Hydrolysis of Glycero-1,2-cyclic Phosphate

Lea Kugel and M. Halmann

Contribution from the Isotope Department, The Weizmann Institute of Science, Rehovoth, Israel. *Received January 28, 1967*

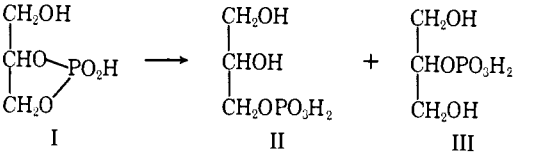
Abstract: The kinetics of hydrolytic ring opening of the five-membered cyclic phosphate ester glycero-1,2-cyclic phosphate was measured at 0–26° in aqueous acid solutions up to 0.5 *M* perchloric acid, and in alkaline solutions up to 3 *M* sodium hydroxide. The hydrolysis is subject to considerable acid and base catalysis, but is extremely slow in the pH range 3–10. Both in acid (0.1 *M* $HClO_4$) and in alkaline (1 *M* NaOH) solutions, the activation energy is only 15.0 and 15.8 kcal/mole, respectively. It is thus much lower than for the hydrolysis of open-chain dialkyl hydrogen phosphates. The deuterium oxide solvent isotope effect on the rates of hydrolysis in acid (0.2 *M* $HClO_4$) and alkaline (1.5 *M* NaOH) solutions is appreciable: $k_{D_2O}/k_{H_2O} = 2.25$ and 1.5, respectively. In alkaline solutions the rate of hydrolysis is linearly related to the function $(H_- + \log [H_2O])$, where H_- is the basicity function and $[H_2O]$ is the concentration of water.

Five-membered cyclic esters of phosphoric acid are important as intermediates which can be isolated in the enzymatic, acid, and alkaline hydrolysis of ribonucleic acid.[1] Unsubstituted dialkyl phosphates, such as dimethyl hydrogen phosphate, are stable to alkali,[2] but dialkyl phosphates containing a hydroxy group in the β position to the phosphoryl group undergo very rapid hydrolysis in alkaline media.[3] Also, the loss in optical activity in the degradation of L-glyceryl-phosphorylcholine and of L-α-lecithin was more rapid than could be accounted for by simple hydrolysis.[4] A rapid reversible migration of the phosphoryl group to the neighboring hydroxy substituent was therefore assumed to occur in this reaction, as well as in the hydrolysis of phospholipids.[5] The reversible ring closure to five-membered cyclic phosphates is common to a wide variety of natural compounds and has been studied on several model systems.[6] The mechanism proposed involves ring formation by nucleophilic attack of the β-hydroxy group on the phosphorus atom with displacement of the alkoxy group. This scheme is in agreement with the ^{18}O-tracer experiments, which proved that the hydrolysis product contains one oxygen atom from the solvent.[7] It also explains the lability of ribonucleic acid to alkaline hydrolysis, in contrast to the stability of deoxyribonucleic acid, which lacks the hydroxy group.

Monoalkyl dihydrogen phosphates, with a hydroxy group in the β position to the ester bond, such as the glycero cyclic phosphates,[8] and sugar phosphates[9] undergo rapid reversible rearrangement in acid solution to the neighboring position. The intramolecular nature of this reaction was revealed by tracer studies.[10]

The ring opening of ethylene hydrogen phosphate in acid and alkaline solutions was studied extensively in order to understand its rapid hydrolysis in contrast to the stability of open-chain and six-membered cyclic phosphate esters.[11] The remarkable reactivity of ethylene phosphate was explained at first by the strain in the five-membered ring,[11b] and later by weakening of bonds, which is caused by the smaller double-bond character in the ring[11e] compared with the acyclic and six-membered cyclic phosphates. Simultaneously with hydrolysis, ethylene hydrogen phosphate in acid solutions was found to undergo oxygen exchange with the solvent at comparable rates.[11d] A common mechanism for the hydrolysis and exchange was therefore postulated, which assumed the reversible formation of an intermediate complex or transition state involving pentacovalently bonded phosphorus. None of the previous results seems to provide a completely satisfactory explanation of the special reactivity of the five-membered cyclic phosphates.

