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Comparisons of Phosphorothioate with Phosphate Transfer Reactions for a Monoester, Diester, and Triester: Isotope Effect Studies

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Abstract: Phosphorothioate esters are sometimes used as surrogates for phosphate ester substrates in studies of enzymatic phosphoryl transfer reactions. To gain better understanding of the comparative inherent chemistry of the two types of esters, we have measured equilibrium and kinetic isotope effects for several phosphorothioate esters of *p*-nitrophenol (*p*NPPT) and compared the results with data from phosphate esters. The primary ^{18}O isotope effect at the phenolic group ($^{18}k_{bridge}$), the secondary nitrogen-15 isotope effect (^{15}k) in the nitro group, and (for the monoester and diester) the secondary oxygen-18 isotope effect ($^{18}k_{nonbridge}$) in the phosphoryl oxygens were measured. The equilibrium isotope effect (EIE) $^{18}k_{nonbridge}$ for the deprotonation of the monoanion of *p*NPPT is 1.015 ± 0.002, very similar to values previously reported for phosphate monoesters. The EIEs for complexation of Zn^{2+} and Cd^{2+} with the dianion *p*NPPT^{2-} were both unity. The mechanism of the aqueous hydrolysis of the monoanion and dianion of *p*NPPT, the diester ethyl *p*NPPT, and the triester dimethyl *p*NPPT was probed using heavy atom kinetic isotope effects. The results were compared with the data reported for analogous phosphate monoester, diester, and triester reactions. The results suggest that leaving group bond fission in the transition state of reactions of the monoester *p*NPPT is more advanced than for its phosphate counterpart *p*NPP, while alkaline hydrolysis of the phosphorothioate diester and triester exhibits somewhat less advanced bond fission than that of their phosphate ester counterparts.

Introduction

Phosphorothioate esters are counterparts of phosphate esters in which an oxygen atom in the phosphoryl group has been replaced by sulfur. Such compounds have been used as stereochemical probes and in mechanistic studies of enzymatic phosphoryl transfer. The uncatalyzed reactions of phosphorothioate esters have been less extensively studied than phosphates. We previously compared the kinetic and thermodynamic parameters, and solvent effects, for hydrolysis reactions of phosphate and phosphorothioate monoesters.[1] The present study extends our investigation of the chemistry and the hydrolysis mechanisms of phosphorothioate esters. We report the equilibrium ^{18}O isotope effects (EIEs) for deprotonation and for zinc and cadmium ion complexation with the monoester *p*-nitrophenyl phosphorothioate (*p*NPPT) and the kinetic isotope effects (KIEs) for the hydrolysis of the dianion and monoanion of *p*NPPT, the diester ethyl *p*-nitrophenyl phosphorothioate (EtO*p*NPPT), and the triester dimethyl *p*-nitrophenyl phosphorothioate ((MeO)$_2$*p*NPPT). Figure 1 shows the positions at which isotope effects in each compound were measured and the notation used. Comparisons of these data with previously determined results for kinetic isotope effects and equilibrium isotope effects with phosphate esters can shed light on the consequences of sulfur substitution on the comparative mechanistic chemistry and the transition states of these compounds and supplement information from linear free energy relationships (LFER).

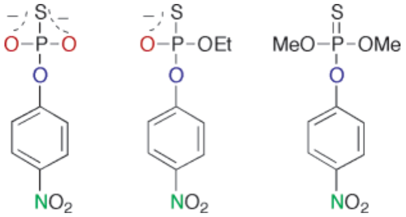

Figure 1. Phosphorothioate esters for which isotope effect measurements are reported: red, nonbridge phosphoryl oxygen atoms ($^{18}k_{nonbridge}$); blue, bridge oxygen atom, site of bond fission ($^{18}k_{bridge}$); green, nitrogen atom of the leaving group (^{15}k).

