Stereochemistry of Nucleophilic Displacement on Two Phosphoric Monoesters and a Phosphoguanidine: The Role of Metaphosphate

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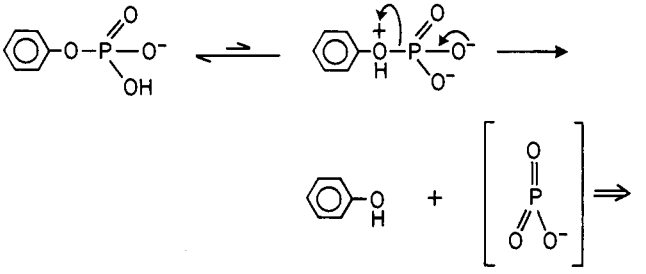
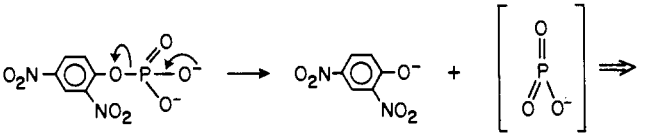
Abstract: For the role of monomeric metaphosphate and the nature of the transition states in the alcoholysis of phosphoric monoesters to be examined, phenyl [(*R*)-^{16}O,^{17}O,^{18}O]phosphate and 2,4-dinitrophenyl [(*R*)-^{16}O,^{17}O,^{18}O]phosphate have been synthesized and the stereochemical course of the methanolysis of phenyl phosphate monoanion and of dinitrophenyl phosphate dianion has been evaluated. [(*R*)-^{16}O,^{17}O,^{18}O]Phosphocreatine has also been synthesized and the stereochemical course of the methanolysis of this molecule determined. In each case, complete *inversion* of configuration at phosphorus is observed. It is clear that metaphosphate, if it exists as a true intermediate in these reactions in protic solvent, does not leave the solvent cage in which it is generated. Indeed, product formation occurs more rapidly than rotation of the putative metaphosphate intermediate. These displacements must therefore occur by *preassociative* mechanisms in which there may be some assistance from the incoming nucleophile. The present results do not allow a distinction to be made between a "preassociative concerted" path (that is, an S_N2-like displacement via a very loose transition state) and a "preassociative stepwise" path via a metaphosphate intermediate of very short lifetime.

Independent reports from the laboratories of Westheimer[1] and of Bunton[2] in 1955 showed that the rate of hydrolysis of phosphoric monoesters is maximal near pH 4, where the monoanionic form of the ester is the predominant species. This observation led both groups to propose the mechanistic pathway shown in Scheme I as the favored route for the hydrolysis of phosphoric monoesters. Since that time, much evidence has accumulated in support of this path and of the intermediacy of the monomeric metaphosphate ion, PO_3^-.[3] These data can be summarized as follows. (1) For monoesters of phenols that have a pK_a higher than 5.5 the monoanion is the most reactive species, while if the phenol has a pK_a lower than 5.5 the ester is more reactive in its dianionic form.[4] As Kirby and Varvoglis remark, "It is difficult to see how the removal of a proton from the monoanion" (e.g., of 2,4-dinitrophenyl phosphate, the second pK_a of which is about 4.6) "could make the phosphorus center in the dianion *more* electrophilic."[4] Moreover, the reactivity of phosphoric monoanions is often greater than that of the corresponding neutral species,[1] which argues against a rate-limiting attack of the nucleophile at phosphorus. It therefore seems likely that for esters of phenols that have $pK_a > 5.5$, pre-equilibrium protonation allows heterolysis to generate metaphosphate and the neutral phenol (as in Scheme I), whereas if the leaving group is good (as for esters of phenols having $pK_a < 5.5$) the heterolytic cleavage occurs most readily from the dianion directly (see Scheme II). (2) Monoester hydrolyses have values of $\Delta S^{\ddagger}$ close to zero eu,[4,5] which is typical of dissociative processes. Associative reactions commonly have $\Delta S^{\ddagger}$ values around −20 eu. While deductions based upon the size of activation parameters for reactions in structured solvents are questionable, this result is certainly consistent with a dissociative pathway. (3) When solvolyses of monoesters are performed in aqueous alcoholic media, there is usually a reasonable correlation between the mole fraction of alcohol in the solvent and the mole fraction of alkyl phosphate in the product.[6,7] Although these correlations are rarely perfect, and it was early recognized[6] that metaphosphate monoanion could not be a *free* intermediate in these reactions, the data suggest the intermediacy of a highly reactive phosphorylating agent. (4) The Brønsted coefficient for the leaving group, β_{lg}, for substituted phenolic phosphoric monoesters is −1.2 for dianions and −0.27 for monoanions,[4] consistent with a high degree of bond cleavage at the transition state and departure of phenolate from dianions and of the neutral phenol from monoanions (see Schemes I and II). (5) In contrast, the β_{nuc} for attacking nucleophiles in the aminolysis of 4-nitrophenyl phosphate is small, at 0.13.[8] (6) The solvent

Scheme I. Pathway for the Solvolysis of the Phosphoric Monoester Monoanion of a Phenol of $pK_a > 5.5$



Scheme II. Pathway for the Solvolysis of the Phosphoric Monoester Dianion of a Phenol of $pK_a < 5.5$



isotope effects, k_{H_2O}/K_{D_2O}, for monoester hydrolyses are small, being 1.4 for 2,4-dinitrophenyl phosphate monoanion and negligible for the dianion.[4] This suggests that the rate-limiting transition state does not involve a proton in flight. (7) The kinetic isotope effect, k_{16}/k_{18}, in the hydrolysis of 2,4-dinitrophenyl phosphate containing a bridging ^{18}O is 1.020 ± 0.004, which indicates substantial P–^{18}O bond cleavage at the transition state.[9] All the above data relate to experiments in aqueous or mixed aqueous media, but most discussions of the metaphosphate question include the following evidence that derives from studies in aprotic solvents such as dioxane, acetonitrile, or chloroform. (8) It has proved possible to demonstrate the transfer of a phospho group between polymer beads in a three-phase reaction where associative

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(1) Butcher, W. W.; Westheimer, F. H. *J. Am. Chem. Soc.* **1955**, *77*, 2420–2424.
(2) Barnard, P. W. C.; Bunton, C. A.; Llewellyn, D. R.; Oldham, K. G.; Silver, B. L.; Vernon, C. A. *Chem. Ind. (London)* **1955**, 760–763.
(3) Benkovic, S. J.; Schray, K. F. In "Enzymes"; 3rd ed.; Boyer, P. D., Ed., Academic Press: New York, 1971; Vol. 8, pp 201–238.
(4) Kirby, A. J.; Varvoglis, A. G. *J. Am. Chem. Soc.* **1967**, *89*, 415–423.
(5) Di Sabato, G.; Jencks, W. P. *J. Am. Chem. Soc.* **1961**, *83*, 4400–4405.
(6) Jencks, W. P.; Gilchrist, M. *J. Am. Chem. Soc.* **1964**, *86*, 1410–1417.
(7) Haake, P.; Allen, G. W. *Bioorg. Chem.* **1980**, *9*, 325–341.
(8) Kirby, A. J.; Jencks, W. P. *J. Am. Chem. Soc.* **1965**, *87*, 3209–3216.
(9) Gorenstein, D. G.; Lee, Y-G.; Kar, D. *J. Am. Chem. Soc.* **1977**, *99*, 2264–2267.

