The Nonenzymic Hydrolysis of Nucleoside 2′,3′-Phosphates*

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ABSTRACT: Nucleoside 2′,3′-phosphates are subject to hydrolysis by second-order hydrogen ion catalysis, first-order hydroxide ion catalysis, and at elevated temperatures in the pH range 4–9 and yield kinetically controlled mixtures of the 2′- and 3′-phosphates. No evidence of rate enhancement due to the presence of a pyrimidine group is observed. At high temperatures below pH 7 the phosphomonoester products are rapidly hydrolyzed to the nucleosides and inorganic phosphate. Cytidine 2′,3′-phosphate deaminates and hydrolyzes at 100° in 0.10 F NaCl. The acid-catalyzed rate constants at 30° are: uridine 2′,3′-phosphate, 0.276 ± 0.016 sec^{-1} M^{-2} ($E_{act} = 13.5 \pm 2.2$ kcal/mole); cytidine 2′,3′-phosphate, 0.173 ± 0.020 sec^{-1} M^{-2}; and adenosine 2′,3′-phosphate, 0.059 ± 0.003 sec^{-1} M^{-2}. The base-catalyzed rate constants are: uridine 2′,3′-phosphate, $8.9 \pm 0.8 \times 10^{-3}$ sec^{-1} M^{-1} at 40° ($E_{act} = 13.1 \pm 2.2$ kcal/mole); cytidine 2′,3′-phosphate, $6.9 \pm 1.5 \times 10^{-3}$ sec^{-1} M^{-1} at 40°; and adenosine 2′,3′-phosphate, *ca.* 1.5×10^{-3} sec^{-1} M^{-1} at 30°. The rates of hydrolysis of uridine 2′,3′-phosphate at 100° are $3.0 \pm 0.4 \times 10^{-6}$ and $1.1 \pm 0.1 \times 10^{-4}$ sec^{-1} in 0.10 F NaCl and pH 8.5 phosphate buffer, respectively.

The mechanism for ribonuclease action proposed by Witzel (Witzel and Barnard, 1962; Witzel, 1963) is based on the presumed action of the 2-oxygen of the pyrimidone group of a pyrimidine nucleoside 3′-phosphodiester as a general base. Part of the evidence cited in support of this mechanism is the report that ribonuclease substrates are more susceptible to nonenzymic hydrolysis than nonsubstrates (Witzel, 1960; Dekker, 1960). This report and our previous report (Cheung and Abrash, 1964) that Urd-2′,3′-P[1] is hydrolyzed to pure Urd-3′-P at neutrality at remarkably rapid rates have stimulated this present study of the nonenzymic hydrolysis of nucleoside 2′,3′-phosphates.

Experimental Section

Adenosine 2′,3′-Phosphate. Adenosine 2′,3′-phosphate was prepared by the action of cyclohexylcarbodiimide on adenylic acid (mixed isomers, Sigma) according to the procedure of Tener and Khorana (1955) and purified by fractional elution from a cellulose column as described by Brown *et al.* (1952). The eluted product was converted to the sodium salt by elution through a column of Dowex 50W-X8 (Na^+) resin. The product migrates as a single spot in 5% Na_2HPO_4 saturated with amyl alcohol on Whatman No. 1 paper (R_F 0.55) and in isopropyl alcohol–water–ammonia (70:5:25) on Whatman No. 1 paper (R_F 0.40). The material exhibits no buffering tendencies above pH 5. This is taken as evidence for the absence of adenylic acid and ammonium ion contamination. Its ultraviolet spectrum is very similar to those of Ado-2′-P and Ado-3′-P and shows λ_{max} at 258.5 mμ and $A_{280}:A_{260}$ (pH 7) of 0.15.

