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Energetics of Ribonuclease A Catalysis. 2. Nonenzymatic Hydrolysis of Cytidine Cyclic 2′,3′-Phosphate†

Maurice R. Eftink* and Rodney L. Biltonen

ABSTRACT: Various kinetic aspects of the nonenzymatic hydrolysis of cytidine cyclic 2′,3′-phosphate and uridine cyclic 2′,3′-phosphate have been studied in order to provide a basis for comparison with the ribonuclease A catalyzed hydrolysis reaction. Studies of the pH dependence of the nonenzymatic reaction reveal mechanisms that are first order in hydroxide concentration and second order in hydrogen ion concentration, in addition to a "water" reaction. The rate constant for the water reaction was found to be very small, approximately equal to 2.5×10^{-6} min^{-1}. General base catalyzed hydrolysis reactions were also studied with imidazole as the catalyst. At pH values in which both the protonated and neutral forms of imidazole are present, a kinetic mechanism was observed that appears to be second order in total imidazole concentration, thus suggesting that bifunctional catalysis occurs. The activation enthalpy for the hydroxide, hydrogen ion, water, and imidazole catalyzed reactions was determined.

The hydrolysis of the cyclic 2′,3′-phosphate esters of pyrimidine nucleotides is catalyzed by the enzyme ribonuclease A (RNase A).[1] Much is understood about the mechanism of this catalyzed reaction (Richards & Wyckoff, 1971; Roberts et al., 1969), but a complete explanation for the forces responsible for the catalytic power of this enzyme remains to be established, as is the case for enzymes in general. Analysis of the action of enzymes in terms of the so-called transition-state theory of enzyme catalysis has become popular in recent years (Wolfenden, 1973; Lienhard, 1973; Jencks, 1975; Schowen, 1978). The strategy of this approach is to relate the rate constants for the enzymatic and nonenzymatic reactions to the association constant, K_{TS}, for the reaction transition state to the enzyme. In the accompanying papers of this series (Eftink & Biltonen, 1983a,b), we employ the transition-state approach to analyze the energetics of RNase A catalysis.

A key point in the transition-state analysis is the choice of the appropriate nonenzymatic reaction to be used as a reference. In order to discern the nature of the forces responsible for the interaction of the transition state with the enzyme, it is necessary to have as much insight as possible concerning the chemical structure of the transition state of the reference reaction. The reference reaction transition state and the enzymatic transition state may be quite similar in structure. On the other hand, it is possible that the transition state, when bound to the enzyme's active site, will be significantly different

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[1] Abbreviations: cCMP, cytidine cyclic 2′,3′-phosphate; cUMP, uridine cyclic 2′,3′-phosphate; RNase A, bovine pancreatic ribonuclease A; k_H, second-order rate constant for hydrogen ion catalyzed hydrolysis reaction; k_{OH}, rate constant for hyroxide reaction; k_w, rate constant for "water" hydrolysis reaction; k_{Im}, rate constant for imidazole-catalyzed reaction; k_{Im^+}, rate constant for imidazolium-catalyzed reaction; k_{Im^2}, second-order rate constant for imidazole-catalyzed reaction; K_{Im}, acid dissociation constant of imidazole.

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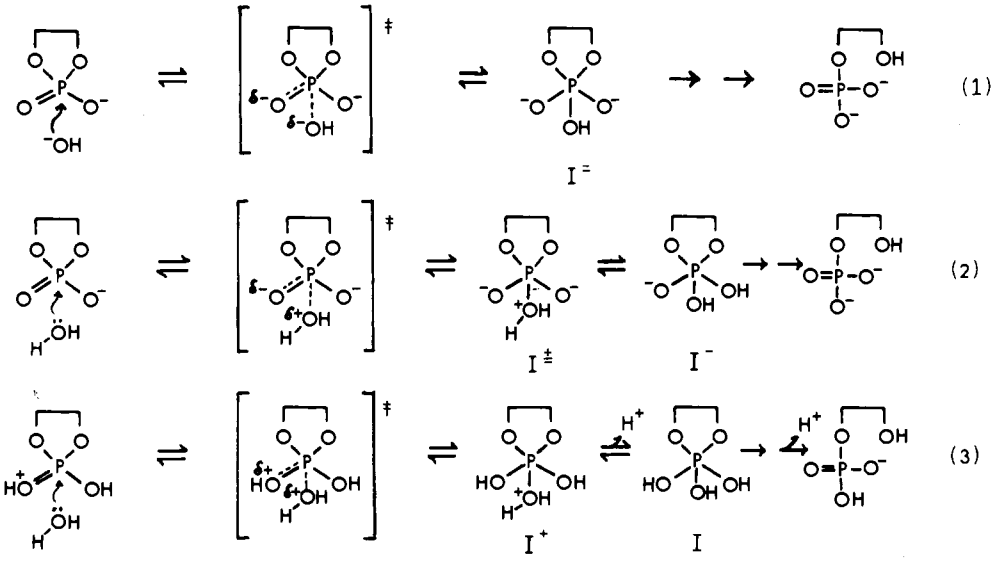

from the nonenzymatic transition state. In such a case, any changes in the chemical structure (state of protonation etc.) will be included in the value for the association constant for the transition state, K_{TS}.

