[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY, CAMBRIDGE, MASSACHUSETTS 02139]

Relaxation Spectra of Ribonuclease. I. The Interaction of Ribonuclease with Cytidine 3′-Phosphate[1]

BY RENATA E. CATHOU AND GORDON G. HAMMES

RECEIVED MARCH 18, 1964

Kinetic studies of the interaction of bovine pancreatic ribonuclease with cytidine 3'-phosphate have been made at high enzyme concentrations ($>10^{-5}$ M) using the temperature jump method. Two different relaxation processes with characteristic relaxation times ranging from about 1 msec. to less than 20 μsec. have been observed. One process is concentration dependent and is observed at all pH's in the range studied (pH 5.0–7.5). This effect is due to the formation of the enzyme–substrate complex; the rate constants for both complex association and dissociation were determined and interpreted in terms of elementary mechanistic steps involving an enzyme group with a pK of 6.7 in the free enzyme. The other relaxation process is observed only when the enzyme is essentially all in the form of the enzyme–cytidine 3'-phosphate complex. The relaxation time is concentration independent, suggesting an intramolecular process. Moreover, the variation of the relaxation time with pH is much greater than that expected for the participation of a single ionizing group; *i.e.*, a cooperative process is occurring. The rate of this reaction in D_2O at the low pH plateau is one-seventh as large as the corresponding rate in H_2O, indicating that a proton transfer of some sort is probably involved.

Ribonuclease has been the subject of many investigations,[2] but to date no direct information concerning the number and nature of the intermediates involved in the conversion of ribonucleic acid to a 2′:3′-cyclic phosphate or in the conversion of the latter to the 3′-phosphate has been given. Several side-chain groups on the enzyme have been implicated as forming part of the active site. The experimental basis of such conclusions has mainly involved either blocking specific groups on the enzyme or studying the pH dependence of the steady-state kinetic parameters in the hydrolysis of cytidine 2′:3′-cyclic phosphate.[3,4] In the former case, activity is abolished when certain specific side-chain groups are blocked.[5-8] The most impressive evidence to date suggests that at least histidine-12,[5] histidine-119,[5,6] and lysine-41,[7,8] where the amino acid numbering is that in general use, form part of the active site. The interpretation of the apparent pK's measured from the steady-state parameters depends on the mechanism assumed and in general cannot be interpreted as a pK of a single ionizing group.[9,10] In neither case can a given group be identified with a single elementary step in the mechanism.

In order to investigate enzymatic reaction intermediates, high enzyme concentrations generally must be employed. Under these conditions, enzyme processes occur so rapidly that special experimental methods are needed. The recent development of relaxation techniques now permits reactions with time constants as short as 5×10^{-10} sec. to be studied.[11] The method of greatest utility for biological reactions is the temperature jump method. This method consists of quickly raising the temperature of an equilibrium mixture of reactants and then measuring the rates at which the new equilibrium concentrations are established. Theoretical considerations and details of the method have been given elsewhere (*cf.* ref. 11). Because high enzyme and substrate concentrations can be used, reaction intermediates can be directly observed.[12]

As a first step towards elucidating the over-all mechanism, the interaction of ribonuclease and cytidine 3′-phosphate, the end product of the over-all reaction, has been studied. This interaction has been studied by Hummel, *et al.*,[13] and Ross, *et al.*,[14] who found that spectral changes occurred upon complex formation. That cytidine 3′-phosphate binds to the active site of ribonuclease is strongly suggested by the fact that cytidine 3′-phosphate prevents carboxymethylation of the histidine residues at the active site.[15] The pH dependence of the product–enzyme Michaelis constant has been determined from steady-state kinetic studies.[16]

In this work, a kinetic study of the ribonuclease–cytidine 3′-phosphate interaction utilizing the temperature jump method is presented; also, the results permit an independent evaluation of the binding constants over a range of pH. The pH dependence of the kinetic constants suggests that a group on the enzyme with a pK of 6.7 is intimately involved in the binding process. In addition, evidence suggesting an isomerization of the initial complex, possibly involving a protein conformational change, has been obtained.

Experimental

The temperature jump apparatus used has been described in great detail elsewhere[12,17]; the only modification was that the volume of the solution required for the temperature jump cell has been decreased to 12.5 ml. Ribonuclease A was obtained from crystalline pancreatic ribonuclease (Worthington Biochemical Corporation, Lot No. R598-602, R615, and R614C) by the method of Taborsky.[18] The fractions in the main peak corresponding to

(1) This work was supported in part by the U. S. Army Signal Corps, Air Force Office of Scientific Research, and Office of Naval Research, and in part by the National Institutes of Health (MG-07803).

(2) See, for example, the recent reviews by H. A. Scheraga and J. A. Rupley, *Advan. Enzymol.*, **24**, 161 (1962), and C. B. Anfinsen and F. H. White, Jr., in "The Enzymes," Vol. 5, P. D. Boyer, H. Lardy, and K. Myrbäck, Ed., Academic Press, New York, N. Y., 1961, p. 95.

(3) D. G. Herries, *Biochem. Biophys. Res. Commun.*, **3**, 666 (1960).

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

(5) A. M. Crestfield, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **238**, 2413 (1963).

(6) W. D. Stein and E. A. Barnard, *J. Mol. Biol.*, **1**, 350 (1959).

(7) C. B. Anfinsen, *Brookhaven Symp. Biol.*, **15**, 184 (1962).

(8) C. H. W. Hirs, *ibid.*, **15**, 154 (1962).

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

(10) T. C. Bruice and G. L. Schmir, *J. Am. Chem. Soc.*, **81**, 4552 (1959).

