Phosphoryl-Transfer Reactions of Phosphodiesters: Characterization of Transition States by Heavy-Atom Isotope Effects

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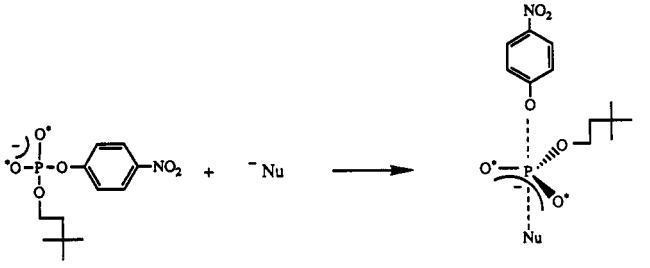
Abstract: The secondary ^{18}O and ^{15}N isotope effects have been measured for phosphoryl-transfer reactions of the phosphodiester 3,3-dimethyl *p*-nitrophenyl phosphate under conditions of acid and alkaline hydrolysis, cleavage by β-cyclodextrin, and cleavage by snake venom phosphodiesterase. Isotopic labeling and other experiments show that these reactions proceed by an $S_N2(P)$ mechanism. The secondary ^{18}O isotope effect measures changes in transition state bonding to the nonbridge oxygen atoms in the central phosphoryl group in these S_N2 reactions. The ^{15}N isotope effects measure transition-state bond cleavage to the leaving group *p*-nitrophenol. The isotope effect data indicate that weaker nucleophiles lead to a more associative transition state with respect to the central phosphoryl group (decreased bonding to the nonbridge oxygens) while the extent of bond cleavage to the leaving group is affected little. Comparison of isotope effect data from the attack of hydroxide on a phosphodiester monoanion with that of a neutral phosphotriester suggests the oxyanion in the diester may assist in expulsion of the leaving group.

Secondry ^{18}O isotope effects for the nonbridge oxygen atoms in phosphate esters can give information about the transition states in phosphoryl-transfer reactions.[1] These measurements can distinguish between associative, dissociative, and S_N2-like mechanisms. An associative mechanism like that which occurs with cyclic phosphotriesters where a phosphorane intermediate is formed results in decreased bond order to the nonbridge oxygen atoms in the transition state and hence gives a normal isotope effect. In a dissociative mechanism like that for monoester hydrolysis, bonding to the nonbridge oxygen atoms is increased in the transition state and an inverse isotope effect would be observed. In an S_N2 mechanism, if the bond order to the nonbridge oxygen atoms is unchanged in the transition state, an isotope effect of unity should be observed. Such might be the case if the total bond order between the phosphorus atom and the nucleophile and between the phosphorus atom and the leaving group were maintained at unity during the reaction. There is no a priori reason for this to be the case, and if for example bond formation were farther advanced in the transition state than bond cleavage, the transition state would begin to resemble that for the associative mechanism with a resultant decreased bond order to the nonbridge oxygens. In contrast, if the charge on the oxyanion of the phosphoryl group were assisting in expulsion of the leaving group, the resulting dissociative character of the transition state would result in increased bond order to the nonbridge oxygens and would manifest itself by an inverse isotope effect.

Phosphodiesters are known to undergo phosphoryl-transfer reactions by an S_N2 mechanism with inversion of configuration at phosphorus. The secondary ^{18}O isotope effects in the nonbridge oxygen atoms of a phosphodiester can give information about the change in bonding of these atoms to the central phosphorus atom in moving from ground state to transition state (Scheme I). This isotope effect can thus reveal changes in transition-state structure as the nucleophile or leaving group is changed in these S_N2 phosphoryl-transfer reactions if the transition state shifts along the associative–dissociative continuum.

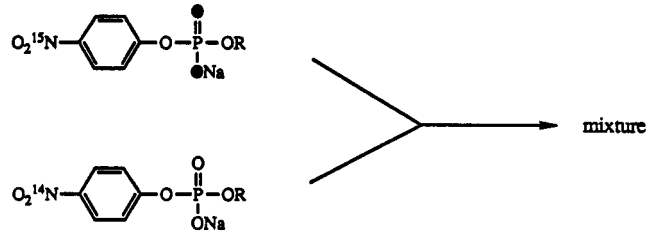
We have examined the secondary ^{18}O isotope effects in the nonbridge oxygen atoms on the reactions of the phosphodiester 3,3-dimethylbutyl *p*-nitrophenyl phosphate (**1**), which has *p*-nitrophenol as the leaving group, under conditions of acid and basic hydrolysis in solution, its reaction with β-cyclodextrin, and its enzymatic reaction with snake venom phosphodiesterase. In addition to the secondary ^{18}O isotope effects for these reactions, we have also measured the ^{15}N isotope effects in the nitrogen atom

Scheme I. Transition State for S_N2 Phosphoryl Transfer of Phosphodiester **1** (Nonbridge Oxygen Atoms Denoted by O*)



1

Scheme II



● = ^{18}O

of the *p*-nitrophenol leaving group. We have shown that this isotope effect gives a measurement of the degree of bond breaking to the leaving group in the transition state.[2] Taken together, the two isotope effect measurements give a good characterization of the transition-state structures for these reactions.

The isotope effects were measured by the competitive method by using an isotope ratio mass spectrometer to measure the change in isotopic composition over the course of the reaction. The isotope ratio mass spectrometer measures gases, so the atom of interest must be converted to N_2 in the case of nitrogen. The nitrogen in *p*-nitrophenol is easily converted into N_2 for subsequent isotopic measurement (see the Experimental Section for details). The secondary ^{18}O isotope effects in the nonbridge oxygen atoms were measured by the remote label method.[3] In this method, 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

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

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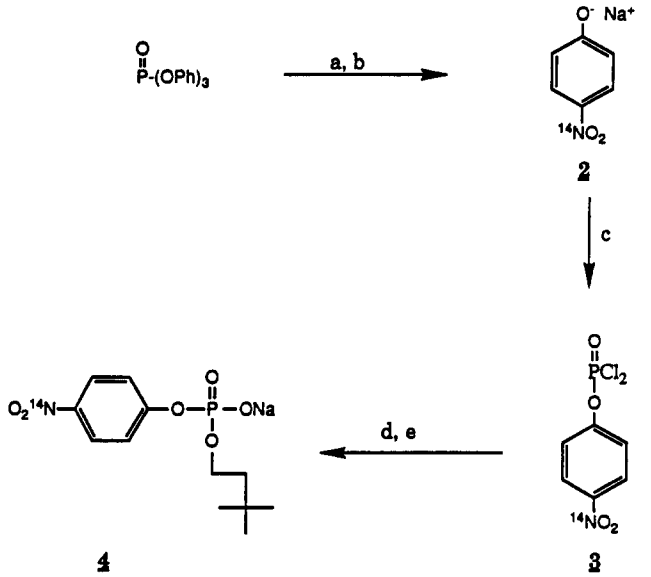
Scheme III[a]