The nonenzymatic hydrolysis of five-membered cyclic phosphate esters has been known for years to proceed as much as 10^6 times faster than the hydrolysis of noncyclic phosphate diesters. Studies by Westheimer and co-workers (Westheimer, 1968; Haake & Westheimer, 1961; Covitz & Westheimer, 1963) have provided evidence that a pentacoordinated phosphate (trigonal-bipyramidal) transition state (or intermediate) forms in the hydrolysis reaction. Studies of the nonenzymatic hydrolysis of the substrates for RNase A have been performed by Abrash and co-workers (Abrash et al., 1967) at several temperatures and pH values. These workers found the hydrolysis of uridine cyclic 2′,3′-phosphate (cUMP) and cytidine cyclic 2′,3′-phosphate (cCMP) to be first order in hydroxide ion concentration and second order in hydrogen ion concentration. At neutral pH, the rate of hydrolysis was found to be quite slow, but their data suggest that a kinetically significant water reaction may occur. Possible mechanisms for the attack of water and hydroxide ion (and the subsequent transition states) for the hydrolysis reaction at low, neutral, and high pH are shown in eq 1–3.

In the RNase A catalyzed reaction, the formation of the transition state is thought to be facilitated by His-119 and -12 acting as general base and general acid catalysts. This being the case, another nonenzymatic reaction which could serve as a useful reference for the enzymatic reaction would be an imidazole-catalyzed hydrolysis reaction. Imidazole is known to catalyze the hydrolysis of methyl ethylene phosphate (Covitz & Westheimer, 1963), but there have been no reports of its ability to catalyze the hydrolysis of ethylene phosphate or of cCMP or cUMP. In this report, we will demonstrate that imidazole can act as a catalyst for the hydrolysis of cCMP and cUMP. Also, we will present data that suggest that a reaction that is second order in imidazole concentration exists. These imidazole-catalyzed reactions, as well as the water-, hydroxide-, and acid-catalyzed reactions, will be characterized as reference reactions for comparison with the enzyme-catalyzed reaction.

Experimental Procedures

cCMP, 3′-CMP, 2′-CMP, cytidine, cUMP, 3′-UMP, 2′-UMP, and uridine were obtained from Sigma Chemical Co. and were used without further purification. Imidazole was twice recrystallized from hexane–chloroform. Other salts and buffers were analytical reagent grade. Water was distilled and deionized. D_2O was 99.8% pure. N-Deuterated imidazole was prepared by freeze-drying imidazole in D_2O.

The rate of hydrolysis of cCMP and cUMP was monitored spectroscopically by measuring the ratio of the absorbance (A) at 286 and 268 nm for cCMP and at 279 and 259 nm for cUMP. The $A_{286/268}$ ($=r$) ratio for cCMP is approximately 0.390; upon hydrolysis to a mixture of 2′- and 3′-CMP, this ratio increases to 0.540. Likewise, for cUMP, the $A_{279/259}$ ratio increases from about 0.250 for cUMP to 0.365 for the hydrolysis product.

In typical experiments, 5-mL solutions of 4×10^{-3} M cyclic phosphate (at appropriate pH, temperature, and buffer composition) were incubated in a water bath. At various times, 50-μL aliquots of the solution were taken and diluted into 2 mL of a 0.1 M acetate buffer (pH 5.0), and the absorbance at the two wavelengths was measured with a Perkin-Elmer 200 spectrophotometer.

As described by Abrash and co-workers (Abrash et al., 1967), the absorbance ratio, r, can be used to determine the pseudo-first-order rate constant, k_{obsd}, for the hydrolysis reaction by utilizing the following equation, where r_0 is the absorbance ratio at zero time (i.e., the ratio for cCMP or cUMP) and r_∞ is the ratio at the reaction completion [i.e., the ratio for the product pyrimidine 2′- and 3′-phosphates]:

$$\log \frac{r_\infty - r_0}{r_\infty - r} = \frac{k_{obsd}}{2.3} t \qquad (4)$$

The r_0 and r_∞ values varied slightly at the different pH values employed and were determined (when feasible) for each set of conditions.

The cCMP hydrolysis product was characterized by paper chromatography using the solvent system of saturated ammonium sulfate–1 M sodium acetate (pH 6.6)–2-propanol (80:18:2 v/v). The $R_f = 0.7$ value of the product was found to be identical with that for 2′-CMP or 3′-CMP, as compared with R_f values of 0.45 and 0.6 for cCMP and cytidine, respectively. Likewise, the cUMP hydrolysis product showed an R_f value characteristic of a mixture of 2′- and 3′-UMP. The absorbance ratios for the hydrolysis products are in line with that expected for a mixture of the pyrimidine 2′- and 3′-mononucleotides, arguing that they are the only reaction products. Also, the absorbance ratios were found to remain

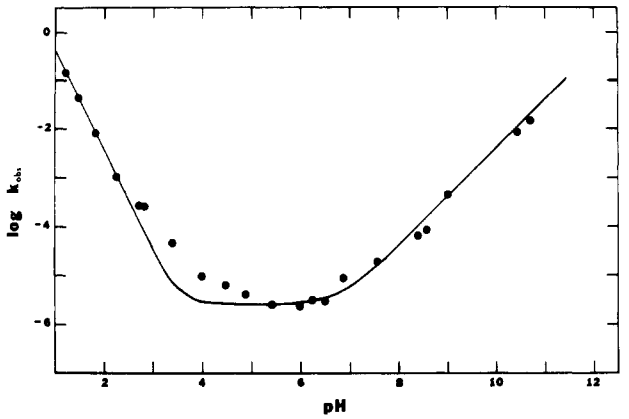

FIGURE 1: pH dependence of the apparent rate constant for the hydrolysis of cUMP at 50 °C, ionic strength 1.0 M. Rate constants (in min^{-1}) were measured as described under Experimental Procedures and were extrapolated to zero buffer concentration. The solid line is the theoretical fit of eq 5 to the data for $k_H = 35\ min^{-1}\ M^{-2}$, $k_{OH} = 40\ min^{-1}\ M^{-1}$, and $k_w = 2.5 \times 10^{-6}\ min^{-1}$. Buffers used were glycine (pH 1.4–2.9), sodium formate (pH 3.5–4.0), sodium acetate (pH 4.5–5.4), sodium phosphate (pH 6.0–6.5 and 10.0–11.5), imidazole (pH 5.5–8.4), sodium borate (pH 9.0), and sodium cacodylate (pH 5.4–6.4).