(11) M. Eigen and L. de Maeyer in "Technique of Organic Chemistry," Vol. VIII, Part II, S. L. Friess, E. S. Lewis, and A. Weissberger, Ed., Interscience Publishers, Inc., New York, N. Y., 1963, p. 895.

(12) G. G. Hammes and P. Fasella, *J. Am. Chem. Soc.*, **84**, 4644 (1962).

(13) J. P. Hummel, D. A. Ver Ploeg, and C. A. Nelson, *J. Biol. Chem.*, **236**, 3168 (1961).

(14) C. A. Ross, A. P. Mathias, and B. R. Rabin, *Biochem. J.*, **85**, 145 (1962).

(15) E. A. Barnard and W. D. Stein, *J. Mol. Biol.*, **1**, 339 (1959).

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

(17) G. G. Hammes and J. I. Steinfeld, *J. Am. Chem. Soc.*, **84**, 4639 (1962).

(18) G. Taborsky, *J. Biol. Chem.*, **234**, 2652 (1959).

Taborsky's fraction D were pooled, desalted with a mixed bed ion-exchange resin (Bio-rad, supplied by California Biochemical Corporation), lyophilized, and stored dry at −29° until used. Care was taken to avoid conditions where aggregation is known to occur. Solutions of the enzyme were prepared fresh for each experiment. The enzyme concentration was determined by spectrophotometric measurement of the absorbancy at 278 mμ with a Beckman Model DU spectrophotometer, using a molar extinction coefficient of 9800.[19] Enzymatic activity was determined by the spectrophotometric method of Crook, *et al.*[20] The catalytic activity was determined before and after temperature jump experiments to check for possible enzyme denaturation. At least 40 high-voltage pulses could be applied without any loss of enzymatic activity. For most of these experiments cytidine 3'-phosphate was purified from a mixture of cytidine 3'-phosphate and cytidine 2'-phosphate (Sigma Chemical Co.) essentially by the method of Cohn[21]; 0.012 *M* HCOOH was used to elute the nucleotides from Dowex-1-formate. Two sequential chromatographic separations were used to obtain pure cytidine 3'-phosphate. Paper chromatography of the latter in a solvent system of saturated ammonium sulfate, 1 *M* sodium acetate (pH 6.6), and 2-propanol (80:18:2),[22] which separates cytidine 3'-phosphate, cytidine 2'-phosphate, cytidine 2':3'-cyclic phosphate, and cytidine, showed that only cytidine 3'-phosphate was present. The compound was lyophilized and stored dry at −29° until used.

For one experiment, cytidine 3'-phosphate was prepared by the action of ribonuclease on cytidine 2':3'-cyclic phosphate. To 1 g. of cytidine 2':3'-cyclic phosphate (prepared by the method of Shugar and Wierzchowski)[23] in 95 ml. of water at pH 6.0 were added 50 mg. of ribonuclease dissolved in 5 ml. of water. The pH of the solution was maintained at 6.0 by the addition of NaOH in a pH Stat (Radiometer, Copenhagen) overnight; nitrogen was bubbled into the solution during this period. Five grams of bentonite (Fisher Scientific Co., U.S.P., powder) was then added and the suspension was stirred for 30 min. at room temperature, after which the bentonite was removed by filtration. This procedure removes all of the ribonuclease from the solution.[24] Paper chromatography showed the presence of only cytidine 3'-phosphate. The solution was diluted to 300 ml. and applied to a 2 × 32-cm. column of Dowex-50 (H^+), 100–200 mesh, in H_2O, at 2°. The cytidine 3'-phosphate was eluted by the addition of 1600 ml. of H_2O. The solution was then lyophilized and the powder stored dry at −29° until used. This method of preparation of cytidine 3'-phosphate was found preferable to the separation of cytidine isomers as gram quantities can easily be prepared. For temperature jump experiments, fresh stock solutions of cytidine 3'-phosphate were prepared as required. The concentration of cytidine 3'-phosphate was determined spectrophotometrically by measuring the absorbancy at 271 mμ at pH 7 using a molar extinction coefficient of 9400.[25]

In practice, solutions for temperature jump experiments were prepared by dissolving the enzyme in 12.5 ml. of freshly boiled, distilled, and deionized water which was also 0.1 *M* in KNO_3 and 2×10^{-5} *M* in a colorimetric pH indicator; the pH of the solution was adjusted to the desired value with the use of a Radiometer pH meter. The pH indicators employed were phenol red in the pH range 6.7–7.5, chlorophenol red in the pH ranges 5.5–6.5, and methyl red at pH 5.0 and in one experiment at pH 5.5. The relaxation times of the protolytic reactions of pH indicators are much shorter than the relaxation times of the reactions involving ribonuclease and cytidine 3'-phosphate. This fact is ascertained by studying the appropriate "blank" solution. Therefore, the progress of the binding process can be followed with the pH indicator provided that the binding process is accompanied by some change in the hydrogen ion concentration. The following wave lengths of the detecting light were employed in temperature jump experiments: 558 mμ for phenol red, 573 mμ for chlorphenol red, and 520 mμ for methyl red.

A blank of enzyme and indicator was always tried first. A typical relaxation effect due to the enzyme–indicator interaction is shown in Fig. 1. Cytidine 3'-phosphate was then added to the solution. A number of concentrations of cytidine 3'-phosphate was used and the corresponding relaxation times were obtained. The method used to compute relaxation times has been described previously.[17] A blank of indicator and cytidine 3'-phosphate in 0.1 *M* KNO_3 was also tested. Temperature jump experiments employing D_2O as solvent were performed in the same manner as experiments employing H_2O. The enzyme was first dissolved in D_2O (99.7%) and then lyophilized. All reagents were dissolved in D_2O; aqueous solutions of HCl and NaOH were used to adjust the pH. The pD of each solution was determined by reading the pH on a Radiometer pH meter and then correcting the values so obtained using the equation[26]

$$\mathrm{pD} = \mathrm{pH} + 0.4$$

Chlorphenol red was used in the pD range 6.5–7.0 and phenol red was used in the pD range 7.5–7.7. The range of enzyme $[E_0]$ and cytidine 3'-phosphate $[S_0]$ concentrations used are given in Table I.

