtained by a least-squares analysis of the data and

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(9) J. E. Erman and G. G. Hammes, *Rev. Sci. Instr.*, 37, 746 (1966).
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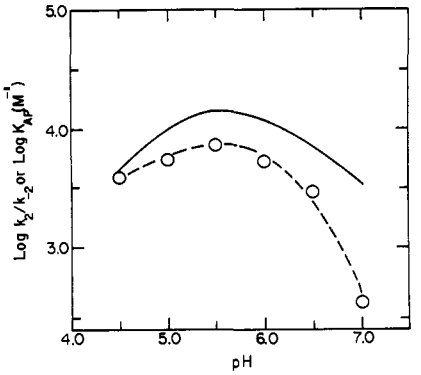

Figure 2. A plot of the logarithm of the equilibrium association constant, K_{AP}, for ribonuclease–uridine 3′-monophosphate binding and the logarithm of k_2/k_{-2} *vs.* pH. The circles represent k_2/k_{-2} calculated from Table II. The solid line represents value of the measured equilibrium association constant;[7] the dashed line has no theoretical significance.

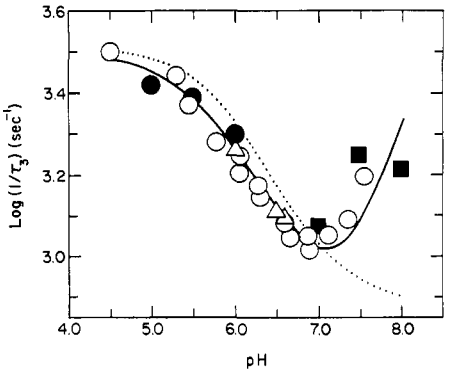

Figure 3. Variation of log $(1/\tau_3)$ with pH for ribonuclease–product complex isomerization. The circles represent experiments with uridine 3′-monophosphate; the triangles represent experiments with cytidine 3′-monophosphate. The filled circles are values of $1/\tau_3$ taken from ref 5, and the filled squares are values of $1/\tau_4$ taken from ref 5 for experiments with cytidine 3′-monophosphate. The point at pH 4.5 was determined by measurement of absorbancy changes at 255 mμ; the point for cytidine 3′-monophosphate at 6.0 was obtained both from measurement of absorbancy changes at 260 mμ and from measurement of pH changes with chlorophenol red indicator. In all other experiments pH indicators were used as described in the Experimental Section. The dotted line represents log $(1/\tau_1)$ taken from ref 11. The solid line is a theoeretical curve for log $(1/\tau_3)$ which was calculated as described in the text for the mechanisms in Figures 5 and 6.

have estimated errors of ±25 and ±15%, respectively. The plots of data at pH 6.0 and pH 7.0 contain data obtained using both pH indicator and ultraviolet absorbance techniques and show that the relaxation times are independent of the detection method employed. Most of the data at pH 7.0 were from experiments using a pH indicator since the reactant concentrations required to determine k_2 prevented the measurement of ultraviolet absorbancy changes: this is due to the fact that the association constant for ribonuclease–uridine 3′-monophosphate binding at pH 7.0 is relatively small.[7] Values of k_2 and k_{-2} for uridine 3′-monophosphate and a few values for cytidine 3′-monophosphate are summarized in Table II. Experiments using cytidine 3′-monophosphate were performed with an identical solvent (*i.e.*, 0.2 *M* KCl) as that for experiments with the uridine nucleotide. The values of k_2 for cytidine 3′-monophosphate are somewhat smaller than those reported previously,[3,4] while the values for k_{-2} are the same within experimental error. The values reported in this work are to be preferred because of the improved apparatus used. The values of k_2 for uridine 3′-monophosphate are only slightly larger than those for cytidine 3′-monophosphate, whereas the values of k_{-2} for the uridine nucleotide are approximately two times those for the cytidine nucleotide at different pH values. These results are sufficient to explain the fact that in the same solvent the over-all association constant for cytidine 3′-monophosphate binding with ribonuclease is approximately two times that for uridine 3′-monophosphate binding.[7]

Table II. Rate Constants for the Ribonuclease–3′-Nucleotide Interaction[a]

pH	$10^{-7}k_2$, M^{-1} sec^{-1}	$10^{-4}k_{-2}$, sec^{-1}	$10^{-3}k_3$, sec^{-1}	$10^{-3}k_{-3}$, sec^{-1}
Uridine 3′-monophosphate				
4.5	4.02	1.01		
5.0	5.69	1.01	1.04	1.77
5.5	7.79	1.05	1.09	1.20
6.0	6.13	1.12	0.97	0.77
6.5	4.01	1.32	0.676	0.583
7.0	0.559	1.64	0.94	0.165
Cytidine 3′-monophosphate				
5.0	4.20	0.420		
6.0	4.61	0.422		
6.6	1.23	0.620		

[a] Calculated according to eq 2 and 4.