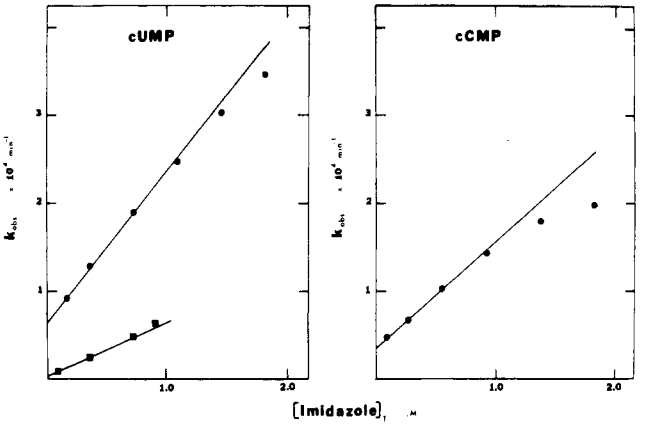

FIGURE 2: Dependence of the pseudo-first-order rate constant for the hydrolysis of cUMP and cCMP on the total concentration of imidazole at pH 8.4, 50 °C, 1.0 M ionic strength (●). At this temperature and ionic strength, the pK_a of imidazole is approximately 6.7. Imidazole will thus be ~98% unprotonated at pH 8.4. Also shown are data for the imidazole-catalyzed hydrolysis of cUMP at pH 5.5 (■, ~94% protonated). The apparent rate constants are corrected for the decrease in water concentrations at the higher imidazole concentrations. At [imidazole] = 0, the water concentration is ~54 M (reduced from 55 M by the added salt); at [imidazole] = 2 M, the water concentration drops to ~46.4 M due to the presence of the added solute. Rate constants were normalized with respect to the case [imidazole] = 0 by multiplying by the factor $54/(54 - g_{Im}/18)$, where g_{Im} is the grams of added imidazole and 18 is the molecular weight of water (it is assumed that the density of imidazole is equal to that of water). At pH ranges where imidazolium chloride substitutes for NaCl to maintain the ionic strength, a similar, but smaller, correction for the decrease in water concentration was applied.

constant at r_∞ for an extended period of time after the hydrolysis reaction had gone to completion (i.e., there does not appear to be a slower side reaction as measured by the absorbance ratio). The only instance in which it was apparent that a product other than the pyrimidine 2′- and 3′-phosphates was being formed was during the hydrolysis of cCMP in the pH range of 3–6, where the absorbance ratio was found to slowly decrease. It is thought that under these conditions deamination of the cytosine ring (Shapiro & Klein, 1966) must be taking place faster than the slow hydrolysis reaction. To avoid this complication, we have primarily used cUMP as the substrate in the pH range 3–6.

Abrash and co-workers (Abrash et al., 1967) also reported that during the hydrolysis of cUMP at neutral pH and high temperatures the nucleoside uridine was formed as a result of the rapid hydrolysis of the 2′- or 3′-phosphomonoester following the rate-limiting hydrolysis of the cyclic phosphate diester. We found no evidence in our studies that uridine is formed as a result of imidazole- (or other buffer) catalyzed hydrolysis of cUMP. Note that our studies were performed at a much lower temperature than those of Abrash and co-workers in this pH range. In addition, as argued by Abrash and co-workers, since the hydrolysis of the cyclic phosphate diester must be rate limiting, the observed rate constant reflects the desired reaction.

The pH of the solutions was measured by using a London PHM 64 pH meter, and, unless specified, the pH values reported refer to the reaction temperature.

Results

The data for the pH dependence of the first-order rate constant for the hydrolysis of cUMP at 50 °C, ionic strength 1.0 M, are shown in Figure 1. The rate constants were obtained by extrapolation to zero buffer concentration. The data for the hydrolysis of cCMP are similar to those shown for cUMP, although differences by a factor of 2 are common.

The smooth line drawn through the data is obtained from a fit of the following equation:

$$k_{obsd} = k_H[H^+]^2 + k_{OH}[OH^-] + k_w \qquad (5)$$

where k_H, k_{OH}, and k_w are the rate constants for the second-order hydrogen ion catalyzed reaction, the hydroxide reaction, and the water reaction, respectively. The values found for these rate constants are $k_H = 35 \pm 3\ M^{-2}\ min^{-1}$, $k_{OH} = 40 \pm 5\ M^{-1}\ min^{-1}$, and $k_w = (2.5 \pm 1.5) \times 10^{-6}\ min^{-1}$. The solid line in Figure 1 represents the theoretical fit obtained by using these rate constants. As found by Abrash et al. (1967), the phosphodiester group of cUMP and cCMP must have a pK_a less than 1 since no significant deviation from second-order hydrogen ion catalysis was observed down to pH 1. We made no attempt to include first-order hydrogen ion catalysis in our analysis. The slight deviation around the pH 3.5–4.5 region suggests that such a catalytic reaction might occur, but the quality of our data does not permit inclusion of an additional term in the rate equation.