In the present study, the hydrolysis of glycero-1,2-cyclic phosphate (I) to glycero-1-phosphate (II) and glycero-2-phosphate (III) was chosen as a model system,



I II III

in an effort to elucidate its mechanism by a detailed kinetic analysis. This cyclic ester I had been shown to be formed during the acid-catalyzed hydrolysis of the

(1) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 52 (1952); 2040 (1953); *Ann. Rev. Biochem.*, 24, 311 (1955); R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552, 558 (1952); H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1960, p 42; H. Witzel and E. A. Barnard, *Biochem. Biophys. Res. Commun.*, 7, 295 (1962); A. Williams, *Chem. Commun.*, 590 (1966).

(2) C. A. Bunton, M. M. Mhala, K. G. Oldham, and C. A. Vernc *J. Chem. Soc.*, 3293 (1960).

(3) (a) O. Bailly and J. Gaume, *Bull. Soc. Chim. France*, **2**, 354 (1935); **3**, 1396 (1936); (b) A. Fono, *Arkiv Mineral. Geol.*, **24A**, 14 (1947).

(4) E. Baer and M. Kates, *J. Biol. Chem.*, **175**, 79 (1948); **185**, 615 (1950).

(5) C. Long and M. F. Maguire, *Biochem. J.*, **54**, 612 (1953).

(6) D. M. Brown and H. M. Higson, *J. Chem. Soc.*, 2034 (1957); D. M. Brown, G. E. Hall, and H. M. Higson, *ibid.*, 1360 (1958); D. M. Brown and D. A. Usher, *Proc. Chem. Soc.*, 309 (1963).

(7) D. Lipkin, P. T. Talbert, and M. Cohn, *J. Am. Chem. Soc.*, **76**, 2871 (1954).

(8) (a) M. Bailly, *Bull. Soc. Chim. France*, **9**, 314, 340, 405 (1942); (b) P. E. Verkade, J. C. Stoppelenberg, and W. D. Cohen, *Rec. Trav. Chim.*, **59**, 886 (1940); (c) W. H. Harrison, P. D. Boyer, and A. B. Falcone, *J. Biol. Chem.*, **215**, 303 (1955); (d) L. Kugel and M. Halmann, *J. Am. Chem. Soc.*, **88**, 3566 (1966), giving further references.

(9) Ch. Degani and M. Halmann, *ibid.*, **88**, 4075 (1966); C. A. Bunton and H. Chaimovitz, *ibid.*, **88**, 4082 (1966).

(10) E. Chargaff, *J. Biol. Chem.*, **144**, 455 (1942).

(11) (a) J. Kumamoto, J. R. Cox, Jr., and F. H. Westheimer, *J. Am. Chem. Soc.*, **78**, 4858 (1956); (b) F. H. Westheimer, Special Publication No. 8, The Chemical Society, London, 1957, p 1; D. A. Usher, E. A. Dennis, and F. H. Westheimer, *J. Am. Chem. Soc.*, **87**, 2320 (1965); (c) J. R. Cox, Jr., R. E. Wall, and F. H. Westheimer, *Chem. Ind.* (London), 929 (1959); (d) P. C. Haake and F. H. Westheimer, *J. Am. Chem. Soc.*, **83**, 1102 (1961); (e) E. T. Kaiser, M. Panar, and F. H. Westheimer, *ibid.*, **85**, 602 (1963); (f) E. A. Dennis and F. H. Westheimer, *ibid.*, **88**, 3431, 3432 (1966); (g) A. Eberhard and F. H. Westheimer, *ibid.*, **87**, 253 (1965).