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attack of the acceptor amine on the donor acyl phosphate is precluded.[10] While the success of these experiments may lie in the use of dioxane as the medium through which the phospho group is transferred, the observed transfer is consistent with a free, relatively long-lived metaphosphate intermediate. (9) When 4-nitrophenyl phosphate monoanion or 2,4-dinitrophenyl phosphate dianion are dissolved in acetonitrile containing the hindered acceptor *tert*-butyl alcohol, significant amounts of *tert*-butyl phosphate are produced.[11] Further, the rates of alcoholysis by *tert*-butyl alcohol and hydrolysis are similar.[11] Since 2,4-dinitrophenyl phosphate *mono*anion gives no *tert*-butyl phosphate under the same conditions,[11] it seems likely that a metaphosphate intermediate could be responsible for the observed product. (10) Finally, Westheimer and his group have demonstrated the existence and chemical competence of monomeric metaphosphate in aprotic solution[12] by its generation from the Conant–Swan fragmentation of β-bromophosphonate dianions. These workers have shown that metaphosphate is indeed a potent electrophile which, for example, reacts with acetophenone to yield the corresponding enol phosphate.[13]

While all the evidence cited above is consistent with the metaphosphate mechanism, much of the data would support any pathway that involved a large degree of bond cleavage at the transition state of the rate-determining step. In order to probe the longevity of the presumed monomeric metaphosphate intermediate, we have investigated the stereochemical course of the alcoholysis of chiral [^{16}O,^{17}O,^{18}O]phospho derivatives under conditions similar to those in the earlier mechanistic studies. Just as stereochemistry has provided essential information concerning nucleophilic displacements at carbon, we hoped to obtain analogously informative results on the nature of nucleophilic reactions at phosphorus in phosphoric monoesters and a phosphoguanidine.

Experimental Section

Materials. All chemicals were from Aldrich, Sigma, or Alfa-Ventron unless otherwise noted and were used as received unless specified otherwise Isotopically enriched water was obtained from either Bio-Rad laboratories or Mound Research Laboratories and was used without purification. Molecular sieves (Linde type 4Å) were washed thoroughly with methanol, dried at 100 °C, and activated by heating at 250 °C under vacuum for 24 h. Benzyl bromide, diisopropylamine, tri-*n*-octylamine, methylene chloride, and carbon tetrachloride were passed through neutral alumina immediately before use. Methanol was distilled from magnesium methoxide prepared in situ; tetrahydrofuran, acetonitrile, diethyl ether, pyridine, and triethylamine were distilled from CaH_2; dimethylformamide was distilled from CaH_2 and stored over molecular sieves; dioxane was distilled from sodium; cyclohexylamine was distilled from CaH_2 under N_2 and stored under N_2. 2,4-Dinitrophenol was recrystallized twice from ethanol; diazomethane was prepared from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Fales et al.;[14] NaH was obtained as a dispersion in oil and was washed with hexane prior to use; 2-phosphopropane-1,2-diol was a gift from Dr. D. H. Pliura.

Alkaline phosphatase (*E. coli*, or calf intestine) was from Sigma. Acid phosphatase (human prostate) was a gift from Dr. R. L. Van Etten. Phosphatases were assayed spectrophotometrically by using 4-nitrophenyl phosphate as the substrate.

Phenyl [(*R*)-^{16}O,^{17}O,^{18}O]phosphate was prepared by the method of Abbott et al.[15] using phenol in place of 2-*O*-benzyl-(*S*)-propane-1,2-diol. The crystalline bis(cyclohexylammonium) salt of phenyl [(*R*)-^{16}O,^{17}O,^{18}O]phosphate was obtained in 42% overall yield (based on $H_2{}^{17}O$). ^{31}P NMR (D_2O) δ −0.064 (s). Mass spectrum of the bis(trimethylsilyl) derivative: *m/z* (M^+ − 15) 303 (2%), 304 (2.7%), 305 (32.3%), 306 (39.8%), 307 (23.3%). These ratios are equivalent to an isotopic composition of the three peripheral phosphate oxygens of ^{16}O, 46.3; ^{17}O, 14.2; and ^{18}O, 39.5%. [The predicted composition, on the basis of the isotopic composition of the labeled water samples used synthetically, is as follows: ^{16}O, 44.5; ^{17}O, 14.2; and ^{18}O, 41.3%.]

2,4-Dinitrophenyl [(*R*)-^{16}O,^{17}O,^{18}O]Phosphate. Lithium 2,4-dinitrophenolate was prepared by the addition by cannula of a suspension of LiH (205 mg, 25 mmol) in dry dioxane (50 mL) at 0 °C to a solution of 2,4-dinitrophenol (4.6 g, 25 mmol) in dry dioxane (25 mL), over 10 min. After the addition was complete, the reaction mixture was stirred at room temperature for 1 h. The solvent was removed by freeze drying under high vacuum, leaving a yellow solid that was used without purification. The syn adduct of [^{17}O]phosphoryl chloride with (−)-ephedrine (**1**) (2-chloro-3,4-dimethyl-5-phenyl-1,3,2-oxaphospholidin-2-[^{17}O]one) was prepared by the method of Abbott et al.[15] in 51% yield (based on $H_2{}^{17}O$) and had ^{1}H NMR ($CDCl_3$) δ 7.37 (s, 5 H), 5.85 (d, *J* = 6.3 Hz, 1 H), 3.83 (m, 1 H), 2.84 (d, *J* = 11 Hz, 3 H), 0.85 (d, *J* = 6.8 Hz, 3 H). To a solution of this adduct (2.4 g, 9.77 mmol) in dry acetonitrile (100 mL) was added solid lithium 2,4-dinitrophenolate (2.23 g, 11.7 mmol). The mixture was stirred for 7 days at 60 °C. The acetonitrile was then removed under reduced pressure to yield a viscous orange oil, from which the syn and anti isomers (in 10:1 ratio) were purified by flash chromatography on silica gel with dry ether as solvent. The syn isomer **2** was obtained as a light yellow oil in 54% yield (based on **1**) and had an R_f of 0.35 on silica gel TLC plates eluting with ether and ^{31}P NMR ($CDCl_3$) δ 13.8 (s) (proton decoupled).

