Transition-State Structures for Phosphoryl-Transfer Reactions of *p*-Nitrophenyl Phosphate

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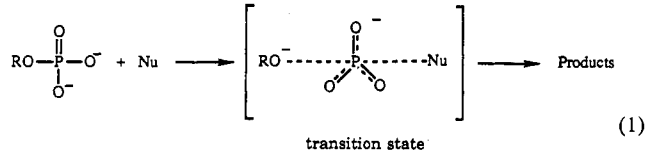
Abstract: Heavy-atom isotope effects have been used to characterize the transition states for the aqueous hydrolysis reactions of the *p*-nitrophenyl phosphate dianion and monoanion, for the reaction of the dianion in neat *tert*-butyl alcohol, and for the reaction catalyzed by alkaline phosphatase. The primary oxygen-18 isotope effect at the phenolic oxygen ($^{18}k_{bridge}$), the secondary nitrogen-15 effect (^{15}k) in the nitrogen atom of the leaving group, and the secondary oxygen-18 isotope effects in the nonbridge oxygen atoms of the phosphoryl group ($^{18}k_{nonbridge}$) have been measured. The isotope effects for the dianion reaction in water at 95 °C were ^{15}k = 1.0028 ± 0.0002, $^{18}k_{bridge}$ = 1.0189 ± 0.0005, and $^{18}k_{nonbridge}$ = 0.9994 ± 0.0005. The dianion reaction in *tert*-butyl alcohol at 30 °C gave values of ^{15}k = 1.0039 ± 0.0003, $^{18}k_{bridge}$ = 1.0202 ± 0.0008, and $^{18}k_{nonbridge}$ = 0.9997 ± 0.0016. When corrected for temperature, the results are very similar, indicating similar late transition state structures for the two reactions with little or no change in bond order between the phosphorus and the nonbridge oxygen atoms. The isotope effects on the aqueous reaction of the monoanion were ^{15}k = 1.0004 ± 0.0002, $^{18}k_{bridge}$ = 1.0087 ± 0.0003, and $^{18}k_{nonbridge}$ = 1.0184 ± 0.0005, suggesting both proton transfer and bond cleavage are rate-limiting. The isotope effects on the alkaline phosphatase reaction are all near unity, indicating that a nonchemical step is rate-limiting for the enzymatic reaction.

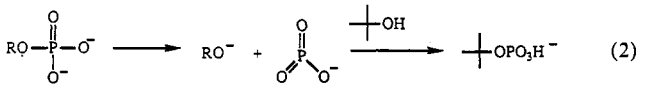
Studies of the hydrolysis reactions of phosphate monoesters show that these compounds react as monoanions or as dianions by dissociative mechanisms, exhibiting transition states with a small amount of bond formation to the nucleophile and a large amount of bond breaking to the leaving group.[1a] The dianion reaction can be represented as a concerted $S_N2(P)$ reaction, described in the IUPAC system[2] as $A_N + D_N$, as shown in eq 1.



(1)

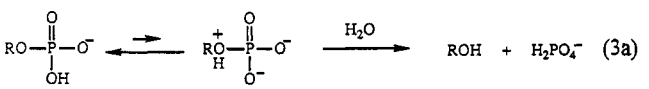
This mechanism is supported by a very small entropy of activation, a large (–1.23) value of β_{lg} and a small β_{nuc}, and the occurrence of inversion of configuration at phosphorus when the phosphoryl group is made chiral.[1]

While the occurrence of inversion of configuration shows that there is no free metaphosphate intermediate in the solvolysis reactions with protic solvents, Knowles et al. have shown that, when an isotopically chiral phosphoryl group is transferred from *p*-nitrophenyl phosphate in neat *tert*-butyl alcohol, the product *tert*-butyl phosphate is completely racemic.[1d] Thus, under these conditions, a viable, diffusible metaphosphate intermediate is generated in a two-step $D_N + A_N$ mechanism as shown in eq 2:[3]

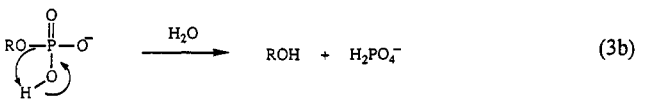


(2)

The reaction of the monoanion is also believed to proceed by a dissociative mechanism, with the proton largely or completely shifted from the phosphoryl group to the leaving group in the transition state, as in eq 3a or 3b:



(3a)



(3b)

This reaction also shows a very small entropy of activation and inversion of configuration at phosphorus in water.[1b,d] The value of β_{lg} is –0.27 in this reaction, reflecting the proton transfer to the leaving group in the rate-limiting transition state. Kirby and Varvoglis considered whether the proton shift occurs in a preequilibrium step, as shown in eq 3a, or if the transfer is concerted with leaving group departure, as in eq 3b. They concluded that, when leaving group basicity drops below that of phenol, proton transfer becomes partially rate-determining and occurs simultaneously with leaving group departure.[1b]