If eq 1 is taken to represent the mechanism of ribonuclease–3′-nucleotide binding then the equilibrium association constant would equal k_2/k_{-2}. Values of k_2/k_{-2} and the observed equilibrium association constants for ribonuclease–uridine 3′-monophosphate binding[7] are plotted as a function of pH in Figure 2. The differences between the values of k_2/k_{-2} and the observed equilibrium constants indicate that the simple mechanism of eq 1 is not sufficient. The disparity between the observed and calculated association constants can be interpreted as indicating the existence of isomeric states of the enzyme–product complex. Furthermore, this isomerization process is pH dependent.

When the concentration of uridine 3′-monophosphate is increased relative to a fixed concentration of ribonuclease the relaxation process associated with τ_2 gets faster until it disappears beyond the resolution time (approximately 15 μsec) of the temperature-jump apparatus. At near-saturating concentrations of the nucleotide, a concentration independent relaxation effect characterized by the relaxation time τ_3 appears. In the case of cytidine 3′-monophosphate this effect at pH 6.0 was readily observed at 260 mμ, as well as with a pH indicator. However, in most cases a pH indicator was employed. At some pH values τ_3 was observed to become shorter with increasing nucleotide concentrations until a concentration of nucleotide was reached above which τ_3 was constant: *e.g.*, the value of τ_3 for cytidine 3′-monophosphate at pH 6.0 was observed to decrease from about 1 msec at a nucleotide concentration of 3.5×10^{-5} *M* to a concentration independent value of 0.52 msec. This behavior of τ_3 is consistent with a mechanism involving an isomerization of the ribonuclease–3′-nucleotide complex following a rapid bimolecular step. The concentration independent values of τ_3 for uridine and cytidine 3′-nucleotides are presented in Figure 3 as a function of pH. The be-

havior of τ_3 at constant pH can be explained by the mechanism

$$E + P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E'P \qquad (3)$$

where EP and E'P are isomers of the enzyme–3'-nucleotide complex; for this mechanism

$$1/\tau_3 = k_{-3} + \frac{k_3}{1 + (k_{-2}/k_2)/[(E) + (P)]} \qquad (4)$$

when $1/\tau_2 \gg 1/\tau_3$. At sufficiently high product concentrations $1/\tau_3$ approaches a concentration independent value of k_3 plus k_{-3}. Since the over-all binding constant is known for the mechanism of eq 3 and is equal to $(k_2/k_{-2})(1 + k_3/k_{-3})$, k_3/k_{-3} can be determined by use of the values of k_2 and k_{-2} obtained from the concentration dependence of $1/\tau_2$. Furthermore these results can be combined with the experimental values of $1/\tau_3$ to determine the individual rate constants k_3 and k_{-3} at a given pH. The rate constants calculated in this manner for uridine 3'-monophosphate are included in Table II. The rate constants could not be calculated at pH 4.5 since the over-all binding constant is approximately equal to k_2/k_{-2} at this pH. This implies k_3/k_{-3} is small at pH 4.5.

In this investigation only one relaxation time was detected which could be associated with an isomerization of the enzyme–uridine 3'-monophosphate complex. Since previous results[4,5] suggested that two isomerizations of the ribonuclease–cytidine 3'-monophosphate complex occur, the relaxation spectra of this system were partially reinvestigated. In brief the results indicate that the relaxation time previously designated τ_4 is, in fact, better assigned as τ_3 at pH values greater than 6.5. At lower pH values τ_4 may be related to the binding of cytidine 3'-phosphate at sites on the enzyme other than the active site: the original experiments[5] were performed at nucleotide concentrations of approximately 10^{-2} *M*; similar experiments were repeated using lower concentrations of the nucleotide, but approximately the same concentration of the enzyme–nucleotide complex, and no relaxation time other than τ_3 was observed. The relaxation time τ_3 which was previously reported for cytidine 3'-monophosphate binding[3-5] is consistent with the present results in the pH range 5.0–6.0 (see Figure 3). However, when experiments using cytidine 3'-monophosphate were repeated around pH 6.5, a longer relaxation time (shown in Figure 3) was found than previously reported. In the previous investigations,[3-5] the two data points around pH 6.5 designated as τ_3 probably represent, for the most part, τ_2 which could not be properly resolved from τ_3 because of the small amplitude of the relaxation effect observed with the previous temperature-jump apparatus. Thus the best available data indicate that only a single isomerization of the ribonuclease–3'-nucleotide complex occurs in the accessible time range; moreover the relaxation time, τ_3, associated with this process is virtually identical for both the cytidine and uridine 3'-nucleotides.

