and as determined experimentally. Plots of $t(R_\infty - R_{obsd})$ *vs.* R_{obsd} for each kinetic experiment are of excellent linearity, and the rate constants as calculated by this method are summarized in Table I.

A detailed kinetic study was then made under conditions in which one of the reactants in each experiment was in sufficient excess to ensure greater than 99.5% neutralization of the minor component. As an example, for neutralization of 1-phenyl-1-nitroethane to be 99.5% complete under the kinetic conditions (eq 7), n must be 1.4; for $n = 10$, neutralization occurs to almost 100%. For use of eq 7 effectively, R_∞ of accuracy is required. It was more reliable and convenient to use excess nitro compound than excess hydroxide ion in each kinetic experiment. Excess hydroxide ion did not lead to a totally constant R_∞ value in any system; with time R_∞ as measured experimentally increased slowly. Excess hydroxide ion could be used reliably upon measuring R_∞ at times corresponding to 8–10 half-lives for neutralization. It was more convenient to use excess nitro compound (n = 1.4–10) because R_∞ stabilized more satisfactorily by this method; upon prolonged storage of the kinetic solutions the resistance of a neutralized solution began to drop slowly but this did not lead to serious inconvenience or experimental complication. A further advantage in using excess nitro compound is that the resistance change is larger than when the base is the reactant in excess. This technique also reduces the effect of any error introduced by approximation of R_0. The rate constants and the kinetic parameters for neutralization of the nitro compounds by this method are summarized in Table I.

Infrared Spectra of Alkanenitronates. Nitromethane, nitroethane, and 2-nitropropane were fractionated and stored over Linde 5A Molecular Sieves. The pure nitroalkanes (1.0 g) were dissolved in pentane (25 ml) and treated, respectively, with 0.5 equiv of butyllithium (Foote Chemical Co., 1 *M* in hexane), sodium methoxide powder (sublimed), and potassium *t*-butoxide powder (MSA Research Corp.). The salts precipitated almost immediately and the mixtures began refluxing. The slurries were stirred for 5 min, filtered, washed with fresh pentane (3 × 25 ml), and vacuum dried.[34]

The white salts were ground with anhydrous potassium bromide crystals (Fisher Chemical Co.) and pressed under vacuum in a pellet die to give clear, transparent wafers. The infrared spectra of each salt were immediately determined on a Perkin-Elmer Infracord spectrophotometer. This position of the major band near 1650 cm^{-1} is reported in Table VI.

Ultraviolet Spectra of Alkanenitronates. For determining the wavelengths ($\pi \rightarrow \pi^*$) of maximum absorption of the various alkanenitronates (Tables V and VII), each nitro compound was treated with excess sodium methoxide or pure sodium salts of the nitronates were dissolved in the various hydrogen bonding or aprotic solvents to give alkaline solutions approximately 10^{-4} *M* in nitro compound. The solutions were always blanketed with nitrogen and analyzed within 0.25 hr after preparation. Cary (Model 14) and Beckman (DU) spectrophotometers were used for the measurements.

For the data in Table IV and Figure 7, solutions from kinetic runs at 0° were used in which the initial concentrations of the reactants were the same. After the resistance of a solution had reached a constant or a maximum value, an aliquot was taken and diluted with 50% (vol) dioxane–water so that the final concentration of the nitronate was $\sim 7.5 \times 10^{-5}$ M. The spectra of the anions were determined immediately with a Beckmann (DU) spectrophotometer. The extinction coefficients are only approximate since neutralization was not complete (>94%) and there was no correction for hydrolysis of the spectral samples upon dilution. Use of excess sodium hydroxide resulted in solutions whose absorptions were time dependent in which the extinction coefficients became less.

(34) Methanenitronates are very dangerous and were used as slightly damp powders.

Models of Ribonuclease Action. II. Specific Acid, Specific Base, and Neutral Pathways for Hydrolysis of a Nucleotide Diester Analog[1,2]

D. A. Usher,[3] David I. Richardson, Jr.,[4] and D. G. Oakenfull[5]

Contribution from the Department of Chemistry, Cornell University, Ithaca, New York 14850. *Received September 18, 1969*

Abstract: The rate of production of phenol from the phenyl ester I has been measured as a function of pH at 50° and ionic strength 0.1. The cyclic phosphate III is the sole initial product at pH values above 4, and it is considered likely that this is also true for the reaction in acid. Four kinetically distinct terms appear in the rate equation: k_1(HEH)(H); k_2(EH)(H); k_3(EH); k_4(EH)(OH), and the four rate constants and the acid dissociation constant of the phosphate were obtained by a weighted nonlinear least squares analysis. A large specific salt effect was shown by k_4. The kinetic pK_a of the neighboring hydroxyl group of I has been measured by stopped-flow kinetics in strong base. Plausible mechanisms for these reactions are considered, and it is shown that these results can be useful in considering some of the elementary steps of ribonuclease action.

Since 1920, when Jones recorded[6] in the *American Journal of Physiology* his recent discovery that a boiled aqueous extract of pig pancreas was able to hydrolyze yeast nucleic acid, ribonucleases from many different sources have come under the close scrutiny of a large number of workers in several disciplines, and today one of the most studied of all enzymes is a member of this group.[7]

Enormous progress has been made since the first crystallization[8] of bovine pancreatic ribonuclease in 1940.

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(2) A preliminary report of a part of this work has appeared: D. G. Oakenfull, D. I. Richardson, Jr., and D. A. Usher, *J. Amer. Chem. Soc.*, **89**, 5491 (1967).

(3) To whom enquiries regarding this paper should be addressed: Career Development Awardee of the National Institutes of Health (1-K4-GM-42,407).

(4) Predoctoral N. I. H. Trainee, 2T01-GM00834.

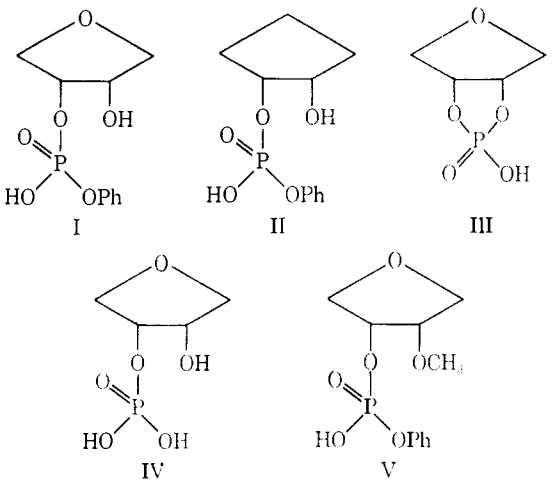
(5) Postdoctoral N. I. H. Trainee, 2T01-GM00834; Division of Food Preservation, C.S.I.R.O., Ryde N. S. W., Australia.

(6) W. Jones, *Amer. J. Physiol.*, **52**, 203 (1920).

(7) For reviews, see E. A. Barnard, *Ann. Rev. Biochem.*, **38**, 677 (1969); H. A. Scheraga, *Fed. Proc.*, **26**, 1380 (1967); J. P. Hummel and G. Kalnitsky, *Ann. Rev. Biochem.*, **33**, 15 (1964); H. Witzel, *Progr. Nucleic Acid Res.*, **2**, 221 (1963); F. H. Westheimer, *Advan. Enzymol.*, **24**, 441 (1962); H. A. Scheraga and J. A. Rupley, *ibid.*, **24**, 161 (1962); C. A. Dekker, *Ann. Rev. Biochem.*, **29**, 453 (1960); D. M. Brown and Sir A. R. Todd, *ibid.*, **24**, 311 (1955).

Among the important advances are determination of the specificity and mode of action of the enzyme,[7] identification of the residues at the active site,[7,9-14] determination of the complete primary sequence[15] and three dimensional structure,[16,17] direct study of the imidazole-C-2 protons of the four histidine residues by nmr spectroscopy,[18] detailed steady-state[7,19] and transient state kinetic analyses,[19] conversion to RNase-S[20] and production of partially synthetic enzyme,[21] and now the first fully synthetic enzyme.[22] At many stages along the way, suggestions of mechanism have been advanced.[7] It is well established that RNase catalyzes the breakdown of a ribonucleic acid by two steps: a transesterification followed by hydrolysis of the resulting cyclic phosphate. Thus, it is surprising that although there have been many investigations of the lability conferred on a phosphate diester by the presence of a neighboring hydroxyl group,[23] no single model of this first reaction that is catalyzed by the enzyme has ever been analyzed in detail. Certainly our knowledge of this reaction is more sketchy than that of the hydrolysis of carboxylic esters. Part of the reason for this may be the slowness of the nonenzymic reaction at other than extremes of pH; extrapolation to pH 7 of the second-order rate constant for the hydroxide ion catalyzed hydrolysis of cytidylylcytidine,[24] assuming no incursion of neutral or acid-catalyzed paths, predicts a half-life for this reaction of about 10^3 to 10^4 days, even at 60°. Indeed, the cyclic phosphate diesters, though remarkable for their ease of hydrolysis in both acid and base,[25] have half-lives of a similar order of magnitude around neutrality at 25°.

In view of the importance of an understanding of the mechanism of participation by neighboring hydroxyl in the hydrolysis of both ribonucleic acids and phospholipids, and since a control reaction was required for our studies of synthetic catalysts, we decided to investigate in detail the hydrolysis of a model of a dinucleoside monophosphate throughout the pH region 1–14. The compound chosen for study was the phenyl ester of *cis*-4-hydroxytetrahydrofuran 3-phosphate (I), the ring closure reaction of which should occur relatively rapidly and be followed conveniently by uv spectrophotometry. The only aryl esters of a similar *cis*-1,2-diol phosphate that have been previously reported are the α-naphthyl esters of uridine 2′- and 3′-phosphates,[26] and these were not obtained in analytically pure form. In this paper a description of the hydrolytic behavior of the model compound at zero buffer concentration is given; the general acid and general base catalyzed reactions of I are discussed elsewhere.[27]



Experimental Section

Paper chromatography was used routinely as a check on product homogeneity and was carried out on Whatman no. 40 paper, using either solvent A (*i*-PrOH:NH_4OH:H_2O 7:1:2) or solvent B (*i*-PrOH:NH_4HCO_3 (1 *M* aqueous, with NH_3 added to pH 9.0):H_2O 7:3:1). Phosphorus was detected on paper chromatograms by the Hanes–Isherwood spray,[28] as modified by Bandurski and Axelrod.[29] Estimation of phosphorus on paper chromatograms was accomplished by wet-ashing,[30] other analyses were by Galbraith Laboratories.

***cis*-Tetrahydrofuran-4-ol 3-Phenyl Phosphate (I).** Phenyl dihydrogen phosphate[31] (1.74 g), triethylamine (1.01 g), *cis*-tetrahydrofuran-3,4-diol[32] (10.4 g), and trichloroacetonitrile[33] (redistilled bp 84–85°, 12 ml) in acetonitrile (10 ml) were allowed to stand at room temperature for 6 days. The progress of the reaction was followed by paper chromatography (solvent B) and by injecting aliquots (5-μl) of the reaction mixture into 0.01 *N* potassium hydroxide solution (3 ml) and following the release of phenoxide by uv spectrophotometry. The reaction mixture was concentrated under vacuum at room temperature and the resulting oil partitioned between water and ether. The phosphate esters in the aqueous phase were separated by ion exchange chromatography on Dowex 1X8-Cl, using a column 2 × 17 cm, and 0.5 *M* lithium chloride as eluent. The fractions containing the phenyl ester were bulked and the solution was concentrated under vacuum, and left at 5° overnight. The crystals of the product were reprecipitated from aqueous methanol by the addition of excess acetone. Yield of