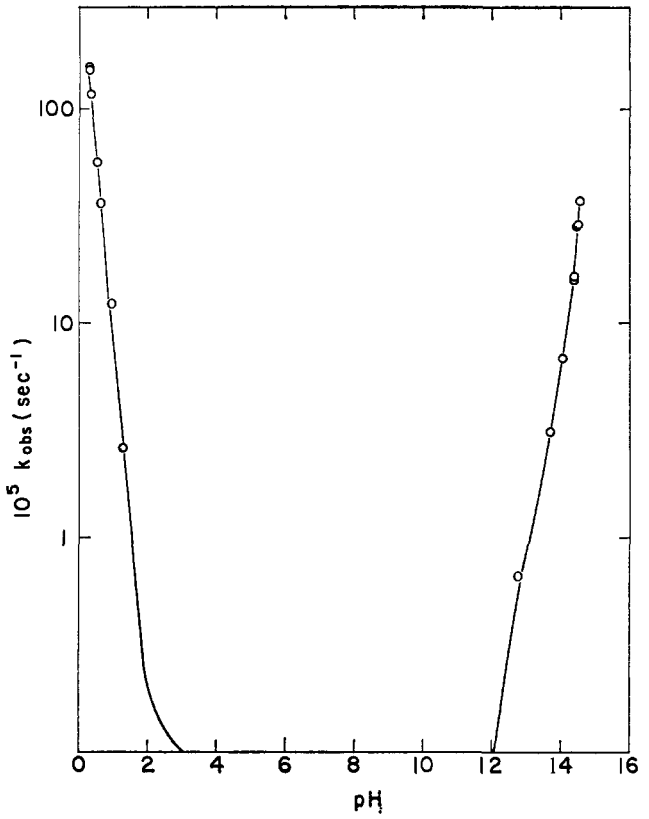


Figure 1. First-order rate constants for the hydrolysis of glycero-1,2-cyclic phosphate at 0° as a function of pH.

open-chain esters II and III.[8,10] Available data on the hydrolytic reactivity of glycero-1,2-cyclic phosphate indicate that it is stable for 72 hr at room temperature in the pH range 3.5–8 and is completely hydrolyzed in 3 hr at pH 1.5 or in 0.1 *N* sodium hydroxide.[12a] In 0.1 *N* hydrochloric acid, the half-time is less than 10 min.[12c]

Experimental Section

Materials. **Glycero-1,2-cyclic phosphate,** which had previously been prepared in low yields by a variety of methods,[12] was obtained in the present work by a procedure used previously for synthesis of cyclic ribonucleoside phosphates.[13] Disodium 2-glycerophosphate (5 g, Sigma Chemical Co.) was converted into the free acid by passing it through the H^+ form of Dowex 50 Wx12 cation exchanger. The solution was diluted with water to 40 ml. After adding concentrated ammonia (6.5 ml), formamide (50 ml, Fisher Reagent), and a solution of dicyclohexylcarbodiimide (25 g, Fluka AG) in *t*-butyl alcohol (150 ml), the mixture was refluxed for 1 hr. The *t*-butyl alcohol was then distilled off; the remaining solution was diluted with water (150 ml) and was extracted four times with ether. The remaining aqueous solution was evaporated under reduced pressure, finally at 10 mm to remove traces of water and formamide. The residue was dissolved in acetone, and a solution of barium iodide in acetone (5 g in 100 ml) was added. The oily precipitate was washed repeatedly with acetone and after drying at 60° under vacuum was turned into a white powder containing barium 1,2-glycerophosphate, yield (based on disodium glycero-2-phosphate) 70%. Its purity was tested by paper chromatography[12b] (ascending, in isopropyl alcohol–5 *N* ammonia, 2:1). Only one spot (R_f 0.7) was found, and none at the position expected for 2-glycerophosphate (R_f 0.35). For purification, the product was dissolved several times in water and precipitated with acetone. Potentiometric titration, using a Radiometer TTT1 pH meter with a SRB2c titrigraph recorder, of a sample of the free acid (prepared using Dowex 50 cation exchanger) with sodium hydroxide resulted in a single break, as required for a monobasic acid. *Anal.* Calcd for $C_6H_{12}O_{10}P_2Ba \cdot H_2O$: equiv wt, 230.7; C, 15.6; H, 3.07; P, 13.4. Found: equiv wt, 224; C, 15.3; H, 3.1; P, 13.5.