[a] Key: (a) $^{14}NH_4{}^{14}NO_3$, sulfuric acid; (b) aqueous NaOH; (c) $POCl_3$; (d) 3,3-dimethylbutanol, pyridine; (e) H_2O, pyridine, ion exchange.

isolation and measurement and functions as a marker. In this work, the *p*-nitrophenyl phosphodiester was synthesized with the nitrogen atom of the nitro group as the remote label. Substrate labeled with ^{18}O in the two nonbridge oxygen atoms and with ^{15}N in the nitro group was synthesized, along with the corresponding $^{16}O,^{14}N$ compound (Scheme II). The two were then mixed in proportion to reconstitute the natural abundance of 0.37% ^{15}N in the nitro group, as the isotope ratio measurements are most accurate when the sample is close to natural abundance in ^{15}N. In the remote-labeled mixture, each ^{15}N in the nitro group is accompanied by two nonbridge ^{18}O atoms in the phosphoryl group. The isotope effect at the nonbridge oxygen atoms can then be measured from the change in isotopic composition in the nitro group. The observed isotope effect in this case is the product of ^{15}N and the ^{18}O isotope effects. Separate determination of the former allows calculation of the latter. No synthesis of labeled substrate is necessary for measurement of the ^{15}N isotope effect, which can be measured with use of the natural abundance substrate.[2]

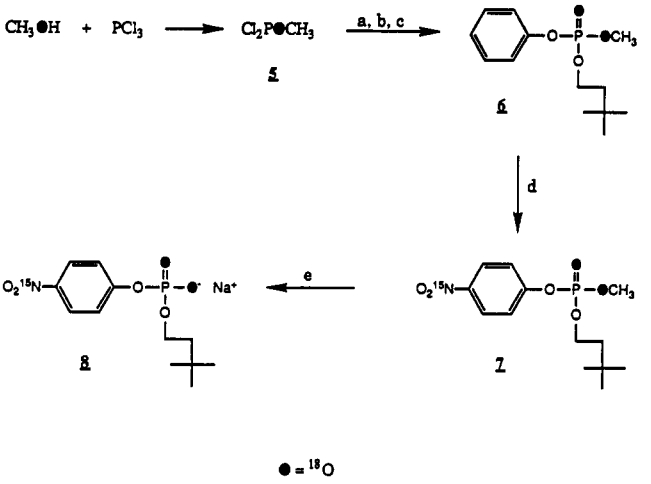
The synthetic routes for the preparation of the remote labeled substrates are shown in Schemes III and IV.

The phosphodiester 3,3-dimethylbutyl *p*-nitrophenyl phosphate (**1**) was originally chosen on the basis of its reaction with β-cyclodextrin.[4] In this cleavage reaction, the nucleophile is a deprotonated secondary hydroxyl group on the cyclodextrin, with a pK_a of about 12.[5] In the cleavage of the phosphodiester by snake venom phosphodiesterase, the nucleophile is an active-site threonine.[6] In the acidic and alkaline solution hydrolysis reactions, the nucleophilic species are water and hydroxide, respectively. Thus, this series of reactions allows us to test the effect of varying the nucleophile on the transition-state structures for these S_N2 phosphoryl-transfer reactions and to determine the participation, if any, of the nonbridge oxygen atoms in the phosphoryl group.

Experimental Section

Materials. Collidine and pyridine were distilled from calcium hydride and stored over molecular sieves. Phosphoryl chloride was distilled under nitrogen. Anhydrous dioxane and tetrahydrofuran were purchased from Aldrich and used as received. Anhydrous analytical reagent grade ethyl ether (Mallinckrodt) was distilled at 34–35 °C to remove higher boiling impurities shortly before use. β-Cyclodextrin was recrystallized from

Scheme IV[a]



● = ^{18}O

[a] (a) collidine, phenol; (b) 3,3-dimethylbutanol; (c) iodine, $H_2{}^{18}O$; (d) $NH_4{}^{15}NO_3$, sulfuric acid; (e) sodium iodide.

water. Methanol-^{18}O and $H_2{}^{18}O$ were purchased from MSD Isotopes. $^{14}NH_4{}^{14}NO_3$ and $NH_4{}^{15}NO_3$ were purchased from Isotec. Other chemicals were of reagent grade (Aldrich) and were used as received. Snake venom phosphodiesterase (phosphodiesterase I, Type II) was purchased as a lyophilized powder from Sigma and used as received. Mercury sulfate solution used in Kjeldahl digestions was made by diluting 24 mL of concentrated sulfuric acid to 200 mL with water and then dissolving 20 g of mercuric oxide.

Preparation of Natural Abundance 3,3-Dimethylbutyl *p*-Nitrophenyl Phosphate (1). *p*-Nitrophenyl phosphorodichloridate (767 mg, 2.99 mmol) was dissolved in 4.5 mL of dry dioxane in a flame-dried 100-mL two-necked flask. One neck of the flask was connected to a nitrogen bubbler, and the other was fitted with a septum cap. Dry pyridine (484 μL, 6.0 mmol) was added via syringe followed by 255 μL (2.1 mmol) of 3,3-dimethyl-1-butanol. The reaction mixture was stirred at room temperature for 2 h, during which time a precipitate of pyridinium salt formed. The reaction mixture was filtered through a sintered glass funnel tube with nitrogen pressure into a 100-mL round-bottom flask, and a mixture of 3 mL of distilled water and 600 μL of pyridine was added followed by stirring for 15 min. The mixture was concentrated to an oil by rotary evaporation and then partitioned between water and methylene chloride. The aqueous layer was extracted a second time with methylene chloride; the organic layers were combined, dried over magnesium sulfate, and concentrated to a viscous oil by rotary evaporation. The diester was purified by anion exchange using DEAE A-25 resin equilibrated with triethylammonium bicarbonate (TEAB) buffer at pH 7.5. After equilibration, the column was washed with 10 column volumes of deionized water. The crude diester was then diluted to 20 mM and loaded onto the column, followed by washing with two column volumes of water, and then eluted with a linear gradient of TEAB buffer at 0–0.3 M. Fractions of 25 mL were collected and monitored at 300 nm. The product-containing fractions were pooled and concentrated in vacuo. The diester was converted to the sodium salt by passage through a 5-fold excess of Sephadex SP-C25 cation-exchange resin. The resin was equilibrated with sodium acetate at pH 5.5 and then washed with 10 column volumes of deionized water, followed by one column of 25% ethanol in water. The oily pyridinium salt of the diester was made up to 20 mM in 25% ethanol in water and passed through the column, followed by one column volume of the ethanol/water mixture. The eluent was concentrated by rotary evaporation to approximately 75 mL and then lyophilized to yield the pure diester sodium salt (512 mg, 1.6 mmol, 76%). Proton NMR confirmed the complete removal of pyridinium ions. ^1H NMR (500 MHz, D_2O): δ 8.28 (d, J = 8.5 Hz, 2 H); 7.36 (d, J = 8.5 Hz, 2 H); 4.07 (overlapping dt, J = 7.5 and 7.5 Hz, 2 H); 1.58 (t, J = 7.5 Hz, 2 H); 0.90 (s, 9 H). ^{31}P NMR (202 MHz, D_2O): δ −2.55 (t, J = 7 Hz).