(8) M. Kunitz, *J. Gen. Physiol.*, 24, 15 (1940); M. R. McDonald, *ibid.*, 32, 33, 39 (1948).
(9) L. Weil and T. S. Seibles, *Arch. Biochem. Biophys.*, 54, 368 (1955).
(10) H. G. Gundlach, W. H. Stein, and S. Moore, *J. Biol. Chem.*, 23, 1754 (1959).
(11) E. A. Barnard and W. D. Stein, *J. Mol. Biol.*, 1, 339 (1959).
(12) D. Findlay, D. G. Herries, A. P. Mathias, B. R. Rabin, and C. A. Ross, *Nature*, 190, 781 (1961).
(13) A. M. Crestfield, W. H. Stein, and S. Moore, *J. Biol. Chem.*, 238, 2413, 2421 (1963).
(14) C. H. W. Hirs, *Brookhaven Symp. Biol.*, No. 15, 154 (1962).
(15) D. G. Smyth, W. H. Stein, and S. Moore, *J. Biol. Chem.*, 238, 227 (1963).
(16) G. Kartha, J. Bello, and D. Harker, *Nature*, 213, 862 (1967).
(17) H. W. Wyckoff, K. D. Hardman, N. M. Allewell, T. Inagami, L. N. Johnson, and F. M. Richards, *J. Biol. Chem.*, 242, 3984 (1967).
(18) J. H. Bradbury and H. A. Scheraga, *J. Amer. Chem. Soc.*, 88, 4240 (1966); D. H. Meadows, J. L. Markley, J. S. Cohen, and O. Jardetzky, *Proc. Nat. Acad. Sci. U. S.*, 58, 1307 (1967); D. H. Meadows, O. Jardetzky, R. M. Epand, H. H. Ruterjans, and H. A. Scheraga, *ibid.*, 60, 766 (1968).
(19) G. G. Hammes, *Accounts Chem. Res.*, 1, 321 (1968), and references therein.
(20) F. M. Richards, *Proc. Nat. Acad. Sci. U. S.*, 44, 162 (1958); F. M. Richards and P. J. Vithayathil, *J. Biol. Chem.*, 23, 1459 (1959).
(21) K. Hofmann, M. J. Smithers, and F. M. Finn, *J. Amer. Chem. Soc.*, 88, 4108 (1966).
(22) B. Gutte and R. B. Merrifield, *ibid.*, 91, 501 (1969); R. Hirschmann, R. F. Nutt, D. F. Veber, R. A. Vitali, S. L. Varga, T. A. Jacob, F. W. Holly, and R. G. Denkewalter, *ibid.*, 91, 507 (1969).
(23) For a review, see T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," Vol. II, W. A. Benjamin, Inc., New York, N. Y., 1966, pp 37–48.
(24) H. Witzel, *Ann.*, 635, 182 (1960).
(25) H. I. Abrash, C. S. Cheung, and J. C. Davis, *Biochemistry*, 6, 1298 (1967).
(26) M. Zan-Kowalczewska, H. Sierakowska, and D. Shugar, *Acta Biochim. Polon.*, 13, 237 (1966).
(27) D. A. Usher, D. I. Richardson, Jr., and D. G. Oakenfull, in preparation.
(28) C. S. Hanes and F. A. Isherwood, *Nature*, 164, 1107 (1949).
(29) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, 193, 405 (1951).
(30) D. A. Usher, *J. Chromatog.*, 12, 262 (1963).
(31) Eastman White Label phenyl dihydrogen phosphate was contaminated with a considerable amount of orthophosphoric acid and required purification. This was accomplished by fractional precipitation of the barium salt.
(32) F. H. Otey and C. L. Mehltretter, *J. Org. Chem.*, 26, 1673 (1961).
(33) F. Cramer, W. Rittersdorf, and W. Bohm, *Ann.*, 654, 180 (1962).

chloride-free product was 15–40%. *Anal.* Calcd for $C_{10}H_{12}LiO_6P$: C, 45.14; H, 4.54; P, 11.64. Found: C, 44.96; H, 4.57; P, 11.80.

cis-**Cyclopentan-2-ol 1-phenyl phosphate (II)** was prepared in essentially the same way by substituting *cis*-cyclopentane-1,2-diol[34] for the tetrahydrofuran diol, yield 20%. *Anal.* Calcd for $C_{11}H_{14}LiO_6P$: C, 50.02; H, 5.34; P, 11.73. Found: C, 49.89; H, 5.43; P, 11.87.

trans-**2-Chlorocyclopentane 1-Phosphate.** A solution of orthophosphoric acid (crystalline, 3.9 g) and triethylamine (11.2 ml) in acetonitrile (30 ml) was added dropwise with stirring to a mixture of *trans*-2-chlorocyclopentanol[35] (9.64 g) and trichloroacetonitrile (12 ml) over a period of 2 hr. The mixture was stirred for a further 2 hr, then *trans*-2-chlorocyclopentane 1-phosphate precipitated as the biscyclohexylammonium salt by the addition of acetone (300 ml) and cyclohexylamine (30 ml). The product was washed with acetone and recrystallized from absolute ethanol, yield 40%.

cis-**Cyclopentane 1,2-Cyclic Phosphate (VI).** A solution of the lithium salt of *trans*-2-chlorocyclopentane 1-phosphate in water at pH 8.5 was heated at 100° for 15 min. The solution was concentrated, the residue dissolved in methanol, and the lithium salt of the *cis*-cyclic phosphate precipitated by the addition of acetone. One further precipitation gave pure material. *Anal.* Calcd for $C_5H_8LiO_4P$: C, 35.32; H, 4.74; P, 18.22. Found: C, 35.35; H, 4.69; P, 18.05.

cis-**Tetrahydrofuran 3,4-cyclic phosphate (III)** was prepared by the method of Carre,[36] and purified from unchanged material and monoester by repeated fractional precipitation of the lithium salt from ethanol solution by the addition of acetone. *Anal.* Calcd for $C_4H_6LiO_5P$: C, 27.93; H, 3.52; P, 18.01. Found: C, 27.90; H, 3.43; P, 17.97.

cis-**Tetrahydrofuran-4-ol 3-Phosphate (IV).** A solution in water of the crude reaction mixture (2 g) from the preparation of the cyclic phosphate was allowed to stand for 3 hr at 25°. Aqueous barium hydroxide was added to bring the pH to 9, the solution was filtered, concentrated to 20 ml, and converted to the lithium salt by passage down a column of Dowex 50-Li^+. The lithium salt was precipitated from concentrated aqueous solution by the addition of acetone, yield 1.0 g. *Anal.* Calcd for $C_4H_7Li_2O_6P$: C, 24.52; H, 3.60; P 15.81. Found: C, 24.37; H, 3.76; P, 15.64.

trans-**2-Hydroxycyclopentane 1-Phenyl Phosphate.** Cyclopentene oxide[34] (10 g) was allowed to react with phenyl dihydrogen phosphate (2.07 g) in chloroform (60 ml) for 24 hr at 25°. The solvent was removed, aqueous barium hydroxide added, and unchanged monoester precipitated by the addition of ethanol. The required diester was converted to the lithium salt and purified by repeated precipitation from ethanol (2 ml) by the addition of acetone (25 ml), yield 300 mg. *Anal.* Calcd for $C_{11}H_{14}LiO_5P \cdot 2H_2O$: C, 44.02; H, 6.04; P, 10.32. Found: C, 43.87; H, 6.37; P, 10.36.

trans-**2-Methoxycyclopentane 1-Phenyl Phosphate.** Diphenyl phosphorochloridate[37] (2.68 g) in anhydrous pyridine (12 ml) was added dropwise to *trans*-2-methoxycyclopentan-1-ol[34,38] (1.00 g) in pyridine (15 ml) over a period of 2 hr. After a further 24 hr, water (1 ml) was added, and the mixture was stirred 1 hour. Benzene (100 ml) was added and the mixture was washed at 0° successively with aqueous sodium bisulfate (twice with 100 ml of 25%), saturated aqueous sodium bicarbonate (60 ml), and water (three times with 50 ml). The benzene soluion was dried with anhydrous sodium sulfate and evaporated to give a yellow oil. Preliminary experiments were carried out to determine the approximate rate of hydrolysis of this triester, and the bulk of the material was then converted directly to the diester. The triester was treated with 100 ml of 0.2 *N* KOH in 60/40 v/v dioxane-water for 24 hr at 25°. The solution was passed down a column of Dowex 50-H^+ and the acid effluent neutralized with aqueous barium hydroxide. The barium salt of *trans*-2-methoxycyclopentane 1-phenyl phosphate was purified by precipitation from 95% ethanol (5 ml) by the addition of acetone, yield 1.028 g. *Anal.* Calcd for $C_{12}H_{16}Ba_{1/2}O_5P$: C, 42.40; H, 4.74; P, 9.11. Found: C, 42.11; H, 4.85; P, 9.34.

cis-**4-Methoxytetrahydrofuran-3-ol.** To a solution of sodium ethoxide in ethanol (from 2.3 g of sodium in 50 ml of ethanol) was added *cis*-tetrahydrofuran-3,4-diol (10 g) followed by methyl iodide (60 g). The solution stopped boiling within 10 min, and was allowed to stand at room temperature for 5 hr, when the mixture was fractionally distilled. *cis*-4-Methoxytetrahydrofuran-3-ol was collected at 73–75° (7 mm) (lit.[39] 77–78° (13 mm)), yield 2 g (phenylurethan mp 92–92.2° (lit.[39] 92–94°). The nmr spectrum confirmed the presence of one methoxy group.

cis-**4-Methoxytetrahydrofuran 3-Phenyl Phosphate (V).** *cis*-4-Methoxytetrahydrofuran-3-ol (1.18 g) was treated with diphenyl phosphorochloridate (3.2 g) in anhydrous pyridine (5 ml) and the reaction mixture treated as described above for the preparation of methoxycyclopentane phenyl phosphate. The yield of crystalline *cis*-4-methoxytetrahydrofuran 3-diphenyl phosphate was 88%. The triester (1.05 g) was converted to the diester by treatment with 0.5 *N* sodium hydroxide in 50:50 dioxane–water for 22 hr at 22°. The *cis*-4-methoxytetrahydrofuran 3-phenyl phosphate was purified by precipitation as the lithium salt from aqueous methanol by the addition of first acetone, then ether, yield 630 mg. *Anal.* Calcd for $C_{11}H_{14}LiO_6P$: C, 47.16; H, 5.04; P, 11.06. Found: C, 47.03; H, 5.15; P, 10.98.

N,N-Dimethylmorpholinium Chloride. Methyl iodide (28.4 g) in diethyl ether (10 ml) was added dropwise with stirring to N-methylmorpholine (20 g) in ether (25 ml). The reaction mixture was filtered after 30 min and again after 16 hr and the combined precipitates were recrystallized from 95% ethanol, yield 21.5 g. The dimethylmorpholinium iodide was converted to the chloride salt by passage of an aqueous solution through a column of Dowex 1-Cl (300 ml of resin per 21.5 g of iodide). The hygroscopic dimethylmorpholinium chloride was crystallized from ethanol–ether and was found to be free from iodide and from any impurity that ionized in the pH range 6.5–11. Solutions of the chloride were standardized by titration with standard silver nitrate using potassium chromate as an indicator.[40]

Purification of Reagents. Morpholine (Aldrich, puriss) was dried over potassium hydroxide and distilled (bp 126°) under nitrogen immediately prior to use. The purity was monitored by vpc. 2-Methoxyethylamine (Eastman White Label), piperidine (Aldrich), acetic acid (Baker, glacial analytical reagent grade), and formic acid (Allied Chemical, CP) were distilled and imidazole and 2-methylimidazole (both Aldrich) recrystallized before use. Monochloroacetic acid (Fisher, certified) was not further purified. Aqueous sodium and potassium hydroxide solution (Fisher, standard) were restandardized against potassium hydrogen phthalate using phenolphthalein or bromothymol blue as an indicator. Hydrochloric acid (Fisher, standard) was then checked against the standard base. Although the base solutions were found by titration to be free from interfering amounts of carbonate, for critical work, carbonate free potassium hydroxide solution was prepared separately.[41] The water used in this work was distilled and deionized (specific resistance greater than 2 Megohms), and in the later work, again distilled from an all-glass apparatus (Corning AG-1b), with protection from atmospheric carbon dioxide. Deuterium oxide (99.7%) was distilled from potassium permanganate and stored in a desiccator.

Preparation of Buffers. Class A volumetric apparatus was used in the preparation and standardization of all solutions. For the amine–amine hydrochloride buffers, the required quantity of amine was weighed directly into the volumetric flask; for the carboxylate buffers, approximately 1 *N* solutions of the acids were standardized against potassium hydroxide, and the required quantity of acid for each buffer ratio added from a buret. For each buffer ratio, four further dilutions were made using 0.100 *M* potassium chloride, and in the case of morpholine only, also using 0.100 *M* N,N-dimethylmorpholinium chloride. The change in concentration of the buffers in going from the temperature of preparation (25°) to the temperature of use was ignored. Buffers were prepared from morpholine that had been freshly distilled as otherwise (even though the amine was kept in wax-sealed bottles) they frequently showed a drift in the optical density at 270 mμ when heated to 50° (possibly due to decomposition of traces of carbamate). This drift, if not recognized, can introduce serious error in the measurement of rate constants.

Products of the Reaction of *cis*-Tetrahydrofuran-4-ol 3-Phenyl Phosphate. (1) In Morpholine Buffer. The phenyl ester I (15 mg) in morpholine–morpholinium hydrochloride buffer (5 ml of 1:1, 0.1 *M* in free base) was heated at 50° for 23 hr (about ten half-lives).