Uridine 2′-Phosphate. The separation of Urd-2′-P from the acidic hydrolysis product of Urd-2′,3′-P was attempted in order to determine its spectroscopic properties, in particular its $A_{280}:A_{260}$ at pH 7. The cyclic phosphate barium salt (50 mg), previously prepared in our laboratory (Cheung and Abrash, 1964), was allowed to stand in 1 F HCl at room temperature overnight. The acid was neutralized with NaOH and the solution was then placed on a 1 × 20 cm column of Bio-Rad AG-2-X8 (formate) 100–200 mesh resin and eluted with a solution that was 0.010 F in formic acid and 0.050 F in sodium formate. The elution was run at a rate of 40 ml/hr and collected in 5-ml fractions by means of a fractionating siphon. We were unable to resolve the mixture into two distinct peaks. Nucleotide started to elute at fraction 170 and reached peak concentration at fraction 250. Nucleotide was still being eluted at fraction 340. We believe that the leading edge of the peak was pure Urd-2′-P. The $A_{280}:A_{260}$ values (pH 7 in Tris buffer) of fractions 170–235 were constant at 0.314 ± 0.002. In fractions 240–340 these ratios rose steadily and by fraction 340 reached a value of 0.356. The ratio previously reported for Urd-3′-P is 0.365 (Cheung and Abrash, 1964). For the purpose of this work, we accept 0.314 as the $A_{280}:A_{260}$ of Urd-2′-P at pH 7.

Other Reagents. Adenosine 2′-phosphate, Ado-3′-P, Cyd-2′,3′-P, and the Urd-2′,3′-P used for the kinetic

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[1] Abbreviations used: Urd-2′,3′-P, uridine 2′,3′-phosphate; Urd-3′-P, uridine 3′-phosphate; Urd-2′-P, uridine 2′-phosphate; Cyd-2′,3′-P, cytidine 2′,3′-phosphate; Cyd-3′-P, cytidine 3′-phosphate; Cyd-2′-P, cytidine 2′-phosphate; Ado-2′,3′-P, adenosine 2′,3′-phosphate; Ado-3′-P, adenosine 3′-phosphate; Ado-2′-P, adenosine 2′-phosphate.

studies were all Sigma reagents. Adenosine, uridine, and uridylic acid were Calbiochem reagents.

Chromatography. All paper chromatographic analyses were carried out using Whatman No. 1 paper and either 5% Na_2HPO_4 saturated with amyl alcohol or isopropyl alcohol–water–ammonia (70:5:25) as solvent. Nucleosides and nucleotides were detected using short-wave ultraviolet light (Mineralight 2537). Phosphates were detected by the method of Bandurski and Axelrod (1951).

Buffer Solutions. Buffer solutions for pH values 4–6 were prepared from sodium acetate trihydrate (Baker analyzed, 0.10 F) and HCl. The pH 8.5 buffer was prepared by dissolving the substrates in 0.10 F Na_2HPO_4 and, if necessary, adding HCl to lower the pH. The pH values of the buffers were measured at room temperature with a Beckman Model G pH meter. No temperature correction is attempted for the work at high temperature since only a preliminary and approximate determination of the effect of pH at these temperatures is attempted.

Spectra. All spectra and $A_{280}:A_{260}$ values were determined using an ac line-operated Beckman DU spectrophotometer with a photomultiplier attachment. The ϵ values for Ado-2′-P and Ado-3′-P were determined by spectrophotometric measurement on samples whose concentration had previously been determined by titration of the secondary phosphate dissociation. Our result of ϵ_{260} 1.5×10^4 M^{-1} cm^{-1} for both compounds agrees well with the available data (Beaven *et al.*, 1955).[2] The ϵ_{260} for Urd-3′(2′)-P (Calbiochem) determined in the same way is 8.4×10^3 M^{-1} cm^{-1}.

