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Kinetic and Equilibrium Studies on the Interaction of Ribonuclease A and 2′-Deoxyuridine 3′-Phosphate*

Frederick G. Walz, Jr.

ABSTRACT: The ionization constants for the secondary phosphate and ring nitrogen groups of 2′-deoxyuridine 3′-phosphate were determined. Kinetic and equilibrium binding studies of the interaction of ribonuclease with this nucleotide were performed using the temperature-jump and difference spectra methods, respectively. Experiments were performed in the pH range 4.5–7.0 at 25°. Association constants and maximum difference extinction coefficients for 2′-deoxyuridine 3′-phosphate binding were determined at six pH values. Two relaxation processes were observed which can be attributed to an initial association–dissociation of the enzyme and deoxynucleotide followed by an isomerization of the enzyme–deoxynucleotide complex. The pH dependence of the individual rate constants was determined for this mechanism. There is no indication that 2′-deoxyuridine 3′-phosphate preferentially binds one of the isomeric states of ribonuclease. A detailed minimal mechanism for the pH dependence of the relaxation processes is presented and requires the participation of two acid-base groups of ribonuclease. Differences in these results with those previously reported for ribonuclease binding with uridine 3′-phosphate are discussed with reference to the participation of the nucleotide 2′-hydroxyl group on interaction with ribonuclease.

The RNase[1]-catalyzed degradation of RNA has been characterized as proceeding *via* a two-step process involving the formation and hydrolysis of pyrimidine 2′:3′-cyclic phosphate intermediates (Markham and Smith, 1952; Brown *et al.*, 1952). The 2′-hydroxyl group of phosphodiester substrates (*e.g.*, RNA and 3′-pyrimidine dinucleoside phosphates) has been postulated to interact with a basic group on RNase in a variety of reaction mechanisms proposed for the enzyme-catalyzed transesterification (Findley *et al.*, 1962; Ramsden and Laidler, 1966; Hammes, 1968; Usher, 1969; Roberts *et al.*, 1969). Likewise, the reverse of the hydrolytic step (*i.e.*, the formation of pyrimidine 2′:3′-cyclic phosphate from the 3′-nucleotide product) in these mechanisms is postulated, at least implicitly, to involve an interaction of the 2′-hydroxyl group of the 3′-nucleotide product with a basic group on the enzyme. The importance of the 2′-hydroxyl group in the binding of nucleosides with RNase was shown by inhibition studies of RNase activity (Ukita *et al.*, 1961), where cytidine was two times more effective as an inhibitor than deoxycytidine. Furthermore, binding studies of RNase with a variety of nucleotides including the phosphobenzyl ester of thymidine 3′-phosphate indicated the importance of a 2′-oxygen atom which apparently was required to give a significant difference optical rotatory dispersion curve for the RNase·nucleotide complex (Deavin *et al.*, 1966).

The present study concerning the pH dependence of the kinetic and equilibrium constants characterizing the interaction of 3′-dUMP with RNase were undertaken in an effort to elucidate the role of the 2′-hydroxyl group in nucleotide 3′-phosphate binding. The results of this study in conjunction with those from similar studies on RNase·3′-UMP binding (Hammes and Walz, 1969) provide, by comparison, an assessment of the participation of the 2′-hydroxyl group in the RNase·3′-nucleotide interaction.

Materials

Ribonuclease A. Phosphate-free, lyophilized ribonuclease A was obtained from Worthington Biochemical Corp. and was used without further purification. The concentration of ribonuclease A was determined as described previously (Hammes and Walz, 1969).

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[1] The abbreviations used in this paper are RNase, bovine pancreatic ribonuclease A; 3′-dUMP, 2′-deoxyuridine 3′-phosphate; 3′-UMP, uridine 3′-phosphate; 3′-CMP, cytidine 3′-phosphate.

5′-Di-p-Methoxytrityl-2′-deoxyuridine. The synthesis of 5′-di-*p*-methoxytrityl-2′-deoxyuridine was accomplished according to the method used in preparing the thymidine derivative (Schaller *et al.*, 1963). The purified product revealed a single spot on thin-layer chromatography.