Figure 2 shows the mechanistic extremes for phosphoryl and thiophosphoryl transfer reactions. In a fully dissociative mechanism (A) a (thio)metaphosphate intermediate is formed in the rate-determining step. In the opposite extreme of a fully associative mechanism (C) a pentacoordinate phosphorane intermediate is formed. Mechanism B is concerted; in such a mechanism the transition state could in principle be loose (resembling that of mechanism A) or tight (resembling mechanism C). Considerable experimental evidence that has been reviewed[2] suggests that phosphate (X = O) monoesters undergo reactions by a concerted mechanism having a very loose transition state, while phosphorothioate (X = S) monoesters are believed to form

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(1) Catrina, I. E.; Hengge, A. C. *J. Am. Chem. Soc.* **1999**, *121*, 2156−2163.

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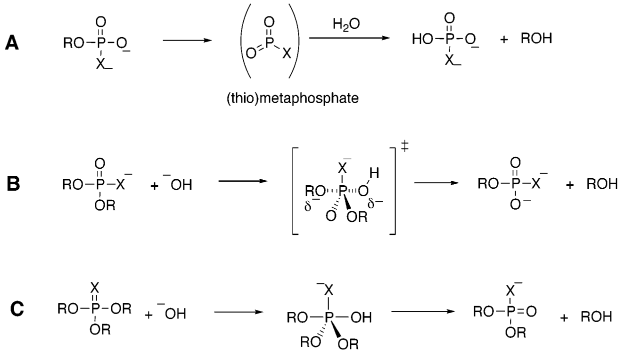

Figure 2. Mechanistic possibilities for phosphoryl (X = O) or thiophosphoryl (X = S) transfer: the fully dissociative mechanism (A) and the fully associative (C) and intermediate concerted (B) mechanisms. The P−O bond order to the nonbridge oxygen atoms increases in the transition state in mechanism A and decreases in C. By contrast the bending modes will loosen in the transition state of A but tighten in C.

a thiometaphosphate intermediate. Both phosphoryl and thiophosphoryl transfer reactions are thought to become more associative in the progression from monoester to diester to triester. Linear free energy relationships (LFER) that examine the interrelationship between β_{nuc} and $\beta_{\text{leaving group}}$ [3] indicate that with good (aryloxy) leaving groups even reactions of phosphotriesters remain concerted, although the transition state is tight and phosphorane-like.

The isotope effects depicted in Figure 1 give information regarding the transition sates for the hydrolysis reactions of the phosphorothioate esters shown. The secondary ^{15}k and the primary $^{18}k_{\text{bridge}}$ isotope effects are sensitive to the charge on the leaving group and the degree of P−O bond fission, respectively. The secondary $^{18}k_{\text{nonbridge}}$ both gives information about hybridization changes occurring at phosphorus during the reaction, and is sensitive to the protonation state of the reactant.

Experimental Section

Materials. Reagents and solvents were commercial products and were used as received unless otherwise noted. Pyridine was distilled from calcium hydride. Tetrahyrofuran (THF) was distilled from sodium and benzophenone.

Isotopically Labeled Compounds Needed for the Measurement of Isotope Effects. The ^{18}O kinetic isotope effects were measured by the remote label method, using the nitrogen atom in the nitro group as a reporter for isotopic fractionation in the labeled oxygen positions.[4] Figure 3 shows the mixtures of isotopically labeled compounds needed for these experiments. For the KIE in the nonbridging oxygen atoms, the compound synthesized with ^{18}O in these positions and ^{15}N in the reporter position is mixed with the isotopic isomer bearing the natural abundance of oxygen and ^{14}N in the reporter position (A for the monoester and C for the diester). For measurement of $^{18}k_{\text{bridge}}$, the mixtures of isotopic isomers represented in B, D, and E are needed. Natural abundance compounds are used for measurements of ^{15}k. All of the synthesized phosphorothioate esters had ^{1}H and ^{31}P NMR spectra consistent with their assigned structures.

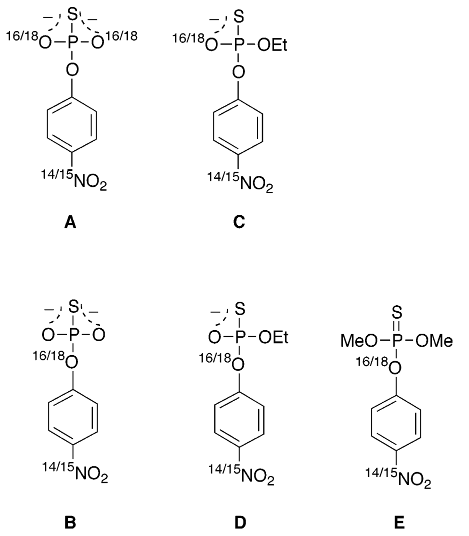

Figure 3. Mixtures of isotopic isomers used for measurements of the ^{18}O isotope effects. A and C are used for measurement of $^{18}k_{\text{nonbridge}}$ in the monoester and diester, respectively. Mixtures represented by B, D, and E are used in measurement of $^{18}k_{\text{bridge}}$ in the monoester, diester, and triester.