pH Dependence of the Relaxation Processes. Before interpreting the pH dependence of τ_2 and τ_3 observed in the present work, the pH-dependent conformational equilibrium of free ribonuclease in solution must be considered. In the absence of ligands ribonuclease exhibits a pH-dependent relaxation process (characterized by the relaxation time τ_1) as determined with a pH indicator[11] or, without an indicator, using iodinated ribonuclease.[12] A mechanism for this relaxation process is

$$H^+ + E \underset{K_A}{\rightleftharpoons} EH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E'H \underset{K_{A'}}{\rightleftharpoons} E' + H^+ \qquad (5)$$

where $pK_A = 6.1$,[11] $K = k_{-1}/k_1 = (EH)/(E'H) = 0.316$,[11] and $pK_A' > 8.5$. Since this mechanism indicates that free ribonuclease can exist in at least two isomeric states in solution,[11] *i.e.*, [(E') + (E'H)] and [(E) + (EH)], three limiting cases are possible for the bimolecular interaction in the free enzyme with the 3'-nucleotide product in the pH range investigated: (1) the nucleotide reacts selectively with the "acid stable" isomer (E'H); (2) the nucleotide reacts selectively with the "base stable" isomer (EH and E); (3) the nucleotide reacts equivalently with both isomers. The graphically determined values of k_2 and k_{-2} are slightly different for the three limiting cases: the total concentration of free ribonuclease must be multiplied by an appropriate factor to yield only the concentration of the *reacting* enzyme species which then must be inserted in the sum [(E) + (P)]. This assumes the rate of interconversion between reactive and nonreactive enzyme species is slow relative to the initial step in enzyme–nucleotide complex formation; *i.e.*, the relaxation time characterizing the isomerization is considerably longer than τ_2. This assumption is consistent with the known values of the relaxation times. The factors for the three cases considered above, in the pH range investigated, are

$$\text{case 1} \quad f_1 = \frac{(E'H)}{(E) + (EH) + (E'H)} = \frac{1}{1 + K[1 + K_A/(H^+)]} \qquad (6)$$

$$\text{case 2} \quad f_2 = \frac{(E) + (EH)}{(E) + (EH) + (E'H)} = \frac{1}{1 + 1/K[1 + K_A/(H^+)]} \qquad (7)$$

$$\text{case 3} \quad f_3 = 1$$

Values of k_2 and k_{-2} which were determined for case 1 and case 3 at different pH values are presented in Figure 4. Under the experimental conditions employed (usually the total 3'-nucleotide concentrations were greater than the total ribonuclease concentrations) the differences between k_2 and k_{-2} for the three cases are small (see Figure 4). The pH dependence of k_2 is determined only by the ionizable groups on the reacting species of free ribonuclease and uridine 3'-monophosphate that are of importance in the bimolecular step.[4] For many mechanisms an independent determination of the pH dependence of k_2 is possible by evaluating the pH dependence of the steady-state kinetic parameter V_P/K_P (V_P is the maximum velocity for the formation of uridine 2':3'-cyclic phosphate and K_P is the Michaelis constant for the uridine 3'-monophosphate product) since the pH dependence of this ratio is also usually

(11) T. C. French and G. G. Hammes, *J. Am. Chem. Soc.*, 87, 4669 (1965).
(12) G. G. Hammes and F. G. Walz, Jr., in preparation.

determined only by the ionizable groups of free enzyme and substrate.[13] The values of V_S/K_S as a function of pH were obtained from a steady-state kinetic investigation of the ribonuclease-catalyzed hydrolysis of uridine 2′:3′-cyclic phosphate,[14] and V_P/K_P was calculated for different pH values using the relation[14,15]

