Kinetic and Equilibrium Studies of the Ribonuclease-Catalyzed Hydrolysis of Uridine 2′,3′-Cyclic Phosphate*

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ABSTRACT: Initial velocity measurements were made of the ribonuclease-catalyzed hydrolysis of uridine 2′,3′-cyclic phosphate to 3′-uridine monophosphate in the pH range 4–8 at 25° in 0.1 M Tris-acetate–0.1 M NaCl. The pH dependence of the ratio of the turnover number to the Michaelis constant, k_s/K_s, for uridine 2′,3′-cyclic phosphate is essentially identical with that previously reported in the literature for cytidine 2′,3′-cyclic phosphate and for several dinucleoside phosphates. This indicates that the same ionizable groups on the enzyme are catalytically important for these substrates, and that the ionization state of the cytosine ring is unimportant in the enzyme-catalyzed hydrolysis of cytidine 2′,3′-cyclic phosphate. An ionizable group in its basic form and an ionizable group in its acid form with pK values of 5.4 and 6.4, respectively, are implicated in the enzymatic reaction. The apparent pK values associated with k_s are 5.8 and 7.5. The equilibrium constant for the hydrolysis of uridine 2′,3′-cyclic phosphate to 3′-UMP at pH 5 and 25° was found to be 440 by an isotope dilution technique. This constant was used to calculate the turnover number for the reverse reaction as a function of pH. The mechanistic implications of these results are discussed.

The mechanism of action of bovine pancreatic ribonuclease A (RNase)[1] has been extensively studied with a variety of methods (*cf.* Scheraga and Rupley, 1962; Hummel and Kalnitsky, 1964). A number of steady-state kinetic studies have been carried out (Herries *et al.*, 1962; Witzel, 1963; Cheung and Abrash, 1964; Ramsden and Laidler, 1966) and relaxation methods have been used to probe the elementary steps of the reaction mechanism (*cf.* Hammes, 1968a,b). Comprehensive steady-state data over a wide range of pH values are only available for the hydrolysis of cytidine 2′,3′-cyclic phosphate (Herries *et al.*, 1962). Although related studies of the corresponding uridine compound have been published (Cheung and Abrash, 1964; Ramsden and Laidler, 1966), the purity of the substrates used is quite suspect so that the results can be regarded in a qualitative sense only. A quantitative comparison of the kinetic parameters characterizing the hydrolysis of uridine and cytidine 2′,3′-cyclic phosphates is of interest in order to assess the role of the pyrimidine base in the reaction mechanism. Also since the cytosine ring can accept a proton (pK_a = 4.32; Anderson *et al.*, 1968), while the uracil ring cannot, the effect of protonation of the ring on the kinetics can be evaluated.

We present here the results of an initial velocity steady-state investigation of the RNase-catalyzed hydrolysis of purified uridine 2′,3′-cyclic phosphate over the pH range 4–8. The pH dependence of k_s/K_s (the turnover number divided by the Michaelis constant) is essentially identical for uridine and cytidine 2′,3′-cyclic phosphates (Herries *et al.*, 1962), as well as for several dinucleoside phosphates (Witzel, 1963), which indicates the same ionizable groups on the enzyme are catalytically important in all cases. However, the values of k_s and K_s are different for the uridine and cytidine cyclic phosphates indicating some degree of base specificity. The equilibrium constant for the hydrolysis reaction has been determined by an isotope dilution technique, and this result has been combined with steady-state and equilibrium binding data to calculate the turnover number of the reverse reaction as a function of pH.

Experimental Section

Materials. The sodium salt of uridine 2′,3′-cyclic phosphate was prepared from 2′(3′)-UMP mixed isomers (Sigma) according to the method of Szer and Shugar (1963). It gave a single spot of R_F 0.51 (the R_F of 3′-UMP is 0.68) when chromatographed on paper in the solvent system saturated ammonium sulfate–1 M sodium acetate (pH 6.6)–2-propanol (80:18:2, v/v; Markham and Smith, 1950). The 280 mμ/260 mμ absorbance ratio in water was 0.21 (lit. (Brown *et al.*, 1952a) value in 0.01 N formic acid–0.05 N formate buffer, 0.195).