Imidazole Catalysis. The rate of hydrolysis of cCMP or cUMP was found to be dependent on the concentration of imidazole as shown in Figure 2. At pH 8.4 where imidazole is ~98% neutral (at 50 °C), the apparent rate of hydrolysis increases with imidazole concentration from 0 to 2 M. The dependence on concentration (after correction for the decrease in the concentration of water in the high [imidazole] solution) shows a slight negative deviation. Deviations from linearity may arise due to reduction in activity coefficients in the highly concentrated solutions (Hand & Jencks, 1975) or to a change in the rate-limiting step (see Discussion). From the linear, low concentration region, values of 1.7×10^{-4} and $1.1 \times 10^{-4}\ M^{-1}\ min^{-1}$ can be obtained for the rate constant for the imidazole-catalyzed hydrolysis (k_{Im}) of cUMP and cCMP, respectively. At pH 5.5, where imidazole is 94% protonated, the rate of hydrolysis of cUMP increases slightly upon the addition of up to 1 M imidazole. The rate constant of the imidazolium-catalyzed hydrolysis (k_{Im^+}) of cUMP is found to be $0.6 \times 10^{-4}\ M^{-1}\ min^{-1}$. (As mentioned under Experimental Procedures, the imidazolium-catalyzed hydrolysis of cCMP cannot be observed, due to a competing side reaction in this pH region.) By comparing the data in these two pH regions,

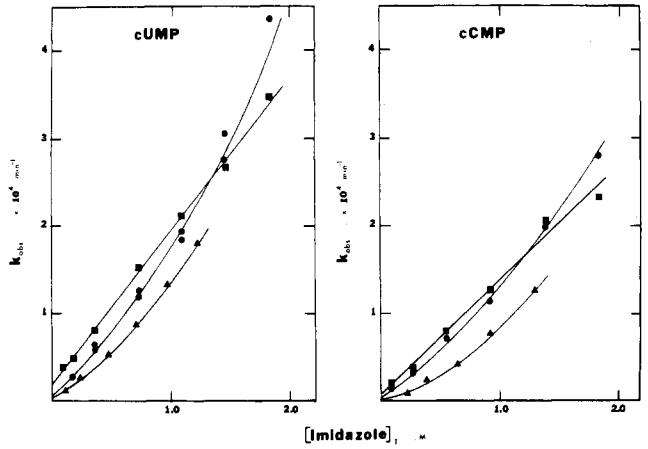

FIGURE 3: Dependence of the apparent rate constant for the hydrolysis of cUMP and cCMP on the total imidazole concentration at pH 7.4 (■), 7.0 (●), and 6.5 (▲). Studies at 50 °C, ionic strength 1.0 M.

it is apparent that the neutral imidazole species is a more effective catalyst than the protonated species.

At intermediate pH values of 6.5, 7.0, and 7.4, where a fraction of the imidazole molecules exists in each state of protonation, the rate of hydrolysis is again dependent on the concentration of imidazole. In addition, plots of the apparent first-order rate constant vs. $[\text{imidazole}]_{\text{total}}$ show an upward curvature as seen in Figure 3. Such deviations suggest that the catalysis is not just a sum of the imidazole and imidazolium terms but that the catalytic process includes a second-order term in imidazole concentration. In particular, the observation that the upward curvature is most apparent at pH values near (or just below) the pK_a of imidazole argues that both a neutral and a protonated imidazole molecule are involved in this second-order catalytic process. If such is the case, the rate equation for the hydrolysis reaction in the presence of imidazole would be (where k_0 is the rate constant in the absence of imidazole, equal to $k_H[H^+]^2 + k_{OH}[OH^-] + k_w$)

$$k_{obsd} = k_0 + k_{Im}[Im] + k_{Im^+}[Im^+] + k_{Im^2}[Im][Im^+] = k_0 + k_{Im}\left(\frac{1}{1 + [H^+]/K_{Im}}\right)[Im]_T + k_{Im^+}\left(\frac{[H^+]/K_{Im}}{1 + [H^+]/K_{Im}}\right)[Im]_T + k_{Im^2}\left[\frac{[H^+]/K_{Im}}{(1 + [H^+]/K_{Im})^2}\right][Im]_T^2 \quad (6)$$

which can be rearranged to give

$$\frac{(k_{obsd} - k_0)(1 + [H^+]/K_{Im})}{[Im]_T} = k_{Im} + k_{Im^+}[H^+]/K_{Im} + k_{Im^2}\left(\frac{[H^+]/K_{Im}}{1 + [H^+]/K_{Im}}\right)[Im]_T \quad (7)$$

and

$$\frac{(k_{obsd} - k_0)(1 + [H^+]/K_{Im})}{[Im]_T} - k_{Im} - k_{Im^+}[H^+]/K_{Im} = k_{Im^2}\left(\frac{[H^+]/K_{Im}}{1 + [H^+]/K_{Im}}\right)[Im]_T \quad (8)$$

where [Im], $[Im^+]$, and $[Im]_T$ are the concentrations of neutral imidazole, imidazolium cation, and total imidazole, respectively; k_{Im}, k_{Im^+}, and k_{Im^2} are the rate constants for the reactions that are first order in imidazole, first order in imidazolium and second order in imidazole, respectively; K_{Im} is the acid dissociation constant of imidazole.