The transition-state structures for these reactions can be probed by measuring the oxygen-18 isotope effects for the phosphoryl oxygen atoms. The magnitude of the secondary oxygen-18 effect in the nonbridge oxygen atoms, $^{18}k_{nonbridge}$, will be dependent on the change in bond order between these atoms and the phosphorus atom. In dissociative mechanisms, such as eqs 1 and 2, an increase in the bond order between the phosphorus atom and the nonbridge oxygens in the transition state would manifest itself in an inverse isotope effect. The primary oxygen-18 isotope effect in the leaving group oxygen atom, $^{18}k_{bridge}$, will depend on the extent of bond cleavage in the transition state. This paper reports the oxygen-18 isotope effects on the phosphoryl-transfer reactions of the

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(1) (a) Benkovic, S. J.; Schray, K. J. The Mechanism of Phosphoryl Transfer. In *Transition States of Biochemical Processes*; Gandour, R. D., Schowen, R. L., Eds.; Plenum Press: New York, 1978; Chapter 13, pp 493–521. (b) Kirby, A. J.; Varvoglis, A. G. *J. Am. Chem. Soc.* **1967**, *89*, 415–423. (c) Herschlag, D.; Jencks, W. P. *J. Am. Chem. Soc.* **1986**, *108*, 7938–7946. (d) Buchwald, S. L.; Friedman, J. M.; Knowles, J. R. *J. Am. Chem. Soc.* **1984**, *106*, 4911–4916.

(2) Guthrie, R. D.; Jencks, W. P. *Acc. Chem. Res.* **1989**, *22*, 343–349. Guthrie, R. D. *Pure Appl. Chem.* **1989**, *61*, 23–56.

(3) As pointed out by Herschlag and Jencks in ref 1c, the initially formed *tert*-butyl phosphate will be bridge-protonated. The given interpretation of the racemization result assumes that proton transfer is faster than diffusional loss of *p*-nitrophenoxide and subsequent attack by a second molecule of *tert*-butyl alcohol on the bridge-protonated *tert*-butyl phosphate.

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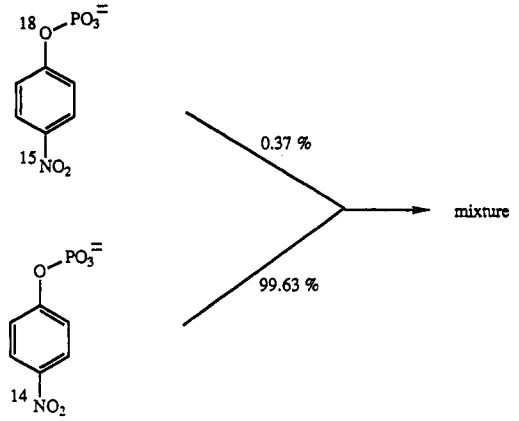
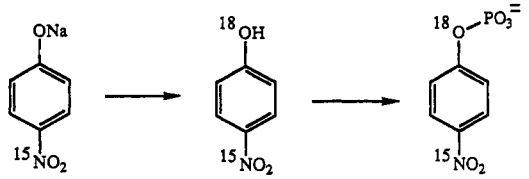
Scheme 1. Mixing Proportions for Reconstituting Close to the Natural Abundance of N-15 in the Mixture of Isotopomers Used for Competitive Isotope Effect Measurements



Scheme 2. Synthetic Route to [^{15}N,bridge-^{18}O]-*p*-Nitrophenyl Phosphate



1 2 3

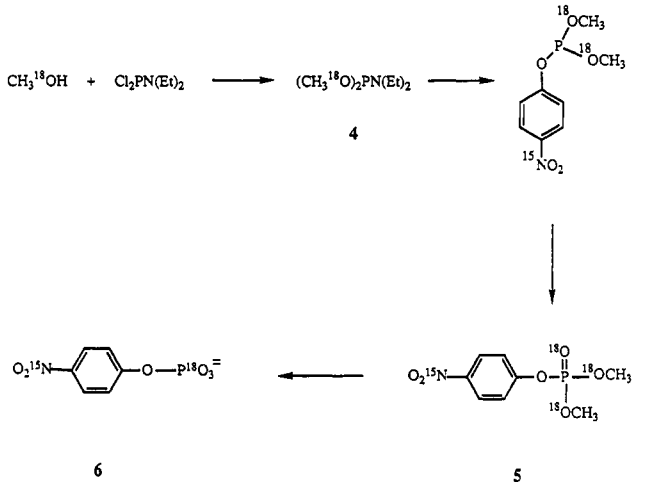
p-nitrophenyl phosphate monoanion and dianion in water, and of the dianion in neat *tert*-butyl alcohol. We have also measured the nitrogen-15 isotope effects, ^{15}k, in the nitrogen atom of the *p*-nitrophenol leaving group. We have previously shown that this effect gives an indication of the degree of bond breaking in the leaving group, by measuring the degree of charge delocalization into the aromatic ring.[4] This same set of isotope effects have been measured for the catalysis of the hydrolysis of *p*-nitrophenyl phosphate by the enzyme alkaline phosphatase from *E. coli*.