TABLE I

RANGE OF ENZYME AND SUBSTRATE CONCENTRATIONS EMPLOYED

pH	$[E_0]$, *M*	$[S_0]$, *M*
5.00	3.00×10^{-5}	0.99×10^{-5}–1.41×10^{-4}
5.50	3.23×10^{-5}–1.27×10^{-4}	8.79×10^{-6}–7.84×10^{-4}
6.00	0.82×10^{-4}–1.57×10^{-4}	5.06×10^{-5}–2.87×10^{-3}
6.50	0.82×10^{-4}–1.07×10^{-4}	5.07×10^{-5}–2.87×10^{-3}
6.70	1.41×10^{-4}	2.87×10^{-4}–1.10×10^{-3}
7.00	1.10×10^{-4}	1.00×10^{-4}–1.50×10^{-3}
7.50	1.82×10^{-4}	2.02×10^{-4}–3.85×10^{-3}

Titrations of cytidine 3'-phosphate were performed on a Radiometer Type TTT 1b automatic titrator at 25.0° in order to determine the acid dissociation constants. A solution containing cytidine 3'-phosphate in 0.1 *M* KNO_3 was titrated with a solution of standardized HCl. A blank solution containing only 0.1 *M* KNO_3 was also titrated with a solution of the same concentration of HCl. Both titrations were performed in duplicate.

Results and Treatment of Data

The p*K*'s for the two most tightly bound protons of cytidine 3'-phosphate were found by first subtracting the titration curve of the blank solution of 0.1 *M* KNO_3 from the curve of cytidine 3'-phosphate and then determining the pH values for half-neutralization from the resulting curve. The p*K*'s at 25.0° were found to be 4.30 ± 0.02 and 5.90 ± 0.02. (These constants are defined in terms of the hydrogen ion activity as determined by the glass electrode.)

Chemical relaxation effects were observed for solutions containing enzyme and indicator as illustrated in Fig. 1. The relaxation times measured for the indicator–enzyme reactions will not be analyzed in detail since they are not directly related to the enzyme mechanism. When cytidine 3'-phosphate was added to the solution another (shorter) relaxation effect was observed, the absorption change being in the opposite direction to that for enzyme and indicator alone (see Fig. 1). With increasing cytidine 3'-phosphate concentrations, the magnitude of the enzyme–indicator effect decreased and finally disappeared, while the second relaxation time decreased until it became too short to measure. No relaxation effects were observed for solutions containing only indicator and cytidine 3'-phosphate. Since this second relaxation time is markedly concentration dependent the reaction is probably at least bimolecular. The simplest mechanism to assume for the binding process is

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \qquad (1)$$

(19) M. Sela and C. B. Anfinsen, *Biochem. Biophys. Acta*, **24**, 229 (1957).

(20) E. M. Crook, A. P. Mathias, and B. R. Rabin, *Biochem. J.*, **74**, 234 (1960).

(21) W. E. Cohn, *J. Am. Chem. Soc.*, **72**, 2811 (1950).

(22) R. Markham and J. A. Smith, *Biochem. J.*, **52**, 552 (1952).

(23) D. Shugar and K. L. Wierzchowski, *Bull. Acad. Polon. Sci., Ser. Sci.*; **6**, 283 (1958); W. Szer and D. Shugar, *Biochem. Prep.*, **10**, 139 (1963).

(24) B. Singer and H. Fraenkel-Conrat, *Virology*, **14**, 59 (1961).

(25) R. J. C. Harris, S. F. D. Orr, E. M. F. Roe, and J. F. Thomas, *J. Chem. Soc.*, 489 (1953).

(26) P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960).