2′-Deoxyuridine 3′-Phosphate. Phosphorylation of 5′-di-*p*-methoxytrityl-2′-deoxyuridine was accomplished by reaction with 2-cyanoethyl phosphate using dicyclohexylcarbodiimide as a condensing agent (Tener, 1961). The nucleotide was isolated as its barium salt which was chromatographed on an AG 1-X10 (formate form) column using 0.05 M formic acid brought to pH 4.8 with ammonium hydroxide as an eluent. The major peak fractions (~98%) were flash evaporated and then repeatedly lyophilized to remove formic acid. The nucleotide was converted into the potassium salt by passage through an AG 50-X10 column (H^+ form) and neutralization of the acid fractions to pH 7.5 with potassium hydroxide solution. Paper chromatography of the final material using Whatman No. 3MM paper and two solvent systems (isobutyric acid–2.5 M ammonium hydroxide (66:34, v/v) and 1-butanol–glacial acetic acid–water (50:25:25, v/v)) revealed a single spot. No significant phosphate was released when the material was treated with 5′-nucleotidase. The ultraviolet absorption of 2′-deoxyuridine 3′-phosphate is characterized by a λ_{max} at 262 mμ and a 280 mμ:260 mμ ratio of 0.29 at pH 6.5. The extinction coefficient at 260 mμ, pH 6.5 was found to be 10,000 M^{-1} cm^{-1}. The ultraviolet absorption spectra is essentially the same in the pH range, 4.5–7.0.

Miscellaneous Reagents. All other chemicals were reagent grade or the best grade available commercially. Glass-distilled water having a specific resistance of greater than one megohm was used in all solutions.

Methods

Difference Spectrophotometry. Difference spectra were determined using a Zeiss PMQ II spectrophotometer. Rectangular split compartment cells (0.44-cm path length) were used in a thermostatted cell compartment. All experiments were conducted at 25 ± 0.1°. The slit width was maintained constant at 0.1 mm for all measurements. All difference absorbance measurements presented are calculated for 1-cm path length. The concentration of RNase was approximately 8×10^{-5} M in every experiment and nucleotide concentrations ranged from 4×10^{-5} to 3.5×10^{-4} M. A stock buffer solution of 0.05 M Tris, 0.05 M sodium acetate, and 0.1 M KCl was used in all experiments. For each experiment the pH was adjusted with acetic acid: the resulting ionic strength was 0.2 M over the pH range investigated. At each pH at least fifteen experiments at different nucleotide concentrations were performed. The difference spectra at each pH was taken from 240 to 300 mμ at 1-mμ intervals.

Temperature-Jump Experiments. The temperature-jump apparatus was similar to that described previously (Hammes and Walz, 1969). All solutions used in temperature-jump experiments were 0.2 M in KCl. The relaxation processes associated with the initial binding of 3′-dUMP and RNase were detected by measurement of absorbance changes at 255 mμ. The concentration ranges employed in these experiments were $0.2–2.0 \times 10^{-4}$ M 3′-dUMP and $3.1–8.5 \times 10^{-5}$ M RNase. This initial process could also be measured by observing associated pH changes using pH indicators. However, the amplitude of the relaxation process was considerably smaller than that obtained by direct observation and therefore, experiments were conducted at 255 mμ to obtain more accurate data. Nevertheless, semiquantitative observations of this process using pH indicators could be extended to higher concentrations of 3′-dUMP which were prohibitive at 255 mμ due to the large absorption of the nucleotide at this wavelength. In experiments designed to study the isomerization of RNase·3′-dUMP complex, the enzyme was brought to at least 85% saturation with nucleotide. The range of nucleotide concentrations used was $0.4–1.0 \times 10^{-3}$ M. 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 methyl red, chlorophenol red, and phenol red and the changes in absorbance were observed at 520, 573, and 558 mμ, respectively. In all temperature-jump experiments the reactants were equilibrated at 19.5° and a 5.5° temperature jump was applied. This latter quantity was determined by measuring the voltage and current across the cell when the capacitor was discharged at 10 kV, and represents approximately 75% of the energy stored in the capacitor.

Ionization Constants. The secondary phosphate and ring nitrogen ionization constants for 3′-dUMP were determined using a Radiometer automatic titration assembly composed of an SBR 2 titratograph, TT 111 titrator, SBU 1A syringe burette assembly, and PHM-26 pH meter. All experiments were performed using carbon dioxide free NaOH, under a nitrogen atmosphere, and were thermostated at 25 ± 0.1°. The nucleotide was titrated in the presence of 0.2 M KCl. Blank titrations were performed and the corresponding corrections were made on the titration curves. The number of separate experiments performed in determining the secondary phosphate and ring nitrogen ionization constants was ten and four, respectively. All pH measurements were performed with a Radiometer pH M-26 pH meter; the error in pH measurement is estimated to be ±0.05 pH unit.