Synthesis. [^{14}N]-*p*-nitrophenol, [^{15}N]-*p*-nitrophenol, and [^{15}N,^{18}O]-*p*-nitrophenol were synthesized as previously described.[5]

(A) Isotopic Isomers of the Monoester *p*-Nitrophenyl Phosphorothioate. Natural abundance *p*-NPPT was synthesized from *p*-nitrophenol and $PSCl_3$ with hydrolysis of the *p*-nitrophenyl thiophosphorodichloridate intermediate as previously reported.[1]

In the same manner, [^{15}N,nonbridge-$^{18}O_2$]-*p*-nitrophenyl phosphorothioate was synthesized, but with the use of [^{15}N]-*p*-nitrophenol and then $H_2{}^{18}O$ to hydrolyze the intermediate. The most economical use of labeled water was obtained when the *p*-nitrophenyl thiophosphorodichloridate intermediate was isolated[6] followed by hydrolysis in the presence of pyridine using a 3:1 ratio of ^{18}O-labeled water:intermediate. ^{31}P NMR analysis showed the product to be 87 ± 1% $^{18}O_2$, with the remainder $^{18}O^{16}O$.

[^{14}N]-*p*-Nitrophenyl phosphorothioate was made in the same way, using [^{14}N]-*p*-nitrophenol and natural abundance water.

To closely reconstitute the natural abundance of ^{15}N (as confirmed by isotope ratio mass spectrometry), [^{15}N,nonbridge-$^{18}O_2$]-*p*-nitrophenyl phosphorothioate and [^{14}N]-*p*-nitrophenyl phosphorothioate were mixed, and this mixture (A in Figure 3) was used to measure $^{18}k_{\text{nonbridge}}$.

To prepare the mixture needed for measurement of $^{18}k_{\text{bridge}}$ (B in Figure 3) [^{14}N]-*p*-nitrophenol and [^{15}N,^{18}O]-*p*-nitrophenol were mixed to closely reconstitute the natural abundance of ^{15}N, and this mixture was converted to the phosphorothioate ester as described above.

(B) Isotopic Isomers of the Diester Ethyl-*p*-nitrophenyl Phosphorothioate. Ethyl [^{14}N]-*p*-nitrophenyl phosphorothioate (EtO*p*NPPT) was synthesized by adding a solution of 5.5 mmol of anhydrous ethanol in 3 mL of dry THF dropwise to a solution of 5.5 mmol of [^{14}N]-*p*-nitrophenyl thiophosphorodichloridate and 5.5 mmol of dry pyridine in 11.4 mL of dry THF. After 10−15 min of stirring at room temperature under nitrogen, a solution of 8.4 mL of water and 5.7 mmol of dry pyridine was added, and this mixture was stirred for an additional 10 min. THF was then removed by rotary evaporation, and the residue was diluted with water. The mixture was filtered, concentrated in vacuo, and then purified by reverse-phase column chromatography,[7] eluting with a solution of 1:1 acetonitrile:TEAB (triethylammonium bicarbonate), 0.1 M buffer, pH 7.5. EtO*p*NPPT elutes first, and after solvent

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evaporation the TEAB is removed by repeated addition/evaporations of methanol. Stock solutions of the diester in water were stored at −20 °C.