$$V_P/K_P = \frac{V_S/K_S}{K_0[1 + K_{AO}/(H^+)]} \quad (8)$$

where K_0 is the equilibrium constant representing the ratio of the monoanionic form of uridine 3′-monophosphate (product) to the monoanionic form of uridine 2′:3′-cyclic phosphate (substrate) and K_{AO} ($= 10^{-5.74}$ M)[7] is the secondary acid dissociation constant of uridine 3′-monophosphate. Since K_0 is a scale factor it does not have to be known to adduce the the pH dependence of V_P/K_P. As mentioned above the bimolecular relaxation process is much faster than the isomerization of free ribonuclease, therefore the bimolecular rate constants obtained from temperature-jump studies would only exhibit the pH dependence of steps rapidly equilibrating relative to τ_1[4] whereas the parameter V_P/K_P would include the pH dependence of the bimolecular reaction of ribonuclease and uridine 3′-monophosphate, *plus* the pH dependence of the isomerization of free ribonuclease. Therefore, a comparison of these two means of assessing the pH dependence of k_2 requires that V_P/K_P be corrected in accordance with which isomeric forms of ribonuclease are assumed to react with the uridine 3′-phosphate. At each pH investigated values of $(V_P/K_P)/f_i$ were compared with those of k_2 which were determined for each case as described above. The best fit between the pH dependence of k_2 and $(V_P/K_P)/f_i$ is given by case 1. Case 3 gives a reasonable but slightly worse fit to the data for k_2, whereas the fit of case 2 appears to exceed any reasonable experimental error. The calculated curves of $(V_P/K_P)/f_i$ for cases 1 and 3 are included in Figure 4. Additional support for case 1 can be derived from the previously published dependence of τ_1 on the concentration of cytidine 2′3′-cyclic phosphate.[5] At high concentrations of this substrate, the limiting value of $1/\tau_1$ is equal to the rate constant characteristic of the rate of formation of the active isomer.[5] For case 1, $1/\tau_1 = k_2/[1 + K_A/(H^+)]$ at high substrate concentrations; this equality is in quantitative agreement with the limited available data. Unfortunately we have been unable to obtain similar data for the case of uridine 3′-phosphate binding so that a definite decision cannot yet be made between these two cases.

Although τ_3 is a complex function of pH, an observation that leads to a considerable simplification of this complexity is that τ_1 shows a marked similarity with τ_3 as illustrated in Figure 3. As will be discussed in detail later, the pH dependence of both τ_1 and τ_3 probably results from the ionization of the same group on the enzyme. Thus, the data for τ_3 in the pH range 4.5–7.0 and the individual rate constants given in Table I can be fit by an equation identical in form with that

(13) L. Peller and R. A. Alberty, *J. Am. Chem. Soc.*, 81, 5907 (1959); R. A. Alberty and V. Bloomfield, *J. Biol. Chem.*, 238, 2804 (1963).
(14) E. J. del Rosario and G. G. Hammes, *Biochemistry*, 8, 1884 (1969).
(15) J. T. B 'ır, R. E. Cathou, and G. G. Hammes, *J. Biol. Chem.*, 240, 3372 (1965).

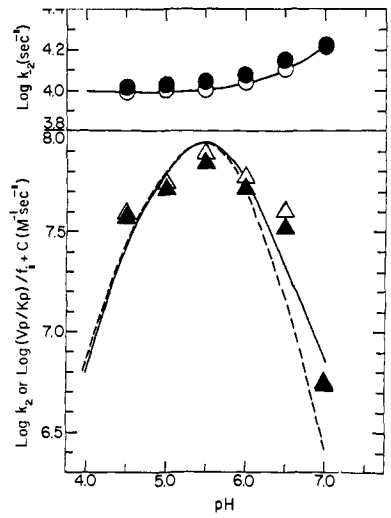

Figure 4. The pH dependence of log k_2, log k_{-2}, and log $(V_P/K_P)/f_i + C$ for ribonuclease-uridine 3′-monophosphate binding. The circles represents k_{-2} and the triangles represent k_2; the filled symbols represent values of k_2 and k_{-2} calculated on the basis that binding only occurs with the "acid stable" isomer of ribonuclease; the unfilled symbols are calculated assuming both isomers bind equivalently. The line through k_{-2} is the theoretical curve, and the solid line through k_2 is a theoretical curve which also coincides with log $(V_P/K_P)/f_i + C_1$ and the dashed line is a theoretical curve which coincides with $(V_P/K_P)/f_i + C_2$ where C_i is an arbitrary constant. See text for details.

which describes the pH dependence of τ_1.[11]

$$1/\tau_3 = k_3 + \frac{k_{-3}}{1 + K_A/(H^+)} \quad (9)$$

k_3 and k_{-3} depend on which isomeric state or states are assumed to bind the 3′-nucleotide since the over-all binding constant is a function of k_2, k_{-2}, k_3, k_{-3}, and f_i ($K = (k_2/k_{-2})(1 + k_3/k_{-3})f_i$); the values of k_2 and k_{-2} also depend slightly on f_i as previously discussed. A reasonable fit of the data is obtained for case 1 with $k_3 = 1300$ sec^{-1}, $k_{-3} = 1200$ sec^{-1}, and p$K_A = 5.8$. A better fit of the data is obtained for case 3 with $k_3 = 1000$ sec^{-1}, $k_{-3} = 2100$ sec^{-1}, and p$K_A = 5.8$. The mechanistic implications of these values and the pH dependence of $1/\tau_3$ above pH 7 will be considered in detail later.