Preparation of [^{14}N]Nitrophenolate (2). A 20-g (76-mmol) portion of triphenyl phosphate was dissolved in 150 mL of concentrated sulfuric acid cooled in an ice bath. Eighteen grams (228 mmol) of ammonium nitrate ($^{14}NH_4{}^{14}NO_3$, 99.9% ^{14}N; Isotec) was added in portions over 30 min. The mixture was stirred for an additional 30 min and then carefully poured over crushed ice. When the ice had melted, the precipitated product was collected by filtration with a Buchner funnel and washed with water. The nitrated triester product was divided into two equal portions, and each was added to 350 mL of 1 N NaOH and stirred at 90 °C for 40 h. The reaction mixture was allowed to cool to room

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temperature and then cooled further in an ice bath. The sodium salt of [^{14}N]-*p*-nitrophenol that precipitated was collected by filtration through a Buchner funnel and washed with several small portions of ice-cold water. Proton NMR of the product showed it to be clean *p*-nitrophenol with no detectable meta-nitrated or dinitrated product. The sodium nitrophenolate from the above procedure was dried over P_2O_5 at 100 °C in a Abderhalden drying apparatus. During drying, the nitrophenolate salt color changed from yellow to dark orange.

Preparation of [^{14}N]-*p*-Nitrophenyl Phosphorodichloridate (3).[7] A 125-mL portion of $POCl_3$ in a 500-mL round-bottom flask under N_2 was cooled in an ice bath. A 6.4-g (39.7-mmol) sample of dry sodium *p*-nitrophenolate was added in small portions over 30 min. After addition was complete, the ice bath was removed and stirring stopped, to allow the NaCl precipitate to settle. The supernatant was poured off into a second dried 500-mL flask. The NaCl remaining was washed with two 30-mL portions of $POCl_3$ and similarly decanted. The excess $POCl_3$ was distilled away under vacuum at 40–45 °C. The product was then isolated by Kugelrohr distillation under vacuum. The product distills at around 128 °C; the first small portion to distill over, along with a small amount of material that distilled at lower temperature, was discarded and a new receiving flask attached to receive the product. The product (4.7 g, 18.3 mmol, 46%) was recovered as a pale yellow oil, which crystallized upon leaving in the freezer (in a desiccator over P_2O_5) overnight. **It is important when distilling this material to use a Kugelrohr or similar ultrashort-path distillation apparatus to avoid explosive decomposition as alluded to in ref 7.**

Preparation of [^{14}N]-3,3-Dimethylbutyl *p*-Nitrophenyl Phosphate (4). This was prepared by the same method as described above for the natural abundance compound, but with [^{14}N]-*p*-nitrophenyl phosphorodichloridate.

Preparation of [^{18}O]Methyl Dichlorophosphite (5).[8] A 2.16-mL (24.7-mmol) portion of PCl_3 in a 50-mL round-bottom flask attached to a nitrogen bubbler was cooled to −78 °C in a dry ice–acetone bath. One milliliter of [^{18}O]methanol was added slowly via syringe through a rubber septum cap. After addition was complete (approximately 3 min), the reaction mixture was removed from the cooling bath and allowed to come to room temperature. The crude product was distilled under nitrogen at 90 °C with a short-path distillation head into a flame-dried 5-mL flask.

Preparation of [^{18}O,^{15}N]-3,3-Dimethylbutyl *p*-Nitrophenyl Phosphate (8). In a dried, two-neck round-bottom flask were cooled 10 mL of anhydrous tetrahydrofuran and 1.7 mL of dried collidine to −78 °C under nitrogen with a dry ice–acetone bath. [^{18}O]Methyl dichlorophosphite (291 μL, 3.14 mmol) was added dropwise over 3 min. After another 2 min a solution of phenol (274 mg, 2.91 mmol) in 10 mL of THF was added dropwise via syringe over 10 min. The resulting mixture was stirred for 25 min, and then a mixture of 3,3-dimethyl-1-butanol (366 μL, 3.0 mmol) and 10 mL of THF was added dropwise via syringe. After being stirred for 30 min, the reaction mixture was allowed to warm to room temperature. Iodine (813 mg, 3.2 mmol) was dissolved in 5 mL of THF with 0.6 mL of [^{18}O]water. The iodine solution was added to the reaction mixture dropwise and the resultant mixture stirred an additional 15 min. The solvent was removed under reduced pressure; 15 mL of a saturated sodium bisulfite solution was added, and the mixture was extracted with methylene chloride (3 × 25 mL). The combined organic layers were dried over magnesium sulfate and concentrated by rotary evaporation. The triester **6** was purified by flash chromatography on silica gel eluting with a 5/1 mixture of ether/cyclohexane to yield 345 mg (1.26 mmol, 44% from **5**).

The triester **6** was dissolved in 0.6 mL of concentrated sulfuric acid and the solution cooled in an ice bath. A solution of $NH_4{}^{15}NO_3$ (154 mg, 1.87 mmol) in 0.6 mL of concentrated sulfuric acid was added. After 25 min, approximately 10 g of crushed ice was added. After most of the ice had melted, the aqueous material was extracted with ether (3 × 25 mL). The ether layers were combined, dried over magnesium sulfate, and concentrated in vacuo. The nitrated triester **7** was chromatographed as described above.

The triester **7** was dissolved in 1.8 mL of dry acetone in a dry 10-mL round-bottom flask under nitrogen. Sodium iodide (105 mg, 0.7 mmol) was added and the reaction mixture heated to reflux for 1 h. After cooling, the solvent was removed in vacuo and the crude diester was purified by anion exchange on DEAE A-25, eluting with a gradient of 0–0.3 M triethylammonium bicarbonate buffer at pH 7.5 (as described above for the ^{14}N-labeled diester). Subsequent cation exchange provided the remote labeled diester **8** as the sodium salt (220 mg, 50% from **6**).

Determination of Oxygen-18 Incorporated into the Remote-Labeled Diester. A 10-mg sample of the sodium salt of the remote labeled diester **8** was dissolved in 1 mL of D_2O in a 5-mm NMR tube. This gave a sufficiently high concentration to afford a high-quality proton-decoupled ^{31}P spectrum with a single acquisition. The spectrum consisted of two signals, a larger more upfield peak for the doubly ^{18}O-labeled compound and a smaller peak 0.033 ppm downfield for the singly ^{18}O-labeled material. No unlabeled diester was detectable. The identity of the NMR signals was confirmed by adding a few milligrams of natural abundance diester, which resulted in the appearance of a new peak downfield from that of the singly ^{18}O-labeled compound. Integration of the two peaks from the remote-labeled diester showed it to consist of 92% doubly ^{18}O-labeled and 8% singly-^{18}O labeled material, or 96% ± 1% total incorporation.