Deuterium oxide solutions were prepared by adding deuterium oxide (99.8 g/100 g of deuterium; Norsk Hydro Electrisk, Kvaelstofaktieselskab) up to 10 ml to a volumetric flask containing 70% perchloric acid or solid sodium hydroxide. The final concentration of deuterium was 99%.

Kinetic Methods. Hydrolytic ring opening of monobasic glycero-1,2-cyclic phosphate resulted in formation of a mixture of the dibasic acids glycero-1- and -2-phosphates. The progress of the reaction was followed by bringing an aliquot of the reaction mixture rapidly with either 1 *N* perchloric acid or 1 *N* sodium hydroxide to approximately pH 3–4, and then titrating potentiometrically with 0.07 *N* sodium hydroxide. The differences in the two breaks in the titration curve were taken as proportional to the concentration of the reaction product. First-order kinetics were observed for each run. The subsequent hydrolysis of the resulting glycerol-1- and -2-phosphates to glycerol and orthophosphate is negligible under the conditions causing rapid ring opening.[8d] The errors in rate constants were about ±3–8%.

Results

The pH dependence of the ring opening of glycero-1,2-cyclic phosphate is presented in Figure 1. The rate is seen to be extremely small in the pH range 3–10 and to rise steeply in more acid and alkaline media. Observed first-order rate constants at several temperatures, and in various solvents, are listed in Tables I, II, and III for the acid, intermediate (pH 3–10), and alkaline regions, respectively. In the acid region, an in-

Table I. First-Order Rate Constants for Hydrolysis of Glycero-1,2-cyclic Phosphate (initially 0.02 *M*) as a Function of Perchloric Acid Concentration[a]

$HClO_4$, *M*	Temp, °C	$10^3 k_{obsd}$, sec^{-1}
0.0505	0	0.025
0.101	0	0.12
0.101[b]	0	0.094
0.202	0	0.37
0.303	0	0.56
0.404	0	1.19
0.500	0	1.51
0.505	0	1.58
0.20	3.8	0.61
0.20[c]	3.8	1.37
0.101	11.9	0.35
0.101	25.8	1.37

[a] Each rate constant is derived from at least two separate kinetic runs. [b] In the presence of $NaClO_4$; ionic strength = 0.505. [c] In deuterium oxide solution.

Table II. Hydrolysis of Glycero-1,2-cyclic phosphate in Moderately Acid and Alkaline Solutions

pH	Temp, °C	Time, hr	% reaction	$10^7 k_{obsd}$, sec^{-1}
3.0	0	65	0	
3.0	55	2	0	
3.0	100	6	<10	
4.9	0	65	0	
4.9	55	2	0	
4.9	100	22	0	
7.3	0	45	0	
10.0	0	60	0	
11.5	0			5.0
11.98	0			8.73
12.15	0			9.57
12.79	0			67.50

(12) (a) T. Ukita, N. A. Bates, and H. E. Carter, *J. Biol. Chem.*, **216**, 867 (1955); (b) T. Ukita, N. Nagasawa, and M. Irie, *Pharm. Bull.* (Tokyo), **5**, 121, 127 (1957); (c) B. Maruo and A. A. Benson, *J. Biol. Chem.*, 234, 254 (1959); (d) D. M. Brown, G. E. Hall, and R. Letters, *J. Chem. Soc.*, 3547 (1959).

(13) M. Smith, J. G. Moffat, and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6204 (1958).