Discussion

Any mechanism that agrees reasonably well with the experimental data must be relatively complex. A number of mechanisms have been considered and we will now delineate the minimal features of possible mechanisms. We first consider the bimolecular reaction characterized by the rate constant k_2. This rate constant is a function of the ionizable groups on the 3′-nucleotide and those on the isomeric form of the enzyme reacting with the product. If the requirement is imposed that the same ionizable groups on the enzyme and the same isomeric form of the enzyme interact with both the 3′-nucleotide products and 2′,3′-cyclic phosphate substrates, only two simple mechanisms are consistent with the data: (1) two ionizable groups are required on the enzyme and monoanionic uridine 3′-phosphate reacts predominantly with the monoprotonated enzyme; (2) two ionizable groups are required on the enzyme and dianionic 3′-nucleotide reacts predominantly with a diprotonated enzyme. These possi-

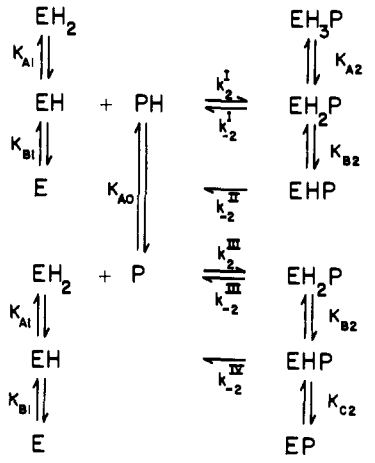

Figure 5. A possible mechanism for the initial interaction between ribonuclease and uridine 3′-monophosphate: the EH_i represent enzyme species (either both isomeric forms or the "acid stable" isomer depending on whether case 1 or 3 is under consideration), PH is monoanionic nucleotide, P is dianionic nucleotide, and EPH_i represent enzyme-nucleotide complexes. Free protons have been omitted for the sake of clarity. The horizontal arrows represent kinetically significant steps in the pH range 4.5–7.0. Values of the rate and ionization constants are given in the text.

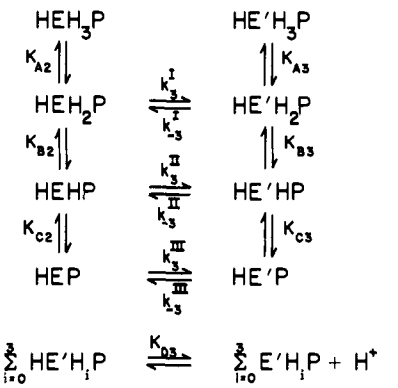

Figure 6. A possible mechanism for the isomerization of the enzyme–uridine 3′-monophosphate complex. The horizontal arrows represent kinetically significant steps in the pH range 4.5–7.5. Values of the rate and ionization constants are given in the text.

bilities follow directly from the pH dependence of k_2 and the relationship between V_P/K_P and V_S/K_S as given by eq 8. Since these mechanisms are kinetically indistinguishable and since ribonuclease probably binds both monoanions and dianions at the active site,[5,7,16] both mechanisms will be assumed to be operative. (No obvious method exists for distinguishing between these mechanistic possibilities at the present time.) This mechanism is depicted in Figure 5; the pH dependence of k_2 is given by

$$k_2 = \frac{k_2' + k_2'''K_{A0}/K_{A1}}{[1 + (H^+)/K_{A1} + K_{B1}/(H^+)][1 + K_{A0}/(H^+)]} \quad (10)$$

If only the "acid stable" isomer of the free enzyme reacts, $pK_{A1} = 5.4$, $pK_{B1} = 6.6$, and $k_2' + k_2'''K_{A0}/K_{A1} = 2.6 \times 10^8\ M^{-1}\ sec^{-1}$ generate a pH profile for k_2 that is very similar to that for $(V_P/K_P)f_1$ given in Figure 4. On the other hand if both isomeric forms of the enzyme react equivalently, the pH dependence of $(V_P/K_P)/f_3$ (also shown in Figure 4) is reproduced with $pK_{A1} = 5.4$, $pK_{B1} = 6.4$, and $k_2' + k_2'''K_{A0}/K_{A1} = 2.5 \times 10^8\ M^{-1}\ sec^{-1}$. The best fit of the data appears to lie between these two limiting cases; *i.e.*, both isomers react, with the "acid stable" isomer being somewhat favored. However, the data are such that a more detailed consideration of this mechanism is not warranted. In any event at least two ionizable groups on the free enzyme are implicated in the bimolecular reaction with pK values of approximately 5.4 and 6.5. The values of other possible second-order rate constants cannot be specified other than to stipulate they must be less that about $10^{-2}k_2' + k_2'''K_{A0}/K_{A1}$.