Results and Treatment of Data

Equilibrium Measurements. The p*K* values for the secondary phosphate and ring nitrogen ionizations of 3′-dUMP were found to be 6.08 ± 0.03 and 9.45 ± 0.06, respectively. The error limits represent the standard deviations of repeated experiments. The p*K* value for the secondary phosphate ionization is slightly greater than the value 5.74 reported for 3′-UMP (Anderson *et al.*, 1968) which was determined at the same ionic strength (0.2 M). The value for the ring nitrogen ionization constant is similar to those reported for other uridine nucleotides (Jordan, 1960).

The binding stoichiometry of 3′-dUMP and RNase was determined by the method of continuous variation (Job, 1928). The total concentration of RNase and 3′-dUMP was held constant and the relative concentrations of the enzyme and ligand were varied. The maximum difference absorbance was achieved at a 1:1 ratio of RNase and 3′-dUMP, which is the same as that for other phosphate ligands binding with RNase (Anderson *et al.*, 1968).

The difference spectra between the RNase·3′-dUMP complex and free RNase plus free 3-dUMP was measured as a function of concentration and pH. The difference spectral curve (not illustrated) was characterized by a broad negative maxima at 255 mμ and a crossover at 279 mμ; furthermore, the curve was invariant at different values of pH. These features of the difference spectrum are the same as those for RNase·3′-UMP binding (Anderson *et al.*, 1968).

TABLE I: Association Constants for RNase·3′-dUMP Binding.[a]

pH	$10^{-3}\ \Delta\epsilon_{max}$ [b] (M^{-1} cm^{-1})	$10^{-4}\ K_{AP}$ (M^{-1})
4.5	3.25	0.601
5.0	3.10	1.79
5.5	3.30	3.79
6.0	3.20	3.98
6.5	3.10	2.23
7.0	3.20	0.839

[a] Conditions were as described under Methods. [b] Maximum absolute values of $\Delta\epsilon$.

At each pH the molar difference extinction coefficient, $\Delta\epsilon$, and the association constant, K_{AP}, were calculated as described previously (Anderson *et al.*, 1968). The errors in the values reported for K_{AP} are estimated to be approximately ±15% and those for the maximum values of $\Delta\epsilon$ ($\Delta\epsilon_{max}$) is about ±10%. A summary of these constants at different values of pH are reported in Table I. The association constants for RNase binding with 3′-dUMP and 3′-UMP (Anderson *et al.*, 1968) are compared in Figure 1. At all pH values the association constants for 3′-dUMP binding were greater than those for 3′-UMP binding and show a different pH dependence.

Kinetic Measurements. Two relaxation processes were found to be associated with RNase·3′-dUMP binding. The faster of these processes had observed relaxation times of 60–160 μsec and is dependent on the concentration of the reactants. This effect can be interpreted as representing the initial binding reaction

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

where E represents RNase and L represents 3′-dUMP. For this mechanism (Eigen and DeMaeyer, 1963)

$$1/\tau_1 = k_1[(\bar{E}) + (\bar{L})] + k_{-1} \qquad (2)$$

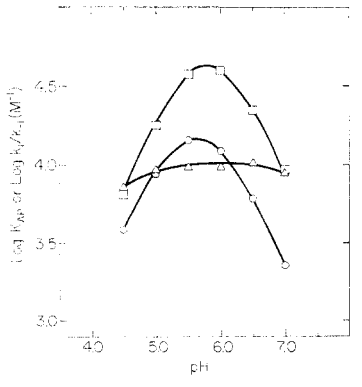

FIGURE 1: A plot of the logarithm of the association constants, K_{AP}, for RNase-ligand binding and the logarithm of k_1/k_{-1} for 3′-dUMP binding as a function of pH. The squares represent values of K_{AP} for 3′-dUMP binding; the circles represent values of K_{AP} for 3′-UMP binding (Anderson *et al.*, 1968); the triangles represent values of k_1/k_{-1} calculated as indicated in the text. Conditions were as described under Methods. The lines have no theoretical significance.