All isotope effects were measured by the competitive method, using an isotope ratio mass spectrometer. The measurement of heavy-atom isotope effects using an isotope ratio mass spectrometer is much more accurate and reliable than utilizing direct spectrophotometric comparisons of rates, due to the small magnitudes of the observed effects. The oxygen-18 isotope effects were measured by the remote label method.[5] In this method, the substrate is synthesized with labels at two positions, one at the site of chemical interest and the other at a position that lends itself to isolation and isotopic measurement, to function as a marker. In this work, the nitrogen atom of the substrate served as the remote label. For measurement of $^{18}k_{\text{bridge}}$, *p*-nitrophenyl phosphate was synthesized with oxygen-18 in the bridge oxygen atom and nitrogen-15 in the nitro group, as well as the compound with nitrogen-14 and containing natural abundance oxygen (Scheme 1). These were then mixed to reconstitute the natural abundance of 0.37% nitrogen-15, as the isotope ratio measurements are most accurate when samples approximate natural abundance. The $^{18}k_{\text{nonbridge}}$ effects were measured similarly, using the corresponding compound with the three nonbridge oxygen atoms labeled with oxygen-18 and with nitrogen-15 in the nitro group. The synthetic schemes for the multiply labeled compounds are summarized in Schemes 2 and 3.

(4) Hengge, A. C.; Cleland, W. W. *J. Am. Chem. Soc.* **1990**, *112*, 7421–7422.

(5) O'Leary, M. H.; Marlier, J. F. *J. Am. Chem. Soc.* **1979**, *101*, 3300–3306.

Scheme 3. Synthetic Route to [^{15}N,nonbridge-^{18}O$_3$]-*p*-Nitrophenyl Phosphate



4 6 5

The general methodology has been previously described in other studies of phosphoryl-transfer mechanisms using isotope effects.[6]

Experimental Section

Materials. Tetrahydrofuran was distilled under nitrogen from sodium and benzophenone ketyl just before use. Cyclohexylamine was distilled under nitrogen shortly before use. *tert*-Butyl alcohol was distilled from calcium hydride under nitrogen and stored over activated molecular sieves. Tetra-*n*-butylammonium *tert*-butoxide was produced as a solution in *tert*-butyl alcohol by adding 1 equiv of dry tetra-*n*-butylammonium chloride to a solution of potassium *tert*-butoxide in *tert*-butyl alcohol and removing precipitated potassium chloride by centrifugation. Methanol-^{18}O, water-^{18}O, $NH_4{}^{14}NO_3$, and $NH_4{}^{15}NO_3$ were purchased from Isotec. Alkaline phosphatase from *E. coli*, type III, was purchased from Sigma and used as received. Natural abundance *p*-nitrophenyl phosphate was purchased from Aldrich and used as received. Thin layer chromatography was performed on polyester plates coated with silica gel containing a fluorescent indicator. Compounds were visualized under ultraviolet light. Column chromatography was performed using silica gel, 230–400 mesh.

Preparation of [^{14}N]-*p*-Nitrophenyl Phosphate. The sodium salt of [^{14}N]nitrophenol was prepared by nitrating triphenyl phosphate with [^{14}N]ammonium nitrate followed by hydrolysis.[6a] The sodium [^{14}N]-nitrophenolate thus obtained (6 g) was stirred as a slurry in water in a beaker cooled by an ice–water bath, and sulfuric acid was added until the pH of the slurry remained below 5. The *p*-nitrophenol was collected by Buchner filtration and dried under vacuum in a dessicator over P_2O_5. *p*-Nitrophenol (4.55 g) was converted to the dicyclohexylammonium salt of *p*-nitrophenyl phosphate by reaction with phosphoryl chloride by the method of Bourne and Williams.[7]

Preparation of [^{15}N,bridge-^{18}O]-*p*-Nitrophenyl Phosphate (3). The sodium salt of [^{15}N]-*p*-nitrophenol was prepared using [^{15}N]ammonium nitrate by the method described for the preparation of the [^{14}N]phenolate.[6a] The phenolic oxygen atom of the sodium salt **1** thus obtained was exchanged in [^{18}O]water under basic conditions.[8] After labeled water was recovered by distillation, water was added and the neutral phenol **2** was isolated by acidification followed by extraction with ether. The ether extracts were dried over magnesium sulfate, and the solvent was removed by rotary evaporation. After it was dried over P_2O_5, [^{18}O,^{15}N]-*p*-nitrophenol (146 mg, 1.03 mmol) was reacted with phosphoryl chloride (168 mg, 1.1 mmol) as for the [^{14}N] compound. After recrystallization from ethanol, 290 mg of **3** was obtained (0.69 mmol, 67%). Mass spectrometry (FAB) showed the product to contain 95% oxygen-18 at the phenolic oxygen atom and 98% nitrogen-15.