Uridine 3′-phosphate-^{14}C (Na salt), used in the equilibrium experiment, was prepared from uridine 2′,3′-cyclic phosphate-^{14}C as previously described for the cytidine compound (Cathou and Hammes, 1964). Mixed 2′ and 3′ isomers of UMP-^{14}C (lot no. 6801, 20 mCi/mmole Schwarz BioResearch) were used to prepare the uridine 2′,3′-cyclic phosphate-^{14}C in the same manner as the nonradioactive material. Most of the

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[1] The abbreviation used in this paper is: RNase, bovine pancreatic ribonuclease A.

radioactive impurities remaining in the 3′-UMP-^{14}C were removed by paper chromatography on Whatman No. 3MM paper using the solvent system 2-propanol–H_2O–concentrated NH_4OH (70:30:0.02, v/v). The 3′-UMP-^{14}C was chromatographically pure in the two solvent systems mentioned above and had a 280 mμ/260 mμ ratio of 0.36 at pH 7 (lit. (Beaven *et al.*, 1955) value, 0.35). The concentrations of both 3′-UMP and uridine 2′,3′-cyclic phosphate were determined spectrophotometrically in a Zeiss PMQII spectrophotometer using extinction coefficients of 10,000 M^{-1} cm^{-1} at 260 mμ, pH 7 (Beaven *et al.*, 1955), and 9570 M^{-1} cm^{-1} at 258.5 mμ in water (Brown *et al.*, 1952b), respectively.

Ribonuclease A, phosphate free, was purchased from Worthington Biochemical Corp. and was used without further purification. Its concentration was determined spectrophotometrically using an extinction coefficient of 9800 M^{-1} cm^{-1} at 278 mμ and pH 6.5 (Sela and Anfinsen, 1957). Crystalline tris(hydroxymethyl)aminomethane was obtained from Nutritional Biochemicals; all other chemicals were standard reagent grade, and deionized distilled water was used to prepare all solutions. Buffer solutions used in the initial velocity experiments were prepared by titration of 0.1 M Tris with acetic acid to the desired pH, using a Radiometer PHM-26 meter, and the solutions were made 0.1 M in NaCl. The 2,5-diphenyloxazole and *p*-bis[2′-(5′-phenyloxazolyl)]benzene used to prepare the scintillation solution (Dintzis, 1961) were purchased from Calbiochem.

Steady-State Kinetics. Initial velocities were measured with a Beckman DK2A ratio recording spectrophotometer. The procedure for a typical kinetic run was as follows: 3 ml of the substrate solution was pipetted into the sample and reference cells in a thermostatted cell holder at a temperature of 25 ± 0.1°. After about 5 min of thermal equilibration, 50 μl of enzyme solution was transferred into the sample cell by means of a 100-μl Hamilton syringe; the solution was stirred with a clean dry Teflon stirrer and recording commenced within 15 sec after mixing. The initial velocity was measured for about 3 min on the 90–100% transmittance scale of the spectrophotometer. Solutions were prepared just prior to use, and in all cases nonenzymatic hydrolysis of the substrate was not detectable during the time between preparation of the solutions and completion of the kinetic run. Measurements were done with light at a wavelength of 286 mμ and the slit was maintained constant in each series of runs (0.2 or 0.4 mm).

The optical density at 286 mμ of the substrate was measured before addition of the enzyme and after the reaction had reached completion. This allowed determination of the molar extinction change, $\Delta\epsilon_{286}$, for the complete hydrolysis of uridine 2′,3′-cyclic phosphate to 3′-UMP.

Equilibrium Experiments. The equilibrium constant for the reaction of 2′,3′-cyclic UMP to 3′-UMP at pH 5 was determined using the procedure of Bahr *et al.* (1965). The sample solutions contained about 7×10^{-3} M 3′-UMP-^{14}C (2.50×10^5 cpm/μmole) and about 3×10^{-5} M RNase. Chromatographic separation of 3′-UMP and uridine 2′,3′-cyclic phosphate was carried out in the $(NH_4)_2SO_4$–sodium acetate–2-propanol solvent system described above. Scintillation counting of the eluates was done with a Packard Tri-Carb (Model 314EX) liquid scintillation spectrometer.