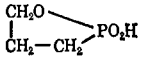
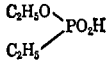
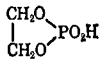
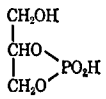
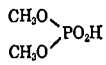
Table III. First-Order Rate Constants for Hydrolysis of Barium Glycero-1,2-cyclic phosphate Initially 0.02 *M* as a Function of Sodium Hydroxide Concentration and of Basicity Function H_-

Temp, °C	NaOH, *M*	H_-[c]	$H_- + \log [H_2O]$	10^5k, sec^{-1}
0	0.5	13.71	13.70	3.20
	1.08	14.05	14.02	6.88
	2.16	14.42	14.37	16.7
	2.16[a]			15.4
	2.7	14.58	14.51	28.8
	2.7[b]			27.5
	3.24	14.72	14.63	36.9
3.6	1.45			11.60
11.9	1.08			21.8
25.8	1.08			87.8

[a] Initial concentration of substrate, 0.04 *M*. [b] The substrate was sodium glycero-1,2-cyclic phosphate. [c] Data for H_- are from G. Yagil, *J. Phys. Chem.*, in press. We wish to thank Dr. Yagil for providing us with these data in advance of publication.

acyclic analog, dimethyl hydroxyethyl phosphate (23.0 kcal/mole).[11c,e] It seems, however, that more consideration should also be given to specific kinetic effects. Only in acid and alkaline solutions are the five-membered cyclic phosphates very sensitive to hydrolytic ring opening (see Table IV). In the intermediate range, pH 3–12, the reactivity is very small, and may be similar to that of open-chain dialkyl phosphates. For dimethyl phosphate[2] at 100° at pH 3.33, $k = 7.2 \times 10^{-8}$ sec^{-1}; for glycero-1,2-cyclic phosphate at the same temperature at pH 4.9, we got an upper limit, $k \leq 10^{-7}$ sec^{-1}. A precise comparison is difficult, due to the very low rates of reaction for both the cyclic and acyclic monoanions and since the point of bond cleavage is unknown for either type of ester in the monoanion state. This result is difficult to explain merely by ring strain. If the five-membered ring is strained, it should

Table IV. Comparison of Kinetic Parameters for Cyclic and Open-Chain Dialkyl Hydrogen Phosphates

	Ref	k_H,[a] l. $mole^{-1}$ sec^{-1}, 50°	ΔE, kcal/mole	$\Delta S_{50°}$,[a] eu	k_{OH},[a] l. $mole^{-1}$ sec^{-1}, 50°	ΔE, kcal/mole	$\Delta S_{50°}$,[a] eu
	*b*	1.87×10^{-3}	16.3[c]	−27	0.2×10^{-3}	16.0	−28
	*b*	5.5×10^{-9}	25.4[c]	−19	1.75×10^{-10}	23.4	−33
	*d, f*	9.0×10^{-2}	15.0	−19			
	*e*	3.8×10^{-1}	15.0	−18	50×10^{-3}	15.8	−22
	*f, g*	1.7×10^{-9}	31	−5	2.4×10^{-10}	28.2	−17

[a] All the rate constants were extrapolated to 50°. [b] Reference 11g. [c] *E* calculated from rate constants given for two temperatures. [d] Reference 11b. [e] Present work. [f] Reference 2; Ph.D. Thesis, J. R. Cox, Jr., Harvard University, 1959. [g] Reference 11a.

crease in the ionic strength (addition of 0.4 *M* $NaClO_4$) had no appreciable effect on the rate of hydrolysis. Also, in the alkaline medium, no difference in the rates of hydrolysis was found for the sodium or barium salts of the substrate. These results are in contrast to reports on the hydrolytic ring opening of ethylene phosphate, which was found to be subject to a considerable ionic strength effect, and to catalysis by barium ions.[11a] The deuterium oxide solvent effect on the acid-catalyzed ring opening of glycero-1,2-cyclic phosphate results in a marked increase in rate in the deuterated solvent (see Table I). In alkaline solutions too (in 1.5 *M* NaOH), the solvent isotope effect is appreciable, $k_{D_2O}/k_{H_2O} = 1.5 \pm 0.1$.