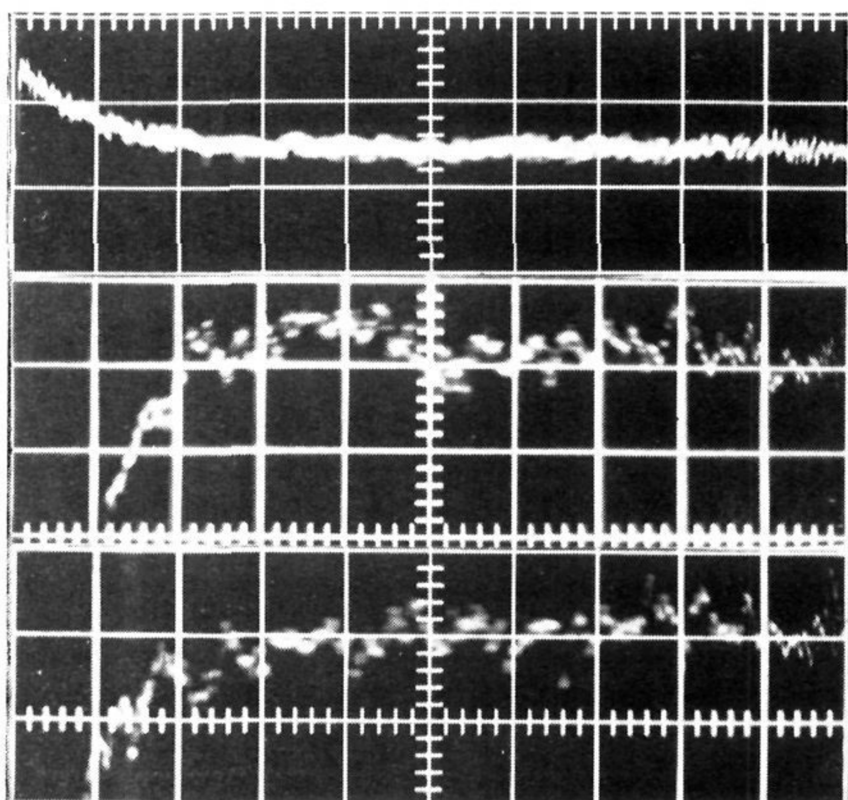


Fig. 1.—Oscilloscope traces of temperature jump effects observed with ribonuclease and cytidine 3′-phosphate at pH 6.7. All solutions contained 0.1 M KNO_3 and 2×10^{-5} M chlorophenol red indicator. Top picture, enzyme–indicator interaction: 1.41 $\times 10^{-4}$ M ribonuclease $[E_0]$; abscissa scale: 1 msec. per large division; τ_1 = 0.792 msec. Middle picture, enzyme–substrate interaction: $[E_0] = 1.35 \times 10^{-4}$ M, $[S_0]$ (cytidine 3′-phosphate) $= 2.87 \times 10^{-4}$ M; abscissa scale: 100 μsec. per large division; τ_2 = 90 μsec. Bottom picture, intramolecular process: $[E_0] = 1.22 \times 10^{-4}$ M, $[S_0] = 1.10 \times 10^{-3}$ M; abscissa scale: 100 μsec. per large division; τ_3 = 113 μsec. The vertical scale in all cases is in arbitrary units of decreasing absorbancy.

where E designates the enzyme and S the substrate, in this case cytidine 3′-phosphate. The reciprocal relaxation time, $1/\tau_2$, of the observed relaxation effect is related to the rate constants k_1 and k_{-1} by[11]

$$1/\tau_2 = k_1(\bar{E} + \bar{S}) + k_{-1} \qquad (2)$$

where $\bar{E}$ and $\bar{S}$ are the equilibrium concentrations of enzyme and substrate, respectively. Therefore, a plot of $1/\tau_2$ *vs.* $(\bar{E} + \bar{S})$ should be a straight line with intercept k_{-1} and slope k_1. In order to calculate $(\bar{E} + \bar{S})$, however, the equilibrium binding constant, K $(= k_1/k_{-1})$, must be known. In practice, a value of the equilibrium constant was assumed and a plot of $1/\tau_2$ *vs.* $(\bar{E} + \bar{S})$ was constructed. The best straight line was then drawn through the points and a new value of the constant K was determined by taking the ratio of the slope to intercept. This new value of K was then used to calculate $(\bar{E} + \bar{S})$ and the entire process was repeated until the value of K used to calculate the equilibrium concentration was identical with that found by taking the ratio of slope to intercept. In Fig. 2 and 3, a plot of $1/\tau_2$ *vs.* $(\bar{E} + \bar{S})$ is shown for several pH's. The error in the relaxation times is about $\pm 15\%$. Values of k_1, k_{-1}, and K obtained in the pH range 5.0–7.5 are given in Table II. The maximum experimental error associated with the rate constants is estimated to be $\pm 25\%$; in the case of the equilibrium constants, the associated error is probably slightly larger.

At substrate concentrations such that an appreciable amount of the enzyme is bound to substrate, a third chemical relaxation was observed in the pH range 5.0–6.7. Above pH 6.7 either the effect became too fast to be observed (*i.e.*, $\tau_3 < 20$ μsec.) or the amplitude of the effect became too small to detect. This relaxation time

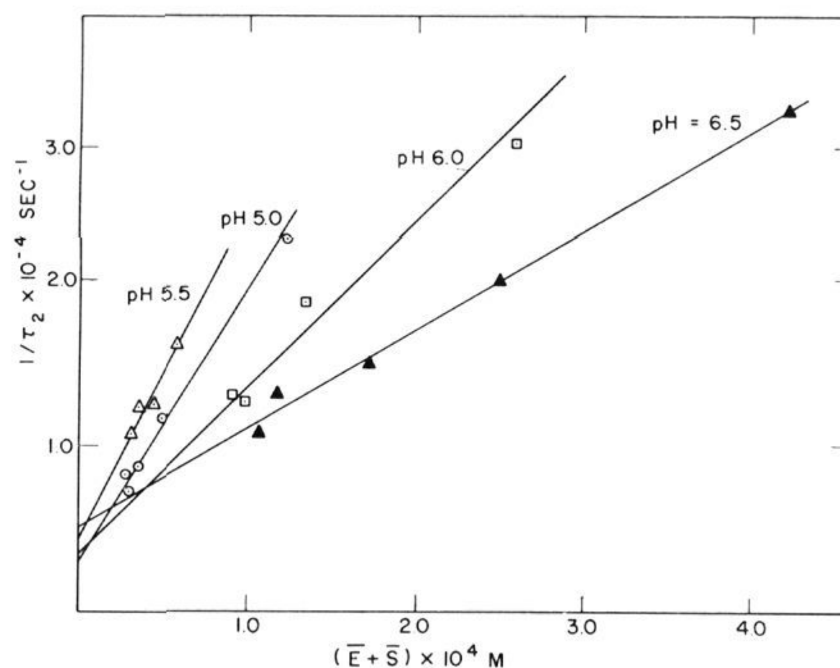


Fig. 2.—Variation of $1/\tau_2$ with $(\bar{E} + \bar{S})$ at several pH's.