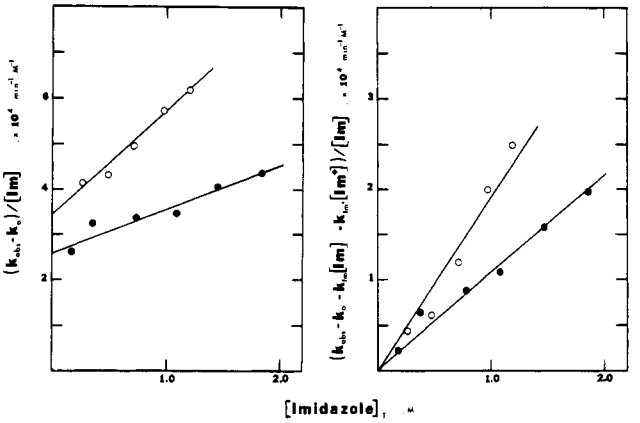

FIGURE 4: Plots of $(k_{obsd} - k_0)/[Im]$ and $(k_{obsd} - k_0 - k_{Im}[Im] - k_{Im^+}[IM^+])/[Im]$ vs. $[Im]_T$ for the imidazole-catalyzed hydrolysis of cUMP at pH 7.0 (●) and pH 6.5 (○), 50 °C, ionic strength 1 M. For the right graph, values of k_{Im} and k_{Im^+} are assumed to be equal to 1.7×10^{-4} M^{-1} min^{-1} and 0.6×10^{-4} M^{-1} min^{-1}, respectively. The Im and Im^+ concentrations were determined from pH measurements made at 25 °C (pH values of 7.3 and 6.8 at 25 °C corresponding to the pH values of 7.0 and 6.5 at 50 °C) and the pK_{Im} value of 7.2 at 25 °C, ionic strength 1 M (Fox & Jencks, 1974).

Table I: Rate Constants for the Imidazole-Catalyzed Hydrolysis of cUMP and cCMP[h]

	k_{Im} ($\times 10^{-4}$ min^{-1} M^{-1})	k_{Im^+} ($\times 10^{-4}$ min^{-1} N^{-1})	k_{Im^2} ($\times 10^{-4}$ min^{-1} M^{-2})
cUMP	1.7[a]	0.6[b]	2.0,[c] 2.2[e]
	2.0[g]	0.5[g]	2.8,[d] 2.4[f]
cCMP	1.1[a]		2.2[c]
	1.0[g]		2.5[d]

[a] From data at pH 8.4 (Figure 2). [b] From data at pH 5.5 (Figure 2). [c] From data at pH 7.0 using eq 7; slope of Figure 4. [d] From data at pH 6.5 using eq 7; slope of Figure 4. [e] From data at pH 7.0, assuming values of k_{Im} and k_{Im^+} as given in Figure 4. [f] From data at pH 6.5, assuming values of k_{Im} and k_{Im^+} as given in Figure 4. [g] From a replot of the intercept of eq 7 vs. $[H^+]/K_{Im}$. [h] All values are for 50 °C, 1.0 M ionic strength.

In order to analyze the data shown in Figure 3 to obtain k_{Im^2}, the plots were first extrapolated to zero imidazole concentration to obtain an estimate of k_0. From this k_0 value and k_{obsd} as a function of $[Im]_T$, a plot of the left-hand side of eq 7 vs. $[Im]_T$ was made in order to obtain k_{Im}' ($=k_{Im} + k_{Im^+}[H^+]/K_{Im}$) and k_{Im^2} from the intercept and slope, respectively. The $[H^+]$ and K_{Im} values used in this treatment were values measured at 25 °C ($K_{Im} = 6.3 \times 10^{-8}$ M at this temperature and an ionic strength of 1 M). Both $[H^+]$ and K_{Im} will increase with an increase in temperature from 25 to 50 °C. However, the ratio $[H^+]/K_{Im}$ (and hence the ratio $[Im]/[Im^+]$) will remain practically unchanged as the temperature is increased. Therefore, to avoid possible errors due to $[H^+]$ and K_{Im} determinations at the higher temperature, values at 25 °C were employed (see the legend of Figure 4).

The rate constants obtained for the imidazole-catalyzed hydrolysis of cUMP and cCMP are listed in Table I. In addition to using eq 7 to treat the data, we also performed the analysis by setting k_{Im} and k_{Im^+} equal to their values determined at pH 8.4 and 5.5 (where second-order catalysis is insignificant) and graphically determining k_{Im^2} by using eq 8 (open symbols in Figure 4). In view of the fact that a downward-curving plot was found at pH 8.4, the accuracy of

all determined rate constants will be poor. Nevertheless, there is consistency in the values of the rate constants, including k_{Im^2}, obtained at all pH values.

Since high concentrations of imidazole were employed, there is a possibility that the formation of an $Im-Im^+$ dimer may account for the increased catalytic efficiency at high $[Im]_T$ (Hand & Jencks, 1975). While this is a possibility, other workers have used similar concentrations of imidazole without a remark concerning the formation of such a dimer. Moreover, the existence of such an active dimeric species does not detract from the interpretations given below.

Studies at lower ionic strength (0.2 M, at pH 8.4 only) show that k_{Im} decreases by a factor of about 2.5-fold. Also studies in D_2O show a solvent isotope effect of 3.8 for k_{Im}.

Other Catalysts. In addition to imidazole, a number of other agents were found to act as catalysts as well. These agents include acetate ($k = 4.5 \times 10^{-5}$ M^{-1} min^{-1}, $pK_a = 4.6$), cacodylate ($k = 1.7 \times 10^{-4}$ M^{-1} min^{-1}, $pK_a = 6.1$), phosphate ($k = 1.8 \times 10^{-4}$ M^{-1} min^{-1}, $pK_a = 6.4$), and borate ($k = 5.5 \times 10^{-3}$ M^{-1} min^{-1}, $pK_a = 9.3$). The rate constants given in parentheses are for the catalyzed hydrolysis of cUMP at 50 °C, ionic strength 1.0 M, and the pK_a values given for the agents are estimated for this ionic strength and temperature from literature values at 25 °C (Fox & Jencks, 1974) and the temperature dependence of the acid dissociation constant of these compounds. No effort was made to determine if second-order catalysis occurs for these agents. The magnitude of the rate constant for catalysis by these agents shows an increase with the pK_a of the catalyst, as expected for Bronsted general base catalysis ($\beta = 0.4 \pm 0.1$). The rate constant for phosphate catalysis is similar to that for imidazole.