Discussion

In previous attempts to understand the remarkable reactivity of five-membered cyclic phosphates, by comparison with the acyclic esters, much emphasis was given to the particular ring strain presumed to exist in the five-membered ring.[11b] This hypothesis of ring strain found support in the higher heat of reaction for alkaline-catalyzed hydrolysis of the cyclic methyl ethylene phosphate (28.5 kcal/mole) by comparison with the

be very reactive even as the monoanion, and not only by acid and alkaline catalysis.

A self-consistent molecular orbital method was used to compute the charge distributions and energies caused by 2p–3d orbital interactions in various acyclic and cyclic phosphate esters.[14] These calculations suggest that the net positive charge on the phosphorus atom is larger for the five-membered cyclic phosphates than for the acyclic esters. Reaction is assumed to be due to attack of nucleophilic reagents, primarily H_2O and OH^-, occurring either *only* on the phosphorus atom (for the cyclic esters), or *partly* on the phosphorus atom (for acyclic esters). The increased rate of hydrolysis of the five-membered cyclic esters would thus seem to be consistent with the larger net positive charge on their phosphorus atom compared with that of the acyclic phosphate esters. These calculations were, however, made for the anions of the cyclic and acyclic dialkyl phosphates. Our results show that there is no marked difference in reactivity of the anions of the cyclic and acyclic dialkyl phosphates toward nucleophilic attack by water. The enormous differences ap-

(14) R. L. Collin, *J. Am. Chem. Soc.*, **88**, 3281 (1966).

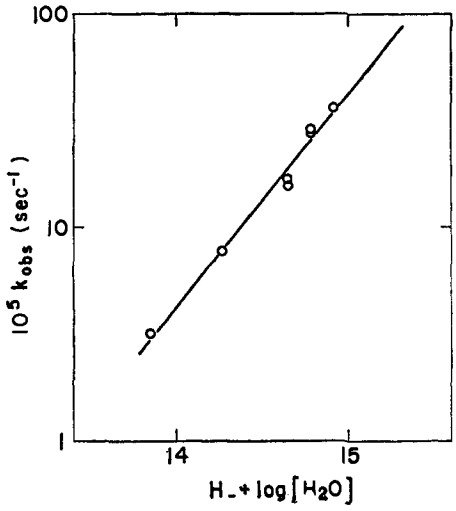


Figure 2. Plot of first-order rate constants for the hydrolysis of glycero-1,2-phosphate at 0° against $H_- + \log [H_2O]$.

pear for nucleophilic attack by OH^-, as well as in the formation or decomposition of the conjugate acid. It would be interesting to extend the molecular orbital calculations also to the neutral molecules and conjugate acids of the cyclic and acyclic esters.

Activation Energies. Additional information can be gained from the activation parameters. In hydrolysis of a dialkyl hydrogen phosphate, the number of product molecules (alkyl dihydrogen phosphate and alcohol) is equal to that in the reactants (alkyl dihydrogen phosphate and water), while the hydrolytic ring opening does involve a *decrease* in the number of molecules from the reactants (glycero-1,2-cyclic phosphate and water) to the product (glycero phosphate). By these considerations, the entropy of activation should be more negative for the ring opening than for the hydrolysis of a dialkyl phosphate. As shown in Table IV, the entropy of activation for both the acid and alkaline hydrolysis of cyclic and open-chain phosphate esters has a very negative value. However, the *energy of activation* is seen to be dramatically lower for the cyclic phosphates, in both acid- and alkali-catalyzed reactions. Thus, the very rapid acid and alkaline ring opening is due to the enormous decrease in activation energy.

