Binding of Phosphate Ligands to Ribonuclease A*

David G. Anderson,† Gordon G. Hammes,‡ and Frederick G. Walz, Jr.§

ABSTRACT: Association constants for ribonuclease (RNase) binding with cytidine 2′-monophosphate (2′-CMP), cytidine 3′-monophosphate (3′-CMP), uridine 3′-monophosphate (3′-UMP), orthophosphate, and pyrophosphate were determined at 25° as a function of pH and the relevant ionization constants of these phosphate ligands were measured. RNase–2′-UMP binding was studied at pH 5.5. The association constants are all maximal around pH 5.6. If the assumption is made that the monoanions of 2′-CMP, 3′-CMP, 3′-UMP, and orthophosphate and the dianion of pyrophosphate are the ionized species that bind with RNase, the pH profiles of the association constants for all ligands are quite similar except for 2′-CMP. The simplest possible interpretation of these results requires participation in the binding process of two ionizable groups on the free enzyme with approximate pK_a values of 5 and 6.5, except for 2′-CMP which requires an additional group having a pK_a around 5. The association constants for 2′-CMP and 3′-CMP are smaller in 0.1 M KNO_3 than in 0.1 M KCl; however this inhibition is somewhat different for the 2′ and 3′ isomers of CMP. The protonated and nonprotonated cytosine ring species of 2′- and 3′-CMP bind equally well with RNase. The association constants at pH 5.5 are in the order: 2′-CMP > 2′-UMP > 3′-CMP > 3′-UMP > pyrophosphate > orthophosphate. The interactions of the nucleotide bases with RNase are discussed in terms of the association constants and the difference spectra of the RNase–nucleotide complex.

Previous studies of RNase[1] binding with a variety of phosphate-containing ligands including 2′-CMP (Hummel *et al.*, 1961; Nelson and Hummel, 1961; Hummel and Dreyer, 1962; Barnard and Ramel, 1962; Hummel and Witzel, 1966), 3′-CMP (Hummel *et al.*, 1961; Hummel and Witzel, 1966; Herries *et al.*, 1962; Hammes and Schimmel, 1965), 3′-UMP (Cheung and Abrash, 1964), orthophosphate (Nelson *et al.*, 1962), and pyrophosphate (Nelson *et al.*, 1962) have been reported. However, comprehensive investigations of the pH dependence of the RNase–ligand association constants have not been undertaken. Kinetic studies of the interaction of some of these ligands with RNase have been carried out (Cathou and Hammes, 1965; Erman and Hammes, 1966), but interpretation of the pH dependence of the kinetic parameters requires a knowledge of which ionized state of the ligands binds to the enzyme. In an effort to determine the ionized state of the phosphate groups that is bound to the enzyme, the association constants of RNase with the above ligands as a function of pH and the relevant ionization constants of the phosphate ligands were determined. The association constants for the nucleotides with RNase were obtained by a difference absorption spectra technique (Hummel *et al.*, 1961; Hammes and Schimmel, 1965), while measurements of the inhibition of the RNase-catalyzed hydrolysis of 2′,3′-cyclic CMP were employed to determine the association constants for orthophosphate and pyrophosphate.

Information with regard to which of the ionized states of the cytosine ring of 2′- and 3′-CMP binds to RNase was obtained from the pH dependence of the difference spectra accompanying the interaction of these nucleotides with RNase. A comparison of the contributions to RNase–nucleotide binding of the protonated and nonprotonated base species of 2′- and 3′-CMP and the uracil moiety of 2′- and 3′-UMP is presented.

Experimental Section

Materials

The 3′ isomer of CMP was prepared from 2′,3′-cyclic CMP as previously described (Cathou and Hammes, 1964). The 280 mμ/260 mμ absorbance ratio at pH 7.0 was 0.93 (reported value, 0.93; Beaven *et al.*, 1955). The concentration of 3′-CMP was determined spectrophotometrically at 260 mμ using an extinction coefficient of 7600 cm^{-1} M^{-1} (Beaven *et al.*, 1955). The 2′ isomer of CMP was prepared from a mixture of 2′- and 3′-CMP (Sigma Chemical Co.) by column chromatography using Dowex 1-X10 (Hummel *et al.*, 1961). The two isomers were quantitatively separated and the isolated 2′-CMP had a 280 mμ/260 mμ ratio at pH 7.0 of 0.86 (reported value, 0.85; Beaven *et al.*, 1955). The concentration of 2′-CMP was determined spectrophotometrically at 260 mμ using an extinction coefficient of 7600 cm^{-1} M^{-1} at pH 7.0 (Beaven *et al.*, 1955). The mixed 2′ and 3′ isomers of UMP (P. L. Biochemicals,

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† Present address: College of Medicine, Department of Gynecology and Obstetrics, Ohio State University, Columbus, Ohio.

‡ To whom reprint requests should be directed.

§ National Institutes of Health postdoctoral fellow.

[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: RNase, bovine pancreatic ribonuclease A.

Inc.) were quantitatively separated by column chromatography using Dowex 1-X10 (Cohn and Khym, 1957). The isolated 3′-UMP had a 280 mμ/260 mμ ratio of 0.36 at pH 7.0 (reported value, 0.35; Beaven *et al.*, 1955); the same ratio for the isolated 2′-UMP was 0.31 (reported value, 0.30; Beaven *et al.*, 1955). The concentrations of both isomers were determined spectrophotometrically at 260 mμ employing an extinction coefficient of 10,000 cm^{-1} M^{-1} at pH 7.0 (Beaven *et al.*, 1955).

Mixed 2′ and 3′ isomers of CMP were used to prepare 2′,3′-cyclic CMP (Szer and Shugar, 1963). Paper chromatography of this material employing a solvent system of isopropyl alcohol–saturated ammonium sulfate–1.0 M sodium acetate (pH 6.6) (2:80:18, v/v; Markham and Smith, 1952) yielded a single spot having a R_F value of 0.42 (2′-CMP and 3′-CMP had R_F values of 0.66 and 0.68, respectively). The frequently used solvent system composed of isopropyl alcohol–water–NH_4OH (Brown *et al.*, 1952) was avoided since it was found to cause significant hydrolysis during chromatography. The concentration of 2′,3′-cyclic CMP was measured spectrophotometrically at 268 mμ using an extinction coefficient of 8400 cm^{-1} M^{-1} at pH 7.0 (Brown *et al.*, 1952).