The diester amidate **2** (2.07 g, 5.27 mmol) was dissolved in dry dioxane (9 mL) and added by cannula to a solution prepared from redistilled trifluoroacetic anhydride (376 μL, 2.66 mmol) and $H_2{}^{18}O$ (2 mL, 98.37% ^{18}O). After 90 min, TLC analysis (on silica gel, eluting with ethyl acetate) showed that no starting material (**2**) remained. The reaction mixture was filtered and the precipitate was washed with dry ether and then dried under vacuum to give the diester **3** as a white solid in 75% yield (based on **2**), having ^{31}P NMR (D_2O) δ −6.5 (d, *J* = 9.7 Hz).

To a suspension of the diester **3** (1.63 g, 3.95 mmol) in $CHCl_3$ (50 mL) was added trimethylsilyl bromide (5 mL, 37.9 mmol), and the mixture was stirred at room temperature for 35 h. Since at this time ^{31}P NMR showed the presence of some remaining **3**, more trimethylsilyl bromide (2 mL, 15.2 mmol) was added and the mixture stirred for a further 12 h. The solvent and excess trimethylsilyl bromide were removed in a brisk N_2 stream. Ether (50 mL) was then added, and the insoluble ephedrine bromide hydrobromide was removed by filtration. The ethereal solution was concentrated under reduced pressure to give an orange oil. Trituration with dry methanol gave a solution which, on removal of the methanol under reduced pressure, yielded a white solid. This solid was dissolved in dry ether (50 mL), and dry 2,6-lutidine was added until a faint yellow color persisted in the solution. The white precipitate (of the mono(2,6-lutidinium) salt of 2,4-dinitrophenyl [(*R*)-^{16}O,^{17}O,^{18}O]phosphate) was collected by filtration, washed with dry ether, and dried under vacuum. The product (**4**) was obtained in 73% yield (based on **3**) and had mp 138.5–141 °C (Kirby and Varvoglis[4] give 142 °C): ^{1}H NMR (D_2O) δ 8.88–8.80 (m, 3H), 7.83–7.99 (m, 3 H), 2.72 (s, 6 H); ^{31}P NMR (0.05 M CH_3COOD in D_2O) δ −4.6 (s) (proton decoupled); mass spectrum of the bis(trimethylsilyl) derivative, *m/z* (M^+ − 15) 393 (1.5%), 394 (2.2%), 395 (17%), 396 (45.2%), and 397 (34.0%). These ratios are equivalent to an isotopic composition of the three peripheral phosphate oxygens of ^{16}O, 40.7; ^{17}O, 15.8; and ^{18}O, 43.4%. [The predicted composition, based upon the isotopic composition of the labeled water samples used synthetically, is as follows: ^{16}O, 37.8; ^{17}O, 17.7; and ^{18}O, 44.5%.]

(*S*)-Propane-1,2-diol was prepared by the reduction of ethyl *S*-lactate by the method used for the 2-*O*-benzyl derivative, in 63% yield. The product had $[\alpha]_D$ 21.92 (Huff[16] reports $[\alpha]_D$ 22.22); ^{1}H NMR ($CDCl_3$) δ 3.1–4.15 (br m, 5 H), 1.09 (d, *J* = 9 Hz, 3 H). (*S*)-Butane-1,3-diol (88% enantiomeric excess) was from Aldrich.

[(*S*)-^{16}O,^{17}O,^{18}O]Phosphocreatine was prepared from adenosine [γ-(*S*)-^{16}O,^{17}O,^{18}O]triphosphate which was synthesized according to the method described by Blättler and Knowles.[17] The ATP was then used as substrate in the creatine kinase reaction according to Hansen and Knowles.[18] The labeled phosphocreatine was isolated by ion exchange chromatography and then converted into the sodium salt, as described.[18] Solvent was removed by evaporation under reduced pressure, and buffer components were removed by repeated evaporation of added isopropyl alcohol.

Methods. ^{1}H NMR spectra were recorded on a Varian HFT-80 or XL-100 spectrometer or a Bruker WM-300 spectrometer. ^{31}P NMR spectra were measured on a Varian XL-100 or a Bruker WM-300 spectrometer. Chemical shifts are reported in parts per million relative

(10) Rebek, J.; Gaviña, F.; Navarro, C. *J. Am. Chem. Soc.* **1978**, *100* 8113–8117.
(11) Ramirez, F.; Marecek, J. F. *Tetrahedron* **1979**, *35*, 1581–1589. Ramirez, F.; Marecek, J. F. *Ibid.* **1980**, *36*, 3151–3160.
(12) Satterthwait, A. C.; Westheimer, F. H. "Phosphorus Chemistry Directed Toward Biology" Stec, W. J. Ed.; Pergamon Press: New York, **1980**; p 117.
(13) Calvo, K. C.; Westheimer, F. H. *J. Am. Chem. Soc.* **1983**, *105*, 2827–2831.
(14) Fales, H. M.; Jaouni, T. M.; Babashak, J. R. *Anal. Chem.* **1973**, *45*, 2302–2303.
(15) Abbott, S. J.; Jones, S. R.; Weinman, S. A.; Bockhoff, F. M.; McLafferty, F. W.; Knowles, J. R. *J. Am. Chem. Soc.* **1979**, *101*, 4323–4332.
(16) Huff, E. *Biochim. Biophys. Acta* **1961**, *48*, 506–516.
(17) Blättler, W. A.; Knowles, J. R. *Biochemistry* **1979**, *18*, 3927–3933.
(18) Hansen, D. E.; Knowles, J. R. *J. Biol. Chem.* **1981**, *256*, 5967–5969.

to external 85% H_3PO_4. Downfield shifts are positive. ^{13}C NMR spectra were recorded on a Bruker WM-300 spectrometer. Mass spectra were measured on an AEI MS-9 or a Kratos MS-50 spectrometer. Phosphate monoesters were derivatized for mass spectral analysis by heating a small sample in a mixture of an excess of pyridine and bis(trimethylsilyl)trifluoroacetamide (1:2, v/v) at 80 °C for 10 min. The resulting solution was placed directly into the probe for analysis. Ultraviolet spectra were measured on a Perkin-Elmer 575 spectrometer. High-pressure liquid chromatography (HPLC) was performed on a Waters Associates ALC/201/R-401/6000 system. A Waters Associates μ-Porasil column (column A) was used for all separations in organic solvents and a Whatman M-9 Partisil-10 SAX column (column B) was used for all separations using aqueous eluents. Optical rotations were measured on a Perkin-Elmer 451 polarimeter. Melting points were taken on a Thomas Hoover melting point apparatus and are uncorrected.