Control experiments showed negligible hydrolysis of uridine 2′,3′-cyclic phosphate during equilibration and chromatography. Bentonite (U.S.P., Fisher) adsorbed about 10% of the uridine cyclic phosphate-^{14}C and about one-third of the 3′-UMP-^{14}C. The concentration of the latter as well as the pH of the solutions were always checked after treatment with bentonite. Ribonuclease assays showed complete removal of the enzyme by bentonite in all runs.

Results and Treatment of Data

The range of substrate concentrations, the number of different concentrations employed, and the difference in molar extinction coefficients between substrate and product, $\Delta\epsilon_{286}$, at each pH are given in Table I. The estimated error in $\Delta\epsilon_{286}$ is about ±4%. The initial velocity at each concentration was always run in duplicate. The initial rate data were plotted according to the usual equation (Lineweaver and Burk, 1934)

$$\frac{[E_0]}{v} = \frac{1}{k_S} + \frac{K_S}{k_S}\frac{1}{[S_0]} \qquad (1)$$

where $[E_0]$ and $[S_0]$ are the total enzyme and substrate concentrations, respectively, v is the initial velocity, k_S is the turnover number, and K_S is the Michaelis constant. A weighted least-squares analysis of the data was used at each pH (*cf.* Cleland, 1967), and the kinetic parameters obtained are summarized in Table II. A typical plot of the data according to eq 1 is given in Figure 1. The estimated uncertainty in the calculated kinetic parameters obtained from such a plot is about ±15%.

TABLE I: Range and Number of Substrate Concentrations Used for the Kinetic Runs.

pH	Substrate Concn Range (10^3 M)	No. of Concn	$10^{-2}\Delta\epsilon_{286}$[a] ($M^{-1}$ cm^{-1})
4.0	0.420–3.48	10	5.0
4.5	0.420–3.53	12	5.2
5.0	0.271–2.01	11	5.2
5.5	0.348–1.81	12	5.5
6.0	0.291–1.81	12	6.0
6.5	0.286–3.28	17	6.0
7.0	0.579–3.68	15	6.0
7.5	0.440–5.38	17	6.0
8.0	0.854–5.13	12	6.0

[a] Extinction coefficient of the product minus that of the substrate.

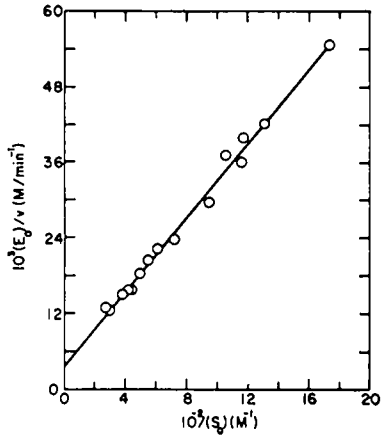

FIGURE 1: Lineweaver–Burk plot for the RNase-catalyzed hydrolysis of uridine 2′,3′-cyclic phosphate at 25° and pH 7 in 0.1 M Tris-acetate–0.1 M NaCl; other conditions were as described under Experimental Section. The straight line was obtained by a weighted least-squares analysis.

The observed equilibrium constant, K_{obsd}, for the hydrolysis of uridine 2′,3′-cyclic phosphate to 3′-UMP was calculated from the equation (Bahr *et al.*, 1965)

$$K_{obsd} = \frac{v_1 c_1 (SA_1 - SA_2)}{v_2 c_2 (SA_2)} \quad (2)$$

Here, v_1 and v_2 are the volumes of the chromatographed equilibrium mixture and added cyclic carrier solution, respectively, c_1 is the concentration of 3′-UMP-^{14}C before addition of carrier, c_2 is the concentration of uridine cyclic phosphate in the carrier solution, and SA_1 and SA_2 are the final corrected specific activities of 3′-UMP and uridine 2′,3′-cyclic phosphate, respectively.