Bovine pancreatic RNase A (EC 2.7.7.16) was obtained as a phosphate-free lyophilized powder from the Worthington Biochemical Corp. and was used without further purification. The concentration of RNase was determined spectrophotometrically at 278 mμ using an extinction coefficient of 9800 cm^{-1} M^{-1} (Sela and Anfinsen, 1957). Sodium dihydrogen phosphate monohydrate and sodium pyrophosphate decahydrate (100.0%) were reagent grade chemicals obtained from the J. T. Baker Chemical Co.; Tris was a three-times-crystallized product of the Nutritional Biochemical Co. All other chemicals were reagent grade. Distilled, deionized water was used to prepare all solutions.

Methods

Difference Spectrophotometry. Difference spectra were determined using a Zeiss PMQ II spectrophotometer. Rectangular tandem cells (Pyrocell Manufacturing Co.) were employed in a thermostated cell compartment. All experiments were conducted at 25 ± 0.1°. The slit width was maintained at 0.2 mm for all measurements. A stock buffer solution of 0.05 M Tris, 0.05 M sodium acetate, and 0.1 M KNO_3 was used in all experiments except when 0.1 M KCl was employed in place of 0.1 M KNO_3. For each experiment the pH was lowered to the desired value by the addition of acetic acid; the resulting ionic strength was 0.2 M over the pH range investigated. All pH measurements were made at 25° using a Radiometer PHM-26 meter; the error in pH measurement is estimated to be ±0.03 pH unit.

The difference spectra were determined directly. One set of tandem cells contained RNase and nucleotide in separate compartments, while the other set contained the same concentrations of RNase and nucleotide in one compartment and buffer in the other. The concentration of RNase was approximately 7×10^{-5} M for each experiment and nucleotide concentrations ranged from 1×10^{-5} to 2.5×10^{-4} M. At each pH at least fifteen experiments at different nucleotide concentrations were performed. The difference spectra for cytidine nucleotides were taken from 255 to 300 mμ and for uridine nucleotides from 240 to 290 mμ at 1-mμ intervals. The spectra of the nucleotides were determined under identical conditions of buffer and pH.

Inhibition Kinetics. The kinetics of the hydrolysis of 2′,3′-cyclic CMP catalyzed by RNase was studied by the differential spectrophotometric method (Crook *et al.*, 1960) using the 90–100% transmission scale on a Beckman DK-2A recording spectrophotometer. The slit width was held constant at 0.2 or 0.4 mm for any given series of experiments. The temperature was maintained at 25 ± 0.1° for all measurements by employing a thermostated cell holder. A pen speed of 9 cm/min was used to record the progress of the reaction. Although the difference spectrum of 2′,3′-cyclic CMP and 3′-CMP is maximal at 285 mμ (Crook *et al.*, 1960), measurements were performed at 292 mμ. At this wavelength higher substrate concentrations could be employed because the magnitude of the difference spectrum decreased less at this longer wavelength than the absorption of the substrate.

The buffer solution used was 0.05 M Tris, 0.05 M sodium acetate, and 0.1 M NaCl. The pH was adjusted to the desired value by the addition of acetic acid. At every pH three different concentrations of substrate were employed at five different inhibitor concentrations, and each experiment was performed in duplicate. The concentration range of 2′,3′-cyclic CMP was 2×10^{-4} to 1.3×10^{-3} M; for pyrophosphate, 2×10^{-4} to 2×10^{-3} M; and for orthophosphate, 4×10^{-3} to 4×10^{-2} M. The initial velocity was determined in all experiments and had an estimated uncertainty of ± 5%.

Ionization Constants. Carbon dioxide free NaOH solutions were prepared (Kolthoff and Sandell, 1952) and standardized against potassium acid phthalate. HCl solutions were standardized using the standardized NaOH solutions. All solutions were maintained at 25 ± 0.1° and were titrated under nitrogen. The pH of the solutions was measured to the nearest 0.01 pH unit using a Radiometer PHM-26 pH meter. The titrations of sodium dihydrogen phosphate and sodium pyrophosphate were performed in the presence of 0.2 M NaCl and the titrations of 2′-CMP and 3′-UMP were performed in the presence of 0.2 M KNO_3. Blank titrations were performed and the corresponding corrections were made on the titration curves. The pK_a values obtained represent an average of six to ten pK_a values calculated according to the method described by Albert and Serjeant (1962). All titrations were reversible and were performed in duplicate or triplicate. The pK_a values from repeated titrations agreed to within 0.01 and the estimated accuracy is ±0.02.

Results

The relevant ionization constants of the ligands were measured at the same ionic strength (0.2 M) as the association constants. The ionization constants (K_a) reported in Table I are defined in terms of the hydrogen

TABLE I: Ligand Ionization Constants.[a]

Ligand	Dissociation	pK_a (M)
Orthophosphate	Secondary	6.67
Pyrophosphate	Tertiary	5.97
3′-UMP	Secondary phosphate	5.74
2′-CMP	Secondary phosphate	6.02
	Ring nitrogen-3	4.32
3′-CMP[b]	Secondary phosphate	5.90
	Ring nitrogen-3	4.32

[a] At 25°, other conditions as described under Experimental Section. [b] Bahr *et al.* (1965).

ion activity (a_{H^+}) as measured by the glass electrode where $K_a = c_A a_{H^+}/c_{HA}$, and c_A and c_{HA} are the concentrations of nonprotonated and protonated ligand species, respectively. The phosphate groups of the ligands studied have only one ionizable proton in the pH range where the association constants were determined. The variation of pK_a among the ligands is small except for orthophosphate which has a pK_a 0.7–0.9 unit higher than the other ligands. The ionization constants of the ring nitrogen-3 were found to be the same for the 2′ and 3′ isomers of CMP.