Thin-layer chromatography (TLC) was performed using Analtech silica gel GHLF plates (250 μm) for analytical separations and Analtech Silica Gel GF (1000 or 2000 μm) for preparative separations. Compounds were visualized either under ultraviolet light or by spraying with 6% phosphomolybdic acid in ethanol, followed by heating. Column chromatography was performed using either E. Merck Silica Gel 60 (63–200 μm) or (for "flash" chromatography) E. Merck Silica Gel 60 (40–63 μm). Ion-exchange chromatography was performed using Bio-Rad AG1-X8 or Dowex 1 (200–400 mesh) for anion exchange and Dowex 50 (200–400 mesh) for cation exchange. For a column of n mL of ion-exchange resin (wet volume), a linear gradient of $5n$ mL each of 50 and 400 mM triethylammonium bicarbonate buffer, pH 7, was used. In instances where aromatic phosphates were being separated, the final buffer concentration was 500 mM. Phosphate-containing fractions were located in the eluate by treatment of 50-μL portions of every other fraction with alkaline phosphatase (0.5 unit) for 15 min at room temperature. Fractions containing phosphate were then located by the method of Ames.[19] Fractions containing the organic phosphates were pooled and evaporated to dryness. The resulting syrup was dissolved in a small volume of water and loaded onto a Dowex 50 (H^+) column. The column was then washed with 5 column volumes of water. The pH of the eluate was raised to >10 with cyclohexylamine, and the eluate was freeze dried to yield the white crystalline bis(cyclohexylammonium) salt. These salts were recrystallized from acetone–water (20:1, v/v).

Unless otherwise specified, all mention of phosphate monoesters, including weights of material, refer to the bis(cyclohexylammonium) form of the compound.

Methanolysis of Phenyl [(*R*)-^{16}O,^{17}O,^{18}O]Phosphate. Phenyl [(*R*)-^{16}O,^{17}O,^{18}O]phosphate (4.0 mmol) was converted to the disodium salt by passage down a column (50 mL) of Dowex 50 (Na^+). The eluate was concentrated under reduced pressure to yield a white solid. This material was dissolved in a mixture (1:1, v/v) of methanol and 0.5 M sodium acetate buffer (100 mL; the "pH" of resulting solution was 4.7 as measured with use of a glass electrode) and placed in a resealable tube with a Teflon-lined cap. The tube was immersed in a constant temperature oil bath at 100 °C for 2.5 h. At this point, ^{31}P NMR of a portion of the reaction mixture showed that the reaction had proceeded to 60% completion. The reaction mixture contained approximately 20% methyl phosphate, 40% inorganic phosphate, and 40% phenyl phosphate. The methyl phosphate was isolated by ion-exchange chromatography on AG1-X8 in about 20% yield (based on phenyl phosphate). The mass spectrum of the bis(trimethylsilyl) derivative of methyl phosphate showed that no label loss had occurred in the reaction or during the subsequent isolation procedure. ^{31}P NMR (D_2O) δ 3.5 (s) (proton decoupled).

Alkaline Phosphatase Catalyzed Transfer of the Phospho Group from Methyl [^{16}O,^{17}O,^{18}O]Phosphate to (*S*)-Propane-1,2-diol. The phospho group of the methyl [^{16}O,^{17}O,^{18}O]phosphate produced in the methanolysis reaction was transferred to (*S*)-propane-1,2-diol with retention of configuration at phosphorus using *E. coli* alkaline phosphatase, as described by Jones et al.[20] 1-[^{16}O,^{17}O,^{18}O]Phospho-(*S*)-propane-1,2-diol (containing 4% of the 2-phospho isomer) was isolated in approximately 5% yield after purification by ion-exchange chromatography on AG1-X8 and subsequent recrystallization of the bis(cyclohexylammonium) salt from acetone–water. The ratio of 1-phosphopropanediol to 2-phosphopropanediol in the sample was determined by measuring the ratio of the signals in the ^1H NMR spectrum of the disodium salt (in D_2O) at δ 1.20 (2-phosphopropanediol) and δ 1.14 (1-phosphopropanediol). ^{31}P NMR (D_2O) δ 4.24 (s) (proton decoupled). Mass spectrum of the tris(trimethylsilyl) derivative: m/z (M^+ – 15) 357 (6.6%), 358 (6.6%), 359 (35.1%), 360 (32.8%), 361 (18.7%). These data provide the isotopic composition of the peripheral phospho group oxygens as ^{16}O, 51.7; ^{17}O, 35.1; and ^{18}O, 13.2%. About 18% of the isotopic label had therefore been lost during the transfer and product isolation.

Alkaline Phosphatase Catalyzed Transfer of the Phospho Group from Phenyl [^{16}O,^{17}O,^{18}O]Phosphate to (*S*)-Propane-1,2-diol. The phospho group of the substrate phenyl [^{16}O,^{17}O,^{18}O]phosphate, reisolated from the methanolysis reaction, was transferred to (*S*)-propane-1,2-diol with retention of configuration at phosphorus with use of *E. coli* alkaline phosphatase, as described by Jones, et al.[20] 1-[^{16}O,^{17}O,^{18}O]Phospho-(*S*)-propane-1,2-diol (containing 3% of the phospho isomer) was isolated in approximately 12% yield after purification by ion-exchange chromatography on AG1-X8. The mass spectrum of the tris(trimethylsilyl) derivative showed that no label loss had occurred in the transfer and product isolation. ^{31}P NMR (D_2O) δ 4.24 (s) (proton decoupled).

Methanolysis of 2,4-Dinitrophenyl [(*R*)-^{16}O,^{17}O,^{18}O]Phosphate. A solution of 2,4-dinitrophenyl [(*R*)-^{16}O,^{17}O,^{18}O]phosphate mono(2,6-lutidinium) salt (705 mg, 1.89 mmol) in methanol and 0.3 M K_2CO_3–$KHCO_3$ (50 mL; 1:1 v/v; "pH" of 10.2 as measured with use of a glass electrode) was stirred for 32 h at 22 °C. At this point, ^{31}P NMR showed that no substrate remained. The reaction mixture was acidified with Dowex 50 (H^+) and the pH adjusted to about 7 with triethylamine. Concentration under reduced pressure left a white solid which contained the triethylammonium salts of methyl phosphate and of inorganic phosphate in approximately 2:1 ratio as judged by ^{31}P NMR. The mass spectrum of the bis(trimethylsilyl) derivative of the methyl phosphate revealed that no label loss had occurred in the methanolysis reaction or the isolation procedure. This mixture was used in the transfer reaction without further purification.