The rate constant for catalysis of the hydrolysis of cCMP by histamine (at pH 8, 50 °C) was found to be 3.5×10^{-4} M^{-1} min^{-1}. This rate constant is significantly larger than that for the imidazole-catalyzed reaction ($k_{Im} = 1.1 \times 10^{-4}$ M^{-1} min^{-1}) and suggests that the neighboring protonated amino group of histamine might assist in the catalytic process.

Temperature Dependence Studies. The temperature dependence of the various cCMP and cUMP hydrolysis reactions was also studied. The hydrogen ion (pH 1.8–2.0) and hydroxide ion (pH 10.5–10.8) catalyzed hydrolyses of cCMP were studied between 30 and 60 °C and were found to be characterized by activation enthalpies of 21 ± 2 and 20.5 ± 2 kcal/mol and activation entropies of 5.4 ± 0.5 and 4.1 ± 0.4 cal/(mol·deg), respectively (see Figure 5). These activation enthalpies are in general agreement with, although slightly larger than, those reported by Abrash et al. (1967). The activation enthalpy for the water reaction was also determined by measuring the rate of hydrolysis of cUMP at pH 6 [using 2-(*N*-morpholino)ethanesulfonic acid (Mes) buffer and extrapolating to zero buffer concentration] at five temperatures over the range 50–90 °C. (Note that at this pH the water reaction accounts for >90% of the observed rate, extrapolated to zero buffer concentration.) A value of 17 ± 5 kcal/mol was found for the activation enthalpy [40 ± 18 cal/(mol·deg) for the activation entropy]. The imprecision in the value for the water reaction is due to the great difficulty in measuring the very small rate constants, particularly at the lower temperatures.

For the imidazole-catalyzed hydrolysis of cCMP, an activation enthalpy over the range 40–70 °C of 11 ± 2 kcal/mol [activation entropy of −50 ± 9 cal/(mol·deg)] was found for the reaction that is first order in the neutral imidazole species.

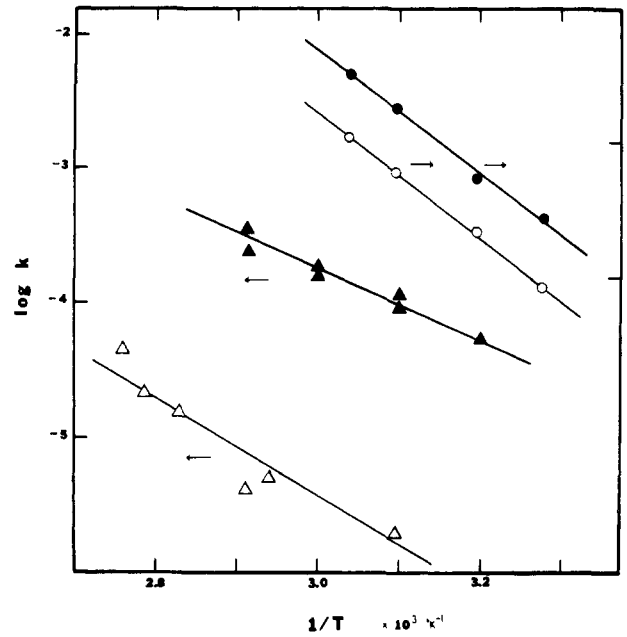

FIGURE 5: Arrhenius plots for various hydrolysis reactions of cCMP and cUMP. (●) Hydrogen ion catalyzed hydrolysis of cCMP; (○) hydroxide ion catalyzed hydrolysis of cCMP; (▲) imidazole-catalyzed hydrolysis of cUMP at pH 8.4; (△) hydrolysis of cUMP at pH 6.0 ("water reaction"). For the hydrogen ion and hydroxide ion catalyzed reactions, the pH was measured at the experimental temperature.

Discussion

The hydrolysis of cCMP and cUMP is found to be both specific acid and specific base catalyzed. The rate constant for the water reaction is very small. A k_w value of 2.5×10^{-6} M^{-1} min^{-1} is found at 50 °C, ionic strength 1.0 M.

The ability of imidazole to act as a catalyst is of interest due to the involvement of two active-site histidine residues in the RNase A catalyzed hydrolysis of these same cyclic phosphate esters. The solvent isotope effect of 3.8 strongly suggests that neutral imidazole acts as a general base catalyst, assisting the attack of H_2O. (Of course, general acid catalyzed attack of hydroxide ion is kinetically indistinguishable.) Since other chemically dissimilar buffers, such as phosphate, catalyze the hydrolysis reaction equally well, it seems certain that imidazole is acting as a general base catalyst rather than as a nucleophilic catalyst.