Mixing of the ^{14}N and ^{15}N Remote-Labeled Material. Quantities of the labeled diesters **4** and **8** were weighed and mixed in proportions calculated to duplicate the 0.37% natural abundance of ^{15}N. To assure complete mixing, the mixture was dissolved in water and then lyophilized. The isotope ratio δ value for the diester mixture was 2.975. For the sake of comparison, the "natural abundance" diester synthesized from the commercially obtained *p*-nitrophenyl phosphorodichloridate gave δ 1.209. The δ values obtained from the isotope ratio mass spectrometer are related to the isotopic ratio R by the equation $R = 1 + \delta/1000$. R is the ratio of the $^{15}N/^{14}N$ ratio in the sample to that of the natural abundance nitrogen gas standard. Thus, a sample that is exactly at natural abundance in ^{15}N will have δ 0; a sample with twice the natural abundance of ^{15}N will have δ 1000.

General Procedures for Isotope Effect Determinations. Each of the isotope effect reactions involved isolation and ^{15}N isotopic measurement of *p*-nitrophenol. Reaction mixtures were typically made close to 2 mM in diester substrate with a total volume of 50 mL. After the reactions had run to about 50% completion, the reaction was stopped and the amount of *p*-nitrophenol produced was assayed at 400 nm. The reaction mixtures were acidified as necessary and the *p*-nitrophenol extracted with distilled diethyl ether until the ether extracts no longer showed the presence of *p*-nitrophenol when assayed at 400 nm. The ether extracts were dried over magnesium sulfate and concentrated to dryness.

The aqueous fractions containing the residual substrate were subjected to rotary evaporation to remove dissolved ether, and the residual substrate was completely hydrolyzed. Complete hydrolysis was assured by using reaction times of at least 10 times the half-life for given reaction conditions. The *p*-nitrophenol produced was then assayed and collected as before. The two respective assays were used to calculate the fraction of reaction. The total amount of *p*-nitrophenol produced corresponded in all reactions to that expected on the basis of the known weight of starting material used.

The *p*-nitrophenol samples were subjected to reduction followed by Kjeldahl digestion. Prior reduction is necessary in order to completely recover nitrogen from nitro compounds. A reduction procedure using sodium thiosulfate in sulfuric acid was used. It is important that the samples be dry for the reduction to be successful. Five milliliters of concentrated sulfuric acid was added to the sample in a Kjeldahl flask and swirled until the sample dissolved. Dry sodium thiosulfate was added (75 mg), and the flask was heated in an oil bath at 140 °C for 15 min. The reaction mixture turns from yellow to dark brown during this time. The flask was then heated over a Bunsen burner for an additional 3–4 min. After cooling, potassium sulfate (1.5 grams) and mercury sulfate solution (1.5 mL) were added and the samples subjected to Kjeldahl digestion. The ammonia so produced was isolated by alkaline steam distillation and oxidized to N_2 with hypobromite for isotope ratio analysis. The procedures and equipment used for these steps have been described.[9]

The isotope effects were calculated using the isotopic ratios from the product at partial reaction (R_p), from the residual substrate (R_s), and from the starting material (R_0). Measuring the isotopic ratios of both the residual substrate and product allows two independent calculations of the isotope effect from each experiment, one using R_p and R_0 and one using R_s and R_0.[10]

The specific reaction conditions used for each isotope effect experiment are described below. All isotope effect reactions were run in triplicate, and at least two triplicate sets were performed for each set of conditions. In a control experiment the isotopic ratio in the starting material was determined by completely hydrolyzing samples of the diester substrate and analyzing the product *p*-nitrophenol, and separately by analyzing unreacted substrate. The isotopic ratios were identical within experimental error. This demonstrates that no fractionation occurs during the procedures involved in isolation of the product material.

This control is also necessary to establish that no contaminant is picked up during the product isolation process that can be carried along through

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the subsequent procedures and cause errors in the isotopic ratio measurements. The isotope ratio mass spectrometer measures the mass ratio 29/28, so any species producing fragments with these masses (especially 29) will interfere with accurate measurement of the isotopic ratio. We have encountered this problem if the ethyl ether used for the extractions of *p*-nitrophenol is not distilled before use. Small amounts of a high-boiling contaminant remain even after rotary evaporation and vacuum drying and survive the Kjeldahl, steam distillation, and oxidation procedures and cause spuriously high δ values by giving rise to mass 29 fragments, probably ethyl radicals in the mass spectrometer.

We have noted similar interference from other compounds that can act as a source of ethyl fragments. It is thus desirable to have clean samples for the Kjeldahl procedure and to do control experiments to ensure that no procedures used in product isolation introduce error in the isotopic measurements. The best control experiment is that described above, where the isotopic content of the compound of interest, in the present case *p*-nitrophenol group, is measured separately in unreacted starting material and then from *p*-nitrophenol, which is isolated from completely hydrolyzed starting material. Any sources of error introduced in the product recovery procedures will show up as a disagreement between the two measurements.

Alkaline Hydrolysis. A 100-μmol sample of the diester in 50 mL of 1 N NaOH was stirred at 95 °C; under these conditions the half-life for hydrolysis was about 2.5 h. At this time the reaction mixture was cooled in an ice bath, assayed, and acidified to pH 4, and the *p*-nitrophenol was isolated as described above. The residual substrate solution was then made 1 N in hydroxide by addition of solid NaOH and stirred at 95 °C for 30 h, followed by assay and isolation of the liberated *p*-nitrophenol.

β-Cyclodextrin Reaction. A mixture of 100 μmol of the diester, 2.27 g of β-cyclodextrin, and 930 mg of KCl was brought up to a volume of 50 mL in 0.1 N NaOH, giving concentrations of 40 mM cyclodextrin and 2 mM substrate. The reaction mixtures were stirred at 45 °C for their half-life of about 12 h. In control experiments without the cyclodextrin present, less than 0.5% of the phosphodiester substrate was hydrolyzed under these conditions. The reaction was stopped by cooling to room temperature and titrating the reaction mixture to pH 5. The *p*-nitrophenol product was assayed and isolated as described above.

The slow rate of this reaction made it impractical to use the same reaction conditions to completely hydrolyze the residual substrate. Using harsher conditions (higher pH and higher temperatures analogous to those used in the hydroxide reaction) resulted in formation of cyclodextrin breakdown products that were difficult to separate from the *p*-nitrophenol and interfered with subsequent procedures. Accordingly, the isotope effects for this reaction were measured using the isotopic ratios of the product and the starting material only.