Alkaline Phosphatase Catalyzed Transfer of the Phospho Group of Methyl [^{16}O,^{17}O,^{18}O]Phosphate. The phospho group of methyl phosphate produced in the methanolysis of 2,4-dinitrophenyl [^{16}O,^{17}O,^{18}O]phosphate was transferred to (*S*)-propane-1,2-diol as before.[20] The product 1-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol (containing 5% of the 2-phospho isomer) was isolated in 6% yield after purification by ion-exchange chromatography on AG1-X8 and subsequent recrystallization of the bis(cyclohexylammonium) salt from acetone–water. ^{31}P NMR (D_2O) δ 4.2 (s) (proton decoupled). Mass spectrum of the tris(trimethylsilyl) derivative: m/z (M^+ – 15) 357 (5.6%), 357 (6.2%), 359 (24.7%), 360 (36%), 361 (27.4%). These data provide the isotopic composition of the peripheral phospho group oxygens as ^{16}O, 47.4; ^{17}O, 14.1; and ^{18}O, 38.5%. About 21% of the isotopic label had been lost in the transfer and subsequent product isolation.

Acid Phosphatase Catalyzed Transfer of the Phospho Group of 2,4-Dinitrophenyl [^{16}O,^{17}O,^{18}O]Phosphate to (*S*)-Propane-1,2-diol. 2,4-Dinitrophenyl [(*R*)-^{16}O,^{17}O,^{18}O]phosphate mono(2,6-lutidinium) salt (187 mg, 0.5 mmol) was dissolved in a mixture of (*S*)-propane-1,2-diol and H_2O (1:4, v/v) and the solution cooled to 4 °C. Human prostatic acid phosphatase (200 units) was added and the reaction was allowed to proceed for 15 min at 4 °C. At this point the reaction was 70% complete (as determined from the ultraviolet absorbance of 2,4-dinitrophenol) and was quenched with 1 N NaOH (35 mL) and left for 15 min to inactivate the enzyme. The resulting solution was then incubated at 39 °C for 6 h to hydrolyze the remaining 2,4-dinitrophenyl phosphate. The pH of the solution was then adjusted to 7 with acetic acid. 1-[^{16}O,^{17}O,^{18}O]-Phospho-(*S*)-propane-1,2-diol (containing about 5% of the 2-phospho isomer) was obtained from this solution by ion-exchange chromatography on AG1-X8 in 30% isolated yield. The phospho group in the phosphopropanediol has the same configuration as the donor ester, 2,4-dinitrophenyl phosphate.[21] ^{31}P NMR (D_2O) δ 3.9 (s) (proton decoupled). Mass spectrum of the tris(trimethylsilyl) derivative: m/z (M^+ - 15) 357 (2.2%), 358 (2.7%), 359 (16%), 360 (44.8%), 361 (34.3%).

Methanolysis of [(*S*)-^{16}O,^{17}O,^{18}O]Phosphocreatine. A solution of [(*S*)-^{16}O,^{17}O,^{18}O]phosphocreatine disodium salt (1.16 mmol) in 426 mM sarcosine buffer (26.2 g), containing NaCl (256 mM), was adjusted to pH 2.0 with 1 M HCl. Freshly distilled methanol (72.5 g) was added, and the solution was incubated at 30 °C. The final concentrations were as follows: methanol, 60 mol %; phosphocreatine, 10 mM; sarcosine, 100 mM; and NaCl, 60 mM. After 24 h, the reaction was quenched by the addition of solid NH_4HCO_3. ^{31}P NMR showed the ratio of methyl phosphate to inorganic phosphate to be 55:45. The methyl phosphate was isolated by ion-exchange chromatography at 4 °C on a column (150 mL) of AG1-X8 equilibrated with 50 mM triethylammonium bicarbonate buffer, pH 7.0, eluting with a linear gradient (50–200 mM; 800 + 800 mL) of the same buffer.

Alkaline Phosphatase Catalyzed Transfer of the Phospho Group of Methyl [^{16}O,^{17}O,^{18}O]Phosphate. The phospho group of methyl phosphate produced in the methanolysis of [(*S*)-^{16}O,^{17}O,^{18}O]phosphocreatine was

(19) Ames, B. N. *Methods Enzymol.* **1966**, *8*, 115–118.

(20) Jones, S. R.; Kindman, L. A.; Knowles, J. R. *Nature (London)* **1978**, *275*, 564–565.

(21) Buchwald, S. L.; Saini, M. S.; Knowles, J. R.; Van Etten, R. L. *J. Biol. Chem.* **1984**, *259*, 2208–2213.

transferred to (*S*)-butane-1,3-diol by a modification of the method used for transfer to propanediol.[20] Methyl [^{16}O,^{17}O,^{18}O]phosphate (700 μmol) was dissolved in H_2O (3 mL) and D_2O (0.9 mL) containing K_2CO_3 (300 mM), $KHCO_3$ (300 mM), NaCl (1 M), $Zn(OAc)_2$ (7.5 μM), and $Mg(OAc)_2$ (0.75 mM). *E. coli* alkaline phosphatase (15.2 units, in 1 mL of the above buffer) was added, the pH rapidly adjusted to 10.0 with NaOH (1 M), and (*S*)-butane-1,3-diol (4 mL) added immediately. The mixture was vortexed and left for 30 h at room temperature. ^{31}P NMR showed that 40% of the methyl phosphate had been consumed. The mixture was filtered through a column (10 mL) of Dowex 50 (H^+ form), and the eluate was neutralized with solid NH_4HCO_3. After dilution with water to 1.6 L, the sample was applied at 4 °C to a column (150 mL) of AG1-X8, equilibrated with 50 mM triethylammonium bicarbonate buffer, pH 6.9. The column was eluted with a linear gradient (50–150 mM, 2 + 2 L) of the same buffer. The mixture of 1- and 3-phosphobutane-1,3-diols was collected, and the remaining methyl phosphate was isolated and resubjected to another transfer reaction. The combined samples of [^{16}O,^{17}O,^{18}O]phosphobutanediols (41.7 μmol) were found to contain the 1- and 3-phospho isomers in a ratio of 13.5:1 by ^{31}P NMR.

^{31}P NMR Analysis of the Configuration at Phosphorus. The configuration and the enantiomeric excess at phosphorus in the derived samples of 1-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol were determined by high-resolution ^{31}P NMR with a Bruker WM-300 instrument, as described earlier.[22] The configuration at phosphorus in phosphobutanediol was determined by a method very similar to that used for phosphopropanediol.[22,29]

Results and Discussion

On the basis of the evidence summarized in the introduction, we selected two phosphate monoesters, the solvolysis of which in protic media appeared most likely to proceed via monomeric metaphosphate as a reaction intermediate. Phenyl phosphate behaves like a typical phosphoric monoester of an alcohol (or phenol) of $pK_a > 5.5$ and hydrolyzes most rapidly at around pH 4, where the proportion of monoanion is greatest.[1,2,4] The fact that this rate is much faster than that for the hydrolysis of the corresponding diester at the same pH,[23] and since oxygen isotope studies argue against an addition–elimination pathway (for the monoanion) analogous to that followed by esters of carboxylic acids,[23] the mechanism of Scheme I was proposed.[1,2] Proton transfer within the phosphoric monoester monoanion yields a dipolar species from which the neutral alcohol (in this case, phenol) can depart, leaving behind what is formally the monomeric metaphosphate ion. As has been argued by Kirby and Varvoglis,[4] as the pK_a of the phenol moiety falls below 5.5, the phosphoric ester *di*anion becomes the more reactive species. With dianions, the rate of solvolysis becomes very sensitive to the pK_a of the leaving phenol (β_{lg} is 1.2), and the mechanism appears to involve the direct heterolytic breakdown illustrated in Scheme II.[4] On the basis of the known behavior of phosphoric monoesters, therefore, we chose to investigate the monoanion of phenyl phosphate and the dianion of 2,4-dinitrophenyl phosphate.