(6) (a) Hengge, A. C.; Cleland, W. W. *J. Am. Chem. Soc.* **1991**, *113*, 5835–5841. (b) Caldwell, S. R.; Raushel, F. M.; Weiss, P. M.; Cleland, W. W. *Biochemistry* **1991**, *30*, 7444–7450. (c) Cleland, W. W. *Methods Enzymol.* in press. (d) Weiss, P. M. Heavy Atom Isotopes Effects Using the Isotope Ratio Mass Spectrometer. In *Enzyme Mechanisms from Isotope Effects*; Cook, P. F., Ed.; CRC Press: Boca Raton, FL, 1991; Chapter 11.

(7) Bourne, N.; Williams, A. *J. Org. Chem.* **1984**, *49*, 1200–1204.

(8) Hengge, A. C. *J. Am. Chem. Soc.* **1992**, *114*, 2747–2748.

A small portion of **3** was mixed with an appropriate quantity of the [^{14}N]-labeled compound so that the mixture contained close to the natural abundance of nitrogen-15. The mixture was then converted to the disodium salt by passing it through a column of Sephadex SP-C25 cation exchange resin which had been equilibrated with sodium phosphate at pH 7.5. The eluate was concentrated by rotary evaporation to a small volume, and the *p*-nitrophenyl phosphate was precipitated by addition of acetone, then collected by Buchner filtration.

Preparation of [^{15}N,nonbridge-$^{18}O_3$]-*p*-Nitrophenyl Phosphate (6). [^{18}O]Methanol (480 μL, 11.5 mmol) was added to a mixture of diethylphosphoramidous dichloride (1 g, 5.7 mmol) and triethylamine (1.3 g, 12.5 mmol) in 2 mL of dry THF in a flask under nitrogen, cooled in a dry ice–acetone bath. After the addition was complete (about 10 min), the reaction mixture was allowed to warm to room temperature and stirred for another 3 h. A solution of 5% aqueous sodium bicarbonate (5 mL) was added and the crude product extracted with ether (3 × 20 mL). The ether layers were dried with magnesium sulfate, and the solvent was removed by rotary evaporation. The product was distilled under reduced pressure using a water aspirator at 55 °C to yield **4** (780 mg, 4.6 mmol, 81%).

In 2 mL of THF, **4** (200 mg, 1.2 mmol) was dissolved followed by [^{15}N]-*p*-nitrophenol (170 mg, 1.2 mmol) and tetrazole (151 mg, 2.1 mmol). TLC showed the reaction to be complete after 10 min (product R_f = 0.6 eluting with methylene chloride). The reaction mixture was cooled in an ice bath. A mixture of collidine (285 μL), iodine (330 mg), and [^{18}O]water (150 μL) in 1.5 mL of dry THF was added by syringe. After 5 min, the ice bath was removed and the reaction mixture allowed to come to room temperature with stirring. After 10 min TLC showed oxidation to be complete. Twenty milliliters of 10% aqueous sodium bisulfite was added and the mixture extracted with ether, 2 × 30 mL. The ether layers were combined, dried over magnesium sulfate, and concentrated by rotary evaporation. The product was purified by flash chromatography using silica which had been dried for 12 h in a drying oven at 100 °C, eluting with a mixture of methylene chloride and ethyl acetate (20:1). The triester **5** was obtained as a crystalline solid (207 mg, 0.81 mmol, 67% yield from **4**).

The triester **5** was dissolved in 2 mL of carbon tetrachloride in an ice bath and treated with trimethylsilyl iodide (356 mg, 1.78 mmol). After 15 min, the reaction mixture was concentrated by rotary evaporation, then treated with 3 mL of dry methanol and stirred for 20 min in an ice bath. After the methanol was removed by rotary evaporation, 5 mL of water was added and the solution treated with cyclohexylamine to pH 9. The crude product was lyophilized and recrystallized twice from ethanol to give **6** (160 mg, 0.38 mmol, 47%). Mass spectral analysis (FAB) of the product showed it to consist of 84% [$^{18}O_3$] and 16% [$^{18}O_2$] material.

A small portion of the compound was mixed with an amount of the [^{14}N]-labeled compound as described for the bridge-labeled material, then similarly converted to the disodium salt.

Isolation of Molecular Nitrogen for Isotopic Analysis. Combustion was used to convert the nitrogen in the sample to be analyzed to N_2. In a 25-cm quartz tube (7-mm i.d., 9-mm o.d.) sealed at one end was added a sample containing at least 30 μmol of nitrogen, either as a solid or evaporated from ether in the tube. This was followed by 10 g of CuO wire (previously baked at 850 °C for 1 h), 0.5 g of copper powder (previously heated at 550 °C for 15 min under an atmosphere of hydrogen and cooled to room temperature), and a 4-mm square piece of silver foil to trap halogens. The tube was evacuated under high vacuum, sealed, and heated to 850 °C for 2 h, then cooled over 1 h to 550 °C and held at that temperature for 8 h. After cooling, the tube was cracked open on the vacuum line, and the nitrogen gas was separated by using a dry ice–isopropyl alcohol trap to remove water and a liquid nitrogen trap to remove CO_2. The nitrogen was then adsorbed on molecular sieves in a tube sealed with a vacuum stopcock and cooled in liquid nitrogen (the sieves were previously heated for several hours under vacuum at 200 °C to free them from nitrogen or other adsorbed gases, then cooled and stored under vacuum). The sample tube containing the sieves was then heated to 200 °C to release the nitrogen for analysis.