The observed equilibrium constant at pH 5 was found to be 440. This is essentially identical with the equilibrium constant found for the analogous reaction involving cytidine phosphate (Bahr *et al.*, 1965). This value is the result of four trials in duplicate and has an estimated error of ±25%. The pH dependence of K_{obsd} in the pH range 2–8 can be written as

$$K_{obsd} = K^\circ[1 + K_a/(H^+)] \quad (3)$$

where K_a is the ionization constant of the secondary phosphate hydrogen in 3′-UMP and K° is a constant equal to the concentration ratio of monoanionic 3′-UMP to monoanionic uridine 2′,3′-cyclic phosphate. From the value of K_{obsd} at pH 5 and a pK_a value of 5.74 (Anderson *et al.*, 1968), K° can be calculated to be 370.

Discussion

For a single substrate–single product enzymatic reaction with one predominant pathway, the pH dependence of k_s/K_s reflects only the ionization states of the free substrate and enzyme, regardless of the number of reaction intermediates (Peller and Alberty, 1959; Alberty and Bloomfield, 1963). The symmetrical bell-shaped

TABLE II: Kinetic Parameters for the RNase-Catalyzed Hydrolysis of Uridine 2′,3′-Cyclic Phosphate.[a]

pH	k_s (sec^{-1})	$10^4 K_s$ (M)
4.0	0.254	3.23
4.5	0.435	1.29
5.0	0.812	0.93
5.5	1.37	0.85
6.0	2.26	1.40
6.5	4.20	3.87
7.0	4.16	7.30
7.5	1.88	8.65
8.0	1.14	15.8

[a] 25°; 0.1 M Tris-acetate–0.1 M NaCl.

curve observed in the log (k_s/K_s)–pH plot of Figure 2 is consistent with the mechanism

$$\begin{array}{ccccccc} EH_2 & & X_1H_2 & & X_nH_2 & & EH_2 \\ \uparrow\downarrow K_a & & \uparrow\downarrow & & \uparrow\downarrow & & \uparrow\downarrow \\ S + EH & \rightleftharpoons & X_1H & \rightleftharpoons \cdots\cdots & X_nH & \rightleftharpoons & EH + P \\ \uparrow\downarrow K_b & & \uparrow\downarrow & & \uparrow\downarrow & & \uparrow\downarrow \\ E & & X_1 & & X_n & & E \end{array} \quad (4)$$

where K_a and K_b represent ionization constants of the free enzyme and an arbitrary number of reaction intermediates has been assumed. For this mechanism

$$\frac{k_s}{K_s} = \frac{\bar{k}_s}{\bar{K}_s[1 + (H^+)/K_a + K_b/(H^+)]} \quad (5)$$

where $\bar{k}_s$ and $\bar{K}_s$ are the pH-independent turnover number and Michaelis constant, respectively. Equation 5 can be fit to the experimental data in the usual manner (*cf.* Frieden and Alberty, 1955). The curve shown in Figure 2 for uridine 2′,3′-cyclic phosphate, which fits the experimental points closely, was calculated with eq 5 and pK_a = 5.4, pK_b = 6.4, and $\bar{k}_s/\bar{K}_s$ = 2940 M^{-1} sec^{-1}. According to the mechanism given by eq 4, the pH dependence of k_s and K_s can be written as (Peller and Alberty, 1959)

$$k_s = \frac{\bar{k}_s}{1 + (H^+)/K_a' + K_b'/(H^+)} \quad (6)$$

$$K_s = \frac{\bar{K}_s[1 + (H^+)/K_a + K_b/(H^+)]}{1 + (H^+)/K_a' + K_b'/(H^+)} \quad (7)$$

where K_a' and K_b' are apparent ionization constants which are dependent upon the number and rates of interconversion of the intermediates. In particularly fortunate cases, the pK_a's can be identified with particular intermediates. The best fit of the experimental points to eq 6 and 7 is shown by the dashed lines in Fig-