In a similar way, [^{15}N,^{18}O-nonbridge]-ethyl *p*-nitrophenyl phosphorothioate was synthesized. A solution of 0.83 mmol ethanol in 0.45 mL of dry THF was added dropwise to a solution of 0.83 mmol of [^{15}N]-*p*-nitrophenyl thiophosphorodichloridate and 0.83 mmol of dry pyridine in 1.9 mL of dry THF. This final solution was stirred for 10 min under nitrogen. Subsequently a mixture of 600 μL of $H_2{}^{18}O$ with 70 μL of dry pyridine was added and the reaction stirred for another 10 min. The purification procedure is the same as that described above. This compound and [^{14}N]-EtO*p*NPPT were mixed to reconstitute the ^{15}N natural abundance (C in Figure 3) and were the reactants for determining $^{18}k_{\mathrm{nonbridge}}$.

The mixture used to measure $^{18}k_{\mathrm{bridge}}$ with ethyl *p*-nitrophenyl phosphorothioate (D in Figure 3) was prepared in the same manner as ^{14}N-EtO*p*NPPT but with use of the mixture of [^{14}N]-*p*-nitrophenol and [^{15}N,^{18}O]-*p*-nitrophenol (at ^{15}N natural abundance).

To measure the ^{15}N isotope effect, the natural abundance diester was used, which was synthesized from natural abundance *p*-nitrophenol as described for [^{14}N]-EtO*p*NPPT.

(C) Isotopic Isomers of the Triester Dimethyl-*p*-nitrophenyl Phosphorothioate. Natural abundance dimethyl-*p*-nitrophenyl phosphorothioate was prepared by adding a mixture of 8.2 mmol of *p*-nitrophenol, 0.8 mmol of dimethylamino pyridine, and 8.2 mmol of triethylamine in 5 mL of THF to a solution of commercial dimethylchlorothiophosphate (8.2 mmol) in 10 mL of THF dropwise over 5 min under nitrogen. After 4 h the reaction mixture was added to 15 mL of water and extracted with ether (3 × 25 mL). The ether layers were dried over $MgSO_4$ and concentrated by rotary evaporation. The product was isolated as a white solid after purification by flash chromatography eluting with hexane/ethyl acetate.

The mixture used to measure $^{18}k_{\mathrm{bridge}}$ with dimethyl-*p*-nitrophenyl phosphorothioate (E in Figure 3) was prepared in the same manner, but with use of the mixture of [^{14}N]-*p*-nitrophenol and [^{15}N,^{18}O]-*p*-nitrophenol.

Determinations of Equilibrium Isotope Effects for Deprotonation and Metal Ion Complexation of *p*NPPT. The changes in chemical shifts with pH for unlabeled *p*NPPT were used to calculate the second pK_a of the *p*NPPT monoanion (see Supporting Information). No attempt was made to measure the first pK_a, which is <1.[8]

The differences in ^{31}P chemical shift between ^{16}O and nonbridge-^{18}O *p*NPPT were used to calculate the isotope effect for deprotonation and for complexation of *p*NPPT with zinc and cadmium. This technique was introduced by Ellison and Robinson to determine the EIE for deprotonation of formic acid[9] and has also been used to determine the EIE for deprotonation of phosphate esters.[10] See Supporting Information for full details.

Kinetic Isotope Effect Determinations. The experimental procedures used to measure these isotope effects were similar to those used to measure KIEs in phosphoryl transfer reactions in which the leaving group is *p*-nitrophenol.[4] In summary, the reaction is initiated and allowed to proceed to from 30 to 70% of completion. The reaction is then stopped or slowed dramatically by adjusting the pH and/or temperature and assayed to determine the precise fraction of reaction. The *p*-nitrophenol produced in this first portion of the reaction is separated from the unreacted ester. The unreacted ester is then completely hydrolyzed, and the portion of *p*-nitrophenol released in the second portion of the reaction is also isolated. Both samples of *p*-nitrophenol are purified and subjected to isotope ratio analysis. The details of the data analysis and the experimental procedures used for the hydrolysis reactions of the *p*NPPT monoester monoanion and dianion, the ethyl *p*-nitrophenyl phosphorothioate diester, and the dimethyl *p*-nitrophenyl phosphorothioate triester are given in the Supporting Information.