Snake Venom Phosphodiesterase. These reactions were run at 37 °C with 100 μmol of substrate and 330 μL of enzyme solution (containing 0.24 unit of enzyme/mL) in a total volume of 50 mL at 100 mM of TAPS buffer, pH 8.0. Since this enzyme is reported to be activated by magnesium, two sets of experiments were run, one with 25 mM magnesium and one set with no magnesium added. Without added magnesium this reaction had a half-life of about 14 h. At this point the reaction was cooled to room temperature and titrated to pH 4. The *p*-nitrophenol product was assayed and isolated by extraction as previously described. The residual substrate was completely hydrolyzed by making the aqueous solution 1 N in hydroxide by addition of solid NaOH and heating to 95 °C for 30 h. The *p*-nitrophenol produced from the residual substrate was then assayed and isolated as usual.

The reactions with the added magnesium were about 1.5 times faster. The isotope effects for the two sets were the same within experimental error.

Acid Hydrolysis Reactions. The ^{15}N isotope effect was measured in 1.0 N sulfuric acid (pH 0.0) at 100 °C, using 2 mM substrate in 50 mM of solution. Under these conditions the half-life was about 12 h. In this reaction the diester is hydrolyzed to produce *p*-nitrophenol and 3,3,-dimethylbutyl phosphate, which itself is slowly hydrolyzed under these conditions. In a practice run a 2 mM solution of diester was allowed to react until it had been 89% consumed, based on assay for the release of *p*-nitrophenol. The concentration of inorganic phosphate was measured with a malachite green assay and found to be 0.2 mM. Thus, the hydrolysis of the monoester is much slower than the diester, but some hydrolysis of it to produce inorganic phosphate and 3,3-dimethylbuanol occurs.

The isotope effect reactions were run to about 50% hydrolysis, the mixture were cooled to room temperature where hydrolysis is undetectable over several hours, and the *p*-nitrophenol was assayed and isolated by ether extraction. The ether extracts were evaporated, and the *p*-nitrophenol was taken up in dilute sodium hydroxide and washed with dichloromethane (3 × 50 mL). The aqueous layer was acidified and the *p*-nitrophenol reextracted with ether. This procedure served to remove the 3,3-dimethylbutanol produced from monoester hydrolysis. The pure nitrophenol was then prepared for Kjeldahl digestion as previously described.

Table I. Isotope Effects for Reactions of Phosphodiester **1** and for the Alkaline Hydrolysis of Diethyl *p*-Nitrophenyl phosphate (Errors Reported Are the Standard Errors)

reaction	$^{14}k/^{15}k$	$^{16}k/^{18}k$
alkaline hydrolysis	1.0016 ± 0.0002	0.9949 ± 0.0006
β-cyclodextrin	1.0013 ± 0.0001	1.0010 ± 0.0002
phosphodiesterase I	1.0017 ± 0.0002	0.9842 ● 0.0006
acid hydrolysis	1.0009 ± 0.0002	1.0139 ± 0.0004
triester alkaline hydrolysis	1.0007 ± 0.0001[a]	1.0063 ± 0.0001[b]

[a] Data from ref 2. [b] Data fromr ef 21.

Table II. Observed and Corrected Isotope Effects for the Acid Hydrolysis of Diester **1**[a]

	$^{16}k/^{18}k$	
pH	obsd	corrd
0.0	1.0028	1.0139
−0.30	1.0054	1.0139

[a] The corrected numbers are the secondary ^{18}O isotope effects for nucleophilic attack, obtained from the observed values as described in the text.

The residual substrate was hydrolyzed by bringing the solution to pH 13 by addition of NaOH and heating to 95 °C for 30 h. Alkaline solutions were used to hydrolyze the residual substrate to avoid the need to remove 3,3-dimethylbutanol from the *p*-nitrophenol released from the hydrolyzed residual substrate.

Two sets of experiments were run to determine the secondary ^{18}O isotope effect for this hydrolysis, one set in 1 N sulfuric acid (pH 0.0) as described above and one set in 2 N sulfuric acid (pH −0.30). The procedures were the same as described for the ^{15}N isotope effect experiments. The isotope effects observed at each pH are reported in Table II, along with value obtained after correction for the ^{18}O isotope effect for protonation (see Results).

Determination of the pK_a of 3,3-Dimethylbutyl *p*-Nitrophenyl Phosphate. The pK_a of the diester was determined by measuring the shift in λ_{max} as the diester was titrated with sulfuric acid and perchloric acid in separate experiments. The low pK_a of the *p*-nitrophenyl diester and the relative insolubility of the protonated species made it impossible to obtain a standard titration curve. The protonated diester is sufficiently soluble to obtain UV spectra.

A 10 mM stock solution of the sodium salt of the diester was prepared. Aliquots of 25 μL of this solution were added to 3.0 mL of acid solutions at various pH values, and the UV spectra were recorded. The position of λ_{max} was recorded as a function of pH. Two titration curves were obtained with use of sulfuric acid and two with use of perchloric acid as the titrant; good agreement between titrations was obtained. The position of λ_{max} changed from 290.0 to 278.0 nm as protonation occurs. The λ_{max} data were plotted as a function of pH and fitted to the program WAVL of Cleland to obtain the pK_a values.[11] The average value was −0.36 ± 0.05.

Isotope Incorporation Experiments. Acid Hydrolysis. The diester **1** (11 mg) was dissolved in 0.5 mL of 95% [^{18}O]water containing 14.3 μL of concentrated sulfuric acid (to give a concentration of 1 N in acid) and heated to 100 °C in an oil bath for 12 h. After being cooled to room temperature, about one-fourth of the reaction mixture was removed, diluted with 200 μL of D_2O, and examined by proton-decoupled ^{31}P NMR (202 MHz). Integration of the product and residual substrate signals indicated hydrolysis to be about 40% complete. The single resonance observed for the residual substrate was identical with that for unlabeled starting material (see Results). The reaction mixture was reheated to 100 °C for 18 h. After cooling, the mixture was diluted with water and the *p*-nitrophenol product isolated by ether extraction as described for the isotope effect experiments. High-resolution mass spectrometry of the isolated *p*-nitrophenol showed no measurable ^{18}O incorporation.

Alkaline Hydrolysis. Diester **1** (9.2 mg) was dissolved in 300 μL of 95% [^{18}O]water containing 25 μL of 13 N NaOH (giving a concentration of 1 N in hydroxide and 88% oxygen-18) in an NMR tube. In a separate NMR tube, 8.1 mg of the diester was dissolved in a similar mixture with unlabeled water. Both samples were heated to 100 °C for 3 h. After cooling, 250 μL of D_2O was added to each mixture and the signals from the monoester product and from the residual substrate in both samples

(11) Cleland, W. W. *Methods Enzymol.* **1979**, *63*, 103.

were examined by proton-decoupled ^{31}P NMR. An NMR sample was then prepared by mixing an aliquot from the labeled and unlabeled reaction mixtures.