The plots of k_{obsd} vs. $[Im]_T$ at pH 8.4 do show a slight negative deviation. As Hand & Jencks (1975) have discussed, there are a number of possible reasons for such negative deviations. One is that the hydrolysis reaction normally involves more than one step (more than one activation free-energy barrier) and that at high concentrations of catalyst there is a change in the rate-determining step. Considering the proposed mechanism for the attack of H_2O on the cyclic phosphate reactant given by eq 2, one can imagine that the first activation free-energy barrier would involve the attack of a water molecule on the phosphate group and that a second activation free-energy barrier would be associated with the breaking of the P–3′-O (or P–2′-O) bond to form the product. The existence of a barrier for both the addition and elimination steps is also thought to be a feature of the hydrolysis of carboxylic acid esters (Jencks, 1969; Johnson, 1967; Bender, 1960). For the present system, a possible free-energy diagram corresponding to the proposed mechanism is given in Figure 6. Possible intermediates that are expected to form are $I^{\pm}$ and I^- (with $I^{\pm}$ being converted to I^- by a rapid proton transfer). The existence of pentacoordinated phosphate intermediates, such as $I^{\pm}$ or I^-, is supported by the studies of Westheimer and co-workers [see Westheimer (1968) and references cited therein] demonstrating the occurrence of

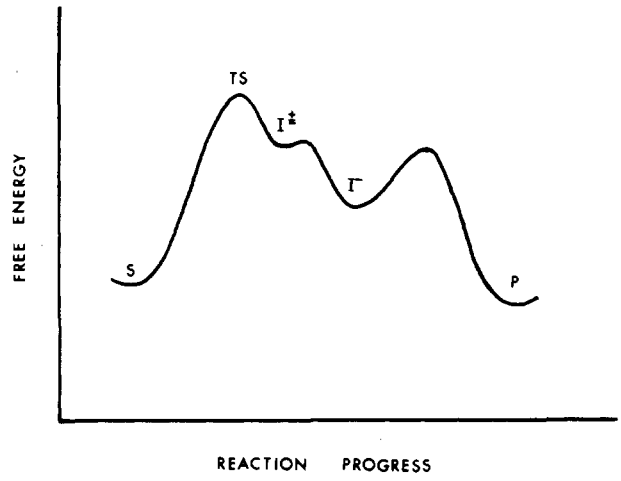

FIGURE 6: Possible free-energy/reaction-coordinate profile for the uncatalyzed hydrolysis of cyclic phosphate esters.

pseudorotation during the hydrolysis of cyclic phosphate esters. Returning to the data in Figure 2, we observe that the negative deviation is consistent with a mechanism in which imidazole catalyzes the attack of a water molecule (i.e., imidazole lowers the barrier for the normally rate-limiting addition step) but that at high $[Im]_T$ the second (elimination) barrier becomes the rate-limiting one. While such a mechanism is reasonable, our data cannot be taken as proof of it, particularly since the negative deviation in Figure 2 could also be due to a decrease in the activity coefficient of the reactants in the concentrated imidazole solutions (Hand & Jencks, 1975). Also, it is possible that the elimination step may be catalyzed by imidazole as well, for example, by partial donation of a proton to the leaving 3′- (or 2′-) oxygen.

The putative second-order catalysis by imidazole is quite interesting in light of the known involvement of the two histidines in the action of RNase A. The bifunctional catalysis (general acid/general base) by imidazole and imidazolium ion is expected to be much like the mechanism of the enzymatic reaction. Multiple catalysis is of course a rare finding due to the expense in entropy needed to incorporate an additional molecule into a transition state (Bruice & Benkovic, 1964). In a well-known study of the mutarotation of glucose, Swain & Brown (1952) found bifunctional catalysis by phenol and pyridine for the reaction carried out in benzene. In aqueous solution, bifunctional catalysis has most frequently been observed by using a single catalyst that has both proton-donating and proton-accepting functional groups (Bender, 1971; Kunitake, 1977; Breslow et al., 1978). One of the few examples of second-order bifunctional catalysis in aqueous solution is the acetate–acetic acid catalyzed enolization of acetone (Swain et al., 1958; Dawson & Spivey, 1930). The unique feature of the present system is that not only it appears to exhibit second-order bifunctional catalysis in aqueous solution but also it closely mimics an enzymatic reaction.

A bit of caution must be exercised concerning this second-order catalysis, however, since the upward-curving plots in Figure 3 may also be due to changes in activity coefficients at the higher imidazole concentrations (Hand & Jencks, 1975). In this context, we note that Drey & Fruton (1965) found no catalytic effect of 4,4′(5,5′)-bis[imidazolyl]methane on the hydrolysis of cUMP. If the second-order imidazole term we have observed is real, one would expect 4,4′(5,5′)-bis[imidazolyl]methane to be a more effective catalyst of the hydrolysis of cUMP than imidazole itself. In their studies with 4,4′-(5,5′)-bis[imidazolyl]methane, Drey and Fruton used a catalyst concentration and temperature that were both much lower than we find necessary to employ in our present studies. Their negative results are therefore not unexpected, and further studies with 4,4′(5,5′)-bis[imidazolyl]methane would be of interest.

The activation enthalpies for the acid- and base-catalyzed hydrolysis reactions were both found to be about 21 kcal/mol. For the water reaction, an activation enthalpy of 17 kcal/mol was found, and, as expected (Bruice & Benkovic, 1964), a lower value of 11 kcal/mol was found for the imidazole-catalyzed reaction. Due to the imprecision of our k_{Im^2} determinations and the above-mentioned caution concerning this term, no attempt was made to determine the activation enthalpy for this rate constant.

In the preceding paper (Eftink & Biltonen, 1983a), the pH-independent rate constant for the RNase A catalyzed hydrolysis of cCMP was determined to be 28 s^{-1}. From the values determined in the present study for the rate constant and activation enthalpy for the water reaction, one can calculate k_w to be approximately 4×10^{-9} s^{-1} at 25 °C. The rate enhancement for the enzymatic reaction can therefore be calculated to be 7×10^9. This value for the rate of enhancement and the above information on the various nonenzymatic hydrolysis reactions of cCMP and cUMP will be used in analyzing the energetics of RNase A catalysis in the following paper in this series.