(34) L. N. Owen and P. N. Smith, *J. Chem. Soc.*, 4026 (1952).
(35) H. B. Donahoe and C. A. Vanderwerf, "Organic Syntheses," Coll. Vol. IV, John Wiley & Sons, Inc., New York, N. Y., 1963, p 157.
(36) P. Carré, *Ann. Chim. Phys.*, [8] 5, 394 (1905).
(37) D. M. Brown, *Advan. Org. Chem.*, 75, (1963).
(38) S. Winstein and R. B. Henderson, *J. Amer. Chem. Soc.*, 65, 2196 (1943).
(39) E. G. E. Hawkins, *J. Chem. Soc.*, 248 (1959).
(40) A. I. Vogel, "Quantitative Inorganic Analysis," 2nd ed, Longmans, Green and Co., London, 1955, p 251.
(41) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," Methuen and Co., London, 1962, pp 24–27.

Samples (25 μl) of the reaction mixture before and after heating were spotted together with appropriate controls, on Whatman no. 40 paper, and the chromatograms developed in solvent A. Quantitative estimation of the spots[30] showed that the phenyl ester had been converted in at least 98% yield to a compound having the same R_f as the cyclic phosphate (III). Authentic cyclic phosphate was shown to be stable under the conditions of the hydrolysis. The buffer solution was concentrated to remove excess morpholine, dissolved in water, and passed down a column of Dowex 50-Li. The lithium salt of the cyclic phosphate was precipitated from methanol solution by the addition of acetone and ether, and identified by comparison of its infrared spectrum with that of an authentic sample. A similar hydrolysis was carried out using a pH-stat to maintain the pH at 8.1 (50°) instead of the buffer. Again the cyclic phosphate (III) was the only product.

(2) In Acetate Buffer. The phenyl ester (I) (7.6 mg of the lithium salt) was heated in acetate buffer (2 ml of 1:1 sodium acetate–acetic acid, 0.1 *M* in free acid) for 18 hr at 95° ± 0.5°. A control reaction consisting of the cyclic phosphate (III) (3.8 mg of the lithium salt) in the same buffer (2 ml) was run simultaneously. Paper chromatography showed that both solutions contained largely cyclic phosphate, together with small amounts of tetrahydrofuran-4-ol 3-phosphate (IV) and inorganic orthophosphate.

A solution of the phenyl ester that had been heated for 4 hr showed essentially only cyclic phosphate (about 80%) and unchanged material. A rough kinetic analysis of the scheme I → III → IV → P_i showed that these results were consistent with the phenyl ester going solely to cyclic phosphate.

(3) In 0.1 *N* Hydrochloric Acid. A solution of the phenyl ester (I) (3.5 mg of the lithium salt) in 0.1 *N* hydrochloric acid was heated at 50° for 1 hr (about seven half-lives). Paper chromatography showed that the only product was tetrahydrofuran-4-ol 3-phosphate (IV).

(4) Products of the Kinetic Runs. At the end of each kinetic run, the uv absorption spectrum of the solution was compared to that of a mixture of phenol (at the same concentration) with the appropriate buffer. The relatively slow reaction of the cyclopentane phenyl phosphate (II) in amine buffers allowed a further reaction (presumably an oxidation) of the phenol to occur before all the ester had reacted. The product of the oxidation had λ_{max} at 315 mμ, but did not significantly affect the optical density at 270 mμ. A similar result was obtained with solutions of phenol in morpholine buffers. Although this effect could be alleviated by exclusion of oxygen (freeze–thaw cycles under vacuum) in practice it was found impracticable to remove it completely. This unsatisfactory situation was resolved by the use of the tetrahydrofuran phenyl ester (I), in this case the release of phenol was much faster, and a negligible degree of oxidation of the phenol took place before a good infinity reading was obtained. The reverse reaction of phenol with cyclic phosphate was shown by paper chromatography and spectrophotometry to be insignificant.

pH Measurement. pH measurements were made using either a Radiometer PHM-26, or TTT-1c titrator equipped with a scale expander (Model PHA 630T), together with a microtitration assembly (Radiometer TTA 31) and thermostated vessel (V 521/526). Approximately 5 ml of water was placed in the space between the outer and inner vessels to serve as a heat transfer fluid. For work at 25°, a Radiometer G2222B glass electrode was used with a K4001 calomel reference electrode, but for work at 50°, the calomel electrode was thermostated at 25° in a separate bath and connected to the test vessel by means of a salt bridge containing saturated potassium chloride. The temperature in the test solution, controlled by circulation of water from a Haake Model F thermostat, was measured by means of a calibrated thermistor (see below), and was constant to better than ±0.02°. Atmospheric carbon dioxide was excluded from the titration vessel by a stream of nitrogen gas that had been passed through a column of soda lime and then through water. The meter was standardized before and after each determination with at least two buffers [pH 4 and pH 10 (Matheson Coleman and Bell) and pH 7 (Corning)]; if the reading had changed by more than 0.01 unit the procedure was repeated. A less than theoretical response of the glass electrode was compensated for by adjusting the sensitivity of the meter; a sluggish response was corrected by replacing the glass electrode. The test solution was stirred throughout the measurement by means of a magnetic disk; any variation in junction potential was ignored. The pH values of the standard buffers at various temperatures were obtained from Bates.[42]

pH Measurement in D_2O Solutions. Since there is some suggestion[43] that the correction factor[44] for converting "pH" readings to true pD values above 25° is dependent on the particular glass electrode, the method of Fife and Bruice[43] was applied to a Radiometer glass electrode type G2222B. Five readings each on 0.095 and 0.0095 *N* deuterium chloride in deuterium oxide, and hydrogen chloride in water, were made and the whole experiment was then repeated. The equation relating true pD with the reading of the pH meter (standardized in the usual way with light-water standard buffers) was pD = (pH meter reading) + (0.275) at 50°. (Fife and Bruice found[43] 0.287 at 50°.) However, it is expected that this correction, applicable to acid solutions, will be somewhat in error when applied to more alkaline solutions.[44]

pK_a Determination. The pK_a of the phosphate group of the methoxy ester (V) was determined by potentiometric titration of 2.0 ml of 0.0982 *M* aqueous solution at 50°. The free acid form of the ester, prepared by passing a solution of the lithium salt down a column of Dowex 50-H, was titrated with 1 equiv of 1.00 *N* potassium hydroxide, added in nine equal increments: the pH was recorded after each addition. Ten values of the pK_a were calculated from the titration data by the iterative process described by Albert and Serjeant:[45] from the pH was calculated the activity and therefore approximate concentration of H, a first value for the ionic strength of the solution could then be found, and hence an approximate value for the activity coefficient of H. A better value for the concentration of H was calculated, and the cycle repeated until there was no further change. Corrections for the volume of the added titrant were included in the calculations. The average value for the "mixed" pK_a of the phosphate, applicable to 50° and an ionic strength of 0.07–0.09, was 0.70 with a spread of ±0.15 using values of from 0 to 0.8 equiv of base added. This figure is less than −log (concentration of ester), and is not regarded as highly reliable.

Temperature Measurement. A NBS calibrated thermometer was used to establish the true temperature (±0.1°) of a 50° thermostated water bath, and other temperatures relative to this were measured with high precision by means of a 5° Beckman thermometer or a calibrated thermistor (YSI Model 402). The resistance of the latter was measured by means of a bridge (unbalanced only for direct recording); a resolution of 0.001° was readily achieved. The thermistor was used for measuring the temperature of spectrophotometer cuvets and of the titration vessel; a surprisingly large "emergent stem" effect (due presumably to the transfer of heat along the connecting leads) was cured by folding the flexible vinyl covered lead several times so that an effective lead length of at least 9 cm could be immersed.

Measurement of Rates. A Cary 15 spectrophotometer, calibrated for optical density, wavelength, and time, was used for the determination of all spectrophotometric rates. A specially constructed thermostated cell block was used; the half-time for heating a Teflon-stoppered 1 cm silica cell containing 3 ml of water from room temperature to 50° was 50 sec. The temperature of the block (49.85 ± 0.02°) was controlled by the circulation of water from a Haake Model FT thermostat. Time was measured by stopwatch and by "Timeit" (Precision Scientific).

The method used for following the release of phenol from the phenyl ester (I) varied according to the half-life of the reaction. For half-lives less than 20 min, the reaction was allowed to go to completion (ten half-lives) in the Cary spectrophotometer. For half-lives between 20 min and about 2 hr, the reaction was followed in the Cary for the first three to four half-lives, and the cuvette was then wrapped in polyethylene film and transferred to an external water bath (49.85 ± 0.02°) in order to obtain an infinity reading. For half-lives between 2 hr and 3 days, the cuvettes were kept as above in the external water bath, and were transferred to the spectrophotometer at fixed intervals (24) to make absorption readings. The short exposure to a lower temperature had a negligible effect on the rate. For half-lives longer than 3 days, the reaction was carried out in Pyrex tubes or volumetric flasks, which were equipped with Teflon-lined screw caps. Aliquot samples were removed at intervals for making absorption readings. For some of these long reaction times (in imidazole buffers when using once distilled and deionized water) it was found necessary to sterilize the solutions by a short initial heating to 100°. For the reactions that were carried

(42) R. G. Bates, "Determination of pH," John Wiley & Sons, Inc., New York, N. Y., 1964.

(43) T. H. Fife and T. C. Bruice, *J. Phys. Chem.*, 65, 1079 (1961).

(44) P. K. Glasoe and F. A. Long, *ibid.*, 64, 188 (1960); A. K. Covington, M. Paabo, R. A. Robinson, and R. G. Bates, *Anal. Chem.*, 40, 700 (1968).

(45) Reference 41, p 63.

out in the cuvettes, the buffer solution (3 ml) was equilibrated at 50° for sufficient time to ensure that the optical density was stable, that no bubbles were forming, and that the contents had reached thermal equilibrium, then an aqueous solution of the phenyl ester (typically 5–10-μl) was introduced by means of a syringe (glass and stainless steel) and the contents of the cell were stirred rapidly by means of a Teflon "mushroom." Part way through the run, the cell was checked for the growth of bubbles on the wall, and if found, the run was discarded. Teflon stoppered matched silica cells of 1 cm path length were used and were placed in the cell block always with the same orientation. For very long reactions, the final optical density reading was sometimes obtained by heating the solution to a higher temperature (75–99°) and allowing a factor of about ten in rate for each additional 25°. This procedure was only used when it could be demonstrated that a similar run that had been kept longer at the lower temperature gave an equivalent result. "Infinity" readings were checked several times for constancy. Wavelengths used were 270 mμ (phenol) or 289 mμ (phenoxide); complete spectra of the products were also run. The substrate concentration varied from 7×10^{-5} to 7×10^{-4} M; no effect of a change in substrate concentration on the rate constant was found. The rate was also unaffected by the addition of 0.0011 M EDTA. Plots of ln ($OD_\infty - OD_t$) *vs.* time were straight for at least four and a half half-lives. First-order rate constants were calculated either by the method of Guggenheim[46] (using a Δt of at least two half-lives, and data collected over at least three and a half half-lives), or by plotting ln ($OD_\infty - OD_t$) *vs.* time (using data from one and a half to three half-lives). In the most accurate work (with morpholine) the best straight line to fit either of these plots was obtained by a weighted least squares method, with weighting factors proportional to $(OD_\infty - OD_t)^2$. This weighting factor assumes a constant variance in optical density measurements. As a check on these methods, a direct analysis of the OD_t *vs.* time plot was occasionally made using a weighted nonlinear least squares method,[47] and including OD_∞ as an unknown parameter. The values of OD_∞ obtained in this way agreed with the experimental values to within 0.01 unit.

A few rough kinetic runs were made to determine the approximate rate of ring opening of the cyclic phosphates III and VI. The rate of production of monoester was determined by a previously described titration method.[48]

Stopped Flow Kinetics. The rate of breakdown of the phenyl ester (I) in sodium hydroxide solution of strength up to 1.00 N was measured at 25.3° using a Durrum-Gibson stopped-flow apparatus. The ester concentration after mixing was about 1.3×10^{-4} M and the ionic strength was maintained at 1.0 by the addition of sodium chloride. The production of phenoxide ion was followed at 287 nm (1 mm slit width, 2 cm path length) by recording the output of the photomultiplier tube on a calibrated Tektronix no. 549 storage oscilloscope. Also recorded was the transmission of the cell after at least ten half-lives and a trace corresponding to zero transmission. The rate constants were calculated by means of a weighted linear least squares program using weighting factors proportional to $(OD_\infty - OD_t)^2$; data used were between 85 and 30% transmission (about two half-lives). For each concentration of base, four to six runs were recorded, with at least two fillings of the drive syringes; reproducibility was good. Extraneous effects on the trace, due to the mixing of two solutions of different refractive index and density, were shown to be negligible.