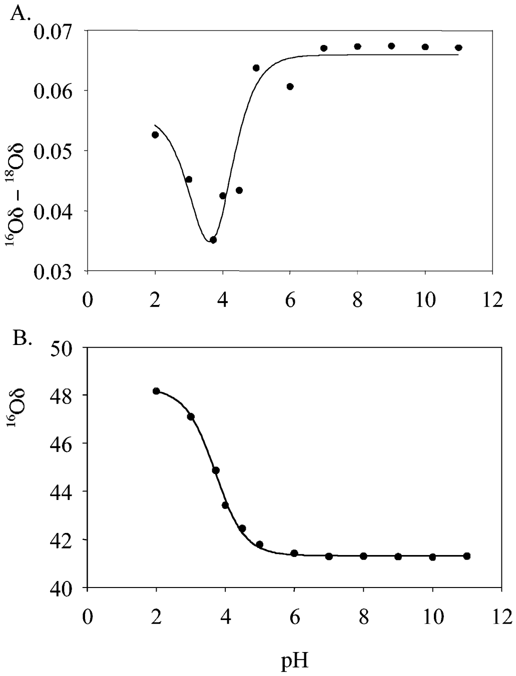

Figure 4. (A) Plot of the difference in chemical shifts between nonbridge $^{18}O_2$-labeled and unlabeled *p*NPPT as a function of pH, used for calculation of the equilibrium isotope effect for the deprotonation of *p*NPPT$^-$. (B) Chemical shift of unlabeled *p*NPPT as a function of pH, used for the pK_a determination.

Results

pK_a of *p*NPPT and the Equilibrium Isotope Effect for Deprotonation. The ^{18}O isotope-induced differences in the ^{31}P chemical shift of unlabeled and nonbridge-$^{18}O_2$-labeled *p*NPPT as a function of pH are shown in Figure 4. If one or both oxygens in a phosphate or phosphorothioate ester are substituted with ^{18}O, the ^{31}P NMR chemical shift is shifted upfield by about 0.02 ppm/atom.[11] Protonation of the phosphorothioate monoester causes the ^{31}P chemical shift to move downfield. Since the ^{18}O-labeled species is preferentially protonated, the isotope effect causes the ^{31}P signals of the ^{16}O- and ^{18}O-labeled *p*NPPT to move together as the thiophosphoryl group is titrated. The separation reaches a minimum at the pK_a and then increases as titration becomes complete.

The rapid hydrolysis of *p*NPPT at low pH[1] prevented collection of data at pH < 2. A fit of the data as described in the Supporting Information gives a value for the EIE for deprotonation of 1.015 ± 0.002. A fit of the variation in ^{31}P chemical shift with pH gave a value for the first pK_a of *p*NPPT of 3.70 ± 0.03, close to a previously reported value of 3.6[8] and within experimental uncertainty of the kinetically determined value of 3.68 ± 0.08.[1]

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Table 1. ^{31}P Chemical Shifts (ppm) of ^{18}O-Labeled and Unlabeled 3 mM *p*NPPT as a Function Zn^{2+} Concentration[a]

Zn^{2+} (M)	$^{16}O\delta$	$^{18}O\delta$	$^{16}O\delta - {}^{18}O\delta$
0	41.235	41.181	0.054
0.5	32.612	32.551	0.061
1.5	32.416	32.354	0.062

[a] The last column is the difference between the two chemical shifts.

Table 2. ^{31}P Chemical Shifts (ppm) of ^{18}O-Labeled and Unlabeled 3 mM *p*NPPT as a Function Cd^{2+} Concentration[a]

Cd^{2+} (mM)	$^{16}O\delta$	$^{18}O\delta$	$^{16}O\delta - {}^{18}O\delta$
0	41.184	41.116	0.068
5	34.971	34.900	0.071
15	32.915	32.844	0.071
45	32.786	32.713	0.073
60	33.290	33.218	0.072
100	33.908	33.834	0.074
130	34.479	34.408	0.071
150	34.268	34.197	0.071

[a] The last column is the difference between the two chemical shifts. From the known stability constant for the Cd^{2+}:*p*NPPT complex,[1] full complexation occurs.