The stoichiometries of RNase complexes with 2′-CMP, 3′-CMP, and 3′-UMP were investigated by the method of continuous variations: the total concentration of RNase and nucleotide was held constant and the relative concentrations of the enzyme and ligand were varied. The difference absorbance was used as a measure of complex formation. As shown in Figure 1 the maximum difference absorbance for all nucleotides was achieved at a 1:1 ratio of RNase and nucleotide. The extrapolated straight-line portions of the curve intersect at this same ratio. The 1:1 stoichiometry found for RNase–2′-CMP binding corroborates the results of previous studies (Barnard and Ramel, 1962; Nelson *et al.*, 1962). Unfortunately the stoichiometry of the orthophosphate and pyrophosphate interactions with RNase cannot be determined in a similar manner; however the fact that these compounds are simple competitive inhibitors of the catalytic reaction suggests a 1:1 stoichiometry.

The difference spectra between RNase–nucleotide and free RNase plus free nucleotide solutions were measured as a function of concentration and pH for 2′-CMP, 3′-CMP, and 3′-UMP. RNase-2′-UMP binding was studied at pH 5.5 only. At each pH the molar difference extinction ($\Delta\epsilon$) and the association constant (K) were calculated using the equation (Hammes and Schimmel, 1965)

$$K = \frac{\Delta a/\Delta\epsilon}{[(E_0) - \Delta a/\Delta\epsilon][(L_0) - \Delta a/\Delta\epsilon]} = \frac{(EL)}{(E)(L)}$$

where (E_0) and (L_0) are the total concentrations of RNase and nucleotide, respectively, Δa is the measured difference absorbance at a given wavelength, (EL) is the concentration of the RNase–nucleotide complex, and (E) and (L) are the concentrations of free RNase and nucleotide, respectively. Usually Δa was measured at the wavelength at which its magnitude was a maximum. After assuming a value of $\Delta\epsilon$, K was calculated for all nucleotide concentrations using the measured values of Δa. The best value of $\Delta\epsilon$ was taken to be that which minimized the standard deviation (as per cent) in K. At pH 4.5 and 7.0 for the 3′-nucleotides and at pH 7.5 for 2′-CMP, the binding of the ligand to the enzyme is so weak that a wide range of degree of saturation of the enzyme could not be investigated. In these cases the minimization technique was insufficient to obtain $\Delta\epsilon$ and K. Instead a value of $\Delta\epsilon$ was assumed which was equal to $\Delta\epsilon$ at the wavelength corresponding to the isobestic point of difference spectra found at the other pH values for cytidine ligands; $\Delta\epsilon$ was assumed invariant with pH for 3′-UMP. The error in the values reported for K are estimated to be approximately ±15%, while the error associated with the maximum absolute values of $\Delta\epsilon$ ($\Delta\epsilon_{max}$) is about ±7%. A summary of the values of K and $\Delta\epsilon_{max}$ for the various nucleotides investigated are reported in Table II. The only reported value of $\Delta\epsilon$ for RNase–2′-CMP binding (Hummel *et al.*, 1961) is in good agreement with those reported in the present study. Likewise, the association constants for RNase–2′-CMP

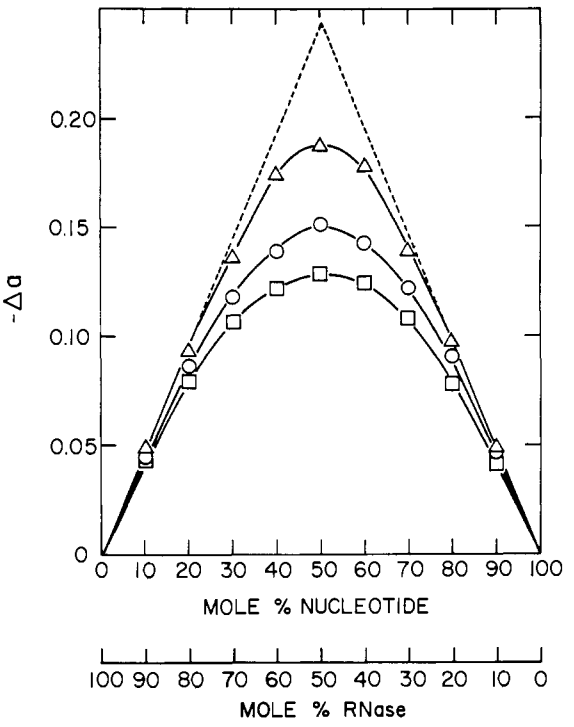

FIGURE 1: Plot of the difference absorbance as a function of the relative concentrations of RNase and nucleotide. Experiments were conducted at pH 5.5 in 0.1 M KNO_3 for 2′-CMP (△) and in 0.1 M KCl for 3′-CMP (○) and 3′-UMP (□). The difference absorbance was measured at 262 mμ for 2′- and 3′-CMP and 255 mμ for 3′-UMP; the total concentration of RNase plus nucleotide was 1.76×10^{-4} M for 2′-CMP, 1.88×10^{-4} M for 3′-CMP, and 1.86×10^{-4} M for 3′-UMP; other conditions were as described under Experimental Section.