Calculations. Numerical calculations in this work were made on various machines; early work on curve fitting was performed on an IBM 1800 with CRT output and a set of potentiometers connected to the computer *via* an A to D converter.[49] Most of the later work used a PDP-9 with buffered display, light pen, pushbutton control box, and Houston Instruments digital plotter. Other calculations were carried out on a IBM 360-65, and the routine calculation of rate constants was done on a Hewlett-Packard 9100A or Wang 360 calculator.

Results

Products of the Reaction of I. In morpholine or acetate buffer the phenyl ester reacted to give initially only the cyclic phosphate and phenol, within the limits of experimental error. In the amine buffer (1:1, 0.1 M free base) specific base catalysis accounts for 60% of the observed rate, and general base/acid catalysis the remaining 40%. Thus, *both* the specific and general catalyzed reactions give rise to cyclic phosphate. In the acetate buffer at 50° general acid catalysis accounts for 57% of the observed rate, and 92% of the remainder can be assigned to the uncatalyzed reaction of the monoanion (see below). Thus, if it is assumed that the products at 50° would be the same as those at 95°, these two reactions also give cyclic phosphate and phenol. The comparison ester (V) with a neighboring methoxy group was stable in morpholine buffer under conditions that represented seventy half-lives for the reaction of the phenyl ester (I). In formate buffer (1:5, 0.5 M formic acid) no reaction was observed for the equivalent of at least 26 half-lives. In 0.1 N hydrochloric acid hydroxy ester I gave the monoester (IV) and phenol; however, it is possible that this reaction also went by way of the cyclic phosphate. It can be calculated[50] that the maximum concentration (6%) of this intermediate would occur in about 3 min after the start of the reaction (at 50°), and would be barely detectable by paper chromatography. In support of the involvement of the cyclic phosphate as an intermediate, the comparison ester (V) was unchanged for at least 90 min (the equivalent of eight half-lives for I) in 0.1 N hydrochloric acid at 50°. The phenyl ester of *trans*-2-hydroxycyclopentyl phosphate in 1.0 N potassium hydroxide gave a considerable amount (~75%) of phenyl phosphate in 31 hr at 50° (presumably by formation of epoxide), but no detectable phenoxide ion, even in 10 days. The related ester with a *trans*-2-methoxy group was stable for more than 24 hr at 25° in 0.2 N potassium hydroxide in dioxane water (60/40).

pH–Rate Profile for I at Zero Buffer Concentration. For each buffer and buffer ratio, rates were determined in five different concentrations of buffer at a constant ionic strength of 0.1;[52] in the most accurate work (with morpholine buffers), each rate was determined four or more times. The pH of each buffer was measured both before and after the reaction; agreement was generally within 0.02 unit. Except when sources of error (bacterial growth, incomplete reaction, carbamate formation) were known to be present (and then the results were discarded) reproducibility in the calculated rate constants was generally better than 5%, and with the morpholine buffers 2%. In any set of dilutions of a given buffer at constant buffer ratio the spread of pH values was generally less than ±0.02 and in no case was a significant trend apparent. Calculation of the pK_a of the buffer component from the experimental pH and buffer ratios gave a spread that was generally less than ±0.03 and again showed no trend.[27] The pH values of the 1:1 and 5:1 morpholine buffers changed by less than 0.01 unit, on replacing potassium chloride by N, N-dimethylmorpholinium chloride. Plots of k_{obsd} *vs.* free base concentration at constant pH were extrapolated back to zero buffer concentration to give the

(46) E. A. Guggenheim, *Phil. Mag.*, 2, 538 (1926).
(47) W. E. Wentworth, *J. Chem. Educ.*, 42, 96 (1965).
(48) J. Kumamoto, J. R. Cox, Jr., and F. H. Westheimer, *J. Amer. Chem. Soc.*, 78, 4858 (1956).
(49) New York State Veterinary College Computer Facility supported by Grant FR-00326 from the National Institutes of Health.
(50) We have not measured the rate of hydrolysis of III in acid but in view of the similarity of rates for uridine-2′,3′-cyclic phosphate (1.68×10^{-3} sec^{-1} in 0.1 N HCl at 25°)[51] and ethylene phosphate (2.06×10^{-3} sec^{-1} in 0.1 N $HClO_4$ at 30°) it appears safe to assume that a similar figure would apply to III.
(51) M. R. Harris, D. A. Usher, H. P. Albrecht, G. H. Jones, and J. G. Moffatt, *Proc. Nat. Acad. Sci. U. S.*, 63, 246 (1969).
(52) The actual ionic strength in the imidazole, methoxyethylamine, and piperazine buffers was 0.098 ± 0.003.

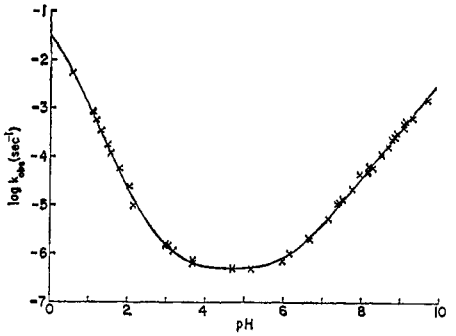


Figure 1. The zero-buffer pH–rate profile for the reaction of I at 50° and ionic strength 0.1 (potassium chloride). The theoretical curve and experimental points were drawn by a digital plotter using the values in Tables I and II.

rate constants listed in Table I and shown in Figure 1. The replacement of potassium by N,N-dimethylmorpholinium ion had a considerable effect on the rate, so that unless otherwise noted the particular values of the rate constants given in Tables I and II refer only to 0.1 *M* aqueous potassium chloride solution at 50°. The shape of the pH–rate profile indicated the involvement of four kinetically distinct terms: (EH)(H)^2, (EH)·(H), (EH), and (EH)(OH), where (EH) is the monoanion of the phenyl ester. In the pH range 1–10, at least 99.8% of the ester is present as either the monoanion (EH) or uncharged free acid (HEH), thus the observed rate of production of phenol can be expressed by the equation

$$\text{rate} = k_1(\text{HEH})(\text{H}) + k_2(\text{EH})(\text{H}) + k_3(\text{EH}) + k_4(\text{EH})(\text{OH}) = k_{obsd}(E_t)\cdot$$

where E_t is the total concentration of ester, and H and OH the activities of hydrogen and hydroxyl ions, respectively.

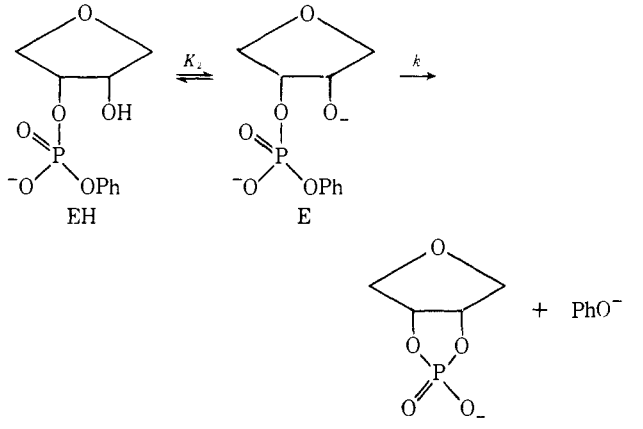
Since (HEH) = $(E_t)(\text{H})/(\text{H} + K_1)$ and (EH) = $(E_t)\cdot K_1/(\text{H} + K_1)$

$$k_{obsd} = \frac{K_1}{(K_1 + \text{H})}(k_1(\text{H})^2/K_1 + k_2(\text{H}) + k_3 + k_4K_w/(\text{H})) \quad (1)$$

where K_1 is the acid dissociation constant of the phosphate group of the ester under the conditions of the reaction (50° and ionic strength 0.1) and K_w has the value $10^{-13.27}$[2] (molarity scale; 50°).[53] Note that k_4 has the dimensions of activity^{-1} sec^{-1} and should be multiplied by the appropriate activity coefficient[54] to calculate the rate at a given concentration of hydroxide ion. The four rate constants and one equilibrium constant of eq 1 were calculated from the experimental data by a nonlinear weighted least squares method[47] as described in the Appendix to this paper. Under conditions such that (H) $\gg K_1$ the first term of the expression for k_{obsd} reduces to k_1(H), and for the reverse inequality, $k_1(\text{H})^2/K_1$. The second-order dependence of k_{obsd} on (H) between pH 2 and 1 thus indicates that pK_1 is significantly less than 1.0, and separate values of k_1 and K_1 should not be well defined by the least-squares analysis. Indeed the standard deviations of both parameters were twice the values of the parameters themselves. In order to estimate k_1 two methods were used: (1) K_1 was removed from the iteration process and was assumed to be equal to the known K_a of the methoxy ester. (2) k_{obsd} was measured in hydrochloric acid solutions (~0.3 *M*) of different ionic strength ($\geq$0.3) but constant pH 0.56; the hypothetical value of the rate constant at this pH, but ionic strength 0.1, was obtained by back extrapolation. At this pH, a sufficiently large fraction of the monoanion has been protonated that the second-order dependence of the rate on (H) no longer holds, the pH–rate profile deviates from a straight line, and the values of K_1 and k_1 are now separable. This procedure appears reasonable since (a) the rate is insensitive to a change in the ionic strength, so that the correction introduced by the extrapolation is small, (b) the uncorrected value of k_{obsd} in 0.3 *M* hydrochloric acid is too large but already shows a negative deviation from the straight line, (c) the value of K_1 obtained in this way (0.27) agrees well with K_a for the methoxy ester (0.20). Values of pH at 50° for solutions of hydrochloric acid of concentration between 0.01 and 0.1 *M* at ionic strength 0.1 were calculated by adding 0.087 to the values of $-\log$(concentration). The correction was calculated from the Debye–Huckel equation[42] using an ion size parameter of 9 Å.

pK_a of the Neighboring Hydroxyl Group. If we assume[23] that the hydroxide ion catalyzed reaction of ester I involves nucleophilic attack on phosphorus by the anion of the neighboring hydroxyl group (see Scheme I) then a determination of the kinetic pK_a of this group may be made by measuring the dependence of k_{obsd} on pH in the region of pH about pK_a.

Scheme I. A Mechanism for the Base-Catalyzed Reaction of I



$$\text{rate} = k(\text{E}) = k(E_t)/(1 + (\text{H})/K_2)$$

where K_2 = (E)(H)/(EH), (H) is the activity of hydrogen ion, and E_t = E + EH (concentrations). Thus, $k_{obsd} = k/(1 + (\text{H})/K_2) = k/(1 + K_wa_{H_2O}/(\gamma_{OH}[\text{OH}]K_2))$ where [OH] is the concentration and γ_{OH} the molar activity coefficient of hydroxide ion and a_{H_2O} is the activity of water. Values of k_{obsd} were measured at 25.3° in five solutions of sodium hydroxide of concentration 0.05–1.0 *M* at constant ionic strength of 1.0 maintained with sodium chloride; under these conditions a_{H_2O} (0.97) and γ_{OH} (taken as 0.67) remain sensibly constant.[55] The standard deviation of k_{obsd} was less than

(53) A. K. Covington, R. A. Robinson, and R. G. Bates, *J. Phys. Chem.*, 70, 3820 (1966).

(54) From the Debye–Huckel equation,[42] using an ion size parameter of 3.5 Å for hydroxide, γ_{OH} = 0.75 at 50° and ionic strength 0.1.

(55) H. S. Harned and M. A. Cook, *J. Amer. Chem. Soc.*, 59, 1890 (1937). Molar activity coefficients were calculated from the molal ones by the equation given by E. J. King ("Acid-Base Equilibria," Macmillan Co., New York, N. Y., 1965, p 17); values of the density of solutions of

5%. The "mixed" constant ${}_2K(1.2 \pm 0.2 \times 10^{-14})$ and k (5.5 ± 0.7 sec^{-1}) were obtained from a nonlinear weighted least squares fit to the above equation.[56] Error limits are standard deviations.

Activation Parameters. Approximate figures for $\Delta H^{\pm}$ and $\Delta S^{\pm}$ for the base- and the acid-catalyzed reactions of I were obtained by repeating a number of the rate measurements at 35°, and 25° (base only). For the acid-catalyzed reaction, no information is available on the constancy or otherwise of the activation parameters with a change in temperature.