The ^{18}O-isotope-induced differences in ^{31}P chemical shift data of unlabeled and nonbridge-$^{18}O_2$-labeled *p*NPPT as a function of Zn^{2+} concentration are given in Table 1 and data for Cd^{2+} complexation in Table 2. In both experiments, the negligible changes observed in $^{16}O\delta - {}^{18}O\delta$ compared to the deprotonation experiment (compare with Figure 4A) indicate the absence of a significant EIE for complexation in aqueous solution. Analysis of the Cd^{2+} data as described in the Supporting Information yields a calculated EIE of unity within experimental error (1.000 ± 0.001). The ratios of Cd^{2+}:*p*NPPT used are more than sufficient to achieve full complexation, based on the previously determined stability constant for complexation.[1]

Kinetic Isotope Effects. The KIE results for phosphorothioate esters are presented in italics in Table 3. For comparison, previously reported KIE results for the hydrolysis of the phosphate monoester *p*NPP, the diester EtO*p*NPP and the range of KIE data for other phosphodiesters with the same leaving group, and the triester diethyl-*p*NPP are also shown.

Discussion

What Is Known from Linear Free Energy Relationships. The Brønsted correlation of rate constants with leaving group pK_a (β_{lg}) is one measure of the degree of leaving group bond fission in the rate-limiting transition state. To normalize the β_{lg} value for a specific reaction, the Brønsted correlation of the equilibrium constant as a function of leaving group (β_{eq}) for the reaction of interest is needed. As has been reviewed,[15] a generally used approach is to take the ratio of these values (β_{lg}/β_{eq}) as an indication of the fraction of bond fission to the leaving group in the transition state.[16]

The β_{lg} for the uncatalyzed hydrolysis of aryl phosphorothioate monoester dianions is -1.1,[17] similar to the value of -1.2 for aryl phosphates.[18] The β_{eq} value for aryl phosphate dianions is $+1.36$, reflecting an "effective charge" of $+0.36$ felt by the aryl group in such compounds.[15] The β_{eq} for phosphorothioate dianions has not been reported, precluding a direct comparison of the fraction of bond fission in the two reactions, though the large magnitudes of both β_{lg} values suggest a high degree of bond fission in both reactions. Reported values of β_{lg} are also similar between phosphorothioate and phosphate diesters. The alkaline hydrolysis of uridine 3′-aryl phosphorothioates gives a β_{lg} value of -0.55 ± 0.05,[19] within experimental error of the $\beta_{lg} = -0.54$ for the reaction of the corresponding 3′-aryl phosphates.[20] A value of $\beta_{lg} = -0.35$ is observed for the alkaline hydrolysis of diethyl aryl phosphorothioate triesters, while the value for phosphate analogues is -0.43.[21]

As for monoesters, β_{eq} values have not been reported for phosphorothioate diesters or triesters to our knowledge. The similarity of the data is suggestive of at least generally similar transition states. However, there is reason to suspect a different β_{eq} value may hold for the sulfur analogues. Phosphorothioate monoesters have ^{31}P chemical shifts that are about 40 ppm downfield of their phosphate counterparts, indicating a difference in the electron density around phosphorus in the two types of compounds. In addition, as noted and discussed elsewhere in this paper, protonation of a phosphorothioate monoester results in a downfield change in the ^{31}P chemical shift, in contrast to a movement upfield in phosphate monoesters. Last, it is known that the charge distribution differs in phosphorothioate dianions in that a full or nearly full negative charge resides on the sulfur atom while the remaining charge is shared among the two oxygen atoms.[11,22] Changes that affect the electron distribution in phosphoryl groups can result in changes in β_{eq}; for example, the effective charge on the aryl group in aryl diethyl phosphate triesters (+0.87) is significantly different than that in aryl diphenyl analogues (+0.33).[15] Thus the KIE measurements reported here add to existing knowledge from LFER data. The EIE data also adds to our understanding of the differences and similarities between phosphates and phosphorothioates, as discussed below.

Expected Ranges of the Isotope Effects. The small absolute magnitudes of heavy-atom isotope effects, which are never more than a few percent, necessitate the use of an isotopic counting method, such as isotope ratio mass spectrometry, for their accurate measurement. Such isotope effects have proven very useful as mechanistic tools in the study of many reactions, including those of phosphate esters.[4] This prior work gives benchmarks for evaluations of the isotope effects shown in Figure 1.