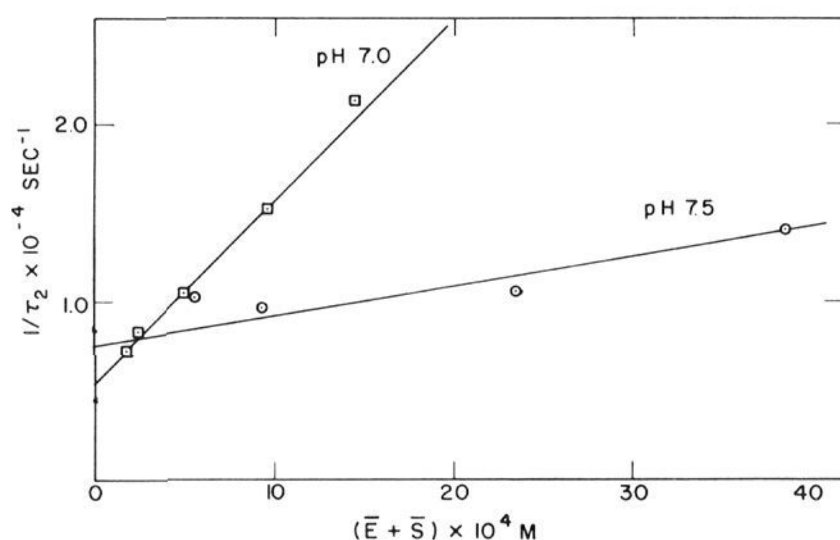


Fig. 3.—Variation of $1/\tau_2$ with $(\bar{E} + \bar{S})$ at pH 7.0 and 7.5.

was independent of substrate and enzyme concentration. Values of $1/\tau_3$ obtained in the pH range 5.0–6.7 are given in Fig. 4. In order to rule out the possibility that this relaxation effect was due to enzyme–indicator interactions, the curve in Fig. 4 was obtained using

TABLE II

SUMMARY OF KINETIC PARAMETERS[a]

pH	k_1, M^{-1} sec.$^{-1}$	k_{-1}, sec.$^{-1}$	K, M^{-1}
5.0	1.7×10^8	3.0×10^3	5.7×10^4
5.5	2.0×10^8	4.3×10^3	4.6×10^4
6.0	1.1×10^8	3.4×10^3	3.2×10^4
6.5	6.3×10^7	5.0×10^3	1.3×10^4
7.0	1.1×10^7	5.3×10^3	2.1×10^3
7.5	1.8×10^6	7.3×10^3	2.5×10^2

[a] 25°, 0.1 M KNO_3.

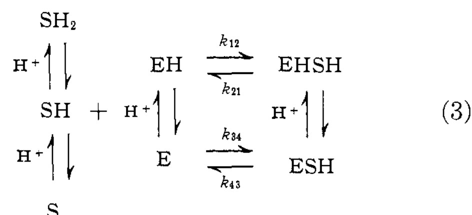
three different indicators; in one case two different indicators were used at the same pH. Also, the observed absorbancy change was in the opposite direction to that found for the enzyme–indicator "blanks." A similar series of relaxation effects was observed in solutions containing D_2O in the pD range 6.5–7.7. The corresponding values of $1/\tau_3$ obtained are also shown in Fig. 4. The relaxation time for the enzyme–indicator reaction is essentially unchanged in D_2O, in contrast to the behavior of τ_3.

Around pH 6.5, at high substrate concentrations, a fourth relaxation effect with a relaxation time of about 2 msec. has been observed. As the magnitude of this effect was very small under the experimental conditions used, a quantitative evaluation of the data could not be

made. The significance of this effect is uncertain at the present time.

Discussion

The most convenient way to explain the pH dependence of k_1 and k_{-1} is to present a plausible mechanism and then show how well the data agree with the proposed mechanism. The mechanism postulated for enzyme–cytidine 3′-phosphate binding is



(3)

In this mechanism, only the singly protonated form of the substrate, SH (*i.e.*, the primary phosphoryl hydroxyl group is ionized and the amino group is not protonated), is assumed to interact with the enzyme. In order to derive the pertinent relaxation time for this mechanism in the desired form, two assumptions will be made. First, all protolytic reactions will be assumed to have relaxation times much shorter than that characteristic of complex formation. This is really not an assumption since temperature jump studies of the indicator–enzyme and indicator–substrate solutions indicate this is indeed so. Secondly, it will be assumed that the protein acid–base groups sufficiently buffer the hydrogen ion so that the approximation $\delta[\mathrm{H}^+] = 0$ can be used. Here δ designates the deviation of the concentration from its equilibrium value. A somewhat circumspect experimental justification for this is that the observed relaxation effects are quite small relative to other (nonprotein) systems indicating a substantial part of the change in hydrogen ion concentration is being buffered.

The equations relating the observed relaxation time, τ_2, to the pH-independent rate constants k_{12}, k_{21}, k_{34}, and k_{43} are (details are given in the Appendix)

$$1/\tau_2 = \left\{\frac{k_{12}}{(1 + K_{S1}[\mathrm{H}^+] + 1/K_{S2}[\mathrm{H}^+])(1 + 1/K_{E1}[\mathrm{H}^+])} + \frac{k_{34}}{(1 + K_{S1}[\mathrm{H}^+] + 1/K_{S2}[\mathrm{H}^+])(1 + K_{E1}[\mathrm{H}^+])}\right\}(\bar{\mathrm{S}} + \overline{\mathrm{SH}} + \overline{\mathrm{SH}}_2 + \bar{\mathrm{E}} + \overline{\mathrm{EH}}) + \frac{k_{21}}{1 + 1/K_{E2}[\mathrm{H}^+]} + \frac{k_{43}}{1 + K_{E2}[\mathrm{H}^+]} \quad (4)$$