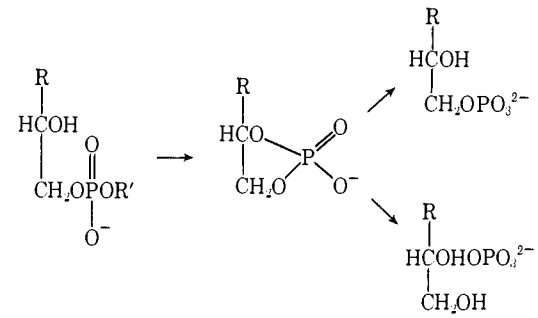
Acid. Values of k_{obsd} in five concentrations of hydrochloric acid between 0.02 and 0.1 M were measured at constant ionic strength at 50 and 35°. The values of k_2 and k_1/K_1 were obtained as above and activation parameters calculated from the transition state theory equation.[57] For k_2, $\Delta H^{\pm}$ is 28 kcal/mol and $\Delta S^{\pm}$ about +10 eu; for k_1/K_1, $\Delta H^{\pm}$ is about 20 kcal/mol and $\Delta S^{\pm}$ about zero. These figures are only approximate; in the weakest acid solution the term in k_2 contributes only 26% of the total rate, and in the strongest acid 6.8%.

Base. Values of k_{obsd} at zero buffer concentration and ionic strength 0.1 were obtained for two buffer ratios of piperidine–piperidinium hydrochloride (five dilutions for each ratio) and k_4 at 35° (3.95 M^{-1} sec^{-1}) was calculated from the expression $k_{obsd} = k_4K_w/(H)$ where K_w[53] is $10^{-13.685}$ and (H) is the hydrogen ion activity. The value of k_4 at 23.3° was obtained from measurements in potassium hydroxide (0.01 M) at an ionic strength of 0.1 maintained with potassium chloride. The concentration rate constant was divided by the appropriate activity coefficient for hydroxide ion (0.76 at 25°) to convert it to the same units as k_4. The average values of $\Delta H^{\pm}$ and $\Delta S^{\pm}$ were about 8 kcal/mol and −30 eu, respectively.

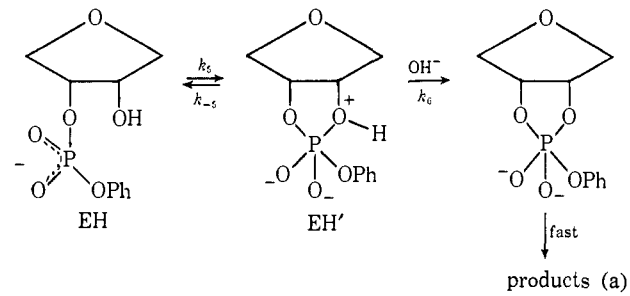
Deuterium Kinetic Isotope Effect. Values of $k_{obsd}{}^D$ at six values of pD (= pH meter reading + 0.275) were obtained by extrapolation to zero buffer concentration of rates measured in morpholine–morpholine–DCl buffers at 50°. Values of pD and $k_{obsd}{}^D$ are given in Table I. $k_{obsd}{}^D = k_4{}^DK_D/a_D$ where K_D is the autoprotolysis constant for D_2O and a_D the activity of deuterium ion, so that a plot of $k_{obsd}{}^D$ *vs.* $1/a_D$ (correlation coefficient 0.999) and knowledge of K_D at 50° ($10^{-14.103}$)[53] allowed calculation of $k_4{}^D$ as 13.6 sec^{-1}. Thus, $k_4{}^H/k_4{}^D = 0.51$. If the mechanism is as shown in Scheme I, $k_{obsd}{}^D = k^DK_2{}^D/a_D$ (below about pD 13) so that $k^D = k_4{}^DK_D/K_2{}^D$. The value of K_2 is not known at 50° in either light or heavy water, we shall therefore assume that the temperature dependence of $K_2{}^D$ is the same as that of K_D and likewise for K_2 and K_w[53] (see below). The equation of Bell[58] gives an estimate of 14.60 for $pK_2{}^D$ at 25°, and subtraction of 0.77 (the change in pK_D with temperature)[53] results in a figure of 13.83 at 50°. Thus, the ratio of rate constants for the first-order ring closure of the alkoxide ion (k^H/k^D) would be $(0.51) \times (10^{-13.272}/10^{-14.103}) \times (10^{-13.83}/10^{-13.2}) \cong 0.8$ which is not unreasonable for this process. Although the above calculation contains many assumptions, a number of the errors (*e.g.*, neglect of the effect of ionic strength on K_2 and $K_2{}^D$) would be expected to cancel partially in the final ratio.

Discussion

Hydroxide Catalysis. It has been assumed for many years that the mechanism of base-catalyzed hydrolysis of a 2-hydroxyalkyl phosphate diester involves a pre-equilibrium formation of the neighboring oxide anion, which then attacks the phosphorus displacing the exocyclic leaving group (Scheme I).[23] The decrease in the second-order rate constant for the hydroxide-catalyzed reaction in strong base is consistent with an increasing equilibrium concentration of the dianion E. In the earliest work it was suggested[59] that a cyclic triester was an intermediate in the reaction, and although this was quickly corrected,[60] the possible existence of a pentaoxyphosphorane intermediate has continued to receive varying support.[61] No evidence either for or against



such an intermediate can be obtained from an examination of the pH–rate profile of Figure 1. A different type



products (a)

of mechanism that avoids the unfavorable electrostatic repulsion of Scheme I, but that nevertheless seems less probable, since it invokes attack of the less nucleophilic hydroxyl group, is shown in (a). The intermediate EH′ is formed in a slow step and can either return to EH or go on to products in a base-catalyzed step. If we assume a steady-state concentration of EH′, $k_{obsd} = k_5k_6(OH)/[k_{-5} + k_6(OH)]$, and comparison with the related expression for the mechanism of Scheme I shows that $k_5 = k$ and $k_{-5}/k_6 = K_w/K_2$. Thus, at 25°, $k_5 = 5.5$ sec^{-1}, and $k_{-5} \approx k_6/1.24$ sec^{-1}. According to this

sodium chloride and hydroxide were taken from the Handbook of Chemistry and Physics, Chemical Rubber Publishing Co., 43 ed, 1961–1962.

(56) The K_a of the unesterified *cis*-glycol would probably be greater than this (in addition to the purely statistical factor of 2).

(57) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," John Wiley & Sons, Inc., New York, N. Y., 1962, p 99.

(58) A. O. McDougall and F. A. Long, *J. Phys. Chem.*, 66, 429 (1962).

(59) A. Fono, *Arkiv Kemi, Min., Geol.*, 24A, 14 (1947); D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 52 (1952).

(60) D. M. Brown and A. R. Todd, *ibid.*, 2040 (1953).

(61) With a poor leaving group the ring opening reaction becomes faster than the ring closure so that little cyclic phosphate may be detected in the hydrolysis products. Proof of the involvement of the *cyclic phosphate* as a major intermediate then relies on the product distribution being in agreement with that found when the cyclic phosphate is hydrolyzed under the same conditions.[62]

(62) See, for example, D. M. Brown, G. E. Hall, and R. Letters, *J. Chem. Soc.*, 3547 (1959).

Table I[a]

Compd	Method[b]	pH	−Log (k_{obsd})	Temp, °C	No. of runs used
I	Piperazine	9.69	2.801	49.85	5
I	Piperazine	9.31	3.185	49.85	5
I	Piperazine	9.09	3.384	49.85	5
I	Piperazine	8.68	3.777	49.85	5
I	Methoxyethylamine	9.13	3.254	49.85	10
I	Methoxyethylamine	9.08	3.303	49.85	10
I	Methoxyethylamine	8.90	3.489	49.85	5
I	Methoxyethylamine	8.78	3.616	49.85	7
I	Methoxyethylamine	8.52	3.947	49.85	7
I	Methoxyethylamine	7.95	4.347	49.85	8
I	Morpholine	8.86	3.567	49.85	5
I	Morpholine	8.84	3.562	49.85	5
I	Morpholine	8.51	3.917	49.85	5
I	Morpholine	8.17	4.289	49.85	10
I	Morpholine	8.15	4.307	49.85	10
I	Morpholine	7.75	4.650	49.85	5
I	Morpholine	7.43	4.932	49.85	5
I	2-Methylimidazole	8.28	4.215	49.85	5
I	2-Methylimidazole	8.19	4.179	49.85	5
I	2-Methylimidazole	7.51	4.860	49.85	5
I	2-Methylimidazole	7.51	4.873	49.85	5
I	2-Methylimidazole	6.63	5.666	49.85	5
I	Imidazole	7.38	4.963	49.85	5
I	Imidazole	7.14	5.268	49.85	5
I	Imidazole	6.67	5.703	49.85	5
I	Imidazole	6.15	5.987	49.85	5
I	Imidazole	5.97	6.155	49.85	5
I	Acetate	5.17	6.307	49.85	10
I	Acetate	4.69	6.302	49.85	10
I	Formate	3.67	6.190	49.85	5
I	Formate	3.67	6.126	49.85	5
I	Formate	3.17	5.933	49.85	10
I	Formate	3.04	5.820	49.85	5
I	Formate	2.98	5.824	49.85	5
I	Formate	2.98	5.790	49.85	5
I	Chloroacetate	2.14	4.992	49.85	5
I	Hydrochloric acid	2.04	4.602	49.85	2
I	Hydrochloric acid	1.79	4.234	49.85	2
I	Hydrochloric acid	1.56	3.914	49.85	2
I	Hydrochloric acid	1.48	3.740	49.85	2
I	Hydrochloric acid	1.31	3.445	49.85	2
I	Hydrochloric acid	1.18	3.234	49.85	2
I	Hydrochloric acid	1.11	3.073	49.85	2
I	Hydrochloric acid	1.08	3.049	49.85	2
I	Hydrochloric acid	0.56	2.256[f]	49.85	6
I	Morpholine-D_2O	8.93[c]	4.042	49.85	5
I	Morpholine-D_2O	8.60[c]	4.419	49.85	5
I	Morpholine-D_2O	8.16[c]	4.893	49.85	5
I	Morpholine-D_2O	7.96[c]	5.097	49.85	5
I	Morpholine-D_2O	7.63[c]	5.415	49.85	5
I	Morpholine-D_2O	7.53[c]	5.527	49.85	5
I	Piperidine	10.82	2.264	34.85	5
I	Piperidine	10.13	2.928	34.85	5
I	Hydrochloric acid	1.78	4.896	34.85	1
I	Hydrochloric acid	1.48	4.377	34.85	1
I	Hydrochloric acid	1.31	4.072	34.85	1
I	Hydrochloric acid	1.18	3.845	34.85	1
I	Hydrochloric acid	1.08	3.654	34.85	2
I	KOH[d]	11.88	1.784	23.3	2
I	KOH[d]	11.88	1.774	23.3	2
I	Morpholine[e]	8.83	3.688	49.85	10
I	Morpholine[e]	8.11	4.424	49.85	10
I	Morpholine[e]	7.36	5.107	49.85	10
II	Morpholine	8.42	5.887	49.85	5
II	Morpholine	8.12	6.153	49.85	5
II	Hydrochloric acid	2.09	4.648	49.85	1
II	Hydrochloric acid	1.79	4.302	49.85	1
II	Hydrochloric acid	1.61	3.932	49.85	1
II	Hydrochloric acid	1.48	3.724	49.85	1
II	Hydrochloric acid	1.39	3.548	49.85	1
II	Hydrochloric acid	1.31	3.413	49.85	1
II	Hydrochloric acid	1.24	3.286	49.85	2
II	Hydrochloric acid	1.18	3.175	49.85	1
II	Hydrochloric acid	1.13	3.093	49.85	1
II	Hydrochloric acid	1.09	3.026	49.85	1

Table I (*Continued*)

Compd	Method[b]	pH	−Log (k_{obsd})	Temp, °C	No. of runs used
II	KOH	11.55	3.346	34.85	2
II	KOH	12.88	2.583	25	1
II	KOH	12.87	2.608	25	2
II	KOH	12.72	2.803	25	2
II	KOH	12.63	2.839	25	1
II	KOH	11.87	3.595	25	2
II	KOH	13.22	2.919	15	2
II	KOH	13.07	3.088	15	2
III	second-order rate constant for base-catalyzed hydrolysis is 6.7×10^{-3} M^{-1} sec^{-1} at 60°[g]				

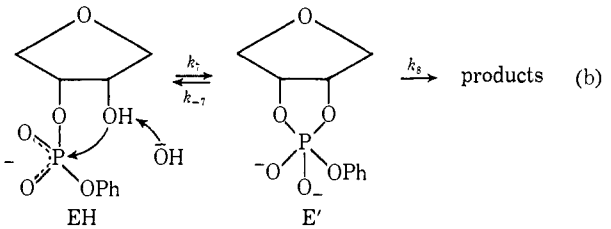
[a] First-order observed rate constants at zero buffer concentration and ionic strength 0.1 maintained with potassium chloride unless otherwise noted. k_{obsd} is in sec^{-1}. [b] Where appropriate the type of buffer used for extrapolation to zero buffer concentration is indicated. Amine buffers were amine-amine hydrochloride; carboxylate buffers were acid-potassium salt. [c] pD values are given (50°). [d] Ionic strength 0.095. [e] Ionic strength maintained at 0.1 by N,N-dimethylmorpholinium chloride. [f] Value extrapolated from data at higher ionic strength (see text). [g] J. R. Cox, Jr., and J. P. Cleveland, Abstracts of the International Symposium on Naturally Occurring Phosphoric Esters, Newcastle, 1967, p 18.