Phenyl [(*R*)-^{16}O,^{17}O,^{18}O]phosphate was solvolyzed in 0.5 M sodium acetate buffer containing 50% (v/v) methanol, at a "pH" of 4.7 and 100 °C. After 150 min, 40% of the phenyl phosphate remained, and the ratio of methyl phosphate and inorganic phosphate was 1:2. The product methyl [^{16}O,^{17}O,^{18}O]phosphate and the remaining phenyl [^{16}O,^{17}O,^{18}O]phosphate were isolated, and each was subjected to stereochemical analysis.[15] This was achieved by using *E. coli* alkaline phosphatase to catalyze the phospho group transfer to (*S*)-propane-1,2-diol with retention of the configuration at phosphorus.[20] Stereochemical analysis by the ^{31}P NMR method described earlier[22] showed that the configuration at phosphorus in the remaining phenyl phosphate was 80 ± 1% *R*, and that in the product methyl phosphate was 86 ± 7% *S* (Figure 1). Within experimental uncertainty, *the methanolysis of the monoanion of phenyl phosphate proceeds with complete inversion at phosphorus.*

2,4-Dinitrophenyl [(*R*)-^{16}O,^{17}O,^{18}O]phosphate was synthesized by our original route[15] via the ephedrine adduct with phosphoryl chloride, modified to overcome the lack of nucleophilic reactivity

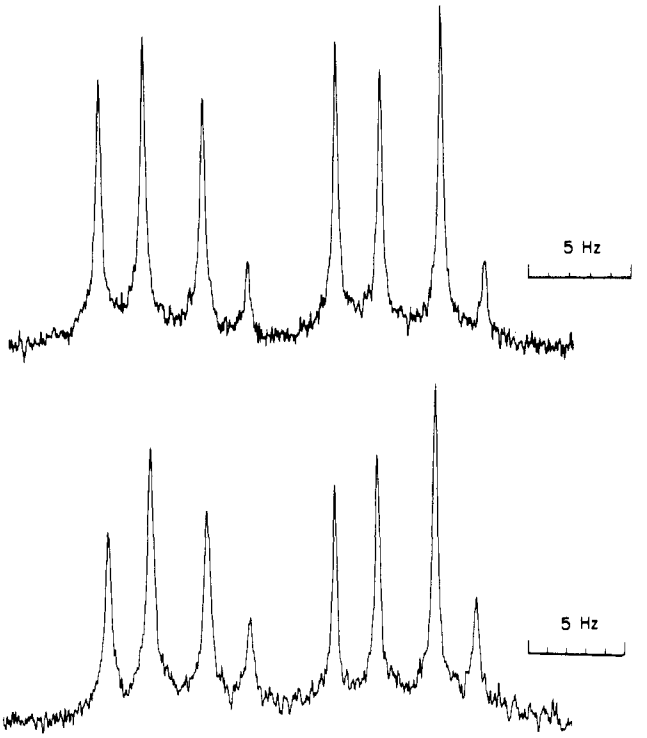
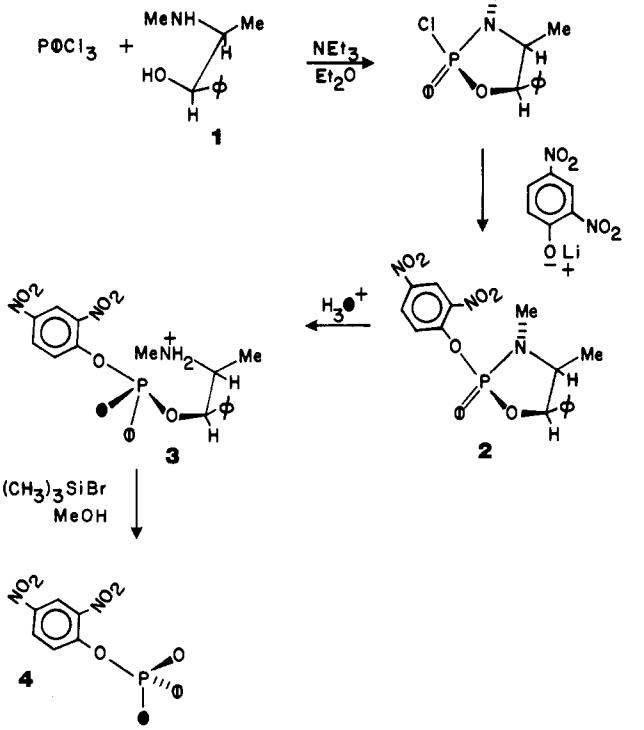


Figure 1. ^{31}P NMR spectra of the products from the "in-line" ring closure and methylation[15,22] of 1-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol obtained by phospho group transfer[20] from samples of methyl phosphate: upper spectrum, product deriving from the methanolysis of phenyl phosphate monoanion; lower spectrum, product deriving from the methanolysis of 2,4-dinitrophenyl phosphate dianion. The spectra were taken[22] on a Bruker WM-300 WB instrument at 121.5 MHz.

Scheme III. Synthetic Route to 2,4-Dinitrophenyl [(*R*)-^{16}O,^{17}O,^{18}O] Phosphate



of 2,4-dinitrophenolate, the instability of the 2,4-dinitrophenyl derivatives once formed, and the sensitivity of the nitro groups to the hydrogenolytic deprotection of the chiral phosphoric monoester (Scheme III). The ester was solvolyzed in 300 mM potassium carbonate buffer containing 50% (v/v) methanol, at a

(22) Buchwald, S. L.; Knowles, J. R. *J. Am. Chem. Soc.* **1980**, *102*, 6601–6602.
(23) Bunton, C. A.; Llewellyn, D. R.; Oldham, K. G.; Vernon, C. A. *J. Chem. Soc.* **1958**, 3574–3587.