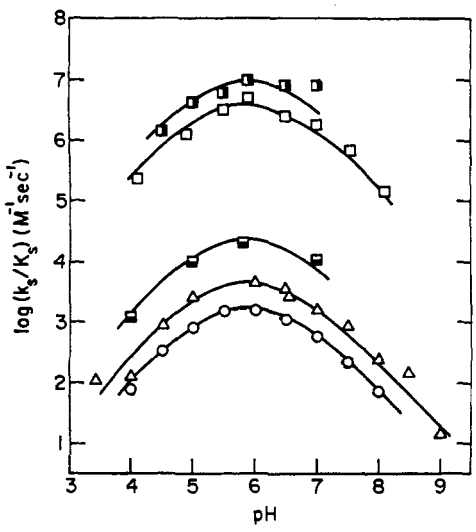

FIGURE 2: Logarithm of the ratio of the turnover number to the Michaelis constant as a function of pH at 25° for the reaction of RNase with several substrates: 2',3'-cyclic UMP (○), 2',3'-cyclic CMP (△, Herries *et al.*, 1962), UpU (▬), UpA (▭), and CpA (◨) (Witzel, 1963). The solid curves were calculated from eq 5 of the text using $pK_a = 5.4$, $pK_b = 6.4$, and the following values of $\bar{k}_S/\bar{K}_S$: uridine 2',3'-cyclic phosphate, 2940 M^{-1} sec^{-1}; cytidine 2',3'-cyclic phosphate, 7540 M^{-1} sec^{-1}; UpU, 3.88 × 10^4 M^{-1} sec^{-1}; UpA, 6.80 × 10^6 M^{-1} sec^{-1}; CpA, 1.59 × 10^7 M^{-1} sec^{-1}.

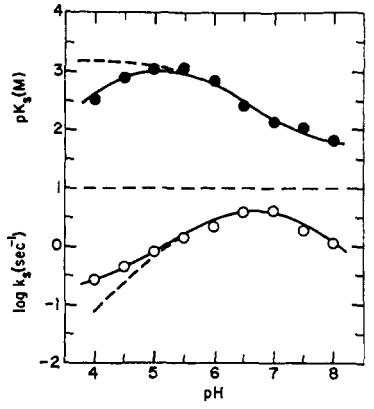

FIGURE 3: Variation with pH of the logarithm of the turnover number, k_S (○), and the negative logarithm of the Michaelis constant, K_S (●), for the RNase-catalyzed hydrolysis of uridine 2',3'-cyclic phosphate. The dashed lines and the solid lines were calculated from eq 6 and 7 and eq 8 and 9, respectively, using $pK_a = 5.4$, $pK_b = 6.4$, $pK_a' = 5.8$, $pK_b' = 7.5$, $\bar{k}_S = 4.90$ sec^{-1}, $\bar{K}_S = 1.67 \times 10^{-3}$ M, and $k_A = 0.19$ sec^{-1}. See text for details.

ure 3. The parameters used to calculate the theoretical curves are $pK_a = 5.4$, $pK_b = 6.4$, $pK_a' = 5.8$, $pK_b' = 7.5$, $\bar{k}_S = 4.90$ sec^{-1}, and $\bar{K}_S = 1.67 \times 10^{-3}$ M. Clearly the fit between theory and experiment is unacceptable at pH values below 5. If the plots of log (k_S/K_S) and log k_S *vs.* pH are to be consistent with the mechanism of eq 4, the curves should be bell shaped with slopes approaching unity at low and high pH values. This implies the pK_S–pH curve should have zero slope at low and high pH values. This appears to be true at high pH values but not at low pH values. Moreover, pK_S does not approach a constant value at low pH values for any substrate (Herries *et al.*, 1962; Witzel, 1963). A possible explanation for this behavior is that a parallel path exists at low pH values such that one of the diprotonated forms of the enzyme–substrate complex is catalytically active. The second proton could go onto the substrate or onto an ionizable group on the enzyme. The occurrence of parallel pathways produces a complex situation which has been analyzed in detail elsewhere (Alberty and Bloomfield, 1963). Equations of the form

$$k_S = \frac{\bar{k}_S[1 + k_A(H^+)/\bar{k}_S K_a']}{1 + (H^+)/K_a' + K_b'/(H^+)} \quad (8)$$