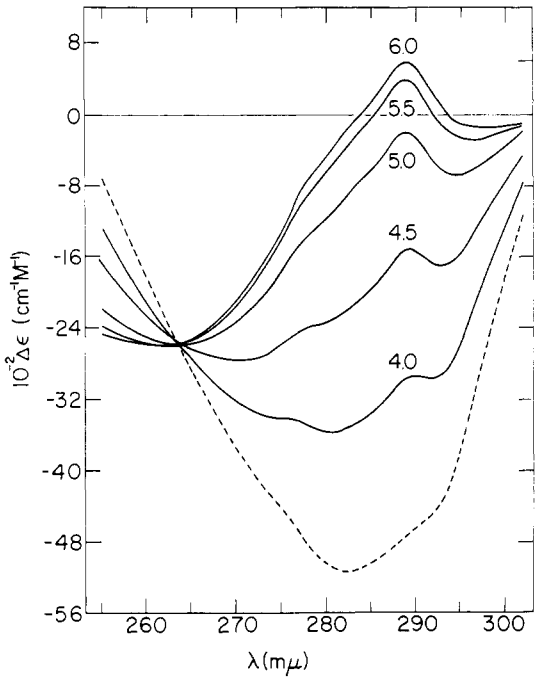

FIGURE 2: Difference absorption spectra of RNase–2′-CMP binding as a function of pH. The numbers represent the pH value for the corresponding spectrum; other conditions were as described under Experimental Section; the dashed line is the difference absorption spectra calculated for the protonated ring nitrogen-3 species of 2′-CMP (see text for details).

binding reported previously (Hummel *et al.*, 1961; Herries *et al.*, 1962) are consistent with the present data; however the association constants reported for 3′-CMP (Herries *et al.*, 1962) and 3′-UMP (Cheung and Abrash, 1964) binding differ significantly from those reported here. These deviations can be explained, in part, by the fact that different anions were present in the various binding experiments. The present data indicate a specific anion effect on ligand binding. The association constants for 2′- or 3′-CMP, at the same pH, are always significantly larger when determined in the presence of 0.1 M KCl as compared with those determined in the presence of 0.1 M KNO_3. The binding constants for 3′-CMP at 25° previously reported from this laboratory (Hammes and Schimmel, 1965) differ significantly (~50%) from those reported here only at pH values of 5.5 and below. The constants reported here are considered more reliable since more comprehensive data were obtained, and the experimental arrangement employed in the present investigation is considerably improved over that previously used.

The molar difference extinction coefficient characterizing RNase–2′-CMP binding as a function of wavelength and pH is presented in Figure 2. Note that the difference spectra at different pH values have an isosbestic point around 264 mμ. Furthermore, the more complex spectra (*e.g.*, at pH 4.5) can be considered as composite spectra which can be resolved into two simple difference spectra. In order to obtain the molar extinction coefficient of the RNase–nucleotide complex from $\Delta\epsilon$ the molar extinction coefficients of RNase and the nucleotide must be known since (Hammes and Schimmel, 1965) $\Delta\epsilon = \epsilon_E + \epsilon_L - \epsilon_{EL}$, where ϵ_E, ϵ_L, and ϵ_{EL} are the molar extinction coefficients of RNase, nucleotide, and RNase–nucleotide complex, respectively. The spectrum of RNase was found to be invariant with pH in the range pH 4.0–7.5. In this same pH range the spectra of 2′-CMP and 3′-CMP showed considerable variation due to protonation of the ring nitrogen-3, while the spectra of 3′-UMP changed insignificantly. As seen in Figure 3 the spectra of 2′- and 3′-CMP as a function of pH have isosbestic points around 263 and 265 mμ, respectively. (Previously reported spectra of 3′-CMP at 25° from this laboratory are erroneous; Hammes and Schimmel, 1965.) Similarly, the calculated spectra of the RNase–2′-CMP and RNase–3′-CMP complexes as a function of pH (Figure 4) have isosbestic points around 263 and 264 mμ, respectively. Moreover, in most other respects RNase–CMP complexes have spectra as a function of pH that are closely analogous to their corresponding free nucleotide spectra as a function of pH. This *prima facie* evidence that the protonated

TABLE II: Association Constants for RNase–Nucleotide Binding.

Nucleotide	pH	λ_{max} (mμ)	$10^{-3}\cdot\Delta\epsilon_{max}$[a] ($cm^{-1}$ M^{-1})	$10^{-3}K$ (M^{-1})
2′-CMP	4.0	275	3.43	3.83
	4.5	269	2.78	28.5
	5.0	264	2.60	111
	5.5	262	2.60	180
	5.5[b]	262	2.88	296
	6.0	262	2.60	142
	6.5	262	2.50	49.8
	7.0	262	2.55	6.75
	7.5	262	2.55	0.881
3′-CMP	4.5	269	3.82	2.95
	5.0	264	3.47	7.53
	5.5	262	3.40	12.1
	5.5[b]	262	3.15	26.8
	6.0	262	3.45	9.68
	6.5	262	3.40	5.23
	6.5[b]	262	3.05	9.80
	7.0	262	3.40	1.61
3′-UMP[b]	4.5	255	3.50	3.86
	5.0	255	3.50	8.96
	5.5	255	3.60	14.2
	6.0	255	3.65	12.2
	6.5	255	3.65	6.06
	7.0	255	3.65	2.27
2′-UMP[b]	5.5	254	3.13	140

[a] Maximum absolute value of $\Delta\epsilon$. [b] Experiments performed in the presence of 0.1 M KCl in place of 0.1 M KNO_3; other conditions were as described under Experimental Section.

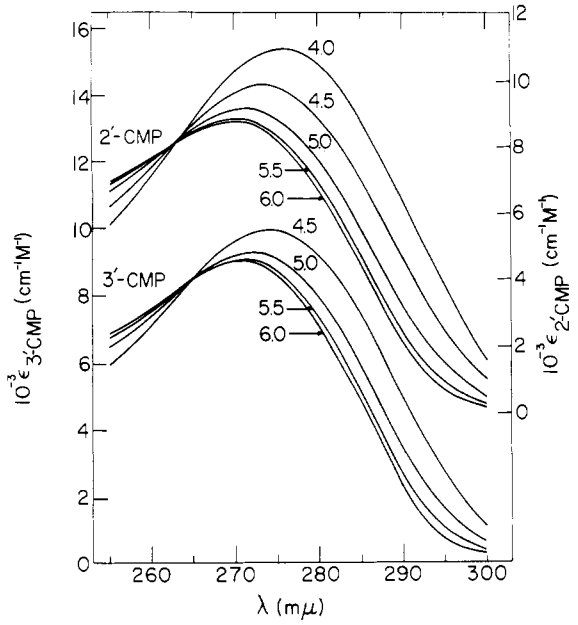

FIGURE 3: The absorption spectra of 2′- and 3′-CMP as a function of pH. The numbers represent the pH value for the corresponding spectrum; other conditions were as described under Experimental Section.