With the *p*-nitrophenyl leaving group, the secondary ^{15}k isotope effect is a measure of the negative charge borne in the rate-determining transition state.[4] Thus, it gives information as to whether the leaving group departs as the anion or if protonation of the leaving group has neutralized all or part of the negative charge resulting from P−O bond fission. In a transition state with extensive P−O bond fission and no charge neutralization ^{15}k reaches a maximum value of about 1.003 at room temperature.[4]

The primary isotope effect $^{18}k_{bridge}$ gives a measure of the degree of fission of the P−O bond in the transition state. If P−O bond fission is extensive, $^{18}k_{bridge}$ is around 1.03 at room

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Table 3. Kinetic Isotope Effects for the Hydrolysis of Phosphate and Phosphorothioate Esters (Phosphorothioate Data in Italics)[a]

reactant	^{15}k	$^{18}k_{\text{bridge}}$	$^{18}k_{\text{nonbridge}}$
*p*NPP$^-$ (95 °C)	1.0004 ± 0.0002 [5]	1.0087 ± 0.0003 [5]	1.0184 ± 0.0005 [5]
*p*NPP$^-$ (30 °C)[12]	1.0005 ± 0.0001	not determined	1.0199 ± 0.0003
*p*NPP^{2-} (95 °C)	1.0028 ± 0.0002 [5]	1.0189 ± 0.0005 [5]	0.9994 ± 0.0005 [5]
pNPPT$^-$ (30 °C)[b]	*1.0005 ± 0.0001*	*1.0091 ± 0.0007*	*1.0221 ± 0.0004*
pNPPT^{2-} (50 °C)[b]	*1.0027 ± 0.0001*	*1.0237 ± 0.0007*	*1.0135 ± 0.0013*
EtO*p*NPP$^-$ (95 °C)	1.0010 ± 0.0001	1.0042 ± 0.0009	not determined
range of phosphodiester data[4]	1.0007 − 1.0016	1.0042 − 1.0063	1.0028 − 1.0056
EtOpNPPT$^-$ (95 °C)	*1.0010 ± 0.0002*	*1.0020 ± 0.0006*	*1.0019 ± 0.0004*
(EtO)$_2$*p*NPP (25 °C)	1.0007 ± 0.0001 [13]	1.0060 [14]	1.0063 ± 0.0002 [14]
(MeO)$_2$pNPPT (30 °C)	*1.0004 ± 0.0002*	*1.0045 ± 0.0006*	

[a] Referenced data are from previously reported work. The KIEs for the monoester monoanion reactions were measured at the pH optima for these reactions (pH 2.0 for pNPPT, pH 3.5 for pNPP). The monoester dianion KIEs were measured within the respective pH-independent ranges (at pH 10.0 and 11.0 for *p*NPPT and pH 11 for pNPP). KIEs for the diester and triester reactions were measured in the region where the hydrolyses are first order in [$^-$OH]: 1 N [$^-$OH] for EtO*p*NPP and EtO*p*NPPT, 0.2 N [$^-$OH] for (MeO)$_2$*p*NPPT, and 0.1 N [$^-$OH] for (EtO)$_2$*p*NPP. [b] The large difference in reactivity between the monoanion and dianion of *p*NPPT prevented measurement of the KIEs for these two reactions at the same temperature.

temperature; protonation of the leaving group in the transition state reduces this value.

Secondary $^{18}k_{\text{nonbridge}}$ isotope effects have proven useful in distinguishing the loose transition states such as those in mechanism A of Figure 2, which are typical of uncatalyzed monoester reactions, from associative transition states typical of triesters.[4] This is an α-secondary KIE adjacent to an atom undergoing a hybridization change. In general, such isotope effects are determined by alterations in bond order as well as in bending and torsional vibrational modes; the latter effects can be the dominant contributors to α-secondary KIEs.[23] For example, α-secondary deuterium isotope effects are normal for carbon hybridization changes of the type sp^3 to sp^2 or sp^2 to sp due to loosening of bending modes.[23] The P−O bond order changes of the nonbridge oxygen atoms in the mechanisms of Figure 2 would lead one to expect an inverse $^{18}k_{\text{nonbridge}}$ for dissociative transition states and a normal value for associative ones. The contributions to the KIE from the bending modes should be in the opposite direction. From the trends in reported $^{18}k_{\text{nonbridge}}$ data for phosphate mono-, di-, and triesters, bond order changes seem to be the dominant contributors. Values for $^{18}k_{\text{nonbridge}}$ are slightly inverse (near unity) for reactions of monoesters, which have dissociative transition states, while $^{18}k_{\text{nonbridge}}$ becomes normal for diester (with a single exception, which may be anomalous) and larger normal for triester reactions, respectively.[5,14,24]