$$K_S = \frac{\bar{K}_S[1 + k_A(H^+)/\bar{k}_S K_a'][1 + (H^+)/K_a + K_b/(H^+)]}{1 + (H^+)/K_a' + K_b'/(H^+)} \quad (9)$$

are possible and fit the experimental data quite well as shown by the solid lines in Figure 3 which were calculated with the parameters given above and with the turnover number for the "acid" pathway, k_A, equal to 0.19 sec^{-1}. In view of the complexity introduced by the assumption of parallel pathways, and the small amount of data suggesting parallel pathways, a more detailed analysis of the data is not warranted. An alternative explanation of the deviation from eq 6 and 7 is that the increased concentration of un-ionized acetic acid in the buffer at low pH values is affecting the reaction rate.

Included in Figure 2 are plots of k_S/K_S taken from the literature for various substrates of ribonuclease, namely, cytidine 2',3'-cyclic phosphate (Herries *et al.*, 1962), UpU, UpA, and CpA (Witzel, 1963). Within experimental error, all of these curves can be fit by eq 5 with pK values of 5.4 and 6.4, $\bar{k}_S/\bar{K}_S$ being different for each case (7540 M^{-1} sec^{-1} for cytidine 2',3'-cyclic phosphate, 3.88 × 10^4 M^{-1} sec^{-1} for UpU, 6.80 × 10^6 M^{-1} sec^{-1} for UpA, and 1.59 × 10^7 M^{-1} sec^{-1} for CpA). (These pK values are reasonably close to the values of 5.22 and 6.78 reported by Herries *et al.* (1962) for cytidine 2',3'-cyclic phosphate; in fact both sets of constants fit their data quite well.) *These results imply that the same ionizable groups on the free enzyme are involved in hydrolysis and transesterification.* The two ionizable groups on the enzyme are probably the imidazole side chains of histidine residues 12 and 119.

The ionization state of the cytosine ring appears to be unimportant since the pH dependence of k_S/K_S for both cytidine and uridine derivatives is similar around pH 4, and the pK of the ring nitrogen-3 of cytidine is 4.32 (Anderson *et al.*, 1968). Equilibrium binding studies of cytidine and uridine 3'-phosphates also suggested that the two possible ionized states of the cytidine ring bind essentially identically with ribonuclease (Anderson *et al.*, 1968).

The turnover number of the reverse reaction, k_P, can be calculated from the equation (Haldane, 1930)

$$K_{obsd} = \frac{k_S K_P}{K_S k_P} \quad (10)$$

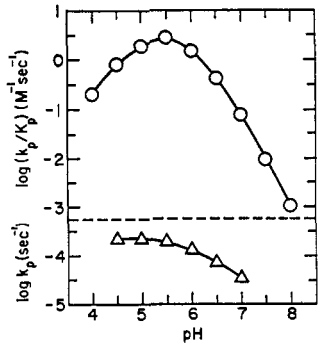

FIGURE 4: Logarithm of the steady-state kinetic parameters k_P/K_P (○) and k_P (△) as a function of pH at 25° for the RNase-catalyzed hydrolysis of uridine 2′,3′-cyclic phosphate. The lines have no theoretical significance.

since K_{obsd}, k_S, K_S, and K_P (Anderson *et al.*, 1968) are known. Although K_P in eq 10 is a Michaelis constant, it has been shown in the case of cytidine 3′-phosphate that the equilibrium dissociation constant is essentially identical with the Michaelis constant (Hammes and Schimmel, 1965); therefore the identity of the Michaelis constant and equilibrium dissociation constant for uridine 3′-phosphate is almost certainly a good approximation. In Figure 4, k_P/K_P and k_P are shown as a function of pH. The error in these quantities is considerable since four experimentally determined quantities were used to derive them. The pH dependence of k_P/K_P reflects the ionization states of 3′-UMP and free enzyme involved in the enzymatic reaction, and will be discussed in detail in a forthcoming paper concerned with relaxation spectra of the 3′-UMP-ribonuclease system (G. G. Hammes and F. G. Walz, Jr., in preparation). The pH dependence of k_P is obviously considerably different from k_S, although because of the considerable imprecision in k_P a quantitative interpretation in terms of apparent pK values will not be attempted. However, the fact that k_P and k_S vary with pH in a different manner implies that more than one intermediate is involved in the enzymatic reaction.