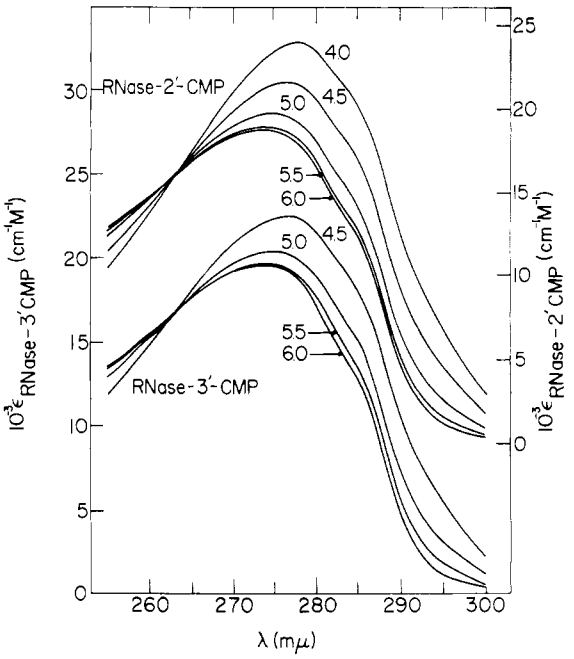

FIGURE 4: The absorption spectra of RNase complexes with 2′- and 3′-CMP as a function of pH. The numbers represent the pH value of the corresponding spectrum; spectra were calculated as indicated in the text, and conditions were as described under Experimental Section.

nitrogen-3 species of the cytidine nucleotides is bound to RNase can be elaborated by calculating the ring nitrogen-3 ionization constants of the bound cytidylic acid isomers using the equation

$$K_a = a_{H^+}\left(\frac{\epsilon_{HA} - \epsilon}{\epsilon - \epsilon_A}\right)$$

where ϵ is the observed molar extinction coefficient at a given value of a_{H^+}, and ϵ_{HA} and ϵ_A are the molar extinction coefficients of the protonated and nonprotonated ring nitrogen-3 species of the RNase–CMP complexes (or the corresponding free nucleotides). Values of K_a were calculated for different values of ϵ_{HA} (a_{H^+}, ϵ, and ϵ_A being known). The best value of ϵ_{HA} was taken to be that which minimized the standard deviation (as per cent) of K_a calculated for the experimental values of a_{H^+} and ϵ. This fitting procedure gave calculated values of K_a for RNase–2′-CMP and RNase–3′-CMP complexes (and their corresponding free nucleotides) which all agreed, within experimental error, with the value determined independently by pH titration of the free nucleotides (see Table I).

Using a pK_a value of 4.32 for the ring nitrogen-3 ionization of the RNase–2′-CMP complex, the molar difference extinction coefficient of the protonated ring nitrogen-3 species of the complex, $\Delta\epsilon_{HA}$, can be calculated as a function of wavelength employing the equation

$$\Delta\epsilon_{HA} = \frac{K_a}{a_{H^+}}(\Delta\epsilon - \Delta\epsilon_A) + \Delta\epsilon$$

where $\Delta\epsilon$ is the observed molar difference extinction coefficient for a given value of a_{H^+}, and $\Delta\epsilon_A$ is the molar difference extinction coefficient of the nonprotonated ring nitrogen-3 species of the complex. The calculated difference spectrum of the protonated ring nitrogen-3 species of the RNase–2′-CMP complex is included in Figure 2; the difference spectrum of the nonprotonated ring nitrogen-3 species of the complex is essentially identical with the difference spectrum at pH 6.0. Unlike 2′- and 3′-CMP, the difference spectra resulting from 3′-UMP binding with RNase and the spectra of free 3′-UMP are essentially invariant with pH in the range pH 4.5–7.0; consequently the calculated spectra of the RNase–3′-UMP complex is the same throughout this pH range. A representative difference spectrum and a

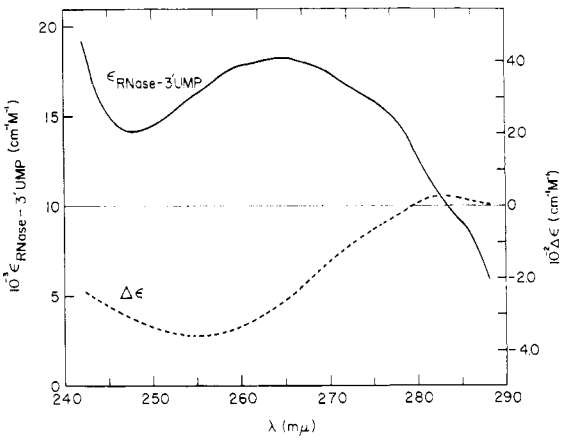

FIGURE 5: A difference absorption spectrum of RNase-3′-UMP binding and a spectrum of the RNase-3′-UMP complex. Conditions for the difference spectra determinations were as described under Experimental Section. The spectrum of the RNase-3′-UMP complex was computed as described in the text.

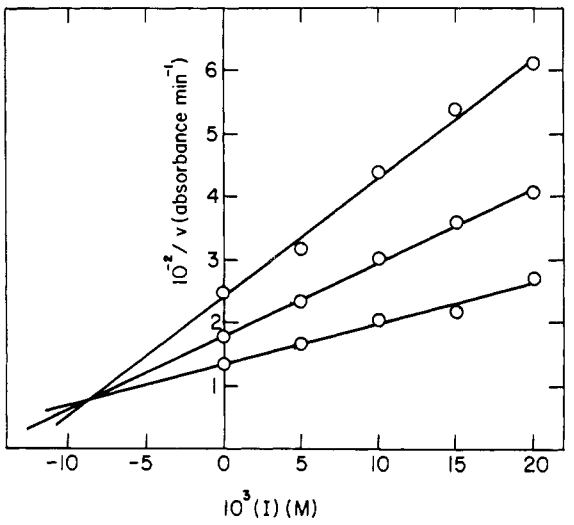

FIGURE 6: Plot of the reciprocal of the initial velocity of the RNase-catalyzed hydrolysis of 2',3'-cyclic CMP *vs.* inhibitor concentration at pH 5.0 with orthophosphate as the inhibitor and 10^{-7} M ribonuclease; substrate concentrations were 3.76×10^{-4} M (top line), 6.26×10^{-4} M (middle line), and 1.13×10^{-3} M (bottom line); other conditions were as described under Experimental Section. The lines were obtained by a least-squares analysis.

spectrum of the RNase–3'-UMP complex are presented in Figure 5.