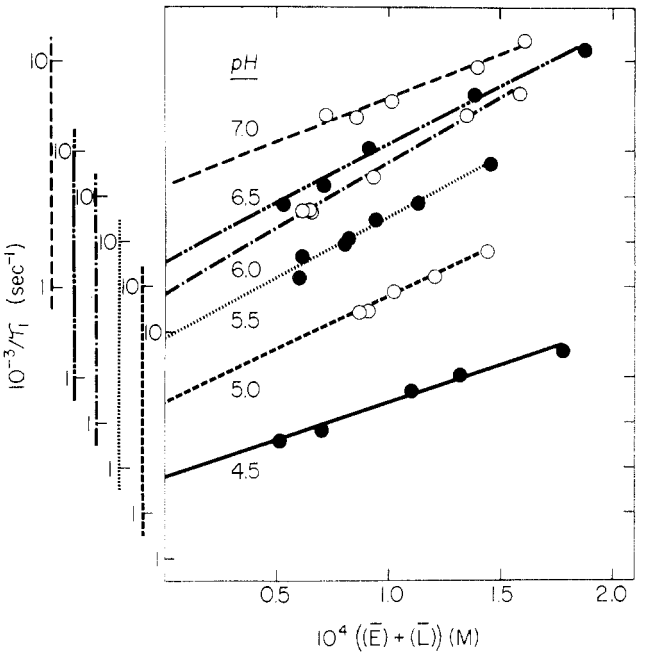

FIGURE 2: A plot of $1/\tau_1$ as a function of [(Ē) + (L̄)] at different pH values. Conditions were as described under Methods. The ordinate has been shifted and different symbols were used for clarity.

where $1/\tau_1$ is the observed reciprocal relaxation time, (Ē) is the equilibrium concentration of free RNase, and (L̄) is the equilibrium of free 3′-dUMP. Knowing the association constant, (Ē) and (L̄) can be calculated and values of k_1 and k_{-1} can be obtained from plots of $1/\tau_1$ *vs.* [(Ē) + (L̄)]. Such plots for different values of pH are shown in Figure 2. At each pH at least 50 individual temperature-jump experiments were performed. For a given pH at least three points had nonoverlapping experimental standard deviations. The values of k_1 and k_{-1} were obtained by a least-squares analysis of the data and have estimated errors of ±25 and ±15%, respectively. Values for k_1 and k_{-1} determined in this way are listed as observed constants in Table II and are shown as a function of pH in Figure 3. When this process is observed using pH indicators, it was found that at higher concentrations of 3′-dUMP τ_1 decreases (as predicted by eq 2) until it is at least as fast as the resolution time (about 15 μsec) of the temperature-jump apparatus under the experimental conditions employed.

If eq 1 is taken to represent the mechanism of RNase·3′-

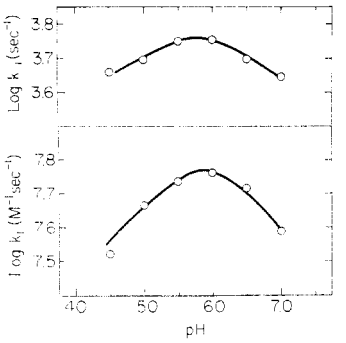

FIGURE 3: The pH dependence of log k_1 and log k_{-1} for RNase·3′-dUMP binding. The lines represent theoretical values calculated as indicated in the text for the mechanism in Figures 5 and 6.

TABLE II: Rate Constants for the RNase·3′-dUMP Interaction.[a]

	10^{-7} k_1 (M^{-1} sec^{-1})		10^{-3} k_{-1} (sec^{-1})		10^{-3} k_2 (sec^{-1})		10^{-3} k_{-2} (sec^{-1})	
pH	Obsd	Calcd	Obsd	Calcd	Obsd	Calcd	Obsd	Calcd
4.5	3.30	3.66	4.54	4.41				
5.0	4.63	4.60	4.96	4.99	2.13	2.26	2.34	1.88
5.5	5.43	5.73	5.64	5.61	2.05	1.45	0.700	0.994
6.0	5.77	5.82	5.97	5.65	1.55	1.20	0.500	0.727
6.5	5.19	4.88	5.03	5.08	1.20	1.75	1.04	1.27
7.0	3.99	4.07	4.46	4.53				

[a] Conditions were as described under Methods.

dUMP binding, then the equilibrium association constant would equal k_1/k_{-1}. Values of k_1/k_{-1} at different values of pH are shown in Figure 1. The differences between the values of k_1/k_{-1} and the observed equilibrium constants from pH 5.0 to 6.5 indicate that the mechanism of eq 1 is not sufficient to reconcile the kinetic and equilibrium data at these pH values. The disparity in the equilibrium and kinetic data can be interpreted as indicating the existence of isomeric states of the enzyme–ligand complex which equilibrate slowly relative to the initial step. Furthermore, this isomerization is pH dependent.