The steady-state kinetic parameters differ for uridine 2′,3′-cyclic phosphate and cytidine 2′,3′-cyclic phosphate (Herries *et al.*, 1962) in the following way. The k_S values are roughly the same for both compounds at pH 4–5. Above pH 5, k_S of the cytidine compound becomes increasingly greater, as pH increases, than that of the uridine compound, being nine times greater at pH 8. The K_S values are esentially identical at pH 6–6.5, are two to three times greater for the uridine compound at pH 4–5, and are two to three times greater for the cytidine compound at pH 7–8. On the other hand, 3′-CMP binds slightly stronger to ribonuclease than 3′-UMP at pH 4.5–7 (Anderson *et al.*, 1968). Thus the base appears to play a small but detectable role in the binding process, and can have a greater effect on the turnover number.

Temperature-jump experiments have indicated that a large number of reaction intermediates are involved in the ribonuclease-catalyzed hydrolysis of cytidine 2′,3′-cyclic phosphate (Erman and Hammes, 1966; Hammes, 1968a,b). Preliminary measurements indicate a similar situation exists in the case of the analogous reaction involving uridine phosphate. The steady-state parameters provide lower bounds for the actual rate constants: k_S/K_S and k_P/K_P give lower bounds for the bimolecular rate constants, while k_S and k_P provide lower bounds for all of the first-order rate constants in the forward and reverse reactions, respectively (Peller and Alberty, 1959). Several plausible chemical mechanisms are consistent with available structural and kinetic data (*cf.* Hammes, 1968a,b). The results presented here strongly suggest the same ionizable groups of the enzyme are involved in the transesterification and hydrolysis steps. A concerted acid–base mechanism and an essentially base-catalyzed mechanism involving histidine residues 12 and 119 of ribonuclease are attractive possibilities (*cf.* Hammes, 1968b).

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References

Alberty, R. A., and Bloomfield, V. (1963), *J. Biol. Chem. 238*, 2804.

Anderson, D. G., Hammes, G. G., and Walz, F. G., Jr. (1968), *Biochemistry 7*, 1637.

Bahr, J. T., Cathou, R. E., and Hammes, G. G. (1965), *J. Biol. Chem. 240*, 3372.

Beaven, G. H., Holiday, E. R., and Johnson, E. H. (1955), *in* The Nucleic Acids, Vol. 1, Chargaff, E., and Davidson, J. N., Ed., New York, N. Y., Academic, 5513.

Brown, D. M., Dekker, C. A., and Todd, A. R. (1952a), *J. Chem. Soc.* 2715.

Brown, D. M., Margrath, D. I., and Todd, A. R. (1952b), *J. Chem. Soc.*, 2708.

Cathou, R. E., and Hammes, G. G. (1964), *J. Am. Chem. Soc. 86*, 3240.

Cheung, C. S., and Abrash, H. I. (1964), *Biochemistry 3*, 1883.

Cleland, W. W. (1967), *Advan. Enzymol. 29*, 1.

Dintzis, H. M. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 247.

Erman, J. E., and Hammes, G. G. (1966), *J. Am. Chem. Soc. 88*, 5607.

Findlay, D., Herries, D. G., Mathias, A. P., Rabin, B. R., and Ross, C. A. (1962), *Biochem. J. 85*, 152.

Frieden, C., and Alberty, R. A. (1955), *J. Biol. Chem.* 212, 859.

Haldane, J. B. S. (1930), Enzymes, London, Longmans, p 80.

Hammes, G. G. (1968a), *Accounts. Chem. Res. 1*, 321.

Hammes, G. G. (1968b), *Advan. Protein Chem. 23*, 1.

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

Herries, D. G., Mathias, A. P., and Rabin, B. R. (1962), *Biochem. J. 85*, 127.