Titration. Titration was carried on in 3.0 ml of *ca.* 10^{-3} F nucleotide and 1.0 ml of 0.10 F NaCl using 0.0300 F NaOH as titrant. An International Instrument Co. difunctional recording titrator, with Leeds and Northrup 124138 miniature electrodes, was used. Water-saturated nitrogen gas was passed over the titration solution and the temperature was maintained at 30.0 ± 0.1° by use of a Haake ultracirculator. The solutions were titrated for secondary phosphate from pH 5.0 to 8.5. When solutions had been subjected to alkaline hydrolysis or when solutions had been subjected to acid hydrolysis and then neutralized with 1 F NaOH, carbonate contamination was a problem. In these cases the titration curves were examined for buffering above pH 9 due to bicarbonate dissociation. Corrections were made for relatively small degrees of contamination (less than 5×10^{-4} M) by use of standard carbonic acid titration curves. If excessive carbon dioxide contamination was indicated, the titration was discarded.

Kinetic Runs. Two techniques were used, a spectrophotometric method and a titration method. The spectrophotometric method was used for the hydrolysis of Urd-2′,3′-P and Cyd-2′,3′-P. A solution that was *ca.* 10^{-3} F in substrate and which had an appropriate concentration of either HCl or NaOH and whose ionic strength was maintained at 0.10 M by the appropriate concentration of NaCl was prepared. This solution was prepared from stock solutions that were maintained at the desired temperature in a constant temperature bath as was the reaction solution after mixing. At recorded time intervals after the addition of HCl or NaOH, a 1.0-ml aliquot was removed and added to 9 ml of 0.10 F Tris buffer of 0.10 M ionic strength such that the pH of the final solution was between 7.0 and 8.0. The $A_{280}:A_{260}$ value was then determined.

This method was also used for the hydrolysis of Urd-2′,3′-P at intermediate pH values and high temperature. Aliquots were removed at measured times, cooled, and buffered to pH 7–8 in Tris and the $A_{280}:A_{260}$ was determined.

The titration method was used for the acid-catalyzed hydrolysis of Ado-2′,3′-P and Cyd-2′,3′-P and to make an approximate estimate of the rate of alkaline hydrolysis of Ado-2′,3′-P. The reaction solution was prepared from 3.0 ml of 10^{-3} F substrate and 0.30 ml of a solution of the appropriate HCl or NaOH concentration. The reaction mixture was stirred rapidly in a nitrogen atmosphere and the desired temperature maintained with the Haake ultracirculator. At a measured time after mixing, the reaction was quenched by careful addition of HCl or NaOH to bring the pH to the range 4–5. Nitrogen was passed over the well-stirred solution for an additional 10 min and the solution was then titrated for secondary phosphate. Several such runs were carried out for different reaction times for each concentration of HCl or NaOH. Crude estimates of the rates of hydrolysis of Ado-2′,3′-P at high temperature were made by elution of chromatographic spots and the determination of the relative A_{260} values of the spots.

Calculations. Individual first-order decay constants (k') were determined from plots of ln $(S)_0/(S)$ *vs.* time. In the spectrophotometric method $(S)_0/(S) = (r_\infty - r_0)/(r_\infty - r_t)$, where r refers to the $A_{280}:A_{260}$ value at the appropriate time. For the titration method $(S)_0/(S) = (S)_0/[(S)_0 - (ROPO_2H^-)]$. The third-order acid-catalyzed rate constants (k_a) were determined by least-squares fits of plots of k' *vs.* $(H^+)^2$. The second-order base-catalyzed rate constants (k_b) were determined by least-squares fits of plots of k' *vs.* (OH^-).

Results

The data for the kinetic behavior of the nucleoside cyclic phosphates are shown in Table I. The second-order dependence on hydrogen ion concentration holds without significant deviation up to 0.080 M H^+ for Urd-2′,3′-P at 35° and up to 0.100 M H^+ for Cyd-2′,3′-P at 30 and 40°. This is shown by plots of $(H^+)/k'$ *vs.* $1/(H^+)$ which pass, within experimental uncertainty, through the origin. This indicates that the phosphodiester groups of Urd-2′,3′-P and Cyd-2′,3′-P have dissociation constants considerably higher than the observed hydrogen ion concentration. As the hydrogen ion concentration approaches the value of the dissociation constant, we would expect a trend

[2] W. E. Cohn (1955).

from second-order to first-order dependence of rate on hydrogen ion concentration. Our estimate for the minimum value for the dissociation constant of the cyclic phosphodiester group is 0.3 M.