At near-saturating concentrations of 3′-dUMP a concentration-independent relaxation process characterized by the relaxation time τ_2 appears. This process is kinetically distinguishable from the faster process characterized by τ_1, since at the concentration of enzyme and ligand used τ_1 was observed to range from 15 to 35 μsec, whereas, τ_2 ranged from 200 to 500 μsec. Concentration-independent values of τ_2 are presented as a function of pH in Figure 4. At the extremities of the pH range where this process was observed the amplitude of this effect became vanishingly small. Repeated experiments under a variety of conditions failed to detect this process beyond the pH range shown in Figure 4. At a given pH the behavior of τ_2 can be explained by the mechanism

$$E + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EL \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E'L \qquad (3)$$

where EL and E′L are isomers of the enzyme–ligand complex; for this mechanism

$$1/\tau_2 = k_{-2} + \frac{k_2}{1 + (k_{-1}/k_1)/[(\bar{E}) + (\bar{L})]} \qquad (4)$$

when $1/\tau_1 \gg 1/\tau_2$. At sufficiently high ligand concentrations $1/\tau_2$ approaches a concentration-independent value of $k_2 + k_{-2}$. The overall association constant is equal to $(k_1/k_{-1})(1 + k_2/k_{-2})$ for the mechanism of eq 3. Since the overall association constant and k_1/k_{-1} are known as well as values for $1/\tau_2$, the individual rate constants k_2 and k_{-2} can be determined at a given value of pH. The rate constants determined in this manner are included in Table II. These rate constants could not be determined at pH 4.5 and 7.0 since τ_2 was not observed at these pH values.

If free 3′-dUMP preferentially interacts with the "acid-stable" or "base-stable" isomers of RNase, the relaxation time, τ_1, would not be a function of the total free enzyme concentration, $(\bar{E})$, but of $(\bar{E})f_i$, where f_i is an appropriate factor determining the free concentration of the *particular* isomeric form assumed to react (Hammes and Walz, 1969). This preferential binding can be ascertained experimentally by determining τ_1 while varying the relative values of $(\bar{E})$ and $(\bar{L})$ at a constant value of $[(\bar{E}) + (\bar{L})]$. To elucidate this aspect of the RNase·3′-dUMP interactions, τ_1 was determined at pH 6.0 under the following experimental conditions: (1) the ratio of total RNase concentration to the total 3′-dUMP concentration was 4.6, (2) the ratio of the total 3′-dUMP concentration to the total RNase concentration was 4.6; in both experiments $[(\bar{E}) + (\bar{L})]$ was 1.42×10^{-4} M. The anticipated differences in τ_1 for expt 1 compared to expt 2 for preferential binding of 3′-dUMP to the acid-stable and base-stable isomers was calculated to be minus 38% and plus 24%, respectively. Twelve values for the relaxation time were determined under both experimental conditions: the value of τ_1 for expt 1 was 77 ± 9 μsec and for expt 2 was 78 ± 12 μsec. This result clearly indicates that there is no significant preferential binding for 3′-dUMP and thereby considerably simplifies the interpretation of the pH dependence of τ_1, and τ_2.

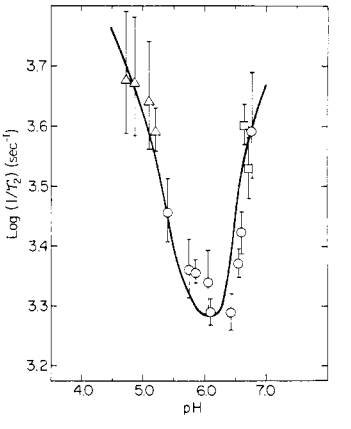

FIGURE 4: Plot of log $(1/\tau_2)$ as a function of pH. Experiments were performed in the presence of methyl red (△), chlorophenol red (○), and phenol red (□) as a means of detecting changes in pH accompanying the reaction. Over 100 separate experiments were performed and the error bars represent the standard deviation for each experimental point. Other conditions were as described under Methods. The line is a theoretical curve calculated as described in the test for the mechanism in Figures 5 and 6.

TABLE III: Theoretical Rate and Acid Dissociation Constants for the Mechanism in Figures 5 and 6.

i	$10^{-7}\ k_1^i$ (M^{-1} sec^{-1})	$10^{-3}\ k_1^i$ (sec^{-1})	$10^{-3}\ k_2^i$ (sec^{-1})	$10^{-3}\ k_2^i$ (sec^{-1})	pK_{1i}	pK_{2i}	pK_{3i}
1	3.0	4.0	3.5	4.00	6.08[a]	6.40	5.20
2	4.0	6.5	3.7	0.200	5.40[b]	5.20	6.40
3	3.5	4.0	0.230	0.200		6.40	6.40
4	9.0	6.5	1.75	1.50		5.20	5.20
5			2.58	3.00		6.40[b]	6.60

[a] Determined in this work. [b] From steady-state kinetics (Del Rosario and Hammes, 1969).