Isotope Effect Determinations. Monoanion Hydrolysis. Approximately 100 μmol of *p*-nitrophenyl phosphate was dissolved in 50 mL of water in a round-bottom flask and titrated to pH 3.5 with sulfuric acid. The flask was sealed with a septum stopper and heated to 95 °C, where the substrate has a half-life of about 1 h. After partial hydrolysis, the reactions were stopped by cooling to room temperature, where no detectable hydrolysis occurs during the time needed for assays and product isolation. The amount of *p*-nitrophenol released was assayed at 400 nm by adding an aliquot of the reaction mixture to 0.1 N NaOH. The *p*-nitrophenol in the reaction mixtures was separated by ether extraction (3 × 50 mL); after it was dried over magnesium sulfate, the ether was removed by rotary evaporation. The *p*-nitrophenol was further purified by vacuum sublimation at 95 °C and transferred to a quartz tube for combustion. The nitrogen gas produced by the combustion was analyzed by isotope ratio mass spectrometry.

The aqueous layers containing the remaining substrate were subjected to the same hydrolysis conditions for 10 half-lives and assayed, in order to determine the exact fraction of reaction at the time the reaction was first stopped. The *p*-nitrophenol produced from the remaining substrate was isolated and treated as described.

Dianion Hydrolysis. These reactions were run at 2 mM substrate in 50 mL of 100 mM sodium carbonate buffer, pH 10, at 95 °C in septum-sealed flasks. The half-life for this reaction is about 8 h; after partial hydrolysis, the reactions were stopped by cooling to room temperature and assayed for *p*-nitrophenol as described above for the monoanion reaction. After acidifying to pH 4, the *p*-nitrophenol was isolated by ether extraction and purified as described above. The aqueous layers were heated to 95 °C at pH 3.5 for 12 h to completely hydrolyze the remaining substrate and assayed, and the *p*-nitrophenol produced from the remaining substrate was isolated.

Reaction of the Dianion in *tert*-Butyl Alcohol. *p*-Nitrophenyl phosphate (1.6 g) was converted to the diacid form by being passed through a column (100 mL) of Dowex 50W-X8 in the proton form. After lyophilization, the substrate was further dried just before use by warming in an Abderhalden drying apparatus under vacuum. The conditions used for generating the dianion in *tert*-butyl alcohol were those of Friedman et al.[9] All steps were conducted in a glovebox under anhydrous conditions. About 100 μmol of the diacid form of the substrate was dissolved in 5 mL of dry *tert*-butyl alcohol; the resulting 20 mM solution was treated with 2.2 equiv of tetra-*n*-butylammonium hydroxide. The solution was heated to 30 °C for 12 h, which was the approximate half-life for reaction. Reactions were then stopped by cooling and addition of 25 mL of water. For determination of the fraction of reaction, a 0.5-mL aliquot was removed and diluted in 1 mL of 1 M formate buffer (pH 3.5); this solution was assayed for *p*-nitrophenol by adding an aliquot to 3 mL of 0.4 N NaOH and measuring the absorption at 400 nm. The 1.5-mL samples were then subjected to conditions to completely hydrolyze remaining *p*-nitrophenyl phosphate (95 °C for 10 h), then assayed again. The reaction mixtures were concentrated by rotary evaporation to a volume of about 10 mL, to remove the *tert*-butyl alcohol. The *p*-nitrophenol was then isolated as described for the previous isotope effect experiments. The aqueous layers, after removal of dissolved ether by brief rotary evaporation, were treated with approximately 3 units of alkaline phosphatase at pH 9 and allowed to stand at room temperature overnight. The *p*-nitrophenol thus produced from residual substrate was then isolated by ether extraction, as usual, and prepared for isotopic analysis.

Alkaline Phosphatase Experiments. The isotope effect experiments with alkaline phosphatase were run at pH 8 and also at pH 6 using HEPES and MES buffers. Reaction concentrations were 100 mM buffer, 2 mM substrate, and 1 mM Zn^{2+} and Mg^{2+}. Reactions were initiated by addition of 0.15 units of enzyme, and progress was monitored by periodic spectrophotometric assays at 400 nm. When the fraction of reaction had reached at least one-third, the reaction was stopped by titration of the solution to pH 3, and after addition of an aliquot to formate buffer for assay (as described previously), the *p*-nitrophenol product was isolated by ether extraction and the residual substrate in the aqueous fraction was completely hydrolyzed at pH 3.5 at 95 °C. The *p*-nitrophenol liberated from the residual substrate was then similarly isolated, and both samples were prepared for combustion and isotopic analysis.