The only product of the acidic hydrolysis of Urd-2′,3′-P observable by paper chromatography is a mixture of Urd-3′-P and Urd-2′-P (R_F 0.16 in isopropyl alcohol–water–ammonia). This product had an $A_{280}:A_{260}$ of 0.337 ± 0.003 (pH 7.0 in Tris) which was independent of the acid concentration and the temperature of hydrolysis. The spectroscopic data indicate that the product is approximately a 1:1 mixture of the two components. Similar results are obtained for the acid-catalyzed hydrolysis of Cyd-2′,3′-P. The product mixture has an $A_{280}:A_{260}$ of 0.87 while pure Cyd-3′-P, prepared by the ribonuclease-catalyzed hydrolysis of Cyd-2′,3′-P, has an $A_{280}:A_{260}$ of 0.95. The formation of these mixtures appears to be kinetically controlled. Uridine 3′-phosphate, prepared by the ribonuclease-catalyzed hydrolysis of Urd-2′,3′-P, showed a decrease in $A_{280}:A_{260}$ from 0.362 to 0.352 on treatment with 0.10 F HCl at 30° for 1 hr, approximately 10 half-lives for the cyclic phosphate. This indicates that product isomerization is about one-tenth as fast as cyclic phosphate hydrolysis and that, while some isomerization may occur, the rates of production of Urd-3′-P and Urd-2′-P by hydrolysis are approximately equal.

The apparent third-order rate constant, k_a, for acid hydrolysis is not the rate constant for a single reaction. The observed rate law is consistent with the mechanism

$$S^- + H^+ \rightleftharpoons SH \overset{H^+}{\rightleftharpoons} SH_2^+ \xrightarrow{k_0} P$$

$$k_a = k_0/K_{a_1}K_{a_2} \text{ if } (H^+) \ll K_{a_2} > 0.3 \text{ M}$$

where $K_{a_1} = (SH)(H^+)/(SH_2^+)$; $K_{a_2} = (S^-)(H^+)/(SH^+)$.

Since we lack a reliable estimate of $K_{a_1}K_{a_2}$ or of the enthalpies of dissociation, we are unable to translate the observed activation energy for the acid-catalyzed hydrolysis into the thermodynamic activation parameters for k_0.

The rates of acid-catalyzed hydrolysis of Cyd-2′,3′-P were followed by both kinetic techniques. Good agreement between the two methods was obtained.

The hydrolysis of Urd-2′,3′-P at elevated temperatures and pH values from four to seven yields uridine (R_F 0.45, isopropyl alcohol–water–ammonia; phosphate negative; $A_{280}:A_{260}$ = 0.365) and inorganic phosphate (R_F 0.03; phosphate positive; ultraviolet negative). Uridine is formed by a rate-determining hydrolysis of Urd-2′,3′-P to phosphomonoester followed by a rapid hydrolysis of phosphomonoester. A small steady-state concentration of uridylic acid (R_F 0.12; ultraviolet positive) is maintained until all of the cyclic phosphate is consumed. The hydrolysis of uridylic acid appears to be the typical high-temperature hydrolysis of phosphomonoesters (Bunton *et al.*, 1958) and shows no exceptional kinetic behavior. At pH 8.5 the phosphomonoesters are stable and are the final product of hydrolysis. The $A_{280}:A_{260}$ value (0.337) indicates that both the 2′- and 3′-phosphomonoesters are formed.

When subjected to 0.10 F NaCl at reflux for 8 days, Cyd-2′,3′-P shows an initial period in which $A_{280}:A_{260}$ remains nearly constant, followed by a slow decrease of this ratio. This is thought to be the result of concurrent hydrolysis of the phosphodiester group and deamination of the cytosine ring (Shapiro and Klein, 1966). Under these conditions, cytidylic acid slowly forms uridine.