Discussion

The relaxation processes characterizing the interaction of RNase with 3′-dUMP and with 3′-UMP (Hammes and Walz, 1969) can both be explained by the gross mechanism of eq 3. This is not surprising since 3′-dUMP differs from 3′-UMP by a single oxygen atom. Nevertheless, it was generally observed that the values of the relaxation times or the individual rate constants for the two nucleotides were significantly different as a function of pH. For example, at all comparable pH values, $1/\tau_2$ for 3′-dUMP binding is greater than the corresponding value for 3′-UMP binding (Hammes and Walz, 1969); at pH values approaching 7.0 this difference is approximately eightfold. Furthermore, values of the bimolecular rate constants for 3′-dUMP binding are the same (within experimental error) as those for 3′-UMP (Hammes and Walz, 1969) from pH 4.5 to 6.0; whereas, at pH values above 6.0 the bimolecular rate constant for 3′-dUMP increases with respect to that of 3′-UMP such that at pH 7.0 it is approximately seven times greater. This latter difference cannot be simply accounted for by the twofold difference in the secondary phosphate ionization constants for 3′-dUMP and 3′-UMP. It was also observed that the magnitude of the dissociation rate constant, k_{-1}, for 3′-dUMP binding is at most one-half that for 3′-UMP binding (Hammes and Walz, 1969) for all pH values tested.

Possible mechanisms were considered to interpret the pH dependence of the four rate constants in the generalized scheme for RNase·3′-dUMP binding in eq 3. An effort was made to formulate the simplest hypothesis involving a minimum number of acid-base groups at the interaction site of RNase. The protolytic reaction rates of acid-base groups directly participating in the enzyme nucleotide complex could be rate limiting if the associated protons are not continuous with the hydrogen-bonded solvent structure (Eigen and Hammes, 1963; Eigen *et al.*, 1964). Nevertheless, since rate-limiting protolytic reactions are not required to explain the data, all protolytic reactions were presumed to be much faster than the reactions characterized by τ_1 and τ_2.

The bimolecular rate constant, k_1, is considered a function of the ionizable groups on the free nucleotide and those at the interaction site on RNase. The monoanionic and dianionic species of 3′-dUMP are both capable of interaction with RNase (Hammes and Walz, 1969); therefore, the reaction of both forms of the ligand were considered. With this assumption the pH dependence of k_1 can be adequately accounted for by involving only one acid-base group at the interaction site of free RNase. This mechanism is shown in Figure 5. Using values for the various constants from Table III, the pH dependence of k_1 is given by

$$k_1 = \frac{k_1^1(\mathrm{H})^2 + k_1^2K_{11}(\mathrm{H}) + k_1^4K_{12}(\mathrm{H}) + k_1^3K_{11}K_{12}}{(\mathrm{H})^2 + (K_{11} + K_{12})[\mathrm{H}] + K_{11}K_{12}} \quad (5)$$

The calculated values are presented in Table II and generate the curved line in Figure 3. Such a complex mechanism is not necessary to satisfy the observed values of k_1, nevertheless, it is reasonable to suppose that a multiplicity of parallel paths exist considering the relative constancy of k_1 from pH 4.5 to pH 7.0. In any event, only one ionizable group on the free enzyme is implicated in the bimolecular reaction. This group was assigned a pK value of 5.4 as determined from steady-state kinetic studies of the RNase-catalyzed hydrolysis of uridine 2′:3′-cyclic phosphate (Del Rosario and Hammes, 1969). It should be noted that *two* dissociation constants on the free RNase were required to fit the data for the bimolecular reaction in RNase·3′-UMP binding (Hammes and Walz, 1969). Since the conformations of free 3′-UMP and free 3′-dUMP are most likely the same (*i.e.*, the base is in the anti conformation (Haschenmeyer and Rich, 1967; Schweizer *et al.*, 1968; Miles *et al.*, 1969)) and the pH dependence of the bimolecular rate constants for 3′-dUMP binding with RNase are different, it is apparent that some interaction of RNase with the 2′-hydroxyl group occurs during formation of the initial complex.

The pH dependence of the dissociation rate constant,

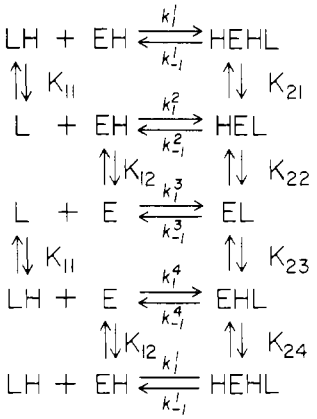

FIGURE 5: A possible mechanism for the initial interaction between RNase and 3′-dUMP. E and EH represent enzyme species, LH and L represent the monoanionic and dianionic species of 3′-dUMP, respectively. HEHL, HEL, and EL represent RNase·3′-dUMP complexes. Free protons were omitted for clarity. Values of the rate and ionization constants are given in Table III.