Data Analysis. The isotope effects were calculated using the isotopic ratios in the product at partial reaction (R_p), in the remaining substrate (R_s), and in the starting material (R_0). Equation 4 was used to calculate the observed isotope effect from the isotopic ratios of the product and starting material at known fractions of reaction, f. Equation 5 was used to calculate the observed isotope effect from the isotopic ratios of the residual substrate and the starting material.

$$\text{isotope effect} = \log(1-f)/\log\,(1-f(R_p/R_0)) \qquad (4)$$

$$\text{isotope effect} = \log(1-f)/\log\,[(1-f)(R_s/R_0)] \qquad (5)$$

(9) Friedman, J. M.; Freeman, S.; Knowles, J. R. *J. Am. Chem. Soc.* **1988**, *110*, 1268–1275.

Table 1. Isotope Effects for Reactions of *p*-Nitrophenyl Phosphate[a]

reaction (t, °C)	^{15}k	$^{18}k_{bridge}$	$^{18}k_{nonbridge}$
monoanion (95)	1.0004 ± 0.0002	1.0087 ± 0.0003	1.0184 ± 0.0005
dianion (95)	1.0028 ± 0.0002	1.0189 ± 0.0005	0.9994 ± 0.0005
dianion in *tert*-butyl alcohol (30)	1.0039 ± 0.0003	1.0202 ± 0.0008	0.9997 ± 0.0016
alkaline phosphatase, pH 8 (22)	1.0003 ± 0.0002	1.0003 ± 0.0004	0.9982 ± 0.0001
alkaline phosphatase, pH 6 (22)	1.0003 ± 0.0003	1.0005 ± 0.0002	0.9994 ± 0.0005[b] 0.9979 ± 0.0005[c]

[a] Uncertainties reported are the standard errors. [b] Observed O-18 isotope effect. [c] Isotope effect after correction for deprotonation; see text.

The isotopic ratio of the starting material was determined from nitrogen obtained from combustion of samples of the *p*-nitrophenyl phosphate substrate and, as a control, by completely hydrolyzing samples of the substrate and analyzing the nitrogen obtained from the *p*-nitrophenol product, which was isolated using the techniques described for the isotope effect experiments. The isotopic ratios from both methods were the same within experimental error. This shows that no isotopic fractionation occurs during the procedures used to recover *p*-nitrophenol.

The oxygen-18 isotope effects were measured using the mixed double-labeled substrates. These experiments yield an observed isotope effect which is the product of the effect due to nitrogen-15 substitution and that due to the oxygen-18. The observed effects from these experiments were corrected for the nitrogen-15 effect and for incomplete levels of isotopic incorporation in the starting material.[6b]

Results

The isotope effect results are tabulated in Table 1. The oxygen-18 isotope effects have been corrected for the nitrogen-15 effect and for isotopic incorporation as described. At least six experiments were run for each set of conditions, which yields 12 determinations for each isotope effect. The effects calculated from R_p and R_0, and from R_s and R_0, gave excellent agreement in all cases and together were averaged to give the mean values recorded in the table, along with the standard errors.

Because the true substrate for alkaline phosphatase is the dianion of *p*-nitrophenyl phosphate, the observed value for $^{18}k_{nonbridge}$ at pH 6 must be corrected for the isotopic fractionation in the proportion of the substrate present as the dianion. This is done by dividing the observed effect of 0.9994 by the appropriate portion of the oxygen-18 equilibrium isotope effect on deprotonation. Using a pK of 4.96 for *p*-nitrophenyl phosphate,[7] then 0.9994 is divided by 10% of the equilibrium effect of 1.0154, assuming the effect for deprotonation is the same as that found for glycerol-3-phosphate.[10] Thus, 0.9994 is divided by 0.1(1.0154 − 1) + 1, to give a final value of 0.9979.

In our hands, the phosphoryl transfer from the dianion of *p*-nitrophenyl phosphate in neat *tert*-butyl alcohol at 30 °C took place with a half-life somewhat longer than that previously reported, 12 h versus 4.[9,11] In order to determine if the slower rate was due to the presence of adventitious water, great precautions were taken, including performing the reaction in a glovebox which had been carefully dried as well as doing the reaction in the presence of added activated molecular sieves. Analysis by ^{31}P NMR showed the reactions to be very clean, containing only residual *p*-nitrophenyl phosphate, and the products *tert*-butyl phosphate and inorganic phosphate in a ratio of about 20 to 1; the latter product results from phosphoryl transfer to water. This is a smaller proportion of inorganic phosphate relative to *tert*-butyl phosphate than previously reported,[9] which means it is unlikely that the presence of water is responsible for the slower rate of this reaction in our hands. In the course of generating the dianion from the acid form of the monoester, using 2 equiv of tetra-*n*-butylammonium hydroxide, 2 equiv of water are formed. In order to eliminate even this source of water, tetra-*n*-butylammonium *tert*-butoxide was used as the base to generate the dianion, but the rate of reaction was unaffected.