where

$$K_{S1} = [\mathrm{SH}_2]/[\mathrm{SH}][\mathrm{H}^+] = 10^{4.30} \quad (5)$$

$$K_{S2} = [\mathrm{SH}]/[\mathrm{H}^+][\mathrm{S}] = 10^{5.90} \quad (6)$$

$$K_{E1} = [\mathrm{EH}]/[\mathrm{E}][\mathrm{H}^+] \quad (7)$$

$$K_{E2} = [\mathrm{EHSH}]/[\mathrm{ESH}][\mathrm{H}^+] \quad (8)$$

Then

$$k_1 = \frac{\dfrac{k_{12}}{1 + 1/K_{E1}[\mathrm{H}^+]} + \dfrac{k_{34}}{1 + K_{E1}[\mathrm{H}^+]}}{1 + K_{S1}[\mathrm{H}^+] + 1/K_{S2}[\mathrm{H}^+]} \quad (9)$$

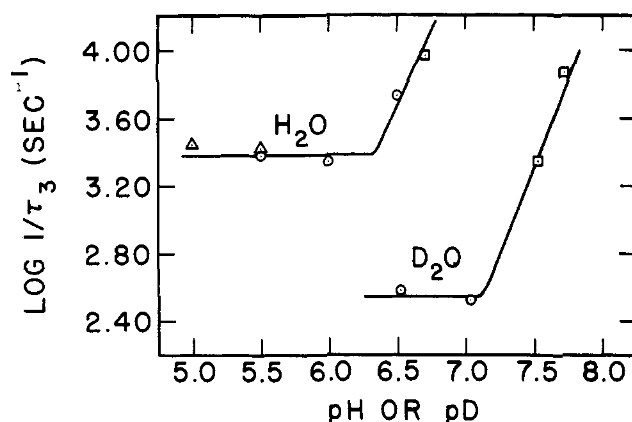


Fig. 4.—Variation of the concentration-independent chemical relaxation time, τ_3, with pH or pD in H_2O and D_2O. The symbols designate the indicators used: methyl red, Δ; chlorophenol red, O; and phenol red, □. The lines have no theoretical significance.

and

$$k_{-1} = \frac{k_{21}}{1 + 1/K_{E2}[\mathrm{H}^+]} + \frac{k_{43}}{1 + K_{E2}[\mathrm{H}^+]} \quad (10)$$

Also, the principle of microscopic reversibility requires that

$$K_{E1}(k_{12}/k_{21}) = K_{E2}(k_{34}/k_{43}) \quad (11)$$

The values of the pH-independent parameters k_{12}, k_{21}, k_{34}, k_{43}, K_{E1}, and K_{E2} were determined by a trial and error procedure until the experimental data were fit as well as possible. The values for k_{12}, k_{21}, k_{34}, k_{43}, K_{E1}, and K_{E2} finally obtained are: $k_{12} = 2.8 \times 10^8$ M^{-1} sec.$^{-1}$; $k_{21} = 3.8 \times 10^3$ sec.$^{-1}$; $k_{34} = 6.0 \times 10^7$ M^{-1} sec.$^{-1}$; $k_{43} = 2.1 \times 10^4$ sec.$^{-1}$; $K_{E1} = 10^{6.7}$ M^{-1}; $K_{E2} = 10^{8.1}$ M^{-1}. Using eq. 9 and 10, theoretical values of k_1, k_{-1}, and K can be calculated as a function of pH. The theoretical curves calculated in this manner together with the experimental points are displayed in Fig. 5 and 6. The agreement is quite satisfactory in view of the rather large experimental errors.

A rather critical look will now be taken at the proposed mechanism and the calculated parameters. The mechanism itself is the simplest found to be consistent with the data. One might question whether or not S and SH_2 bind to the enzyme. The inclusion of this additional complication is not justified by the data; on the other hand such a procedure almost certainly would be consistent with the results. Essentially the same analysis applies to the question of whether or not more enzyme acid–base groups should be included in the mechanism. An alternative, more complex, mechanism which would be consistent with the data is that both ionized forms of the cytidine 3′-phosphate react with the enzyme at comparable rates and that two or more side-chain groups on the enzyme ionizing in the pH range 6–7 are involved in the binding process. In any event, the proposed mechanism should not be accepted too literally.

The parameters determined with the most precision are k_{12}, k_{21}, and pK_{E1}; k_{34}, k_{43}, and pK_{E2} are much less certain since it was not possible to extend these studies to higher pH. However, the relationship given by eq. 11 helps considerably in fixing these parameters. The maximum error in k_{12} and k_{21} is probably about $\pm 30\%$ and that in pK_{E1} about ± 0.1, while the maxi-

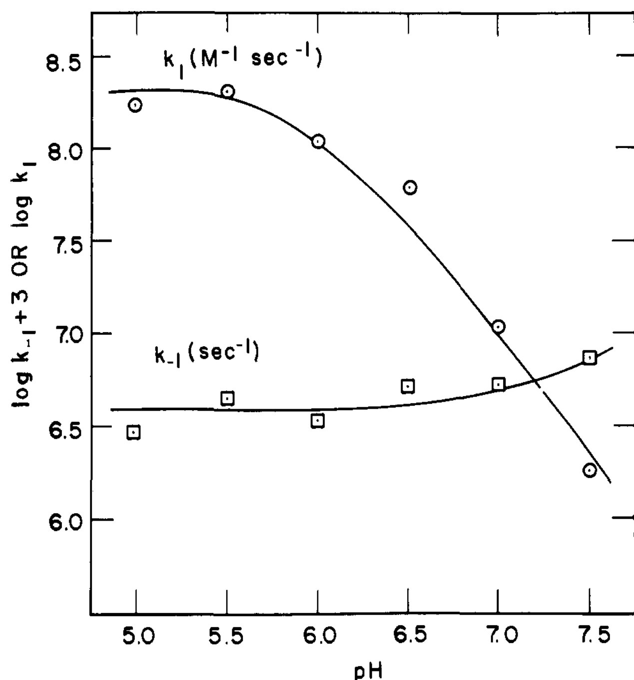


Fig. 5.—Variation of k_1 and k_{-1} with pH. The points are experimentally determined values. The solid lines are theoretical curves calculated according to eq. 9 and 10 as described in the text.