Table II[a] Rate and Equilibrium Constants for I (50°; Ionic Strength 0.1)

Method used	k_1, M^{-1} sec^{-1}	k_2, M^{-1} sec^{-1}	k_3, sec^{-1}	k_4, M^{-1} sec^{-1}	K_1, M	K_2,[b] M
45 points[c]	0.0417 ± 0.0057	$9.00 \times 10^{-4} \pm 0.45 \times 10^{-4}$	$4.53 \times 10^{-7} \pm 0.14 \times 10^{-7}$	6.96 ± 0.08	0.275 ± 0.049	1.24×10^{-14}
44 points[d]	0.0328 ± 0.0009	$8.76 \times 10^{-4} \pm 0.43 \times 10^{-4}$	$4.56 \times 10^{-7} \pm 0.14 \times 10^{-7}$	6.96 ± 0.08	(0.20)	

[a] Error limits are standard deviations. [b] K_a of the neighboring hydroxyl group at 25.3° and ionic strength 1.0. [c] Including the point at pH 0.56 (see text). [d] K_1 was assumed to be 0.20.

simplified form of the mechanism, below pH 14 k_6 would be rate determining, and above this pH, the rate would approach a constant value as k_6 (OH) $\gg k_{-5}$ and k_5 became rate determining. Since the reaction of EH′ with an amine base such as morpholine would be thermodynamically downhill, this alternative route for deprotonation of the intermediate should have a rate constant of about 10^{10} M^{-1} sec^{-1},[63] and observed rates of the order of k_5 should be attainable even at pH 8 say, by the addition of 1.0 M 1:1 amine buffer. This is not found, and argues against this mechanism, at least in this simplified form. A mechanism involving hydroxide as a general base is similar to the mechanism of Scheme I but would require either a concerted reaction, which is probably not consistent with the deuterium solvent kinetic isotope effect (see above), or that the ring closure be more rapid than reprotonation of the alkoxide anion (about 10^{10} sec^{-1}).[63] The latter condition clearly does not hold, as it would imply that observed rates of this same magnitude could be achieved in 1 M



base. If there were an intermediate (E′) it would be expected to partition in favor of product formation ($k_8 > k_{-7}$) so that k_{obsd} should approximate to the value of k_7. Since the experimental rate constant (7.0 M^{-1} sec^{-1}) is much less than that expected for removal of a proton from EH by hydroxide, it follows that, intermediate or not, a preequilibrium ionization must exist, as in Scheme I. At 25° the pseudo-first-order rate constant for deprotonation of EH[63] is likely to exceed the first-order constant for ring closure (k, in Scheme I) down to just below pH 5.

It appears likely then that the major path is as shown in Scheme I and involves an "in-line" SN2 type of displacement of phenoxide ion by the neighboring alkoxide. There is no evidence for a pentacoordinate intermediate in this reaction, the lack of base-catalyzed migration of an alkyl phosphoryl group to a neighboring hydroxyl[64] does not, however, exclude such a species, since the pseudorotation that would be required for such a migration would be expected to be inhibited by the preference of a P–O$^-$ group to maintain a basal position in the trigonal bipyramid.[65-67]

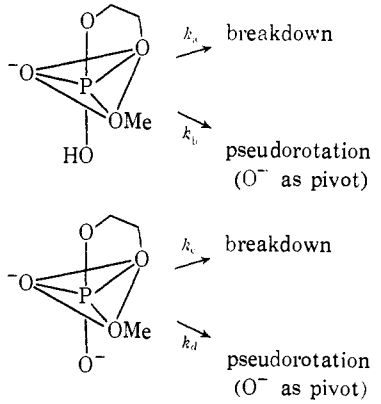
(63) M. Eigen, *Angew. Chem. Intern. Ed. Eng.*, **3**, 1 (1964).

(64) D. M. Brown, D. I. Magrath, A. H. Neilson, and A. R. Todd, *Nature*, **177**, 1124 (1956); D. M. Brown and D. A. Usher, *J. Chem. Soc.*, 6547 (1965).

(65) D. A. Usher, *Proc. Nat. Acad. Sci. U. S.*, **62**, 661 (1969).

(66) R. Kluger, F. Covitz, E. Dennis, L. D. Williams, and F. H. Westheimer, *J. Amer. Chem. Soc.*, **91**, 6066 (1969).

(67) It is possible that the rate of decomposition of a dianionic pentacoordinate intermediate would exceed the rate of its protonation by water. The dependence on pH of the product ratio for hydrolysis of

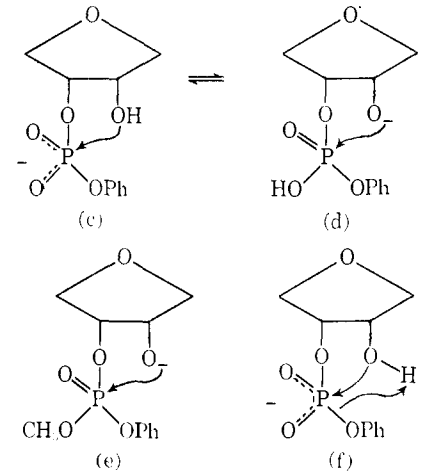


methyl ethylene phosphate[66] is consistent with $k_a > k_b$ for the monoanion and $k_d \geqslant k_c$ for the dianion. In the latter case the pseudorotation is aided by the PO$^-$ group moving from an apical to a basal position. The rate of breakdown of the dianion is likely to exceed that of the

The calculated second-order rate constant for hydroxide ion catalysis (k_4 = 7.0 M^{-1} sec^{-1} at 50°) thus represents a combination of rate and equilibrium constants (kK_2/K_w) and since K_2 is known only at 25.3° in water at an ionic strength of 1, activation parameters are not simply interpretable. However, for the purposes of discussion it will be assumed that the value of K_2 at 50° and ionic strength 0.1 is about $10^{-13.2}$, thus giving it a temperature dependence similar to that of K_w.[53] The value of k at 50° would then be $(K_w k_4/K_2) \cong$ 6 sec^{-1}. The hydroxide ion catalyzed cyclization of a series of esters of propane-1,2-diol monophosphate gave[70] a linear plot of log k_{OH} (80°) *vs.* the pK_a (25°) of the conjugate acid of the leaving group that had a gradient of 0.56; combination of the present result with that of Cox and Cleveland[71] for the methyl ester of tetrahydrofurandiol phosphate defines a line with a gradient of 0.59 (at 50°), though the second-order rate constants are about 1000 times faster than for the propanediol phosphate esters at the same temperature. The rate of hydrolysis of uridylyl(3′-5′)adenosine[24] in base is within a factor of 2 of the rate of the methyl ester of tetrahydrofuran diol phosphate.[71] In both reactions the leaving group is a primary alkoxide anion, thus the more complicated structure of the dinucleoside phosphate has little effect on the rate of reaction in base. However, the ring oxygen has a considerable effect on the rate; *cis*-cyclopentan-2-ol 1-phenyl phosphate (II) reacts in base 70 times slower than does tetrahydrofuran-4-ol 3-phenyl phosphate (at both 25° and 50°). This substitution of CH_2 for O would be expected to decrease K_2 but to increase k. The net effect on the observed rate (which depends on the product kK_2) is a decrease, so that according to this simple analysis the constant, analogous to a Brønsted coefficient for this reaction (intramolecular nucleophilic attack on phosphorus by the alkoxide anion) would be less than one. A similar explanation can be given for the slower rate of base-catalyzed ring closure of some esters of 3-hydroxypropylphosphonic acid compared with the related 2-hydroxyethyl phosphates,[72] as in an intermolecular reaction the phosphonate may be expected to react faster.[73] Cyclopentanediol thus represents a relatively poor model for ribose, as found previously by Zachau and coworkers in the case of models of aminoacyl-*t*-RNA.[74]

In base, as under all other conditions used in this investigation, the *cis*-methoxy phenyl ester (V) was stable, a type of observation that was first made many years ago.[59,75] The rate of hydrolysis of the cyclic phosphate (III) in base is about 1000 times slower than the ring closure reaction of I.[71]

The Neutral Pathway (pH 4–6). In contrast to the base- and the acid-catalyzed reactions of I, there has been no previous clear demonstration of a neutral rate for any 2-hydroxy phosphate diester. Similar restraints on the mechanism operate here as with the base-catalyzed reaction, since the cyclic phosphate and phenol are the sole initial products. A kinetic equivalent to the reaction (EH) (c) is the process d; either of these two mechanisms (and general base or general acid variants of them)[27] could be shown as including a pentacoordinate intermediate. Reaction *via* (c) with a specific rate of 4.53 × 10^{-7} sec^{-1} at 50° would imply that deprotonation of the neighboring hydroxyl group increases the rate of nucleophilic attack on phosphorus by a factor of about 10^7. If we assume $K_2 = 10^{-13.2}$



and that the dissociation constants of the phosphate and hydroxyl groups are independent,[76] the fraction of EH that is present as HE (d) will be 2.3 × 10^{-13}. Reaction by way of the kinetic equivalent (d) would then require that this species decompose with a specific rate of 2 × 10^6 sec^{-1}, so that protonation of the phosphate anion could speed up attack by the neighboring alkoxide by a factor of as much as 10^5. This seems reasonable in view of the reduction in electrostatic repulsion, and it is thus questionable whether the binding of the phosphate group of a substrate to a protonated imidazole of RNase should be described[77] as playing no catalytic role in the enzymic reaction; presumably a part of this possible rate increase could be realized by a favorably located general acid catalyst.

The base-catalyzed decomposition of the triester (e) would constitute a model for the neutral reaction (d). This compound is not yet available, but a substitute triester, uridine 3′-dimethyl phosphate, undergoes what is apparently base-catalyzed hydrolysis[78] with a rate constant of possibly 80 M^{-1} sec^{-1} at 37°. If the sensitivity of this reaction to the pK_a of the leaving group is

monoanion, thus $k_d \geqslant k_c > k_a > k_b$. If k_b were as high as 10^8 sec^{-1}, which is not impossible,[65] then k_c would lie in the region of the diffusion controlled limit,[63] 10^{10} to 10^{11} sec^{-1}, which is faster than the probable rate of protonation of the dianion by water (about 10^9 sec^{-1} assuming a pK_a of 13 for the monoanion[66]). From the above inequality, $k_c > k_b$ and since k_b would be expected to exceed the rate of pseudorotation (k_e) of a dianionic intermediate that had both PO$^-$ groups in basal positions,[65,68] it follows that $k_c > k_e$ as assumed above. An alternative to the inequality $k_a > k_b$ is that the decomposition of the monoanion is highly selective and expels the better leaving group more than 100 times faster than methoxyl. This explanation would be more consistent with the high percentage of base-catalyzed exocyclic cleavage noted[69] with the analogous phenyl ester.

(68) E. L. Muetterties and R. A. Schunn, *Quart. Rev.* (London), 20, 245 (1966), and references therein.

(69) E. A. Dennis and F. H. Westheimer, *J. Amer. Chem. Soc.*, **88**, 3431 (1966).

(70) D. M. Brown and D. A. Usher, *J. Chem. Soc.*, 6558 (1965).

(71) See Table I, footnote *g*.

(72) F. W. Westheimer, personal communication.

(73) J. R. Cox, Jr., and O. B. Ramsay, *Chem. Rev.*, **64**, 317 (1964).

(74) H. G. Zachau and W. Karau, *Ber.*, **93**, 1830 (1960).

(75) O. Bailly and J. Gaumé, *Bull. Soc. Chim. Fr.*, [5] **3**, 1396 (1936).

(76) Calculations of this sort that wrongly employ macroscopic in place of microscopic dissociation constants can obviously give misleading results. In the present case, the true concentration of HE will be larger than the value calculated by using the macroscopic constants (K_1 and K_2) and the true rate constant therefore smaller.

(77) G. C. K. Roberts, E. A. Dennis, D. H. Meadows, J. S. Cohen, and O. Jardetzky, *Proc. Nat. Acad. Sci. U. S.*, **62**, 1151 (1969).