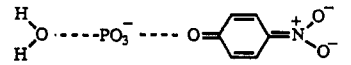


Figure 1. One possible resonance structure of the transition state for the reaction of the dianion of *p*-nitrophenyl phosphate, illustrating the delocalization of charge into the nitrophenolate anion induced by close proximity to the anionic phosphoryl group.

Discussion

Measurement of kinetic isotope effects can give a quantitative analysis of transition-state structure. The $^{18}k_{bridge}$ effects[6b,12] and the $^{18}k_{nonbridge}$[10] and ^{15}k effects[6a] have been determined for several phosphoryl-transfer reactions involving phosphate monoesters, diesters, and triesters. This method can give information about the transition-state bonding in both the leaving group and the central phosphoryl group that is not obtainable by other methods.

Calculations have shown that, for nitrogen and sulfur leaving groups, the magnitude of the kinetic isotope effect increases linearly with the fraction of bond cleavage.[13] A similar relationship is thought to hold for oxygen as the leaving group.[14] Thus the primary oxygen-18 isotope effect should give a reliable indication of the degree of transition-state bond cleavage, although caution must be used in interpreting primary isotope effects solely in terms of changes in bond order since reaction coordinate motion also contributes to primary isotope effects. The ^{15}k effect has been shown to be a useful probe of transition-state bond cleavage in reactions with *p*-nitrophenol as the leaving group.[4] This effect measures the extent of delocalization into the aromatic ring of the partial negative charge resulting from partial cleavage of the bond to the leaving group in the transition state. The $^{18}k_{nonbridge}$ effects in the nonbridge oxygen atoms of the phosphoryl group probe the changes undergone in the phosphoryl group involved in these reactions.

One possible yardstick for interpreting the magnitudes of the primary $^{18}k_{bridge}$ effects is the reported equilibrium effect $^{18}K_{eq}$ on deprotonation of *p*-nitrophenol, 1.0181 ± 0.0019.[15] However, this reaction involves breaking a bond to a proton, not to a phosphoryl group. An indication that these are not equivalent is the finding that an aryl group in a phosphate monoester has an effective charge of +0.36 as measured by Brønsted correlations.[7] This arises from the greater electron-withdrawing power of the phosphoryl group compared to a proton. The reaction of the dianion is a good model for what the magnitude of the $^{18}k_{bridge}$ effect should be for complete bond cleavage, since this reaction is known from Brønsted studies to have a very dissociative transition state with nearly complete bond cleavage to the leaving group. The effect we find (Table 1) for the aqueous reaction is very close to that previously reported on the aqueous hydrolysis of 2,4-dinitrophenyl phosphate, 1.020 ± 0.004, which was determined by direct spectrophotometric methods.[12] Complete loss of the P–O stretch in the transition state for that reaction resulted in a calculated maximum oxygen-18 primary isotope effect of 4.5%.[12] However, delocalization of the partial negative charge on the leaving group will cause some increase in C–O bond order and compensate for some of the loss of O–P bond order (Figure 1). Evidence for such delocalization is seen in the magnitude of ^{15}k, which is larger for the hydrolysis reaction

(10) (a) Knight, W. B.; Weiss, P. M.; Cleland, W. W. *J. Am. Chem. Soc.* **1986**, *108*, 2759–2761. (b) Weiss, P. M.; Knight, W. B.; Cleland, W. W. *J. Am. Chem. Soc.* **1986**, *108*, 2761–2762.

(11) This reaction has also been reported to proceed with a half-life of 5 h at 35 °C, somewhat closer to our observed rate. See: Ramirez, F.; Marecek, J. F. *Pure Appl. Chem.* **1980**, *52*, 1021.

(12) Gorenstein, D. G.; Lee, Y.-G.; Kar, D. *J. Am. Chem. Soc.* **1977**, *99*, 2264–2267.

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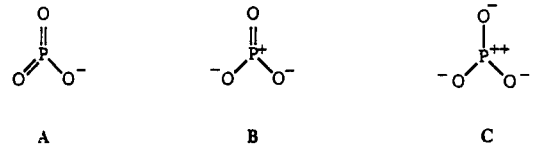

A B C

Figure 2. Possible resonance structures for metaphosphate ion.

(1.0028) than $^{15}K_{eq}$ for the equilibrium deprotonation of *p*-nitrophenol, which is 1.0023.[4] The close proximity in the transition state of the departing phenolate anion and the negatively charged phosphoryl group probably enhances the delocalization, accounting for the greater magnitude of ^{15}k. Thus, the observed 1.89% value for $^{18}k_{bridge}$ should represent nearly complete bond cleavage to the leaving group.

Before comparing the isotope effect data for the reactions of the dianion in *tert*-butyl alcohol and in water, correction must be made for the different temperatures of the two reactions, 30 and 95 °C, respectively. The isotope effects for the aqueous reaction at 30 °C are calculated to be ^{15}k = 1.0034, $^{18}k_{bridge}$ = 1.0230, and $^{18}k_{nonbridge}$ = 0.9993.[16] These are not too different from those observed for the reaction in *tert*-butyl alcohol, indicating similar transition-state structures for these reactions.