The basic hydrolysis of Urd-2′,3′-P and Cyd-2′,3′-P yields kinetically controlled mixtures of the 2′- and 3′-phosphomonoesters. The $A_{280}:A_{260}$ for the basic hydrolysis product of Urd-2′,3′-P is 0.345 ± 0.003, and this value is independent of the temperature and hydroxide ion concentration during hydrolysis. For the alkaline hydrolysis of Urd-2′,3′-P we compute the following activation parameters: ΔH^* = 12.5 ± 2.3 kcal/mole; ΔF^* = 49.5 ± 0.1 kcal/mole; ΔS^* = −122 ± 8 eu/mole. Since the barium salts were used, the (Ba^{2+}) was *ca.* 5×10^{-4} M in most kinetic runs. The addition of 1.0×10^{-3} F $BaCl_2$ had no observable effect on the alkaline hydrolysis of Urd-2′,3′-P.

The data for the hydrolysis of Ado-2′,3′-P are both more sparse and less precise than the data for Urd-2′,3′-P and Cyd-2′,3′-P. This reflects the increased difficulty of the analytic method. The acid-catalyzed hydrolysis shows the same high order in hydrogen ion concentration as for Urd-2′,3′-P and Cyd-2′,3′-P. The k_a is approximately 0.06 sec^{-1} M^{-2}. In addition to our titration data, we have crude confirmation of our results from the chromatography of quenched reaction mixtures. By chromatography we estimate the half-life for Ado-2′,3′-P in 0.10 M H^+ and 30° as 10–20 min. The half-life estimated by titration is 19 min. Hydrolysis in 0.50 M H^+ is complete in 10 min while the half-life predicted on the basis of titration data is 45 sec. The products of both acidic and basic hydrolysis consist only of Ado-2′-P (R_F 0.74, 5% Na_2HPO_4–amyl alcohol) and Ado-3′-P (R_F 0.66) in approximately equal amounts. No isomerization of Ado-2′-P or of Ado-3′-P was observed after 1 hr in 0.1 M H^+ or in 0.1 M OH^-.

The neutral hydrolysis of Ado-2′,3′-P at high temperature is approximately as rapid as that of Urd-2′,3′-P. Below pH 7, adenosine (R_F 0.60, isopropyl alcohol–water–ammonia) is the final product. In refluxing pH 8.5 phosphate buffer, Ado-2′-P and Ado-3′-P are formed in approximately equal amounts.

Discussion

According to Witzel (Witzel and Barnard, 1962), the rates of nonenzymic hydrolysis of pyrimidine nucleoside 3′-phosphodiesters are enhanced by participation of the pyrimidone group. According to this view, the 2-oxygen of the pyrimidone group acts as a general base and, by enhancing the nucleophilicity

TABLE I: Kinetic Parameters for the Hydrolysis of Nucleoside 2′,3′-Phosphates.

I. Uridine 2′,3′-phosphate, Ba^{2+} salt[a]

A. Acid-catalyzed hydrolysis: rate = $k_a(S)(H^+)^2$

Temp (°C)	(H^+) range (M)	k_a (sec^{-1} M^{-2})
25.0	0.010–0.040	0.152 ± 0.009
30.0	0.010–0.040	0.276 ± 0.016
35.0	0.010–0.080	0.327 ± 0.007
40.0	0.020–0.040	0.53 ± 0.04
E_{act} = 13.5 ± 2.2 kcal/mole		

B. Base-catalyzed hydrolysis: rate = $k_b(S)(OH^-)$

	(OH^-) range (M)	$k_b \times 10^3$ (sec^{-1} M^{-1})
25.0	0.005–0.020	2.62 ± 0.11
30.0	0.025–0.100	5.0 ± 0.6
35.0	0.025–0.100	5.5 ± 0.7
40.0	0.025–0.100	8.9 ± 0.8
30.0	0.050–0.100[b]	4.6 ± 0.9
E_{act} = 13.1 ± 2.3 kcal/mole		