The pH dependence of the dissociation rate constant indicates that either all of the initial complexes formed dissociate at essentially the same rate or that none of the initial complexes have characteristic pK values in the range 4.5–7. Only the latter explanation is plausible. The slight rise in k_1 near pH 7 suggests an ionizable group with a pK in the range 7.5–8 is of importance. Since nuclear magnetic resonance data indicate that two protons are associated with the active site imidazole groups in the ribonuclease–3′-nucleotide complex,[17] a reasonable hypothesis is that $pK_{A2} < 3.5$, and pK_{B2}, $pK_{C2} > 7$. According to the mechanism of Figure 5

$$k_{-2} = \frac{(k_{-2}' + k_{-2}''') + (k_{-2}'' + k_{-2}'''')K_{B2}/(H^+)}{1 + K_{B2}/(H^+) + K_{B2}K_{C2}/(H^+)^2}$$

The pH profile of k_{-2} calculated with $(k_{-2}' + k_{-2}''') = 1.0 \times 10^4\ sec^{-1}$, $(k_{-2}'' + k_{-2}'''') = 4.0 \times 10^4\ sec^{-1}$, $pK_{B2} = 7.5$, $pK_{A1} \leq 3.5$, and $pK_{C2} \geq 8.0$ is shown in Figure 4. The values of the other possible dissociation rate constants in the mechanism cannot be assessed. Note that this mechanism specifies the following order of stability of the initial complexes: $EH_2P > EHP > EP$ and $EH_2P > EH_3P$.

The pH dependence of $1/\tau_3$ and k_{-3} strongly suggests an ionizable group with a pK of about 6 is of importance in determining k_{-3}. Two possibilities seem most likely: (1) the pK of one of the ionizable groups implicated in the formation of the initial complex is changed to 6 or (2) a different ionizable group on the enzyme is of importance. We prefer the latter possibility for two reasons. The similarity in both the absolute values and pH dependence of τ_1 and τ_3 suggests the same ionizing group is of importance in both processes. Furthermore, both τ_1 and τ_3 have similar deuterium isotope effects. The pK value of the group implicated in τ_1 ($\sim$6) is different from those required to explain the pH dependence of k_2, thus implying a third ionizable group is of importance. Furthermore, the nuclear magnetic resonance results indicate that neither of the protons interacting with cytidine 3′-phosphate has an associated pK value as low as 6.[17] A simple mechanism for the interconversion of the two enzyme–product complexes consistent with the data is shown in Figure 6. This mechanism implies that the third ionizable group does not interact directly with the 3′-nucleotide, but nevertheless plays a roll in the mechanism. The pH dependence of the rate constants is given by

$$k_3 = \frac{k_3' + k_3''K_{B2}/(H^+) + k_3'''K_{B2}K_{C2}/(H^+)^2}{1 + K_{B2}/(H^+) + K_{B2}K_{C2}/(H^+)^2} \quad (12)$$

(16) C. A. Nelson, J. P. Hummel, C. A. Swenson, and L. Friedman, *J. Biol. Chem.*, 237, 1575 (1962).

(17) D. H. Meadows and O. Jardetzky, *Proc. Natl. Acad. Sci. U. S.*, 61, 406 (1968).

$$k_{-3} = \frac{k_{-3}' + k_{-3}''K_{B_3}/(H^+) + k_3'''K_{B_3}K_{C_3}/(H^+)^2}{[1 + K_{D_3}/(H^+)][1 + K_{B_3}/(H^+) + K_{B_3}K_{C_3}/(H^+)^2]} \quad (13)$$

In order to evaluate the constants in these equations, an assumption with regard to which isomeric form of the enzyme reacts with the nucleotide must be made. We will assume that both isomeric forms react equivalently. The pH profile of $1/\tau_3$ calculated from eq 4, 12, and 13 with $k_3' = 1000$ sec^{-1}, $k_3'' = 420$ sec^{-1}, $k_3''' = 4200$ sec^{-1}, $k_{-3}' = 2100$ sec^{-1}, $k_{-3}'' = 280$ sec^{-1}, $k_{-3}''' = 2800$ sec^{-1}, $pK_{B2} = 7.5$, $pK_{C2} = 8.0$, $pK_{B3} = 7.0$, $pK_{C3} = 8.0$, $pK_{D3} = 5.8$, and pK_{A2}, $pK_{A3} < 3.5$ is shown in Figure 3. This mechanism gives an adequate fit of the pH dependence of this relaxation time. This set of constants is by no means unique; in fact considerable variation of the double and tripled primed rate constants and the pK's associated with the second complex is possible.