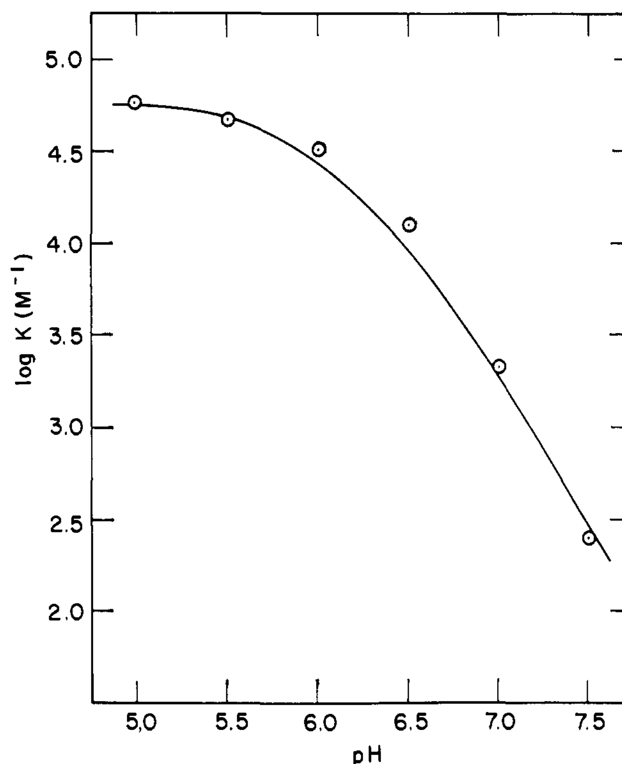


Fig. 6.—Variation of K with pH. The points are experimentally determined values. The line is a theoretical curve calculated according to eq. 9 and 10 as described in the text.

mum error in k_{34} and k_{43} may be as great as $\pm 50\%$ and that in pK_{E2} ± 0.3. The maximum rate constant for complex formation, 2.8×10^8 M^{-1} sec.$^{-1}$, is quite large and is close to the limiting value for a diffusion-controlled reaction[27]; this implies that cytidine 3′-phosphate "fits" the enzyme very well. No evidence for a second binding site for cytidine 3′-phosphate as proposed by Schellman for cytidine 5′-phosphate[28] was obtained although our experiments are probably not precise enough to detect such an occurrence.

The single ionizing group on the free enzyme which is involved in the binding of cytidine 3′-phosphate has a pK of 6.7 and is probably a histidine. Richards and Vithayathil, from studies of the binding of S-peptide to S-protein, have suggested that the site of substrate, *i.e.*, of cytidine 2′:3′-cyclic phosphate, binding is on the S-protein[29]; this would implicate histidine-119. On the other hand, Findlay, *et al.*, have concluded that histidine-119 is a water-binding site.[30] The most likely other possible histidine involved is histidine-12. Experiments are now in progress to determine which particular amino acid residue in ribonuclease is involved in the binding of cytidine 3′-phosphate. (Note that although the binding of substrate to E is considerably weaker than to EH, it is by no means negligible. This implies that other groups on the enzyme are important in the binding process.)

Herries, *et al.*,[16] from steady-state kinetics have obtained values for the product Michaelis constants which are quantitatively different from the K values (which are binding constants, not Michaelis constants) presented here, although they are of the same order of magnitude. They have also reported that ionizing groups on the enzyme having pK's of 6.78 ± 0.2 and 5.22 ± 0.2 in the free enzyme and 8.10 ± 0.09 and 6.30 ± 0.09 in the enzyme–substrate complex are necessary for enzymatic activity. The ionizing group which participates in the binding of cytidine 3′-phosphate thus corresponds to one of the groups found by these authors.

The third relaxation effect, which is independent of concentration, must be due to an intramolecular reaction and cannot be due to the binding of a second molecule of cytidine 3′-phosphate. (Unfortunately the concentration range investigated was quite restricted since at lower enzyme concentrations ($[E_0] < 3 \times 10^{-5}$ M) the relaxation effect became too small to be observed, and at higher enzyme concentrations ($[E_0] > 2 \times 10^{-4}$ M), pH measurements became inaccurate. Within the limits of enzyme concentrations employed, τ_3 showed no dependence on $[E_0]$ or $[S_0]$.) This chemical relaxation is probably due to an isomerization of the enzyme–cytidine 3′-phosphate complex. The variation of $1/\tau_3$ with pH is much greater than that expected for the participation of a single ionizing group; instead, several groups appear to be involved in a cooperative effect. The steep part of the $1/\tau_3$–pH curve varies roughly as $[OH^-]^2$. The rate of this reaction in D_2O at lower pD's is one-seventh as large as the corresponding rate in H_2O, indicating that some type of proton transfer is probably involved. Such an effect could possibly be a conformational change or concerted proton transfer. The proton transfer can directly involve substrate–protein side chains and/or protein side chain–side chain interactions. Actually, because τ_3 shows no concentration dependence, a mechanistic interpretation of the data cannot really be made. Therefore, one cannot even be certain that

(27) M. Eigen and G. G. Hammes, *Advan. Enzymol.*, **25**, 1 (1963).