"pH" of 10.2 and 22 °C. After 32 h, no starting material remained, and the products methyl phosphate and inorganic phosphate had been formed in a ratio of 2:1. The phospho group of methyl phosphate was transferred to (*S*)-propane-1,2-diol for stereochemical analysis using *E. coli* alkaline phosphatase as before.[20] Because of the lability of the substrate, 2,4-dintirophenyl phosphate, as its dianion, the configuration at phosphorus in this ester was checked by transfer to (*S*)-propane-1,2-diol with use of the purified *acid* phosphatase from human prostate. We have shown independently that this enzyme, like the *E. coli* alkaline phosphatase, catalyzes transphosphorylation with overall retention of the configuration at phosphorus.[21] Stereochemical analysis of the samples of 1-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol showed that the configuration at phosphorus in the starting material 2,4-dinitrophenyl phosphate was 86 ± 3% *R*, and that in the product methyl phosphate was 87 ± 3% *S* (Figure 1). Within experimental error, therefore, *the methanolysis of 2,4-dinitrophenyl phosphate proceeds with complete inversion at phosphorus.*

The stereochemical consequence of the methanolysis of phenyl phosphate monoanion and of 2,4-dinitrophenyl phosphate dianion is inversion of the configuration at phosphorus, which is inconsistent with a mechanism involving a free, symmetrically solvated metaphosphate ion intermediate. If such a species were formed, we should expect the product methyl phosphate to have been racemic. Yet the kinetic data reviewed in the introduction are inconsistent with an associative S_N2-like attack of methanol at the phosphorus center, and they require that the transition state be characterized by almost complete bond breaking, with little or no bond making. The apparent conflict between the earlier kinetic data and the present stereochemical results can be resolved by application of the criteria developed by Jencks[24] for distinguishing between mechanisms in the "border line" between dissociative S_N1 processes and associative S_N2-like reactions. Jencks has stressed that the qualitative mechanistic question of whether a reaction proceeds via a discrete reaction intermediate (which may have a lifetime of only one vibration or may be a "liberated" intermediate that escapes fully from the cage in which it was generated) must be separated from the quantitative question of whether the reactant (in our case, the attacking nucleophile) provides kinetic assistance for the reaction.[24] For the reactions under consideration here, experiments in aqueous alcoholic solvents showed that the mol % of products is, for unhindered nucleophiles such as methanol and water, close to the mol fraction of the nucleophiles in the solution. These correlations were held to support the view that a highly reactive and unselective electrophilic species was being generated,[4,25] since methanol is so much more powerful a nucleophile than water. The fact that the correlations were imperfect, however, led Jencks and Gilchrist to suggest that while there may be little participation of the nucleophilic reagent (that is, kinetic assistance is minimal), "the metaphosphate-like intermediate is not completely separated from the attacking or leaving groups".[26] In contrast, Kirby and Varvoglis[4] have preferred to interpret the slight selectivity observed in the phosphorylation of different alcohols in terms of selective solvation, rather than of any degree of nucleophilic participation at the transition state. Indeed, these workers see "non-selective phosphorylation in mixed alcohol–water solvents as sufficient, *but not necessary* (sic), evidence for a metaphosphate intermediate".[4] In summary, the fact that there is a reasonable correlation between the mol % of methyl phosphate formed and the mol fraction of solvent methanol in the reactions of phenyl phosphate monoanion[25] and of 2,4-dinitrophenyl phosphate dianion[4] suggests that there is little nucleophilic assistance in these reactions and is consistent with there being little if any bond formation at the transition state.

It has been shown by Haake and his group,[7] however, that of all phospho group donor species, the product composition correlation in solvolysis reactions is most cleanly obeyed by *N*-phosphoguanidines. Such species appear to be among the most rapidly solvolyzed phosphorylated derivatives[7,27] and among "the most reactive precursors of metaphosphate".[28] Since chemical synthetic routes to chiral [^{16}O,^{17}O,^{18}O]phosphoroguanidines have not been worked out, we opted to study the solvolysis of *N'*-[^{16}O,^{17}O,^{18}O]phosphocreatine, which is accessible in known configuration from chiral [γ-^{16}O,^{17}O,^{18}O]ATP, creatine, and creatine kinase.[18] The hydrolysis of phosphocreatine has been investigated, and it has been established that at pH values higher than 1.0 the main products are creatine and inorganic phosphate.[28] [Only in strongly acidic media does the carboxyl group of phosphocreatine affect the course of the reaction and result in the formation of creatinine.] Accordingly, *N'*-[(*S*)-^{16}O,^{17}O,^{18}O]-phosphocreatine was prepared from [γ-(*S*)-^{16}O,^{17}O,^{18}O]ATP by using the creatine kinase reaction, which we have earlier shown[18] to proceed with inversion of the configuration and to produce phosphocreatine 80 ± 16% *S* at phosphorus. This material was subjected to methanolysis in 78% v/v aqueous methanol at "pH" 2.0, 30 °C. The product methyl [^{16}O,^{17}O,^{18}O]phosphate was isolated and the phospho group transferred to (*S*)-butane-1,3-diol for stereochemical analysis. The configuration at phosphorus was found to be 82 ± 6% *S* (Figure 2). It is evident, therefore, that *the methanolysis of the (phosphorus) monoanion of N'-phosphocreatine proceeds with complete inversion of the configuration at phosphorus.*

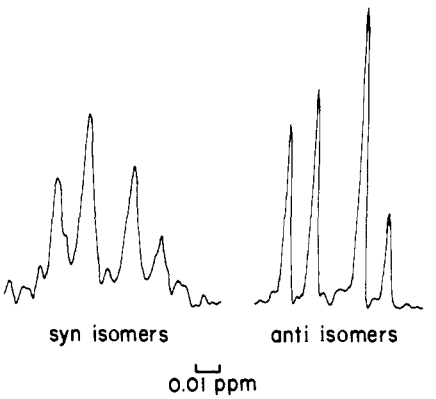


Figure 2. ^{31}P NMR spectra of the products from the "in-line" ring closure and methylation[29] of 1-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-butane-1,3-diol obtained by phospho group transfer[20] from the sample of methyl phosphate deriving from the methanolysis of *N'*-phosphocreatine. The spectrum was taken on a Bruker WM-300 WB instrument at 121.5 MHz.

The finding that a phosphoguanidine (for which kinetic assistance from the nucleophilic reagent must be negligible) suffers clean inversion at phosphorus requires that if a metaphosphate intermediate is formed it is captured before any rotation about a P–O bond can occur. That is, the putative metaphosphate is certainly not a liberated intermediate,[24] nor is it even long-lived enough to rotate within the cage in which it was generated. The reaction must therefore be *preassociative*, in which the only acts of substrate heterolysis that lead to products are when the acceptor nucleophile is in place. Jencks[24] has pointed out that a reaction is *necessarily* preassociative if either the reaction intermediate does not exist or if it is so unstable that it collapses back to starting materials faster than the nucleophilic acceptor can diffuse away from the complex. In terms of Figure 3, the reaction is either *preassociative concerted* or *preassociative stepwise*. Our results cannot distinguish between these alternatives, the first of which involves a loose or "exploded" S_N2-like transition state and the second has a metaphosphate intermediate of extremely short lifetime. In either case, bond breaking dominates the rate-limiting process, and inversion is the stereochemical outcome.