The inhibition constants for orthophosphate and pyrophosphate were determined from the initial velocity data. The reciprocal of the initial velocity was plotted *vs.* the inhibitor concentration at constant substrate concentration according to the equation

$$\frac{1}{v} = \frac{K_M + (S)}{V_S(S)} + \frac{K_M K_I(I)}{V_S(S)}$$

where v is the initial velocity, V_S is the maximal velocity, (S) is the substrate concentration, (I) is the inhibitor concentration (*i.e.*, orthophosphate or pyrophosphate), K_M is the Michaelis constant, and K_I is the inhibition *association* constant. The linear plots for each substrate concentration intersect at an inhibitor concentration equal to the negative reciprocal of the inhibition association constant. A typical plot of the data is shown in Figure 6. In cases where the experimental scatter was such that fitting of the graphical data by eye was ambiguous, a least-squares analysis was employed. The inhibition association constants are summarized in Table III. The estimated uncertainty in these constants is ±15%.

The pH dependence of the association constants for 2'-CMP, 3'-CMP, 3'-UMP, orthophosphate, and pyrophosphate are presented in Figure 7. The association constants for all ligands are maximal around pH 5.6, although the maxima are considerably broader in the cases of pyrophosphate and orthophosphate. The absolute values of the slopes of the ascending and descending limbs of the log K–pH binding curve for 2'-CMP are approximately two, whereas the slopes of all the other curves are approximately one.

TABLE III: Association Constants for Orthophosphate and Pyrophosphate.[a]

pH	K (M^{-1})	
	P[b]	PP[b]
4.02	36.1	1610
4.50	85.0	4300
5.00	116	6500
5.25	164	7100
5.50	235	6700
5.75	231	8000
6.00	217	5800
6.50	154	2560
7.00	69.0	750
7.50	24.4	196
7.98	18.5	139

[a] Determined as inhibition constants of the reaction of RNase with 2',3'-cyclic CMP as described under Experimental Section. [b] P = orthophosphate; PP = pyrophosphate.

Discussion

Previous investigators (Hummel and Witzel, 1966; Hammes and Schimmel, 1965; Deavin *et al.*, 1966) have suggested that the protonated ring nitrogen-3 species of 2'- and 3'-CMP is *not* bound to RNase (this conclusion in one case was based on erroneous spectral data for the nucleotide; Hammes and Schimmel, 1965). The results presented here convincingly indicate that the RNase-bound CMP can accommodate a positive charge on the cytosine ring. The change in absorption of the RNase–CMP complexes in the pH range 5.5–4.0 might conceivably be due to titration of an adjacent charge group on the enzyme which perturbs the spectrum of the bound nonprotonated cytosine moiety of the nucleotide. However, this seems unlikely since the spectrum of the RNase–3'-UMP complex exhibited no alteration in this pH range. Because the calculated K_a values of the RNase-bound ring nitrogen-3 ionizations are essentially the same as the free nucleotides, the association constants of RNase with the protonated ring nitrogen-3 and the nonprotonated ring nitrogen-3 species of the free nucleotide must also be the same according to the principle of detailed balance. The fact that both protonated and nonprotonated cytosine species of 3'-CMP bind equivalently is also suggested by the similar effect of pH on log K for 3'-UMP and 3'-CMP binding in the pH range where the ring nitrogen-3 of 3'-CMP is being protonated (see Figure 7).

Unfortunately, the data are not as clear with regard to which ionized form of the ligand phosphate group is bound to RNase. If the assumption is made that only the monoanions of 2'-CMP, 3'-CMP, 3'-UMP, and orthophosphate and the dianion of pyrophosphate are the ligand species which bind to the enzyme, a corrected binding constant expressed in terms of only the mono-

anion or dianion concentration can be calculated by multiplying the experimentally determined association constant by $(1 + K_a/a_{H^+})$, where K_a is the relevant ionization constant of the ligand. The pH dependence of the resulting constant involves only the ionizable groups of the free enzyme and enzyme–ligand complex and not the ionizable groups of the ligand (if the above stated assumption is correct). The pH dependence of these corrected constants is shown as a dotted line in Figure 7 for 2′-CMP, pyrophosphate, and orthophosphate. The pH dependence of the corrected constants for 3′-CMP and 3′-UMP binding is essentially identical with that of the corrected constants for pyrophosphate above pH 5. With this assumption, the same pH dependence of the association constant is observed for all ligands except 2′-CMP. However, it should be noted that only orthophosphate has an ionizable group with a pK_a value appreciably different from the other ligands. While this finding suggests that the monoanions of 3′-CMP, 3′-UMP, and orthophosphate and the dianion of pyrophosphate are the species initially binding to RNase, it can certainly not be regarded as proof because of the complex manner in which binding constants can depend on pH.