A single ^{31}P resonance was found for the residual substrate from each of the reaction mixtures as well as for the mixed sample, indicating the lack of detectable exchange of the diester phosphoryl oxygens with solvent. The monoester hydrolysis product of the reaction in unlabeled water gave a single resonance. That from the reaction in [^{18}O]water consisted of a pair of signals, a smaller peak at 6.035 ppm and a larger one 0.03 ppm upfield. By integration, the larger more upfield peak was 86% of the total. The mixing experiment showed the smaller peak to be identical with that of the sole resonance from the reaction in unlabeled water.

Results

The ^{15}N and ^{18}O isotope effect data are tabulated in Table I. The ^{15}N isotope effects were measured from reactions using the natural abundance diester. The ^{18}O isotope effects were measured from experiments using the mixed remote-labeled substrate. The observed isotope effect from each experiment was corrected for the ^{15}N isotope effect and for isotopic incorporations in the starting material to obtain the ^{18}O isotope effect.[12]

Correction for Protonation Effects in the Acid Hydrolysis Reaction. The pH profile for phosphodiester hydrolysis in the acid region indicates that the protonated neutral diester is the reactive species.[13] Because of the low pK_a and very low solubility of the protonated diester in solution the isotope effect experiments could not be run under conditions where the diester was completely protonated. Therefore, the observed isotope effect will have a component consisting of the ^{18}O isotope effect for protonation. In order to extract the secondary ^{18}O isotope effect of interest the observed effect must be corrected for the isotope effect on protonation, taking into account the fraction of protonated species present.

The acid hydrolysis reaction in solution where A refers to the diester substrate can be expressed as eq 1.

$$^{16}A \rightleftharpoons {}^{16}AH \xrightarrow{k_{16}} \qquad (1)$$

$$^{18}A \rightleftharpoons {}^{18}AH \xrightarrow{k_{18}}$$

The concentrations of the protonated species AH in solution are related to the total concentrations of A_t by

$$[AH] = [A]_t[[H]/([H] + K)] = [A]_t/(1 + K/[H])$$

Assuming that the isotope effect for protonation of the diester is equal to that for phosphomonoesters,[14] which is 1.016, then $K_{18} = K_{16}/1.016$ and the expressions for the concentrations of the two protonated species are

$$[^{16}AH] = [^{16}A]_t/(1 + K_{16}/[H])$$

$$[^{18}AH] = [^{18}A]_t/(1 + K_{16}/1.016[H])$$

The velocities for the hydrolyses of the two species are then

$$v_{16} = k_{16}[^{16}A]_t/(1 + K_{16}/[H])$$

$$v_{18} = k_{18}[^{18}A]_t/(1 + K_{16}/1.016[H])$$

Dividing v_{16} by v_{18} gives

$$\frac{v_{16}}{v_{18}} = \frac{k_{16}[^{16}A]_t(1 + K_{16}/[H])}{k_{18}[^{18}A]_t(1 + K_{16}/1.016[H])}$$

or

$$\frac{v_{16}/v_{18}}{[^{16}A]_t/[^{18}A]_t} = \frac{k_{16}(1 + K_{16}/[H])}{k_{18}(1 + K_{16}/1.016[H])}$$

The left-hand term in this equation is the observed ^{18}O isotope effect for the overall reaction given in eq 1. The quantity k_{16}/k_{18} is the isotope effect on the second step in eq 1, the secondary ^{18}O isotope effect for the nucleophilic attack. Using the nomenclature ^{18}k to represent the isotope effect k_{16}/k_{18}, the secondary ^{18}O isotope effect of interest, ^{18}k, is related to the observed isotope effect for the whole reaction, k_{obs}, by the relation

$$^{18}k_{obs} = {}^{18}k[(1 + K_{16}/1.016[H])/(1 + K_{16}/[H])]$$

The correction equation requires the value for the pK_a of the phosphodiester. Previous pK_a determinations of phosphodiesters range from 1.29 for dimethyl to 1.72 for dibutyl phosphate.[15] The pK_a for 3,3-dimethylbutyl *p*-nitrophenyl phosphate was determined to be -0.35 ± 0.05 by measuring the shift in λ_{max} as the diester was titrated with acid, as described in the Experimental Section. This pK_a for diester **1** is in the expected range given the electron-withdrawing effect of the *p*-nitrophenyl group.

Two sets of measurements of the ^{18}O isotope effect were made, one at pH 0.0 and the second at pH −0.30. The observed and the corrected isotope effect data are collected in Table II. The match between the corrected isotope effects for the two sets run at different pH adds confidence that the final value of 1.0139 ± 0.0005 obtained is the correct secondary ^{18}O isotope effect for the displacement reaction.

Isotope Incorporation Experiments. If ^{18}O exchange between the phosphodiester and solvent were occurring in these reactions, the apparent isotope effect would not be the intrinsic one and would change with the extent of reaction. In order to test for such exchange, the acid and alkaline hydrolysis reactions were run to partial hydrolysis in [^{18}O]water and the residual phosphodiester substrate was examined by proton-decoupled ^{31}P NMR at 202 MHz. In both reactions only a single phosphorus resonance was observed, which was identical with that of the starting material. The identity of the single peak as being that of diester lacking any ^{18}O incorporation was confirmed by adding a small amount of doubly ^{18}O-labeled diester, which gave rise to a new phosphorus resonance 0.06 ppm upfield. The lack of any detectable ^{18}O incorporation from solvent in either the alkaline or acidic hydrolysis reactions precludes the formation of a reversibly formed pentavalent intermediate in these reactions. Since any such intermediate formed must then be partitioning solely to products, only the first step to form the intermediate will result in an observed isotope effect. Such a step to form a phosphorane intermediate should not produce an ^{15}N isotope effect in these reactions since no bond breaking to the *p*-nitrophenyl leaving group occurs. The fact that significant isotope effects are observed, corresponding to about 57% and 32% bond breakage for the alkaline and acidic hydrolysis reactions, respectively, is inconsistent with a stepwise mechanism and supports the expected S_N2 nature of these reactions.

Position of Bond Cleavage. Although nucleophilic attack at phosphorus is the most common mode of phosphodiester cleavage, it is not the only one possible. In the alkaline hydrolysis of methyl 2,4-dinitrophenyl phosphate, nucleophilic aromatic substitution (attack at carbon) occurs competitively with attack at phosphorus.[16]

In the β-cyclodextrin reaction of the present study, the mode of reaction was shown to be exclusively $S_N2(P)$ by examination of the products of reaction.[4] The phosphoryl transfer was found to result quantitatively in formation of a cyclodextrin 3,3-dimethylbutyl phosphodiester. The competing aromatic substitution reaction would produce a cyclodextrin *p*-nitrophenyl ether and would also result in less than the stoichiometric amount of *p*-nitrophenol being released in the reaction. No such ether product was detected, and the expected amount of *p*-nitrophenol was found.