C. Neutral hydrolysis

	Hydrolyzing solution	$k' \times 10^6$ (sec^{-1})[c]
100 (reflux)	0.10 F NaCl	3.0 ± 0.4
100 (reflux)	pH 5 acetate buffer[d]	3.9 ± 0.4
82	pH 4 acetate buffer[d]	2.3 ± 0.5
82	pH 5 acetate buffer[d]	1.4 ± 0.3
82	pH 6 acetate buffer[d]	0.32 ± 0.04
100 (reflux)	pH 8.5 phosphate buffer[e]	110 ± 10

II. Cytidine 2′,3′-phosphate, Ba^{2+} salt.

A. Acid-catalyzed hydrolysis: rate = $k_a(S)(H^+)^2$

	(H^+) range (M)	k_a (sec^{-1} M^{-2})
30.0	0.040–0.100[a]	0.17 ± 0.02
40.0	0.040–0.100[a]	0.44 ± 0.04
30.0	0.100[f]	0.19 ± 0.03

B. Base-catalyzed hydrolysis: rate = $k_b(S)(OH^-)$

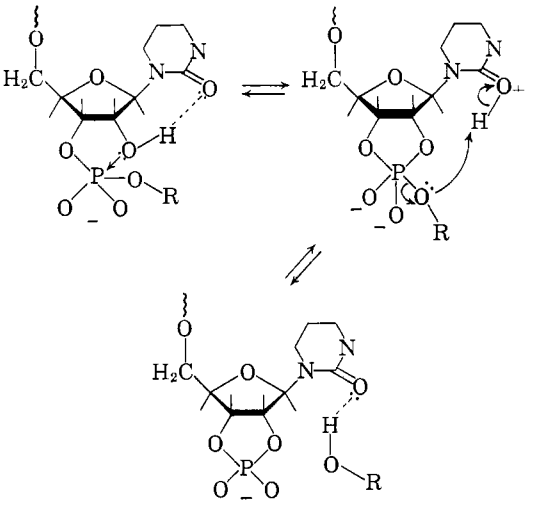
	(OH^-) range (M)	$k_b \times 10^3$ (sec^{-1} M^{-1})
40.0	0.040–0.100	6.9 ± 1.5

III. Adenosine 2′,3′-phosphate, Na^+ salt.

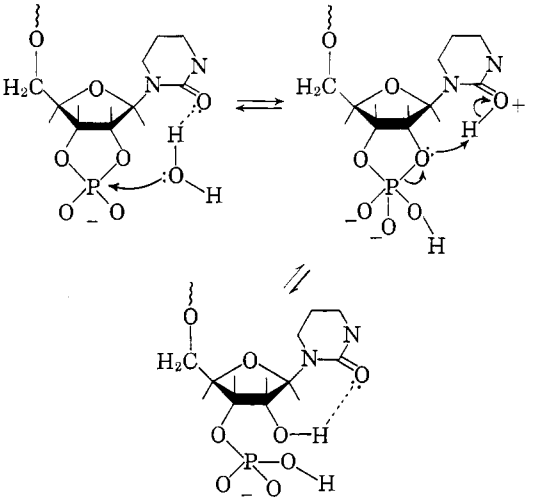
	Hydrolyzing solution (F)	$k' \times 10^6$ (sec^{-1})[c]
30.0	(0.40) HCl[f]	*Ca.* 1×10^{-2}
30.0	(0.100) HCl[f]	$(5.9 \pm 0.3) \times 10^{-4}$
30.0	(0.050) HCl[f]	$(1.8 \pm 0.3) \times 10^{-4}$
30.0	(0.016) HCl[f]	*Ca.* 1×10^{-5}
30.0	(0.050) NaOH[f]	*Ca.* 7×10^{-5}
100 (reflux)	(0.10) NaCl[g]	*Ca.* 2×10^{-6}
100 (reflux)	pH 8.5 phosphate buffer[e,g]	$>3 \times 10^{-5}$[h]

[a] All rates determined by change in A_{280}:A_{260}. [b] In 1.0×10^{-3} F $BaCl_2$. [c] Apparent first-order rate constant. [d] μ = 0.10 M, 0.10 F in sodium acetate trihydrate. [e] μ = 0.20 M, 0.10 F in sodium monohydrogen phosphate. [f] All rates determined by titration of phosphomonoester. [g] Rates determined by semiquantitative paper chromatography. [h] No adenosine 2′,3′-phosphate detectable by paper chromatography after 17 hr, $t_{1/2} < 6$ hr.