k_{-1}, is qualitatively similar to that of k_1 and is given by the values of the relevant constants in Table III and

$$k_{-1} = \frac{k_{-1}^{1}(\mathrm{H})^2 + k_{-1}^{2}K_{21}(\mathrm{H}) + k_{-1}^{4}K_{24}(\mathrm{H}) + k_{-1}^{3}K_{21}K_{23}}{(\mathrm{H})^2 + (K_{21} + K_{24})(\mathrm{H}) + K_{21}K_{23}} \quad (6)$$

The calculated values are presented in Table II and are illustrated as the theoretical curve in Figure 3. The microscopic dissociation constants of the initial complex are independent, *i.e.*, $pK_{21} = pK_{23}$ and $pK_{22} = pK_{24}$. Although macroscopic constants could fit the data reasonably well, microscopic constants were considered as more realistic as well as being compatible with the pH dependence of the isomerization of the initial complex (*vide infra*).

A mechanism for the pH-dependent isomerization of the RNase·3′-dUMP complex characterized by τ_2 is shown in Figure 6. This mechanism involves an additional acid-base group characterized by pK_{25} and pK_{35} which is *not* postulated to be involved in the initial reaction characterized by τ_1. The microscopic dissociation constants for the primed species shown in Figure 6 are dependent: *i.e.*, $pK_{31} \neq pK_{33}$ and $pK_{34} \neq pK_{32}$. For diagrammatic simplicity the dissociating group characterized by pK_{25} and pK_{35} is considered as a macroscopic constant. The values of k_2 and k_{-2} were calculated at different pH values by using the constants in Table III and eq 7, where for $i = 2; j = 2$ and for $l = -2; j = 3$.

$$k_i = \frac{k_i^{1}(\mathrm{H})^3 + k_i^{2}K_{j1}(\mathrm{H})^2 + k_i^{3}K_{j4}(\mathrm{H})^2 + k_i^{4}K_{j3}K_{j4}(\mathrm{H}) + k_i^{5}K_{j3}K_{j4}K_{j5}}{(\mathrm{H})^3 + (K_{j1} + K_{j4})(\mathrm{H})^2 + K_{j3}K_{j4}(\mathrm{H}) + K_{j3}K_{j4}K_{j5}} \quad (7)$$

Some of these calculated values are shown in Table II and the sum of the calculated values of k_2 and k_{-2} $(1/\tau_2)$ at different pH values is illustrated as the curved line in Figure 4. The good fit to the experimental data by the mechanism in Figures 5 and 6 using the constants in Table III is noteworthy since the constants are not independent and must reasonably satisfy eleven detailed balance relationships. Mechanisms utilizing only macroscopic dissociation constants required at least four dissociating groups in the isomerization reaction to approximately fit the sharp inflections characterizing the pH dependence of τ_2. Nevertheless, the proposed mechanism is not considered unique since, in addition to the assumptions made previously, multiple pH-dependent isomerizations of the RNase·3′-dUMP complex which are very rapid or closely coupled with τ_2 would escape detection but could contribute to the apparent rate constants. Furthermore, it is obvious that more complex mechanisms than those in Figures 5 and 6 could satisfy the data.

The pK values assigned to groups in the RNase·3′-dUMP complex as designated in Figure 6 are considerably lower than those comparably assigned for RNase·3′-UMP binding (Hammes and Walz, 1969) and are not obviously reconciled with the simple chemical mechanisms proposed to explain the pK perturbations associated with 3′-UMP binding (Hammes and Walz, 1969) and 3′-CMP binding (Meadows *et al.*, 1969). In an effort to clarify this point nuclear magnetic resonance studies are planned to independently assess the pK values of the active site imidazole groups of the RNase·3′-dUMP complex.

The acid-base groups on RNase which are proposed to participate in the binding interaction are tentatively identified as the imidazole groups of His-119 and His-12 (Crestfield *et al.*, 1963; Heinrikson *et al.*, 1965; Kartha *et al.*, 1967; Wykoff *et al.*, 1970; Meadows *et al.*, 1969). The assigned pK values for these groups (*i.e.*, pK_{12} and pK_{25}) are those reported from steady-state kinetics (Del Rosario and Hammes, 1969). The proposed mechanism for the pH-dependent isomerization of the RNase·3′-dUMP complex requires that the critical dissociating groups are present only at the interaction site. This proposal differs from that made for the isomerization of the RNase·3′-UMP complex which was postulated to be dependent, for the most part, on a dissociating group (His-48 imidazole) extraneous to the interaction site (Hammes and Walz, 1969). Since such a group need not be implicated in the isomerization of RNase·3′-dUMP complex, it is possible that the isomerization process is linked with His-48 only when a 2′-hydroxyl group is present in the nucleotide.