(78) D. M. Brown, D. I. Magrath, and Sir. A. R. Todd, *J. Chem. Soc.*, 3496 (1955).

similar to that of the diester reaction, the rate constant for (e) would be about 10^5–10^6 M^{-1} sec^{-1}, and with the pK_a of the neighboring hydroxyl probably also about 13–14, this is of the right order for the process d. If the reaction were as shown in (d) and went *via* a pentacoordinate intermediate, the rate of protonation at 25° of this initially monoanionic intermediate would probably be about 10^6 sec^{-1} by solvated hydrogen ion at pH 4, and 10^5 sec^{-1} by water[63] (assuming a pK_a of 9 for the fully protonated pentaoxyphosphorane).[66,79] These rates could then be less than the rate of decomposition of the monoanionic intermediate.[67]

Concerted reactions such as (f) can be written for the neutral or acid-catalyzed processes, but since these would involve an "adjacent" type of displacement,[65] the preference rules[80] would require pseudorotation of a pentacoordinate intermediate and it is therefore doubtful whether these could be considered "concerted" in the sense of a four center reaction. The maximum contribution from the neutral pathway to the total rate occurs at pH 4.7 (92.5%); at this pH the terms in H^2, H, and OH account for 0.012, 3.7, and 3.8%, respectively (see Figure 2).

The Reaction Dependent on (H). The contribution of the term in (H) is a maximum at pH 2.8 (63%). At this pH the other terms account for $(H)^2$, 17%; neutral, 20%; base, 0.01%, and a bad fit to the experimental points would result if this term in (H) were omitted, as suggested by the reasonably small standard deviation of the rate constant k_2 (±5%).

By contrast, the acid-catalyzed hydrolysis of nucleoside cyclic phosphates[25] and dinucleoside phosphates[7] apparently shows no evidence for a term in the free acid. It is possible that such a term exists, as found here and for dimethyl[81] and dibenzyl phosphate,[82] but in view of the slowness of the reaction of most of these compounds in the region of pH–2–4, where such a term would be most easily seen (being masked by the $(H)^2$ term at lower pH) its detection would require careful work at high temperatures.

Unlike the neutral and the base terms, the term in (EH)(H) does not necessarily involve reaction *via* the cyclic phosphate, as none was detected among the hydrolysis products of I in 0.1 *M* hydrochloric acid (though this term accounts for only about 7% of the reaction under these conditions). However, in view of the stability of the methylated ester V, and the rather small acceleration that would be expected from the hydroxyl group of I if it participated merely as an intramolecular general acid, we consider the existence of such an intermediate probable. The known acid-catalyzed migration of an alkyl phosphoryl group[64] or of the phosphate group itself[83] to an adjacent hydroxyl clearly involves a cyclic structure, whether the migration goes by way of a cyclic triester, a pentaoxyphosphorane, or a mixture of both as demonstrated recently for glycerophosphate.[83] Our inability to detect the cyclic diester among the products of acid hydrolysis is the expected result of the ring closure now being slower than the ring opening.[50] The value of k_2 (9.00×10^{-4} M^{-1} sec^{-1}) refers to the bimolecular reaction of the anion (EH) with hydrogen ion. This corresponds to a first-order rate constant of 2.5×10^{-4} sec^{-1} for the kinetic equivalent (g), which is not very different from the uncatalyzed rate of hydrolysis of uridine 3′-dimethyl phosphate,[78] allowing for the difference in the leaving group.[84] This process could be drawn as being aided

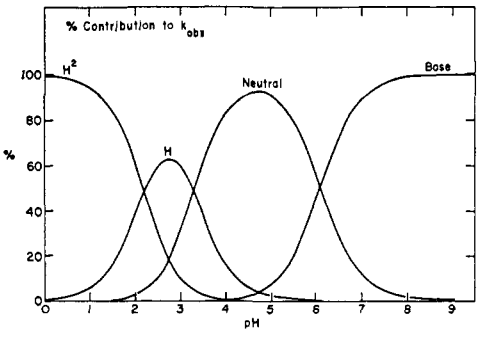
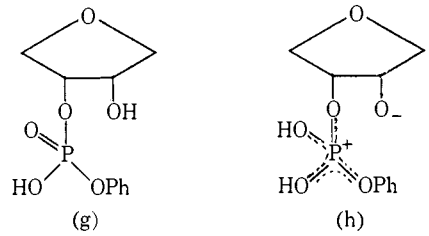


Figure 2. Per cent contributions to k_{obsd} from the terms in k_1, k_2, k_3, and k_4.



by a molecule of water, acting as a general base.[27] Calculation of the specific rate for the alternative process (h) gives a value of about 4×10^{12} sec^{-1}, using an estimate of 10^3 for the acid dissociation constant of the conjugate acid of HEH.[85,86] However, in view of the uncertainties in assigning pK_a values to the phosphate and hydroxyl groups,[76] we may not at present exclude (h) on the grounds that it would need to exceed the accepted maximum rate for the separation of products.

The Reaction Dependent on $(H)^2$. About 93% of the observed rate of hydrolysis of I in 0.1 *M* hydrochloric acid is due to the reaction of the conjugate acid of HEH, or its kinetic equivalents. The products of the reaction were phenol and the acyclic monoester, and again the evidence for the existence of the cyclic phosphate as an intermediate is indirect: the stability of the *cis*-methoxy ester, and the known acid-catalyzed migration of a phosphate group to a neighboring hydroxyl. The migration experiments of Fordham and Wang[83] also suggest that a pentacoordinate intermediate may occur in related reactions in strong acid. The value of k_1 (0.042 M^{-1} sec^{-1}) represents the reaction of the free acid plus a proton (HEH)(H); this would require the kinetic equivalent (i) to decompose with a rate constant of 42 sec^{-1}, assuming a value of -3 for pK_3 as before.

The need to use acid stronger than 0.1 *M* in order to see the dependence of the rate on hydrogen ion start to change from second to first order has been noted pre-

(79) As has been pointed out,[66] the pK_a's of the basal and apical OH groups would be expected to differ, and also therefore their rates of protonation by water (but not by the solvated proton).

(80) F. H. Westheimer, *Accounts Chem. Res.*, **1**, 70 (1968).

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

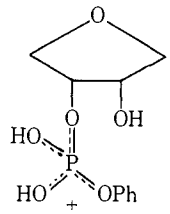
(82) J. Kumamoto and F. H. Westheimer, *J. Amer. Chem. Soc.*, 77, 2515 (1955).

(83) W. D. Fordham and J. H. Wang, *ibid.*, **89**, 4197 (1967).

(84) It is of course possible that the neutral hydrolysis of uridine dimethyl phosphate involves reaction by the equivalent of process h.

(85) P. Haake and G. Hurst, *J. Amer. Chem. Soc.*, **88**, 2544 (1966).

(86) C. A. Vemon, Special Publication No. 8, The Chemical Society, London, 1957, pp 29–30.



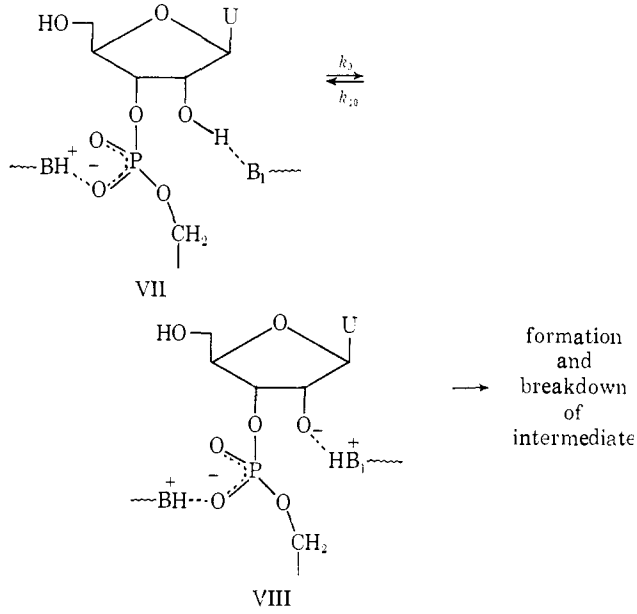
(i)

viously with phosphate esters;[25] apparently the inductive effect of the ribose or tetrahydrofuran ring is sufficient to lower the pK_a of the phosphate group well below 1.[87] In the case of ethylene phosphate, measurements in perchloric acid solutions up to 0.15 *M* were sufficient to define the pK_a as 1.0 (30°; ionic strength 0.2),[73,88] whereas for nucleoside cyclic phosphates Abrash, *et al.*,[25] were unable to detect any departure from the second-order dependence on hydrogen ion up to 0.1 *M* acid. Interestingly, ethylene cyclic phosphate resembled the nucleoside cyclic phosphates in showing no evidence for a term in (H) at lower acidities,[88] and a similar observation has been made for glycerol cyclic phosphate.[89]

Values of k_1/K_1 and k_2 have also been determined for the cyclopentane phenyl ester II at 50°. The observed rate in 0.1 *M* acid depends almost entirely on the value of k_1/K_1, and at this acidity the observed rates are almost identical; K_1 for II is probably less than for I and thus may compensate for a somewhat smaller k_1. This behavior is in contrast to the large reactivity difference between the two esters in base.

Elementary Steps of Ribonuclease Action. An attempt has recently been made to estimate the rate constants for some of the proton transfer reactions in one possible mechanism for ribonuclease action (see Scheme II).[19,90]

Scheme II. A Hypothetical Stepwise Proton Transfer Mechanism for Ribonuclease Action[19,90]



If the ratio of the acid dissociation constant of the 2′-OH to that of the enzyme base B_1 were 10^{-7} then the rate of formation of the active species VIII from VII (k_9) would be about 10^6 sec^{-1}, and the fraction of the enzyme substrate complex present as VIII would be 10^{-7}. Since the overall reaction for a good substrate (*e.g.* CpA) is observed[7] to proceed at a rate of 10^3 to 10^4 sec^{-1}, this would require that the formation and break down of any pentacoordinate intermediate occur with rate constants of the order of 10^{10} to 10^{11} sec^{-1}.[19] This figure is probably rather high since the difference in the dissociation constant of the 2′-OH and of B_1 will be reduced on formation of the enzyme substrate complex.[12] If the effect of a diester substrate on the pK_a of B_1 is similar to that of a monoester inhibitor,[91] and if the pK_a of the 2′-OH is somewhat reduced by the proximity of the positively charged active site, it is possible that the required rate of reaction of VIII may be lowered to about 10^8 to 10^9 sec^{-1}. Some idea of whether such a large rate would normally be possible for VIII can be gained from the neutral reaction of the phenyl ester I. It has been shown above that reaction *via* the kinetic equivalent (d) would require a decomposition rate of about 10^6 sec^{-1} at 50°, *and irrespective of the actual mechanism of the neutral reaction*, the rate of (d) therefore cannot be greater than this value. If the reaction of (d) went by way of an intermediate, present in a low steady state concentration, then since the partitioning of this intermediate would be almost entirely in favor of product formation (phenoxy being a better leaving group than alkoxy) the maximum rate calculated above would refer to the rate of formation of this intermediate. Apart from the different inductive effect of the phenyl group in the model compound, this figure could also represent the upper limit to be expected for the rate of the related reaction of VIII (in which the phosphate group is not "fully" protonated) and according to this analysis would be insufficient to explain the rate of the enzymic reaction.[92] One could conclude that *two* general acids (presumably one histidine and lysine-41)[93] may be required to bind the phosphate group of a diester substrate (the upper limit could now be taken as the rate of the process (h), or that the enzyme binds the dinucleoside phosphate in such a way that the conformation of the groups about phosphorus resembles that in a cyclic phosphate. The molecular orbital calculations of Collin[94] indicate considerable sensitivity of the charge on phosphorus to the orientation of the ester groups, and he has suggested that this factor may account for a good deal of the lability of five-membered cyclic phosphates toward acid or base. It is most interesting, therefore, that the torsion angle around the 3′-OP bond

(87) Sugar phosphates have been known to be stronger acids than monoalkyl phosphates for many years: W. D. Kumler and J. J. Eiler, *J. Amer. Chem. Soc.*, 65, 2355 (1943).

(88) J. R. Cox, Jr., Ph.D. Thesis, Harvard University, 1959; F. H. Westheimer, Special Publication No. 8, The Chemical Society, London, 1957, pp 1–15.

(89) L. Kugel and M. Halmann, *J. Amer. Chem. Soc.*, 89, 4125 (1967).

(90) A similar treatment has been given by J. H. Wang (*Science*, **161**, 328 (1968)).

(91) D. H. Meadows and O. Jardetzky, *Proc. Nat. Acad. Sci. U. S.*, **61**, 406 (1968).