SCHEME I: Witzel Mechanism for the Ribonuclease-Catalyzed Cyclization of Pyrimidine Nucleoside 3′-Phosphodiesters.



SCHEME II: Witzel Mechanism for the Ribonuclease-Catalyzed Hydrolysis of Pyrimidine Nucleoside 2′,3′-Cyclic Phosphates.



of the 2′-hydroxyl, assists cyclization. The pyrimidone oxygen receives a proton from the hydroxyl group and transfers it to the leaving group (Scheme I). Hydrolysis of the cyclic phosphate occurs by the interaction of the pyrimidone oxygen with water, the attacking nucleophile, and subsequent proton transfer to the 2′-oxygen (Scheme II). A similar but more efficient process is thought to occur in the ribonuclease-catalyzed reaction.

Witzel (1960) reported that the rates of hydrolysis of dinucleotides depend on the nature of the base group of the 3′-linked nucleoside. The rates of the acid-catalyzed hydrolysis fell in the order uridine > cytidine > adenosine. Since Witzel's observed rates are much slower than our observed rates for the hydrolysis of the corresponding 2′,3′-phosphates we assume that they represent the rates of acid-catalyzed cyclization and, possibly, direct hydrolysis not proceeding through the cyclic phosphate. Such direct hydrolysis has been reported for the ribonuclease-catalyzed reaction (Williams, 1966). We have found approximately the same relationship between structure and reactivity in the hydrolysis of the cyclic phosphates. The actual differences are fairly small with Ado-2′,3′-P hydrolyzing at rates that are one-fourth those of Urd-2′,3′-P. These effects correspond in magnitude to those found by Witzel.

We cannot, however, draw the same conclusions as Witzel on the basis of these results. There is evidence that the rate differences between the cyclic phosphates are due to rate suppression in the case of Ado-2′,3′-P rather than rate enhancement for Urd-2′,3′-P. Cox (1958) and Haake and Westheimer (1961) reported that ethylene phosphate hydrolyzes in 0.10 F $HClO_4$ at 30° with an apparent first-order rate of 2.1×10^{-3} sec^{-1}. This value falls between our extrapolated value for Urd-2′,3′-P and our measured value for Cyd-2′,3′-P. Thus, there is no evidence for the rate-enhancing effect of the pyrimidone group during the acid-catalyzed hydrolysis of 2′,3′-cyclic phosphates.

The third-order rate constants (k_a's) for the acid-catalyzed hydrolysis are not simple rate constants for a single reaction but are equal to $k_0/K_{a_1}K_{a_2}$, where k_0 is the rate constant for the decomposition of the conjugate acid of the 2′,3′-phosphoric acid and K_{a_1} and K_{a_2} are the two dissociation constants of this conjugate acid. Witzel's explanation assumes that only k_0 is affected by structural change. We propose that the positive ammonium group of Ado-2′,3′-P could sufficiently raise the K_a's by a field effect and lower the rate of hydrolysis by the observed amount.

Acid-catalyzed cleavage of the 3′-phosphate linkage of the cyclic phosphates appears to be as rapid as the cleavage of the 2′-linkage. The product mixtures appear to be the result of kinetic rather than equilibrium control. Although isomerization of the products may occur to some extent during the hydrolysis period, such isomerization is considerably slower than hydrolysis. This is consistent with the view that isomerization occurs *via* a cyclization–hydrolysis mechanism. Witzel's mechanism predicts a strong kinetic preference for the formation of Urd-3′-P over Urd-2′-P. Such a kinetic preference could be masked to some extent by subsequent product isomerization, but it is not likely that it could be completely obscured if it were large.