Implicit in this mechanism is the assumption that the pK value of the ionizing group of the initial enzyme–nucleotide complex corresponding to pK_{D_3} is greater than about 7. The implication of this for the over-all mechanism according to detailed balance considerations inevitably leads to the conclusion that the two isomeric forms of the enzyme do not react exactly equivalently. However, the assumption that only the "acid-stable" isomer of the enzyme reacts gives an adequate but distinctly worse fit of the data for τ_3. A more detailed delineation of the mechanism is not possible at this time.

The mechanism shown in Figures 5 and 6 is one of the simplest consistent with all available data. It postulates that two ionizing groups on free ribonuclease, characterized by K_{A1} and K_{B1}, directly bind the 3′-nucleotide product and one ionizing group, characterized by K_{D3}, is involved in an isomerization of the enzyme–product complex. These can be regarded as the minimal features of possible mechanisms. Although the postulated mechanism includes both monoanion and dianion binding and suggests binding to both isomers of the enzyme, these do not appear to be necessary features of possible mechanisms at the present time. Microscopic acid dissociation constants have not been considered since they would only serve unnecessarily to complicate an already complex mechanism, without changing the basic mechanistic features.

Rate constants associated with mechanisms representing both cases 1 and 3 yield calculated values of the over-all association constant for ribonuclease–uridine 3′-monophosphate at different values of pH that are in reasonable agreement with those determined by equilibrium methods.[7] Thus the proposed mechanism is quantitatively consistent with all of the available data. Considerable more elaborate mechanisms can be constructed which fit the data more precisely, but this serves no useful purpose.

This mechanism is similar to that presented previously for ribonuclease–cytidine 3′-monophosphate binding.[5] Both mechanisms require three ionizing groups on the enzyme having similar pK values and multiple isomeric states for the enzyme–product complex. The principal difference in the two mechanisms is that the previous mechanism required a minimum of three isomeric states of the enzyme–product complex whereas the present mechanism requires only two. The reason for this discrepancy has been discussed in detail earlier Another difference is that the previous mechanism proposed preferential binding of the 3′-nucleotide with the "base stable" isomer of ribonuclease, while the present mechanism includes binding to both isomers. The role of the different isomeric states is still uncertain, but probably a combination of the limiting cases 1 and 3 is involved. The present data are considerably more reliable than available previously because of the technical improvements in the temperature-jump apparatus. Moveover, new information regarding the detailed structure of ribonuclease[18] and ribonuclease-^{19}S recently made available by X-ray crystallographic methods as well as nmr studies[17,20] on the imidazole side chains of ribonuclease have been considered in formulating the present mechanism.

The suggestion that the imidazole groups of histidine-119 and histidine-12 are present at the active site of ribonuclease as deduced from chemical modification of these groups[21,22] has been substantiated by X-ray crystallographic studies of ribonuclease[18] and ribonuclease-S.[19] In both structural studies bound phosphate ligands were adjacent to the imidazole groups of these two histidine residues. In the present work analysis of (V_P/K_P) and k_2 as a function of pH was used to obtain the apparent pK values of two active site ionizable groups on the free enzyme. The groups are most likely the imidazole side chains of histidines 12 and 119. The suggestion has been made[20-22] that the active site imidazole group having the lower pK_A value (pK_{A1}) is histidine-119 while the imidazole group of histidine-12 (pK_{B1}) has the higher pK_A value. The remaining ionizing group (pK_{D3}) involved in the binding process has an apparent pK value of about 5.8, and consequently most likely represents another imidazole side chain group on ribonuclease. Of the two remaining histidine residues (histidine-48 and histidine-105) histidine-48 appears most likely to be involved.

As mentioned above the similarity of τ_1 and τ_3 as a function of pH suggests that the same ionizing group takes part in both processes. The imidazole group of histidine-48 has been previously suggested to be implicated in the isomerization of free ribonuclease as distinguished by τ_1.[23] Histidine-48 is located at the "hinge" of the active site and its environment might easily be altered by conformational changes associated with the active site. Direct evidence supporting this contention is found from nmr studies on ribonuclease[20] and the ribonuclease–cytidine 3′-monophosphate complex[17] which indicate that binding of the 3′-nucleotide perturbs the environment of histidine-48. Moreover, these nmr studies[17,20] indicate that the imidazole group of histidine-105 has the same ionizing properties when ribonuclease is in the free and nucleotide-bound states. Therefore, the histidine-48 imidazole group appears to be involved in both the isomerization of free ribonu-