A detailed interpretation of the pH dependence of the association constants found for the different ligands with RNase is impossible without making simplifying assumptions regarding the acid dissociations of the enzyme–ligand complexes. Nevertheless, in concert with knowledge accumulated from studies of the relaxation spectrum of RNase with various ligands (Cathou and Hammes, 1965; Erman and Hammes, 1966), some suggestive speculations can be made. The curves for the corrected association constants *vs.* pH for orthophosphate and pyrophosphate both inflect upward in the pH region 7.5–8.0 (see Figure 7). The simplest interpretation of this result is that both orthophosphate- and pyrophosphate–RNase complexes deprotonate in this pH range. The pH dependence of the association constants of orthophosphate, pyrophosphate, 3′-CMP, and 3′-UMP are consistent with previous kinetic studies on RNase–3′-CMP binding (Cathou and Hammes, 1965), and can be interpreted as due to the involvement of at least two groups on the free enzyme having pK_a values of approximately 5 and 6.5. The pH dependence of the steady-state parameter V_S/K_S (maximal velocity/Michaelis constant) for the RNase-catalyzed hydrolysis of 2′,3′-cyclic cytidine phosphate (Herries *et al.*, 1962) and transesterification of uridylyl-(3′–5)-uridine and uridylyl-(3′–5′)-adenosine (Witzel, 1963) suggests the participation of ionizable groups on the free enzyme with similar pK_a values. The binding of 2′-CMF with RNase is unique as a function of pH when compared with the above ligands in two respects: first, the assumption that only the monoanion binds does not normalize the pH dependence of the 2′-CMP association constants with respect to those found for the association constants of the other ligands (see Figure 7); second, *two* titratable groups on the free enzyme having pK_a values around 5.0 appear to be necessary to accommodate the pH dependence of the association constants in the pH range 4.0–5.5 (see Figure 7). The fact that correction for

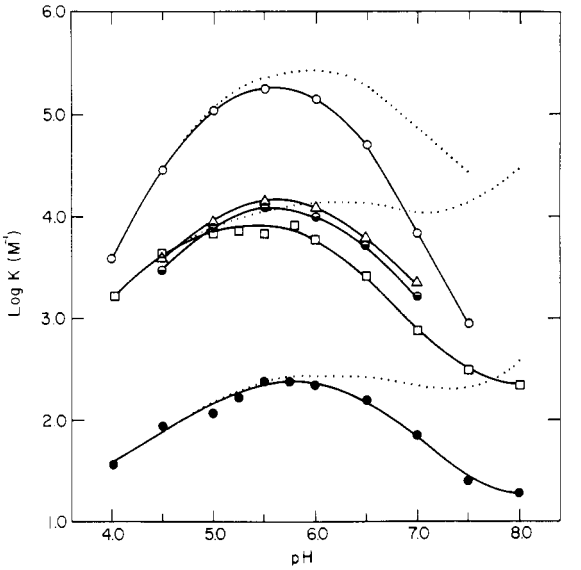

FIGURE 7: The logarithms of the RNase–ligand association constants as a function of pH. All experiments were conducted at 25° in 0.05 M Tris and 0.05 M sodium acetate; association constants for orthophosphate (●) and pyrophosphate (□) were obtained from kinetic inhibition studies, and those for 2′-CMP (○), 3′-CMP (◒), and 3′-UMP (△) from difference spectrophotometry; association constants for orthophosphate and pyrophosphate were measured in 0.1 M NaCl, 2′-CMP and 3′-CMP in 0.1 M KNO_3, and 3′-UMP in 0.1 M KCl; other conditions were as described under Experimental Section. The dotted lines are values of the association constants calculated with the assumption that only monoanion binds for 2′-CMP and orthophosphate, and only dianion binds for pyrophosphate (see text for details).

monoanion binding does not normalize the pH dependence of RNase–2′-CMP binding with that of the other ligands does not preclude monoanion binding but can be simply due to the fact that (unlike the other ligand complexes) the ionization of the RNase–2′-CMP complex does not occur significantly below pH 7.5. This may be related to the fact that 2′-CMP is more tightly bound to RNase than the other ligands. Again the involvement of an ionizable group on the free enzyme having a pK_a of approximately 6.5 is suggested. RNase binding with 2′-CMP appears to require at least one more acid–base group on the free enzyme when compared with 3′-CMP binding. The observation of Breslow and Girotti (1966) that RNase binding with cupric ion is strengthened in the presence of 3′-CMP by formation of a ternary complex, but is little affected in the presence of 2′-CMP, also indicates that 2′-CMP and 3′-CMP do not bind to RNase in an identical manner.

The corrected association constants for pyrophosphate are consistently about 50 times greater than the corrected association constants for orthophosphate over the pH range 4.0–8.0 (see Figure 7). This difference could possibly be interpreted as resulting from an electrostatic interaction between the additional charge on pyrophosphate and a cationic site on the enzyme.

Studies on the anionic inhibition of the urea denaturation of RNase (Nelson *et al.*, 1962) have indicated that nitrate ion binding with RNase was greater than chlo-

ride ion binding. A similar effect was observed in the present study. The association constants for cytidine nucleotides are always greater in 0.1 M KCl than in 0.1 M KNO_3 (see Table II). On the assumption that the binding of nitrate is competitive and that the binding of chloride is negligible, the association constant of RNase with nitrate ion can be estimated as 10 M^{-1} at pH 5.5. The ratios of K(in KCl):K(in KNO_3) for RNase–3′-CMP binding were the same, within experimental error, at pH 5.5 and 6.5, indicating no differential binding of nitrate and chloride ions with RNase as a function of pH in this pH range.

When CMP and UMP binding with RNase were compared in the *same* medium (*e.g.*, 0.1 M KCl at pH 5.5), cytidine 2′- and 3′-nucleotides were bound approximately two times greater than their corresponding uridine nucleotides (see Table II). This clearly indicates a degree of base specificity in the binding process. In this same solvent the ratios of K(2′-nucleotide):K(3′-nucleotide) for CMP and UMP are 11.0 and 9.9, respectively, which are the same within experimental error. However, this same ratio for CMP at pH 5.5 in 0.1 M KNO_3 is 14.9 which appears to be significantly different from the results in 0.1 M KCl. Furthermore, in going from KNO_3 to KCl solutions $\Delta\epsilon_{max}$ for RNase–3′-CMP binding diminished whereas $\Delta\epsilon_{max}$ for RNase–2′-CMP binding *increased* (see Table II). Since the effect of nitrate ion is apparently different for 2′- and 3′-nucleotide binding, then nitrate ion binding *vis-à-vis* chloride ion binding is not strictly competitive with nucleotide binding to RNase.