Acidic Solutions. The rate of ring opening in acid solutions was not linearly related to the acid concentration. The rate constants for the second-order acid-catalyzed reaction (k_H) and for the first-order hydrolysis of the neutral molecule (k_w) could be estimated[11g] from the observed first-order rate constant (k_{obsd}). The rate of the reaction may be represented by

$$v = k_{obsd}\{[AH] + [A^-]\} = \{k_w + k_H[H^+]\}[AH] \quad (1)$$

where [AH] and $[A^-]$ are the concentrations of the neutral molecule and of the monoanion of the substrate, respectively. By introducing in the above equation the ionization constant $K = [H^+][A^-]/[AH]$, we get

$$v' = k_{obsd}\{1 + K/[H^+]\} = k_w + k_H[H^+] \quad (2)$$

From eq 2, k_w and k_H can be obtained as intercept and slope of the plot of v' against $[H^+]$.

Due to the difficulty of direct determination of the ionization constant K, because of the rapid hydrolysis in acid media, various values of K were chosen, from 0.001 to 1.0, in intervals of 0.01. The best fit for the data of Table I, giving the smallest deviations on a least-square plot (run on a CDC-1604 computer), was obtained for $K = 0.38 \pm 0.01$. The resulting rate constants for hydrolytic ring opening of glycero-1,2-cyclic phosphate at 0° are

$$k_w = (-0.06 \pm 0.11)10^{-3} \text{ sec}^{-1}$$

$$k_H = (5.5 \pm 0.3)10^{-3} \text{ l. mole}^{-1} \text{ sec}^{-1}$$

The negligible value for k_w expresses the lack of reactivity in the intermediate pH ranges.

For the acid-catalyzed ring opening of glycero-1,2-cyclic phosphate, another mechanistic clue comes from the deuterium solvent isotope effect (see Table I). Since the rate in deuterium oxide is 2.25 times faster than in normal water, this indicates a rapid reversible proton transfer and a slow decomposition of the conjugate acid to the products.[15] No solvent isotope effect seems to have been reported for hydrolysis of open-chain dialkyl hydrogen phosphates.

The data on the acid-catalyzed reaction of the glycero-1,2-cyclic phosphate are in agreement with a trigonal-bipyramidal structure in the transition state, as suggested for other five-membered cyclic phosphates.[11d,f]

Alkaline Solutions. In alkaline solutions, the rate of ring opening is not linearly related to the OH^- concentration (see Table III). Linear relationships were, however, obtained between log k_{obsd} and the functions H_-, measured at 25°, and $H_- + \log [H_2O]$,[16] with slopes of 1.07 ± 0.04 and 1.15 ± 0.05. Using earlier data[17] measured at 20°, we got slopes of 0.96 ± 0.04 and 1.03 ± 0.05, respectively (see Figure 2).

The above results do not enable us to decide between the various mechanisms. However, the high negative value of the entropy of activation, and the considerable stability of the anion at lower pH, both seem to favor a bimolecular mechanism for the hydrolytic opening of the five-membered cyclic phosphate, in which the hydroxyl anion attack occurs in the rate-determining step. The transition state for the hydrolysis of the cyclic and acyclic phosphates probably occurs in the initial, bond-forming phase, and not in the bond-breaking phase. Thus, for the cyclic esters, it occurs while the ring is still substantially intact.[18]

The extraordinary sensitivity of the five-membered cyclic phosphate esters to acid and base catalysis, as well as their high stability in the intermediate pH range, seem to be a unique feature among phosphorus compounds. It may thus provide a clue to the important role of these cyclic esters in the bifunctional enzymatic catalysis.

(15) K. B. Wiberg, *Chem. Rev.*, **55**, 713 (1955).
(16) (a) M. Anbar and G. Yagil, *J. Am. Chem. Soc.*, **84**, 1790 (1962); (b) G. Yagil and M. Anbar, *ibid.*, **85**, 2376 (1963); (c) M. Anbar, M. Bobtelsky, D. Samuel, B. Silver, and G. Yagil, *ibid.*, **85**, 2380 (1963).
(17) G. Schwarzenbach and R. Sulzberger, *Helv. Chim. Acta*, **27**, 348 (1948).
(18) We are indebted to one of the referees for suggesting this point.