(18) G. Kartha, J. Bello, and D. Harker, *Nature*, 213, 862 (1967).
(19) H. W. Wykoff, K. D. Hardman, N. M. Allewell, T. Ingami, L. N. Johnson, and F. M. Richards, *J. Biol. Chem.*, 242, 3984 (1967).
(20) D. H. Meadows, O. Jardetzky, R. M. Epand, H. H. Ruterjans, and H. A. Scheraga, *Proc. Natl. Acad. Sci. U. S.*, 60, 766 (1968).
(21) A. M. Crestfield, W. H. Stein, and S. Moore, *J. Biol. Chem.*, 238, 2421 (1963).
(22) R. Heinrikson, W. H. Stein, and S. Moore, *ibid.*, 240, 2921 (1965).
(23) G. G. Hammes, *Accounts Chem. Res.*, 1, 321 (1968); *Advan. Protein Chem.*, 23, 1 (1968).

clease and ribonuclease–3′-nucleotide complex as characterized by τ_1 and τ_3, respectively. A possible interpretation of the proposed mechanism is that this imidazole group is "buried" in the "acid stable" isomer of free ribonuclease *and* the initial complex with the product; the pK value of the imidazole group in both of these states is considerably higher than normal. Upon "exposure" (*i.e.*, isomerization) the group assumes a pK value of about 6 for the free enzyme and the enzyme–product complex, respectively (see eq 5 and Figure 6). While this explanation should not be taken too literally, it does provide a rational explanation of the data. More information on this aspect of the mechanism might be obtained from relaxation studies of ribonuclease-S where it does not appear that histidine-48 would be buried.[19]

Although structural details cannot be derived from kinetic measurements, the pK values postulated can be understood in a qualitative manner. The low pK assigned to the ionization of species EH_3P and $E'H_3P$ to EH_2P and $E'H_2P$, respectively, is due to the proximity of three protons associated with groups of similar base strength in a cluster having a net positive charge. On the other hand, the relatively high values of pK_{B2} and pK_{C2} can be understood on the basis that the protons in the complex are bound by hydrogen-bonding interactions involving the imidazole nitrogen atoms of the enzyme *and* phosphate oxygens of the nucleotide. The fact that such hydrogen bonds can be formed is suggested by nmr studies on the interaction of phosphoric acid and imidazole in dimethyl sulfoxide.[17] The pK values derived from this work are similar to, but not identical with, those derived from nmr studies.[17,20]

The second-order rate constants are similar in magnitude for cytidine and uridine 3′-monophosphates and also to values found for the formation of other enzyme–substrate complexes.[23] Apparently the interaction of the base moiety of the 3′-nucleotide with ribonuclease is important in stabilizing the initial complex as witnessed by the significantly different values of k_{-2} for the cytidine and uridine 3′-nucleotides. On the other hand, the different nucleotides have very little, if any, effect on the values of k_3 and k_{-3}.

The suggestion has been made that the isomerization involving histidine-48 also involves lysine-41 in a conformational equilibria which is perturbed by binding of the 3′-nucleotide.[17] In the present mechanism this suggestion could be visualized as follows: the conformational change which exposes the "buried" histidine-48 imidazolium group, *i.e.*

$$\sum_{i=0}^{3} HEH_iP \longrightarrow \sum_{i=0}^{3} HE'H_iP$$

also brings the ϵ-amino group of lysine-41 into position to affect the active site. The function of this positively charged group could be to relieve the imidazole group of histidine-119 or 12 from its binding function so that it may freely serve as a catalytic base species for the cyclization of the 3′-nucleotide product in the reverse reaction. Moreover, the participation of lysine-41 in such a manner agrees with the observation that when lysine-41 is guanidinated the resulting ribonuclease derivative is devoid of enzymatic activity but is still capable of binding cytidine 2′-monophosphate.[24]

To summarize, the relaxation spectra of the interaction of ribonuclease with cytidine and uridine 3′-phosphate are consistent with a two-step mechanism involving formation of an initial complex and isomerization of this complex. In the pH range 4.5–7.5, three ionizable groups on the enzyme have been implicated in the mechanism, one of these groups being associated with an enzyme conformational change rather than with a direct interaction with the nucleotides. This mechanism can be correlated with the three-dimensional structure of ribonuclease and some speculations can be made with regard to the nature of the conformational changes. These findings do not provide a reliable basis for the postulation of a detailed chemical mechanism of action of ribonuclease, but in any event are consistent with previously proposed mechanisms (*cf.* ref 23).

(24) E. A. Barnard and H. Ramel, *Nature*, **195**, 243 (1962).