(28) J. Schellman, as quoted in J. A. Thoma, D. E. Koshland, Jr., J. Ruscica, and R. Baldwin, *Biochem. Biophys. Res. Commun.*, **12**, 184 (1963).

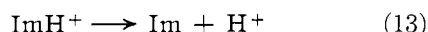
(29) F. M. Richards and P. J. Vithayathil, *Brookhaven Symp. Biol.*, **13**, 115 (1960).

(30) D. Findlay, D. G. Herries, A. P. Mathias, B. R. Rabin, and C. A. Ross, *Nature*, **190**, 781 (1961).

this step is part of the enzymatic reaction sequence. Strictly speaking, the possibility still exists that this relaxation effect may be an artifact due to an enzyme–indicator reaction although the previously cited evidence is against this hypothesis. The possibility that the isotope effect is an artifact of the method of detecting the relaxation process cannot be excluded, but seems unlikely. At the present time, no corroborative data are available to ascertain the significance of the observed chemical relaxation. However, certain provocative statements can be made. The value of $1/\tau_3$ for this reaction at low pH in H_2O, *i.e.*, $\sim 2 \times 10^3$ sec.$^{-1}$, is about the same as that for the reactions

$$\text{Im} + H_2O \longrightarrow \text{ImH}^+ + OH^- \qquad (12)$$

(Im = imidazole) and



$$\text{ImH}^+ \longrightarrow \text{Im} + H^+ \qquad (13)$$

which have rate constants of 2.3×10^3 sec.$^{-1}$ and 1.5×10^3 sec.$^{-1}$, respectively.[27] At high pH $1/\tau_3$ increases markedly. The only way found in model systems for such a dramatic increase in proton transfer rate is if a highly organized water bridge mediates the proton transfer, as found for example in cysteine amine.[27] The possibility suggests itself that the proton transfer rate could be markedly accelerated by the protein folding around the substrate creating a short organized water bridge.

The rotatory dispersion curve for enzyme differs from that for the enzyme–substrate complex, but thus far changes in the rotatory dispersion curve corresponding to the process associated with τ_3 have not been found.[31]

The presentation of a reaction mechanism for the hydrolysis of cytidine 2':3'-cyclic phosphate is not justified on the basis of the data presented here. However, the currently accepted mechanism must be viewed with caution since it is thermodynamically inconsistent if the rate of protolytic equilibration of the enzyme competes with the rate of the over-all enzymatic reaction—this, in fact, could very likely be the case.

Future studies will try to elucidate more of the elementary steps in this reaction, leading hopefully to a detailed reaction mechanism for the over-all process.[31a]

Appendix

For the proposed mechanism the following rate law can be written

$$\frac{-d([S] + [SH] + [SH_2])}{dt} = k_{12}[SH][EH] + k_{34}[SH][E] - k_{21}[EHSH] - k_{43}[ESH] \qquad (A1)$$

This equation can be specialized for the near-equilibrium case by introducing the concentration variables, $c = \bar{c} + \delta c$ where c is the actual concentration, $\bar{c}$ is the equilibrium concentration, and δc is the deviation of the concentration from equilibrium. Also, because of the reaction stoichiometry, the following relationship holds (assuming the protolytic equilibria to be rapidly adjusted).

$$\delta[SH] + \delta[SH_2] + \delta[S] = \delta[EH] + \delta[E] = -\delta[EHSH] - \delta[ESH] \qquad (A2)$$

Substituting into eq. A1, the rate equation is now

$$\frac{-d(\delta[SH] + \delta[SH_2] + \delta[S])}{dt} = \left\{k_{12}\left(\frac{\overline{SH}}{1 + \delta[E]/\delta[EH]} + \frac{\overline{EH}}{1 + \delta[SH_2]/\delta[SH] + \delta[S]/\delta[SH]}\right) + k_{34}\left(\frac{\overline{SH}}{1 + \delta[EH]/\delta[E]} + \frac{\bar{E}}{1 + \delta[SH_2]/\delta[SH] + \delta[S]/\delta[SH]}\right) + \frac{k_{21}}{1 + \delta[ESH]/\delta[EHSH]} + \frac{k_{43}}{1 + \delta[EHSH]/\delta[ESH]}\right\} \times (\delta[SH] + \delta[SH_2] + \delta[S]) \qquad (A3)$$

The derivative ratio $\delta[EH]/\delta[E]$, etc., can be calculated by differentiating the equilibrium constant and setting $\delta[H^+] = 0$. For example

$$K_{E1} = \frac{[\overline{EH}]}{[\bar{E}][\bar{H}^+]}$$

$$\therefore K_{E1}[\bar{H}^+] = \delta[EH]/\delta[E]$$

Carrying out this procedure for all necessary cases and substituting into eq. A3 yields

$$\frac{-d(\delta[SH] + \delta[SH_2] + \delta[S])}{dt} = \frac{1}{\tau}(\delta[SH] + \delta[SH_2] + \delta[S]) \qquad (A4)$$

where τ is defined by eq. 4. Use has also been made of the equalities

$$\overline{SH} = (\overline{SH} + \overline{SH}_2 + \bar{S})/(1 + K_{S1}[\bar{H}^+] + 1/K_{S2}[\bar{H}^+])]$$

$$\overline{EH} = (\overline{EH} + \bar{E})/(1 + 1/K_{E1}[\bar{H}^+])$$

$$\bar{E} = (\overline{EH} + \bar{E})/(1 + K_{E1}[\bar{H}^+])$$

(31) R. E. Cathou and G. G. Hammes, in preparation.

(31a) NOTE ADDED IN PROOF.—A more detailed discussion of the mechanism of formation of the initial enzyme-substrate complex will be given in a forthcoming publication.