The $^{18}k_{nonbridge}$ values for the reactions of the dianion in water and in butanol are both unity within experimental error, a result which is surprising in view of the classical picture of metaphosphate. This species is usually depicted as structure A in Figure 2. In the late transition state for these reactions, the phosphoryl group should resemble metaphosphate and, if this species possesses the bonding as shown in A, calculations predict an isotope effect of about 2% inverse (0.980) resulting from the increased bond order between the nonbridge oxygen atoms and the phosphorus atom.[10b] Recent computational work has challenged the classical representation of metaphosphate and suggests that a more accurate picture is that of a hybrid of structures B and C.[17] The transition state for this reaction is known from Brønsted studies to be highly dissociative, with the bond to the leaving group nearly completely broken and only a small degree of compensatory bond formation to the nucleophile. In order to maintain the total bond order to phosphorus, the metaphosphate-like transition state has usually been depicted with increased bond order to the phosphoryl oxygen atoms. The $^{18}k_{nonbridge}$ effects of unity indicate that the revised view of the structure of metaphosphate is a more accurate representation and support an earlier conclusion that the total bond order to phosphorus is not conserved in the transition state in the dianion reaction.[10b]

The Monoanion Reaction. The isotope effect data for the hydrolysis of the monoanion are consistent with concerted proton transfer and cleavage of the phosphorus–oxygen bond, as in eq 3b, the mechanism predicted by Kirby and Varvoglis for the monoanion reaction with leaving groups less basic than phenol.[1b] The small normal ^{15}k value indicates that proton transfer lags slightly behind P–O bond cleavage. The magnitude of $^{18}k_{nonbridge}$ suggests nearly complete transfer of the proton from the nonbridge oxygen atom in the transition state. Relevant data for comparison are the equilibrium oxygen-18 isotope effects for deprotonation of the glycerol-3-phosphate and dihydrogen phosphate monoanions, which are 1.0139 and 1.0154 at 67 and 65 °C, respectively.[10a] The value for $^{18}k_{bridge}$ should be reduced by protonation, which would compensate somewhat for the loss of the P–O stretch due to partial cleavage of this bond. The measured effect is still about one-half as large as that for the dianion reaction, where cleavage of the bond to the leaving group is nearly complete. This indicates that P–O bond cleavage is also well advanced in the dissociative transition state for this reaction.[18]

Alkaline Phosphatase. Alkaline phosphatase is an excellent catalyst for the hydrolysis of phosphate monoesters. The catalytic rate constant k_{cat} represents rate-limiting hydrolysis of the phosphoenzyme intermediate at pH < 7 or rate-limiting release of inorganic phosphate from the active site at pH > 7.[19] The competitive method measures isotope effects on V/K_m, which will be sensitive to steps up to and including the first irreversible step, which is loss of the leaving group from the *p*-nitrophenyl phosphate substrate. Measurements were made at pH 8, which is at the pH optimum, and at pH 6, where chemistry may be more rate-limiting.

The near-unity values found for the isotope effects, especially for $^{18}k_{bridge}$, which ought to be large, argue that a nonchemical step such as substrate binding or a conformational change is rate-limiting, consistent with recently reported kinetic results.[20] The possibility that diffusion of *p*-nitrophenolate from the active site limits catalysis is ruled out since in this case one should observe the equilibrium primary isotope effect for partitioning between *p*-nitrophenyl phosphate and *p*-nitrophenolate. The magnitude of this effect should be similar to that for partitioning between *p*-nitrophenol and *p*-nitrophenolate (1.0181).

Conclusions. The phosphoryl-transfer reactions of the dianion of *p*-nitrophenyl phosphate in water, where the reaction is concerted, and in *tert*-butyl alcohol, where reaction proceeds through a free metaphosphate intermediate, have similar, late transition state structures. The isotope effects in the nonbridge oxygen atoms suggest that there is little or no increase in P–O bond order for these atoms. Whatever small increase in bond order does occur is evidently barely sufficient to compensate for the loss of bending and torsional vibrational modes between the nonbridge oxygen atoms and the leaving group. This supports a view of metaphosphate as a dipolar structure with relatively weak double bonding to the phosphorus atom. The data for the aqueous hydrolysis of the monoanion also indicate a highly dissociative transition state and are consistent with a predicted mechanism where proton transfer to the leaving group is concerted with cleavage of the P–O bond for the *p*-nitrophenol leaving group. The rate of the alkaline phosphatase-catalyzed hydrolysis reaction is limited by a nonchemical step, such as substrate binding or a conformational change.

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(16) An approximation of the temperature effect on the isotope effects was made using the equation ln(IE at 30 °C) = (368 K/303 K) ln(IE at 95 °C).

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(18) Reviewers have raised the question of whether the isotope effect data can rule out a stepwise mechanism such as (3a). We feel that the ^{15}k isotope effect (1.0004 ± 0.0002) does rule out a stepwise mechanism, in which no normal ^{15}k effect would be expected. Though small, this effect is very reproducible experimentally.

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