To determine the position of bond breakage in the acid hydrolysis reaction, the *p*-nitrophenol produced in an experiment carried out in [^{18}O]water as described in the Experimental Section was isolated and examined for ^{18}O incorporation by mass spectrometry. The possible formation of [^{18}O]-*p*-nitrophenol by an acid-catalyzed exchange between the solvent and free *p*-nitrophenol

(12) Reference 9a. Also see: Weiss, P. M. Heavy Atom Isotope Effects Using the Isotope Rato Mass Spectrometer. In *Enzyme Mechanisms from Isotope Effects*; Cook, P. F., Ed.; CRC Press: Boca Raton, FL; Chapter 11, in press.

(13) Kirby, A. J.; Younas, M. *J. Chem. Soc. B* **1970**, 510.

(14) Knight, W. B.; Weiss, P. M.; Cleland, W. W. *J. Am. Chem. Soc.* **1986**, *108*, 2759.

(15) Kumler, W. D.; Eiler, J. S. *J. Am. Chem. Soc.* **1943**, *65*, 2355.

(16) Kirby, A. J.; Younas, M. *J. Chem. Soc. B* **1970**, 1165.

had to be considered, since partial exchange has been reported to occur under very harsh acidic conditions.[17] No exchange was found to occur under the conditions used in the acidic phosphodiester hydrolysis reactions. Since no detectable amount of [^{18}O]-*p*-nitrophenol was found, attack at carbon is ruled out for this reaction.

This same method could not be used to determine the position of bond cleavage in the alkaline hydrolysis reaction, however, because we have found that the phenolic oxygen in *p*-nitrophenol exchanges with solvent under the reaction conditions used.[18] Therefore, we ascertained the position of nucleophilic attack by examining the other product of hydrolysis, 3,3-dimethylbutyl phosphate, by ^{31}P NMR to check for isotopic incorporation. Samples of the diester were separately hydrolyzed in unlabeled water and in [^{18}O]water. The monoester hydrolysis products were examined by ^{31}P NMR as described in the Experimental Section. The product from the reaction carried out in [^{18}O]water was found to have incorporated one atom of ^{18}O, demonstrating that the alkaline hydrolysis reaction occurs by an $S_N2(P)$ process. The absence of any detectable amount of monoester product containing two or three atoms of ^{18}O indicates further that exchange of the monoester phosphoryl oxygen atoms with solvent does not occur to any measurable extent and rules out exchange as the source of the observed ^{18}O incorporation.

Discussion

In the alkaline hydrolysis reaction, the value for the ^{15}N isotope effect indicates the P–O bond is ~57% broken in the transition state.[2] The slightly inverse secondary ^{18}O isotope effect indicates that bond order to the nonbridge oxygen atoms is slightly higher in the transition state, suggesting a slightly dissociative character.

It is interesting to compare this reaction with the β-cyclodextrin one. We have determined that β-cyclodextrin effects the cleavage of phosphodiesters via a complex in which the leaving group resides outside the cavity, while nucleophilic attack by a deprotonated secondary hydroxyl group of the cyclodextrin produces a cyclodextrin phosphodiester.[4] The secondary hydroxy anion nucleophile in this reaction has a pK of about 12, compared to 15.7 for the hydroxide nucleophile in the alkaline hydrolysis reaction.[5] The isotope effect data reveal bond cleavage to the leaving group to be slightly less advanced in the transition state of the cyclodextrin reaction. In addition, the secondary ^{18}O isotope effect is now slightly normal, indicating that the transition state has become slightly associative in nature. Apparently the weaker cyclodextrin nucleophile approaches more closely and has increased bonding interaction to the phosphoryl group while the leaving group bond breaking is less advanced, relative to the hydroxide reaction. The resulting increased charge on the phosphoryl group is reflected in slightly lessened bond order to the nonbridge oxygen atoms as the charge is delocalized.

Phosphodiesterase Reaction. Snake venom phosphodiesterase (phosphodiesterase I) hydrolyzes phosphodiesters by a ping-pong mechanism with a phosphorylated threonine intermediate.[6] The active site contains a zinc atom, which is necessary for catalysis, and the enzyme is activated by magnesium. The enzyme is reported to have a bell-shaped pH profile with a broad pH–activity optimum. Though typically assayed with bis(*p*-nitrophenyl) phosphate, its natural substrates are oligoribonucleotides, which it cleaves at 3′-hydroxy group to form nucleoside 5′-phosphates.

The diester **1** is a viable substrate for the enzyme. The V/K pH profile obtained at 37 °C is bell-shaped with a broad pH maximum. A fit of the data to the computer program BELL of Cleland[11] gives pK values of 6.3 and 9.6. The K_m for this substrate at the pH maximum of 8.0 was found to be 10.5 mM, about 1 order of magnitude higher than that for bis(*p*-nitrophenyl) phosphate.

The ^{15}N isotope effect for this enzymatic reaction indicates the degree of transition-state bond breaking to the leaving group to be similar to that for the alkaline hyrolysis reaction. The strongly inverse secondary ^{18}O isotope effect is much too large for the known S_N2 nature of this reaction and begs another explanation. The most logical one is that the enzyme is protonating the negatively charged phosphoryl group in order to catalyze the reaction. The ^{18}O isotope effect for protonation of a phosphate monoester has been measured to be 1.6% inverse,[14] which is very close to the value observed for this reaction. Protonation of the diester would serve to greatly increase its reactivity by neutralizing the negative charge, making it electronically equivalent to a triester. Triesters are far more labile toward nucleophilic attack than diesters. For example, diethyl *p*-nitrophenyl phosphate has a half-life in 0.1 N hydroxide at room temperature of about 15 min. The diester **1** by contrast in 1.0 N hydroxide at 100 °C has a half-life of more than 2 h.

The enzyme may thus be activating the phosphoryl group through protonation. Alternatively, a similar activation could be envisaged by coordination of the charged phosphoryl group to the active site zinc, analogous to the role zinc plays in the serine proteases. The isotope effect for the zinc complexation of a phosphate ester has never been measured, but recent work[19] in this laboratory shows that there is no appreciable ^{18}O isotope effect on magnesium complexation to phosphate, thus making this explanation unlikely. Although at first glance protonation of a group with such a low pK_a might seem unlikely, there is some precedence in the work of Breslow who has presented evidence that in the cleavage of dinucleotides by imidazole buffer protonation of the phosphodiester is the first step and has proposed that ribonuclease uses a similar mechanism to cleave phosphodiester bonds in RNA.[20] In addition, solution pK_a values may not reflect the likelihood of an enzymatic proton transfer at the active site of an enzyme.

We favor the interpretation that the relatively large inverse ^{18}O isotope effect arises from protonation of the phosphoryl group on the enzyme. Taken together, the isotope effect data suggest a mechanism involving activation of the phosphoryl group by protonation followed by S_N2 displacement by the active site nucleophile.