The difference spectra resulting from RNase binding with cytidine and uridine nucleotides exhibited significantly lower values of $\Delta\epsilon_{max}$ for the 2′ isomers when compared with the 3′ isomers (see Table II). On the other hand, the binding of the 2′ isomers with RNase, at pH 5.5, is approximately ten times that of the 3′ isomers (see Table II). Since the difference spectra resulting from RNase–nucleotide binding essentially represent perturbations of the nucleotide spectra only (Irie and Sawada, 1967), the different values of $\Delta\epsilon_{max}$ suggest that the base group of the 2′ and 3′ isomers interacts differently with RNase. However, at present no theoretical basis exists for speculating whether this change in $\Delta\epsilon_{max}$ is a significant index of different contributions of the base moiety of the nucleotide to the over-all free energy of binding for the 2′ and 3′ isomers.

Whatever discrete interactions are responsible for base moiety binding with RNase they are discriminating with respect to cytosine and uracil bases. Paradoxically, with 2′- and 3′-CMP the interactions of the enzyme with the cytosine base are not sufficient to differentiate between its protonated and nonprotonated forms. Considering these results, an obvious interpretation of any specific interactions between RNase and the base group of the nucleotide is not possible. However, some consistent features regarding the nature of the difference spectra resulting from RNase–nucleotide binding can be mentioned. In general, all nucleotides studied thus far have difference spectra similar in contour to that illustrated for 3′-UMP binding (see Figure 6) or can be resolved into such (see Figure 2). Moreover, the wavelength of $\Delta\epsilon_{max}$ is approximately 7 mμ lower than that for ϵ_{max} of the corresponding free nucleotide. This has been observed for RNase binding with 2′-CMP, 3′-CMP, 2′-UMP, and 3′-UMP in the present study, with 2′- and 3′-AMP (Hummel *et al.*, 1961), and with a mixture of 2′ and 3′ isomers of 4-thiouridine phosphate (Irie and Sawada, 1967). The only exception involves the difference spectrum calculated for the protonated nitrogen-3 species of 2′-CMP which has a $\Delta\epsilon_{max}$ at about the same wavelength as ϵ_{max} (see Figure 2). These consistent findings seem to point out that a common aspect of enzyme–base interaction prevails, even though the magnitude of this interaction ($\Delta\epsilon_{max}$) is a function of the total structure of the nucleotide.

On the basis of the data presented here, a consistent picture of the binding process can be presented with regard to the ionization states of the ligands which bind to the enzyme and the involvement of ionizable groups on the enzyme. However, these interpretations should be viewed with caution since much of the evidence is circumstantial and the relaxation spectrum characteristic of the 3′-CMP–RNase interaction indicates the binding mechanism is quite complex (Erman and Hammes, 1966).

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Purification and Properties of Uridine Diphosphate Galactose 4-Epimerase from Yeast*

Robert A. Darrow† and Robert Rodstrom

ABSTRACT: Uridine diphosphate galactose 4-epimerase has been isolated from galactose-adapted yeast, by a method which facilitated the preparation of large amounts of the protein. The specific catalytic activity of the final product from five different preparations varied between 41.4 and 87.4 μmoles of substrate converted into product per min per mg of protein, at 23°. The enzyme was free of contaminating protein as judged by chromatography on CM-cellulose, DEAE-cellulose, and gel filtration media. It contained, per mg of protein, 8 mμmoles of bound diphosphopyridine nucleotide and 116 mμmoles of SH titratable with *p*-mercuribenzoate. The preparation migrated as a single boundary upon sedimentation in the ultracentrifuge in buffers of low ionic strength. In 0.01 M Tris-HCl (pH 7.5) a molecular weight of 125,000 ± 5600 was calculated from data obtained by low-speed equilibrium centrifugation, high-speed equilibrium centrifugation, and by combination of sedimentation and diffusion data. The minimum molecular weight calculated from the amount of diphosphopyridine nucleotide bound to the protein was 125,000. The fluorescence of yeast epimerase, with excitation at 350 mμ and emission at 435 mμ, has previously been related to the presence of reduced diphosphopyridine nucleotide (Creveling, C. R., Bhaduri, A., Christensen, A., and Kalckar, H. M. (1965), *Biochem. Biophys. Res. Commun. 15*, 182). In the present studies, the fluorescence decreased markedly on treatment with guanidine hydrochloride, suggesting a strong dependence upon an ordered protein structure.

We report here a new method of preparing UDP galactose 4-epimerase[1] from yeast which has advantages of simplicity, speed, and applicability to large-scale operation. The final product has a specific activity of up to ten times that recorded previously for this enzyme (Maxwell and de Robichon-Szulmajster, 1960) and contains three times as much bound DPN.

The purified protein has a molecular weight of approximately 125,000 as determined by sedimentation studies under conditions of low ionic strength (0.01 M). At higher ionic strengths (0.1 M), the enzyme can be separated into two or more catalytically active components either by ultracentrifugation (Darrow and Rodstrom, 1966) or by chromatography on DEAE-cellulose (R. A. Darrow and R. Rodstrom, unpublished data).

The fluorescence of native epimerase from yeast has been shown to be associated with the presence of DPNH (Creveling *et al.*, 1965) and to be influenced by a number of sugars and uridine nucleotides (Bertland *et al.*, 1966). Studies of the loss of fluorescence in urea and guanidine solutions reported in this paper indicate that the fluorescence is dependent upon a highly ordered protein structure.

* From the Biochemical Research Laboratory, Harvard Medical School, and the Massachusetts General Hospital, Boston, Massachusetts 02114. *Received December 7, 1967.* This investigation has been supported by the National Institutes of Health (AM05507, AM05639), the National Science Foundation (GB3646), and the Joseph P. Kennedy, Jr., Foundation.

† Present address: The Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 45387.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: UDP glucose 4-epimerase (EC 5.1.3.2), UDP galactose 4-epimerase; uridine 5′-(α-D-galactopyranosyl pyrophosphate), UDP galactose; UDP glucose:NAD oxidoreductase (EC 1.1.1.22), UDP glucose dehydrogenase; uridine 5′-monophosphate, UMP; *p*-mercuribenzoate, PMB.