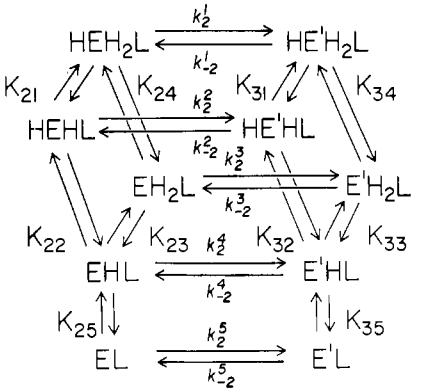

FIGURE 6: A possible mechanism for the isomerization of the RNase. 3′-dUMP complex. Values for the rate and equilibrium constants are given in Table III.

The differences in the kinetic constants for RNase binding with 3′-UMP and 3′-dUMP can be understood on a nonformalistic basis by postulating that the interaction site of RNase consists of a nonpolar locus adjacent to the 2′-hydroxyl group in the RNase·3′-UMP complex and to the 2′-hydrogen in the RNase·3′-dUMP complex. It would be expected that interaction of the polar 2′-hydroxyl group with such a locus would be unfavorable whereas that of the 2′-hydrogen would be favorable. In terms of this hypothesis the different pH dependence in the bimolecular reactions of RNase with 3′-UMP and 3′-dUMP can be understood as resulting from constraints imposed on the 3′-phosphate interaction with the active-site imidazole groups by the interaction of the 2′ substituent with RNase; *i.e.*, the "negative" interactions imposed by the 2′-hydroxyl group in the RNase·3′-UMP complex localizes the phosphate group such that interaction of this group in the bimolecular reaction is dependent on the charged state of *two* imidazole groups, whereas, the "positive" interaction of the 2′-hydrogen in the RNase·3′-dUMP complex localizes the phosphate group such that the charged state of only *one* imidazole group is kinetically significant. The fact that 3′-dUMP shows no preferential binding in the bimolecular step with the different isomeric species of RNase while some evidence suggests that 3′-UMP preferentially binds the acid-stable isomer (Hammes and Walz, 1969) could indicate that the different isomers of RNase accommodate different conformations of the proposed nonpolar locus which are selective when the 2′-hydroxyl group is present (*i.e.*, for 3′-UMP)

and nonselective when a 2′-hydrogen is present (*i.e.*, for 3′-dUMP). The postulated interactions of RNase at the 2′ position of the nucleotide are consistent with the observations that the initial RNase·3′-dUMP complex is more stable than the initial RNase·3′-UMP complex as indicated by the relative values of the dissociation rate constant (*vide supra*). The two isomeric states of the RNase·3′-UMP complex could reflect not only alterations in the phosphate interactions (Hammes and Walz, 1969; Meadows and Jardetzky, 1968) but also the degree of interaction between the 2′-hydroxyl group and the postulated nonpolar locus. This suggestion is fortified by the observation that the rate constants for isomerization of the 3′-dUMP complex (*i.e.*, k_2 and k_{-2}) are significantly greater (approximately twofold) than those for the 3′-UMP complex (Hammes and Walz, 1969).

Most catalytic mechanisms proposed for RNase predict a proton transfer from the 2′-hydroxyl group of the substrate to an imidazole group at the active site of the enzyme. In the context of the present hypothesis it is plausible that the postulated nonpolar locus adjacent to the 2′-hydroxyl group in a given isomeric state of the RNase·3′-UMP complex provides a suitable microenvironment to facilitate proton transfer or otherwise enhance the formation of a negatively charged 2′-nucleophile. This proposal is consistent with a previous suggestion that the RNase-catalyzed transesterification of cytidylyl-3′:5′-adenosine is rate limited by a conformational change (Hammes and Walz, 1970).

This hypothesis regarding the nature of the 2′-hydroxyl group interaction with RNase is consistent with the present kinetic observations and provides some attractive possibilities regarding a catalytic mechanism, nevertheless, these speculations should only be considered tentative since other proposals could equally account for the data. Regardless of the nature of this interaction, the contrast in the binding kinetics of RNase with 3′-dUMP and 3′-UMP gives evidence that the bound 2′-hydroxyl group of 3′-UMP interacts with RNase in a specific fashion that influences the 3′-phosphate group interactions with the enzyme as well as the isomerization process of the RNase·nucleotide complex.

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