Acid Hydrolysis. The ^{15}N isotope effect for the acid hydrolysis reaction of 1.0009 ± 0.0002 is very close to that measured for the alkaline hydrolysis of the triester diethyl *p*-nitrophenyl phosphate, 1.0007 ± 0.0001.[2] Protonation makes the diester electronically equivalent to a triester, and the similar ^{15}N isotope effects indicate a similar degree of bond breaking to the leaving group in the transition states for these two reactions. In the acid hydrolysis reaction of the diester, the bond to the leaving group is ~32% broken in the transition state.[2]

The secondary ^{18}O isotope effect for the acid hydrolysis is 1.0139, which is significantly larger than those oberved in the previous reactions and indicates considerable associative character for the transition state. The effect here is also larger than that reported for the alkaline hydrolysis of the electronically equivalent triester diethyl *p*-nitrophenyl phosphate of 1.0063.[21] By contrast, the degree of transition-state bond breaking to the leaving group is very similar in the two reactions. In the triester reaction, the nucleophile was hydroxide, versus water in the acid diester hydrolysis reaction. The larger isotope effect in the acid reaction seems to indicate once again that the weaker nucleophile gives a more associative transition state, as noted previously in the hydroxide versus cyclodextrin reactions of the diester anion.

Despite its more associative nature, the acid hydrolysis reaction is still clearly an S_N2 process. Hydrolysis via an addition–elimination pathway with a phosphorane intermediate does occur with certain phosphates such as cyclic esters, but the isotope effect data rule out this mechanism for the acid hydrolysis of the diester **1**.

(17) Oae, S.; Kiritani, R.; Tagaki, W. *Bull. Chem. Soc. Jpn.* **1966**, *39* (9), 1961.

(18) Complete exchange occurs within 10 h at 100 °C. This alkaline exchange reaction has not been previously reported to our knowledge. We are examining the scope and utility of this reaction as a method for the preparation of oxygen-18-labeled phenols.

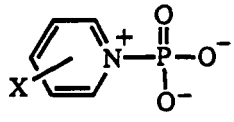
(19) Jones, J. P.; Weiss, P. M.; Cleland, W. W. *Biochemistry* **1991**, *30*, 3634.

(20) Anslyn, E.; Breslow, R. *J. Am. Chem. Soc.* **1989**, *111*, 4473.

(21) Caldwell, S. R.; Raushel, F. M.; Weiss, P. M.; Cleland, W. W. *J. Am. Chem. Soc.* **1991**, *113*, 730.

If one postulates an addition intermediate, this species would surely partition completely in the forward direction by losing nitrophenolate and not revert to starting material by losing hydroxide. The absence of oxygen-18 exchange with solvent when this reaction was run in [^{18}O]water precludes the formation of any reversibly formed addition intermediate. In this case, the only step that would produce an isotope effect is the addition step. There should be no significant ^{15}N isotope effect on this step since there are no bonding changes to the leaving group. The fact that a significant ^{15}N isotope effect is observed, corresponding to about one-third bond cleavage to the leaving group, is thus inconsistent with an addition–elimination pathway.

Comparisons with Phosphorylated Pyridines. In reactions of oxygen nucleophiles with three phosphorylated pyridine monoanions **9**, Herschlag and Jencks found a decrease in the quantity [$-\beta_{lg}$] with increasing pK_a of the nucleophile.[22] This indicates



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less bond cleavage to the leaving group, implying a more associative transition state with increasing pK_a of the nucleophile. This is opposite to the trends seen in the data for diester **1**, where the secondary ^{18}O isotope effect data indicate a trend toward a more associative transition state with decreasing pK_a of the nucleophile. The ^{15}N isotope effect data suggest a slightly more advanced degree of bond breaking for hydroxide than with the weaker nucleophile cyclodextrin alkoxide for attack on the diester monoanion, although the difference is barely significant. The ^{15}N isotope effects are even closer for the acid hydrolysis of the diester and for the alkaline triester hydrolysis, where nucleophiles of drastically differing pK_a attack a neutral phosphoryl group. Within each of these two pairs of reactions there is considerably more difference in the secondary ^{18}O isotope effects, indicating that changes in nucleophile are reflected more in differences in transition-state bonding within the phosphoryl group than to the leaving group.

The type of bond being broken and the leaving group are different in the two studies. In the phosphorylated pyridine reactions, a P–N bond is broken and the leaving group is a neutral pyridine, whereas in this work a P–O bond is broken and a negatively charged phenolate is the leaving group. Another significant difference is in the electronic nature of the two types of substrate. The phosphorylated pyridines **9**, though being monoanionic like diester **1**, differ in having a charge of −2 on the central phosphoryl group and in bearing a positively charged leaving group.

Role of the Oxyanion. In order to compare the attack of hydroxide on the diester monoanion **1** with that for hydroxide attack on the triester *p*-nitrophenyl diethyl phosphate, the isotope effects for the latter reaction are included in Table I. These data allow one to assess the role of the oxyanion in the phosphodiester reaction. Citing unpublished data indicating that the β_{lg} is nearly identical for the attack of phenolate anion on aryl esters of diethyl phosphate and methyl phosphate monoanion ($ArOPO(OEt)_2$ and $ArOPO_2^-OMe$, respectively), Williams has concluded that the oxyanion in the diester does not participate in the expulsion of the leaving group.[23]

The isotope effect data for the attack of hydroxide on the diester used in the present experiments and on the triester *p*-nitrophenyl diethyl phosphate suggest that the oxyanion is involved in this reaction. The ^{15}N isotope effects show bond cleavage to the leaving group is clearly more advanced in the reaction of the diester monoanion (~57%) than for the neutral triester (~25%).[2] The ^{18}O isotope effect is slightly inverse for the diester monoanion reaction, and slightly normal for the neutral triester. This indicates that there is a difference in the involvement of the nonbridge oxygens in these reactions. The slightly inverse ^{18}O isotope effect, along with the greater degree of bond cleavage to the leaving group, is consistent with involvement of the oxyanion in expulsion of the leaving group in the alkaline hydrolysis of the diester monoanion. Such is not the case for the β-cyclodextrin reaction however, where the ^{18}O isotope effect becomes slightly normal with the change to a weaker nucleophile, indicative of a more associative transition state.

More data are needed before the question of the involvement of the oxyanion in phosphodiester displacement reactions can be firmly answered and to confirm the trend observed for weaker nucleophiles to result in more associative transition states in such reactions. The data presented here demonstrate that the nonbridge oxygen atoms in phosphodiesters are not passive bystanders in these S_N2 reactions and that changes in their bonding to the central phosphorus atom revealed by secondary ^{18}O isotope effects can reveal subtle differences in transition-state structure for different nucleophiles.

Acknowledgment. This research was supported by a grant from the National Institutes of Health (GM 18938) to W.W.C. and a National Institutes of Health Postdoctoral Fellowship (GM 11942) to A.C.H. This study made use of the National Magnetic Resonance Facility at Madison, supported in part by NIH Grant RR02301.

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