The same slight trend in rate with structure was observed for the basic hydrolysis of dinucleotides (Witzel, 1960). The reported rates of dinucleotide hydrolysis are about one-tenth our observed rates of cyclic phosphate hydrolysis. The dinucleotides thus appear to undergo a rate-determining base-catalyzed cyclization or possibly a direct hydrolysis, and Witzel's observed rates represent the cyclization and direct hydrolysis

rates. We have again found a parallel relationship between reactivity and structure for the base-catalyzed hydrolysis of cyclic phosphates. Uridine 2′,3′-phosphate is hydrolyzed at rates that are ten times as great as those observed for ethylene phosphate (Kumamoto *et al.*, 1956). This rate difference does not appear to be due to catalysis by the low concentration (5×10^{-4} M) of barium ion introduced by use of the barium salt. The hydrolysis yields considerable amounts of Urd-2′-P under definite conditions of kinetic control (Brown and Todd, 1952). In view of this, we do not believe that the rate enhancement for Urd-2′,3′-P is due to the action of the pyrimidone group as a general base. This is particularly difficult to support since the kinetic behavior indicates that the attacking nucleophile is the hydroxide ion. The uracil ring dissociates above pH 10 so that the Witzel mechanism would require an interaction between a negative uracil ring and a negative hydroxide ion. If the Witzel mechanism were operative under these conditions, one would predict that Cyd-2′,3′-P would be hydrolyzed more rapidly than Urd-2′,3′-P.

There is no evidence for either a rate enhancement or selectivity of phosphate bond hydrolysis in the high-temperature neutral hydrolysis of Urd-2′,3′-P. Our previous report (Cheung and Abrash, 1964) of the production of pure Urd-3′-P when Urd-2′,3′-P is hydrolyzed at 100° is in error. This error was due to our failure to recognize that subsequent hydrolysis of the phosphomonoesters to uridine had occurred. Our report that Ado-2′,3′-P is resistant to hydrolysis at neutral reflux is also incorrect. The chromatographic system used for the analysis (5% Na_2HPO_4–amyl alcohol) does not resolve Ado-2′,3′-P and adenosine.

The hydrolytic reactions in acetate buffers are more rapid than would be predicted on the basis of an extrapolation of the acid- and base-catalyzed hydrolysis. The data indicate general-acid catalysis by acetic acid. The rates of cyclic phosphate hydrolysis are considerably more rapid in phosphate buffers than in acetate buffers. Given the uncertainty in the determination of the pH at high temperature, the extrapolation of the specific-base-catalyzed rate constant to high temperatures, and the effect of the higher ionic strength of the phosphate buffers, we are not sure whether hydrolysis in phosphate buffers is the result of specific base catalysis or general base catalysis by the monohydrogen phosphate ion.

The hydrolysis in phosphate buffers is nonspecific and yields both 2′- and 3′-phosphates. Since we did not isolate the steady-state monophosphates formed during the weakly acid hydrolyses, we are unable to make a direct observation concerning the specificity of cyclic phosphate hydrolysis in the pH range 4–7. However, the similarity in the pH profiles of the hydrolysis of Urd-2′,3′-P and Ado-2′,3′-P and the small difference in the rates of hydrolysis of these two compounds are strong evidence against any anchimeric assistance by the pyrimidone group in the weakly acidic range. In all other cases, the lack of phosphate bond specificity offers additional evidence against pyrimidone assistance. The failure to find any evidence of rate enhancement or phosphate bond specificity due to the presence of a pyrimidone ring over such a wide range of conditions indicates that the nonenzymic hydrolysis of nucleoside 2′,3′-phosphates is not a suitable model system for the determination of the mechanism of ribonuclease action. Any evidence in support of Witzel's mechanistic proposals must be found in spectrophotometric measurements or in the analysis of the structures of substrates and inhibitors.

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