Registry No. RNase, 9001-99-4; cyclic 2′,3′-CMP, 633-90-9; cyclic 2′,3′-UMP, 606-02-0; hydrogen ion, 12408-02-5; hydroxide, 14280-30-9; water, 7732-18-5; imidazole, 288-32-4; L-histidine, 71-00-1; histamine, 51-45-6.

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Energetics of Ribonuclease A Catalysis. 3. Temperature Dependence of the Hydrolysis of Cytidine Cyclic 2′,3′-Phosphate†

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ABSTRACT: Studies of the temperature dependence of the steady-state kinetics of the ribonuclease A catalyzed hydrolysis of cytidine cyclic 2′,3′-phosphate at pH 5.0 are reported. Contributions to the temperature dependence of the apparent Michaelis–Menten parameters from temperature-sensitive protonic equilibria (primarily the coupled protonation/deprotonation of the active-site histidine residues) were incuded in our analysis. The data were interpreted by employing a transition-state approach. By comparing the temperature dependence of the rate constant for the nonenzymatic hydrolysis of the substrate with the temperature dependence of the enzyme-catalyzed reaction, we obtained values for the enthalpy change, entropy change, and heat capacity change for the interaction of the reaction transition state with the enzyme. These thermodynamic quantities were then interpreted by comparison with corresponding values for the binding of cytidine 2′- and 3′-phosphate to the enzyme. A model is presented for the enzyme–transition-state interaction involving the favorable transfer of a proton from the transition state to a histidine residue at the active site and the formation of hydrogen bonds and van der Waals contacts between the pyrimidine ring of the transition state and the enzyme's binding pocket. These elementary interactions are consistent with the determined values of the enthalpy change and entropy change, as well as earlier reported ionic strength and solvent isotope dependence studies. The Gibbs energy contributions from these elementary interactions have also been estimated, giving a sum approximately equal to the experimentally determined value for the stabilization energy of the enzyme–transition-state complex. The model thus provides an explanation for the magnitude of the $\sim 10^{10}$-fold rate enhancement achieved by this enzyme.

Interpretation of the temperature dependence of the kinetics of enzyme-catalyzed reactions has proven to be a difficult task. The activation enthalpy ($\Delta H^{\ddagger}$)[1] and activation entropy ($\Delta S^{\ddagger}$) that are determined for such reactions will reflect not only the electronic and geometric changes taking place within the substrate molecule(s) as it (they) form(s) the transition state but also any concomitant changes in the state of the enzyme, including changes in solvation. The activation parameters for several enzymes have been tabulated (Laidler, 1955; Laidler & Peterman, 1979; Lumry, 1959), and it has been noted that there is a general tendency for the enzyme to lower $\Delta H^{\ddagger}$. Other studies have focused on the observation of curved Arrhenius plots for certain enzyme-catayzed reactions (Massey et al., 1966; Dixon & Webb, 1964; Levy et al., 1959). Such curvature can be attributed to the existence of a thermally induced reversible change in the conformation of the protein (Massey et al., 1966) or to the existence of consecutive reaction steps with different individual activation enthalpies (Kitskiakowsky & Lumry, 1949). Frequently the enzymes displaying anomalous Arrhenius plots happen to be membrane associated (Thilo et al., 1977; Linden et al., 1973; Raison, 1973).

In this paper, we offer an alternative strategy for interpreting activation parameters in terms of the transition-state theory of enzyme catalysis. As will be discussed below, one can convert the information in $\Delta H^{\ddagger}$ (and $\Delta S^{\ddagger}$) to an enthalpy change (and entropy change) for the association of the transition state with the enzyme. One may then attempt to interpret these equilibrium thermodynamic parameters in terms of the elementary interactions responsible for the stabilization

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[1] Abbreviations: cCMP, cytidine cyclic 2′,3′-phosphate; 3′-CMP, cytidine 3′-phosphate; 2′-CMP, cytidine 2′-phosphate; k_c, apparent first-order rate constant for the enzyme-catalyzed reaction ($k_c = V_m/[E]$ where V_m is the maximum velocity); $k_{\bar{c}}$, pH-independent value of k_c as defined in Eftink & Biltonen (1983a); $\Delta H^{\ddagger}_{k_{\bar{c}}}$ and $\Delta S^{\ddagger}_{k_{\bar{c}}}$, activation enthalpy and entropy, respectively, for $k_{\bar{c}}$; k_E, first-order rate constant for an enzyme-catalyzed reaction (equivalent to k_c); k_{NE}, rate constant for the nonenzymatic conversion of the substrate to the product; k_{Im}, rate constant for the imidazole-catalyzed conversion of the substrate to the product; K_m, apparent Michaelis constant for the enzyme, equal to K_S^{-1} in the present studies with ribonuclease and cCMP; $K_{\bar{m}}$, pH-independent value of K_m as defined in Eftink & Biltonen (1983a); $\Delta H^{\circ}_{K_{\bar{m}}}$, $\Delta S^{\circ}_{K_{\bar{m}}}$, and $\Delta C^{\circ}_{p,K_{\bar{m}}}$, enthalpy change, entropy change, and heat capacity change, respectively, for the interaction between cCMP and ribonuclease; K_{TS}, ΔH°_{TS}, ΔS°_{TS}, and $\Delta C^{\circ}_{p,TS}$, association constant, enthalpy change, entropy change, and heat capacity change, respectively, for the interaction of the reaction transition state with the enzyme; RNase A, bovine pancreatic ribonuclease A.

0006-2960/83/0422-5140$01.50/0