(92) Another kinetic equivalent of the neutral reaction involves protonation of the phenolic oxygen and attack by the alkoxide ion. This process has a maximum specific rate much higher than that of (d), and an analog of this alternative process would simply require the enzyme general acid BH^+ (or B_1H^+) to be in close proximity to the 5′-oxygen. The rate of the base-catalyzed reaction of I is a function of the pK_a of the leaving group and presumably this would also be true of the neutral reaction so that partial protonation of the 5′-oxygen in VIII by BH^+ or B_1H^+ could have a large effect on the real rate expected for this reaction.[19,90]

(93) The relationship of the cross-linking experiment of Marfey and coworkers (*J. Biol. Chem.*, 240, 3270 (1965)) to the other lysine modification studies has not yet been satisfactorily resolved.

(94) R. L. Collin, *J. Amer. Chem. Soc.*, **88**, 3281 (1966).

corresponds to a maximum in reactivity for an "in-line" mechanism, and near the minimum for either of the two "adjacent" mechanisms.[65] The considerable specificity of ν_{max} with respect to the 5′-linked nucleoside of a dinucleoside phosphate substrate[7] could be consistent with this variation of the "strain" theory of enzymic catalysis.[95]

However, there is no requirement for this process to occur by way of individual steps as shown in Scheme II, and reaction *via* a "concerted" general base–general acid type of mechanism bypassing VIII could allow formation of a pentacoordinate intermediate or of products at a faster rate than would obtain if reaction had to proceed *via* a vanishingly small concentration of a species with high but finite reactivity.

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Appendix

Least Squares Fitting of pH–Rate Profiles. Methods used to fit an assumed theoretical equation to an experimental pH–rate profile vary widely among different investigators; in many cases no explicit mention is made of the actual curve-fitting process, and no estimate is given of the confidence to be placed in the parameters. This situation is in contrast to other fields in which relatively sophisticated techniques are regularly employed. It is therefore hoped that the following details of the curve fitting, when read in conjunction with the paper of Wentworth,[47] will prove helpful.

The equation for k_{obsd} (eq 1) is rearranged to give

$$F = \frac{K_1}{(K_1 + H)}(k_1H^2/K_1 + k_2H + k_3 + k_4K_w/H) - k_{obsd}$$

For obvious reasons the fit is made with H and k_{obsd} (rather than with pH and log k_{obsd}). If we define $\alpha = (k_1H^2/K_1 + k_2H + k_3 + k_4K_w/H)$ and $\beta = K_1/(K_1 + H)$, then the partial derivatives of F with respect to H, k_{obsd}, k_1, k_2, k_3, k_4, and K_1 are

$$\frac{\partial F}{\partial H} = \beta(2k_1H/K_1 + k_2 - K_wk_4/H^2) - \alpha[\beta/(K_1 + H)]$$

$$\frac{\partial F}{\partial k_{obsd}} = -1$$

$$\frac{\partial F}{\partial k_1} = \beta(H^2/K_1)$$

$$\frac{\partial F}{\partial k_2} = \beta H$$

$$\frac{\partial F}{\partial k_3} = \beta$$

$$\frac{\partial F}{\partial k_4} = \beta(K_w/H)$$

$$\frac{\partial F}{\partial K_1} = \alpha[H/(K_1 + H)^2] - \beta k_1(H^2/K_1^2)$$

(95) The analogy of the ring strain of a cyclic phosphate with the strain possibly introduced in a substrate by an enzyme has been mentioned by several workers (see some of the reviews in ref 7 above). For a recent discussion of this point see W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill Book Co., Inc., New York, N. Y., 1969.

The partial derivatives are calculated for all N experimental points, of which the Jth is $(k_{obsd}, H)_J$.

We estimate the standard deviation of our pH measurements to be a constant 0.01 unit, thus since d(log H) = d(ln H)/2.303 = d(H)/2.303H, the variance of $H = \sigma_H^2 = 5.3 \times 10^{-4}(H)^2$. Similarly, the standard deviation of k_{obsd} is about 5% throughout the pH range, thus the variance of $k_{obsd} = \sigma_{k_{obsd}}^2 = 2.5 \times 10^{-3}(k_{obsd})^2$. The variance of unit weight was arbitrarily chosen as 10^{-13}, so that

$$L = H^2\left[5.3 \times 10^9\left(\frac{\partial F}{\partial H}\right)^2\right] + k_{obsd}^2\left[2.5 \times 10^{10}\left(\frac{\partial F}{\partial k_{obsd}}\right)^2\right]$$

The inclusion of weighting is essential, in a typical pH–rate profile the value of H alone may vary by a factor of more than 10^{10}, and neglect of the weighting factor will not only invalidate the calculated standard deviations of the parameters, but can result in a curve that even to the eye is a poor fit.

If we let the partial derivatives of F with respect to k_1, k_2, k_3, k_4, and K_1, evaluated at the Jth point, be $F(1, J)$, $F(2, J)$, $F(3, J)$, $F(4, J)$, and $F(5, J)$ respectively, then the 5 × 5 matrix (B) of the coefficients of the normal equations for N points can be simply generated by the following Fortran statements (without even taking advantage of the symmetry of (*B*))

```
DO 1 K = 1,5
DO 1 L = 1,5
B(K,L) = 0.
DO 1 J = 1, N
B(K,L) = B(K,L) + F(K,J)*F(L,J)/F(6,J)
1 CONTINUE
```

where $F(6, J)$ is the value of L, calculated at the Jth point. The column matrix (*C*) of the constants from the right-hand side of the normal equations is then calculated

```
DO 2 K = 1,5
C(K) = 0.
DO 2 J = 1, N
C(K) = C(K) + F(K,J)*FI(J)/F(6,J)
2 CONTINUE
```

where *FI*(*J*) is the value of the original function, calculated at the Jth point.

The (*B*) matrix is then inverted and the correction terms and variances and covariances calculated as in Wentworth's article. The corrected values of the parameters are then used in a new round of calculations and the whole process repeated until convergence is obtained. The variance ratio (*F*) test can then be applied, if desired, to test the overall goodness of fit.

In the present work, the use of pH–rate data down to pH 1.1 gave a very poor definition to the separate values of k_1 (0.21 with a standard deviation of 0.42) and K_1 (1.6 with a standard deviation of 3.3). However, for the reasons outlined in the Results, the ratio k_1/K_1 is well defined. This is then an example[47] where the variance of a quantity derived from the least squares parameters is reduced greatly by the inclusion of the covariance term. Let $g = k_1/K_1$, then

$$\sigma_g^2 = \left(\frac{\partial g}{\partial k_1}\right)^2 \sigma_{k_1}^2 + \left(\frac{\partial g}{\partial K_1}\right)^2 \sigma_{K_1}^2 + 2\left(\frac{\partial g}{\partial k_1}\right)\left(\frac{\partial g}{\partial K_1}\right)\sigma_{k_1K_1}$$

where $\sigma_{k_1K_1}$ is the covariance of k_1 and K_1. The three terms of this equation were numerically +0.06491, +0.06992, and −0.13472, so that the value of k_1/K_1 (0.131) has a standard deviation of only 8%.

We feel that the use of a general procedure of this sort is much to be preferred over methods that rely on special characteristics of a particular problem or which obtain a fit "by eye."[96] The present example is instructive in that the investigator would have been warned by the large standard deviations of k_1 and K_1 that the data did not adequately define these two constants even if the reason for this had not been recognized. This warning would not necessarily have been apparent with other more intuitive fitting methods.

(96) This type of rough fit (*e.g.*, with a CRT on-line to a digital computer) is a convenient way of obtaining the initial trial parameters for the least squares program.

Stereochemistry of a Substrate for Pancreatic Ribonuclease. Crystal and Molecular Structure of the Triethylammonium Salt of Uridine 2′,3′-O,O-Cyclophosphorothioate[1]

W. Saenger and F. Eckstein

Contribution from the Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, 34 Göttingen, Germany. *Received December 31, 1970*

Abstract: Uridine 2′,3′-O,O-cyclophosphorothioate is a substrate for pancreatic ribonuclease. Knowledge of the absolute configuration of this substrate and of the reaction product obtained by enzymatic methanolysis is of interest from a mechanistic point of view. The title compound crystallizes in space group $P2_12_12_1$. The structure was solved from three-dimensional X-ray data and refined to an R value of 9.6%. The bicyclic system is "pseudo-mirror" symmetrical with respect to the O–P–S plane. The ribose exhibits the unusual O(1′) *exo* conformation, the bonds C(1′)–C(2′), C(3′)–C(4′) and C(2′)–O(2′), C(3′)–O(3′) being pairwise coplanar. The heterocycle is in *anti* position with respect to the ribose and almost coplanar with the C(1′)–O(1′) bond. The conformation about the C(4′)–C(5′) bond, defined as φ_{oo} and φ_{oc}, is *trans,gauche*. The P–S bond seems to have double bond character, the negative charge being located at the free oxygen atom of the phosphorothioate group. The triethylammonium cation is disordered, its symmetry nearly C_{3v}.

Recently the uridine 2′,3′-O,O-cyclophosphorothioate anion was synthesized[2] and one of the diastereomers isolated by fractional crystallization of the triethylammonium salt (Figure 1). This isomer is a substrate for pancreatic ribonuclease with the same K_m value as uridine 2′,3′-O,O-cyclophosphate.[3] In the presence of methanol it is converted by the enzyme to uridine 3′-O-(O-methyl)phosphorothioate, which we were able to crystallize as well. Knowledge of the absolute configuration of substrate and reaction product would clarify the stereochemistry of the enzymatic methanolysis and would thus yield valuable information as to the stereochemistry of the transesterification and hydrolysis reaction of ribonuclease.[4] In this report the X-ray structural analysis of the triethylammonium salt of the crystalline isomer of uridine 2′,3′-O,O-cyclophosphorothioate will be presented, which is interesting not only from a biochemical but also from a structural point of view since nucleoside 2′,3′-O,O-cyclophosphates have not yet been investigated.

(1) Short communication published in *Angew. Chem. Int. Ed. Engl.*, **8**, 595 (1969). Differences in some angles and bond distances in this and the present publication are due to the disorder of the triethylammonium cation which had not been accounted for at that time.

(2) F. Eckstein and H. Gindl, *Chem. Ber.*, **101**, 1670 (1968).

(3) F. Eckstein, *FEBS (Fed. Eur. Biochem. Soc.) Lett.*, **2**, 85 (1968); δ values for uridine 2′,3′-O,O-cyclophosphorothioate in this publication should be corrected to δ = −68.5 and −69.5 ppm, respectively.

(4) M. R. Harris, D. A. Usher, H. P. Albrecht, G. H. Jones, and J. G. Moffatt, *Proc. Nat. Acad. Sci.*, **63**, 246 (1969).

Experimental Section

When an ethanolic solution of the triethylammonium salt of uridine 2′,3′-O,O-cyclophosphorothioate was allowed to evaporate slowly at 4° stout prismatic crystals formed (mp 204–205°). The orthorhombic space group $P2_12_12_1$ of these crystals was indicated by the mirror symmetries of the X-ray photographs and the systematic absence of reflections $h00$, $0k0$, $00l$ when h,k,l, respectively, were odd. The unit cell dimensions were $a = 12.495 \pm 0.005$ Å, $b = 7.079 \pm 0.003$ Å, $c = 22.574 \pm 0.008$ Å. The observed density of 1.391 ± 0.005 g/cm³ was in good agreement with the calculated value if four formula weights within the unit cell were assumed. We collected 1108 data on a four-circle automatic diffractometer using Zr-filtered Mo radiation and 2θ scan mode. In view of the small crystal dimensions of 0.3 × 0.1 × 0.1 mm and the linear absorption coefficient of 2.83 cm⁻¹ the data were corrected for geometrical factors, not for absorption.

The positions of the two heavy atoms, P and S, could be determined unambiguously from a sharpened Patterson map. Two successive Fourier syntheses, phased first with these two atoms and then with all but three atoms of the structure, revealed the geometry of the molecule. After two cycles of isotropic and four cycles of anisotropic full-matrix least-squares refinement using the 986 observed data and applying Hughes' weighting scheme[5] (Fomin = 4.2), the reliability index, $R = \Sigma||F_o| - |F_c||/\Sigma|F_o|$, dropped to 9.4%. A difference Fourier synthesis computed at this stage revealed a statistical disorder of the three α-carbon atoms of the triethylammonium ion. In a further cycle of refinement the occupation and thermal parameters of the six "ordered" and "disordered" α-carbon atoms of the ethyl groups were set at 0.5 and isotropic, respectively, and varied. Since the occupation parameters fluctuated around 0.5 but did not shift in a definite manner they were reset to 0.5, the isotropic thermal parameters converted

(5) E. W. Hughes, *J. Amer. Chem. Soc.*, **63**, 1737 (1941).