Hummel, J. P., and Kalnitsky, G. (1964), *Ann. Rev.*

Biochem. 33, 15.
Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.
Markham, R., and Smith, J. D. (1950), *Biochem. J. 46*, 513.
Peller, L., and Alberty, R. A. (1959), *J. Am. Chem. Soc. 81*, 5907.
Ramsden, E. N., and Laidler, K. J. (1966), *Can. J. Chem. 44*, 2597.
Scheraga, H. A., and Rupley, J. A. (1962), *Advan. Enzymol. 24*, 161.
Sela, M., and Anfinsen, C. B. (1957), *Biochim. Biophys. Acta 24*, 229.
Szer, W., and Shugar, P. (1963), *Biochem. Prepn. 10*, 139.
Witzel, H. (1963), *Progr. Nucleic Acid Res. 2*, 221.

Effect of Charge on the Carbamylation and Binding Constants of Eel Acetylcholinesterase in Reaction with Neostigmine and Related Carbamates*

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ABSTRACT: The effect of charged quaternary ammonium groups on the carbamylation, k_2, and binding, K_a, constants governing inhibition of eel acetylcholinesterase by neostigmine and 13 related charged and uncharged phenyl *N*-methyl- and phenyl *N,N*-dimethylcarbamates was studied. Carbamates with substituents which bound well to the anionic site, whether charged or uncharged, were characterized by relatively high rates of carbamylation. For example, the k_2 values of *N*-methylneostigmine and of one of its carbon isosteres were 142 ± 4 and 97 ± 4 min^{-1} at 25°, respectively.

The phenyl substituents of the *N*-methylcarbamates appeared to bind more strongly to the anionic site than did those of the comparable *N,N*-dimethyl compounds. Orientation with respect to the anionic site seemed to be the predominant factor determining rates of carbamylation. The coulombic and noncoulombic energies associated with binding of substituents to the anionic site were calculated. The K_a and k_2 values of five carbamates and of diisopropyl phosphorofluoridate in reaction with eel and erythrocyte acetylcholinesterase were compared.

Esters of carbamic acid containing a charged quaternary ammonium group are more potent inhibitors of acetylcholinesterase (EC 3.1.1.7) than their uncharged analogs (Stedman, 1926; Stevens and Beutel, 1941; Kolbezen *et al.*, 1954; Kitz *et al.*, 1967b). The charge is generally considered to increase initial binding and to improve affinity through coulombic attraction to the anionic portion of the active site (Wilson and Bergmann, 1950). It is now widely accepted that inhibition by carbamates occurs by the following reaction

$$\mathrm{EH + CX} \underset{}{\overset{K_a}{\rightleftharpoons}} \mathrm{EHCX} \xrightarrow{k_2} \mathrm{EC + HX} \quad (\text{overall: } \mathrm{EH + CX} \xrightarrow{k_i} \mathrm{EC + HX}) \qquad (1)$$

where EH and CX are the enzyme and carbamate, respectively, and EHCX is an intermediate enzyme–inhibitor complex whose formation is controlled by the equilibrium affinity constant, K_a (Wilson *et al.*, 1960; Main and Hastings, 1966; O'Brien, 1968). EC is the carbamylated enzyme and the rate of its formation is controlled by k_2, the carbamylation rate constant. HX is the leaving group and k_i ($=k_2/K_a$) is the rate constant governing the over-all rate of inhibition.

Although charge has been clearly implicated in initial binding, its effect on the following carbamylation step is uncertain. Metzger and Wilson (1963) reported that certain alkylammonium ions accelerated the inhibition of acetylcholinesterase by dimethylcarbamyl fluoride. Presumably, the quaternary ammonium ions exerted their effect by occupying the anionic site which the relatively small fluoride leaving group of the carbamate would have left empty. Clearly such occupation could have affected either initial binding or the rate of carbamylation or both. However, the issue could not be decided because the criterion used to measure relative rates of inhibition did not separate initial binding from the carbamylation step. Moreover, it is not certain that the influence of the charge on a simple quaternary am-

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