Recently, two groups have investigated the transfer of a phospho group from 3-methoxypyridine[30] or from isoquinoline[31] to a series

(24) Jencks, W. P. *Chem. Soc. Rev.* **1981**, *10*, 345–375.
(25) Chanley, J. D.; Feageson, E. *J. Am. Chem. Soc.* **1963**, *85*, 1181–1190.
(26) Jencks, W. P.; Gilchrist, M. *J. Am. Chem. Soc.* **1964** *86*, 1410–1417.
(27) Allen, G. W.; Haake, P. *J. Am. Chem. Soc.* **1973**, *95*, 8080–8087.
(28) Allen, G. W.; Haake, P. *J. Am. Chem. Soc.* **1976**, *98*, 4990–4996.
(29) D. E. Hansen, unpublished experiments.
(30) Skoog, M. T.; Jencks, W. P. *J. Am. Chem. Soc.* **1983**, *105*, 3356–3357.
(31) Bourne, N.; Williams, A. *J. Am. Chem. Soc.* **1983**, *105*, 3357–3358.

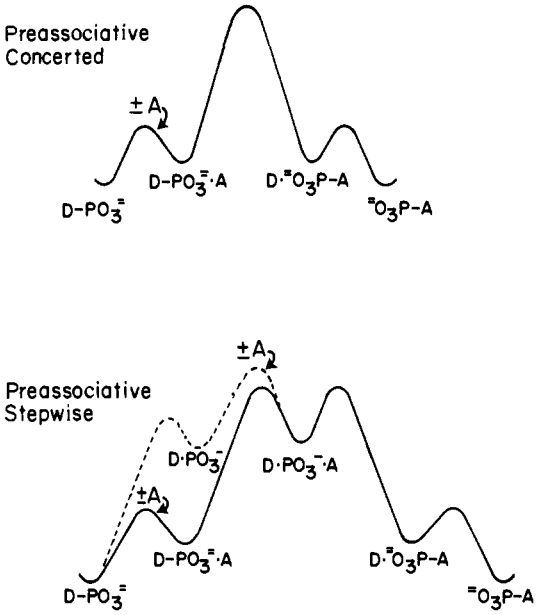


Figure 3. Free energy profiles for preassociative processes. In a preassociative concerted reaction (e.g., an S_N2 process: upper profile) the acceptor A diffuses to the phosphorylated donor D and phospho group transfer occurs via a single associative transition state. A reaction is preassociative stepwise (lower profile, full line) rather than dissociative (lower profile, dashed line) if the intermediate PO_3^- in the complex $D{\cdot}PO_3^-{\cdot}A$ is so unstable that it collapses back to $D{-}PO_3^{2-}$ faster than the acceptor A can diffuse away. [Charges on D and A are omitted for clarity.]

of substituted pyridines and have concluded that the absence of a "break" in the Brønsted plots is most consistent with a concerted reaction, the single, relatively symmetrical transition state involving weak bonding to both the entering and leaving groups. There is no evidence for a change in rate-limiting step as the pK_a of the acceptor nucleophile becomes less than that of the leaving group, as would be expected for a preassociative stepwise path. In so far as these conclusions can be applied more generally, it is likely that the transfer of phospho groups from phospho monoamidates in protic media is best viewed as a preassociative concerted mechanism where the single transition state is a loose one in which neither acceptor nor donor nucleophile is closely associated with phosphorus.

The above conclusion is consistent with the stereochemical results reported in this paper and with the mechanistic evidence cited in the introduction for reactions in protic solvents (items 1-7). For such processes, there are no data that demand the existence of a free metaphosphate intermediate, and it now seems clear that no such postulate is required. In the case of aprotic media, however, the evidence that metaphosphate can be a liberated intermediate is more compelling (see items 8-10 of the introduction). Thus the transfer of phospho groups in the three-phase reactions of Rebek and his group,[10] the phosphorylation of *tert*-butyl alcohol during solvolytic reactions of aryl phosphoric esters studied by Ramirez and collaborators,[11] and the products from the fragmentation reactions of β-halophosphonates investigated by Westheimer and co-workers[12,13] are all strongly suggestive of free (or possibly, in the case of dioxane and acetonitrile, specifically solvated) monomeric metaphosphate ion. Stereochemical investigations on these systems will, it is hoped, provide evidence for the explicit intermediacy of metaphosphate in reactions in aprotic media and set some limits on the freedom of metaphosphate in such solutions.

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Stereochemical Evidence for Pseudorotation in the Reaction of a Phosphoric Monoester

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Abstract: The phospho group of 2-phosphopropane-1,2-diol migrates to the 1-position on heating in aqueous acid. This migration occurs by two routes. The direct route is believed to proceed via a pentacoordinate intermediate that must, by Westheimer's rules, pseudorotate at least once to yield product. In the phospho diester route, the cyclic 1,2-phospho diester is formed as an intermediate. Four experiments have been performed to determine the rate constants for each route. These experiments involve the measurements of (a) the overall equilibrium constant, (b) the partition ratio (to the 1- or 2-phospho monoesters) for the hydrolysis of the cyclic diester intermediate, (c) the overall rate of isomerization, and (d) the rate of solvent ^{18}O incorporation into an equilibrium mixture of 1- and 2-phospho compounds. By using chiral 2-[(*R*)-^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol as substrate and determining the configuration both of the 1-phospho product isomer and of the remaining 2-phospho substrate, the direct route has been shown to proceed with *retention* of configuration at phosphorus, in accord with the predicted behavior for reaction via a pseudorotating pentacoordinate intermediate.

In the mid 1960's, Westheimer[1] laid the foundation for our understanding of many previously enigmatic reactions of phosphate esters, phosphonates, phosphinates, and phosphoramidates. He recognized the importance of and requirements for pseudorotatory processes in these reactions and proposed a set of predictive rules governing the stereochemical disposition of entering groups, departing groups, and other ligands to phosphorus, in the formation and breakdown of pentacovalent phosphorus intermediates.[1] During the past 15 years, much evidence has accumulated in support of Westheimer's proposals for the reactions of phosphoric esters. Many of these data necessarily derive from work on

† National Science Foundation predoctoral Fellow.
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(1) Westheimer, F. H. *Acc. Chem. Res.* **1968**, *1*, 70-78.

0002-7863/84/1506-4916$01.50/0