Equilibrium Isotope Effect for Protonation of *p*NPPT. The equilibrium isotope effect of 1.015 for the deprotonation of *p*NPPT is the same, within experimental uncertainty, of EIEs reported for phosphate monoesters.[10] The data confirm that protonation occurs at oxygen rather than at sulfur, as expected from relative basicity considerations. The similarity of the EIEs indicates that sulfur substitution does not affect this isotope effect. This result confirms the notion that the EIE for deprotonation is dominated by differences in ^{16}O−H and ^{18}O−H stretching modes[25] with minimal influence from bending modes that might be influenced by the larger size of sulfur.

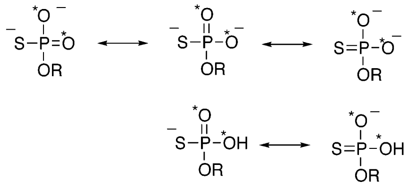

Figure 5. Contributing resonance structures in a phosphorothioate monoester as the dianion (top) and monoanion (bottom). The resonance form with a P−S double bond is a negligible contributor in the dianion but is more important (though still the lesser contributor) in the monoanion species.[26] This would result in a reduction in bond order to the isotopically labeled oxygen atoms (marked with asterisks) in the monoanion compared to the dianion and a smaller isotope shift in the ^{31}P NMR signal.

In this work, as previous researchers have observed, ^{18}O substitution resulted in a small upfield change in the ^{31}P chemical shift, analogous to that seen in phosphate esters. However the chemical shifts respond differently to protonation in the two esters. Protonation of a phosphate monoester, or of inorganic phosphate, causes the ^{31}P chemical shift to move upfield.[10] In contrast, protonation of *p*NPPT causes the ^{31}P chemical shift to move downfield (Figure 4B). It was also noted that the $^{18}\mathrm{O}\delta - {}^{16}\mathrm{O}\delta$ for *p*NPPT at low pH, where it exists as the monoanion, does not return to its value in the dianion (Figure 4A). In similar experiments with phosphate monoesters, this isotopic separation returns to its original value when titration is complete.[10] Both differences in behavior likely originate from dissimilarities in charge localization and bond order in phosphorothioates compared to phosphates. Experimental and computational evidence indicate that in dianions of phosphorothioate esters close to a full negative charge resides on the sulfur atom, while the remaining charge is shared between the two nonbridge oxygens[11,26] (Figure 5). In a computational study of inorganic thiophosphate[26] it was found that essentially no P−S double bond character is present in the trianion, consistent with experimental data.[27] The computational results predict gradually more π bonding to sulfur as the protonation state increases, with the expected P−S double bond manifested in the neutral species.[26] Extending these conclusions to *p*NPPT, somewhat greater P−S bond order is expected in the monoanion than in the dianion, resulting in a decrease in P−O bond order. Because the magnitude of the $^{18}\mathrm{O}\delta - {}^{16}\mathrm{O}\delta$ isotope shift is correlated with P−O bond order, this should result in a smaller isotope shift in the monoanion. The fit of the data in Figure 4 gives a

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reduction in $^{18}O\delta - ^{16}O\delta$ from 0.066 ppm in the dianion to 0.054 ppm in the monoanion.

It is uncertain why protonation of *p*NPPT causes the ^{31}P chemical shift to move downfield, in contrast to the movement upfield observed with phosphates. Calculations have been reported of the comparative Mulliken atomic charges on phosphate and thiophosphate in all four possible protonation states (from the trianion to the neutral species).[26] In these calculations the charge on phosphorus is more altered in phosphate than in thiophosphate by protonation; in phosphate, from the trianion PO_4^{3-} to the neutral species H_3PO_4 the Mulliken atomic charge on phosphorus changed in the sequence 1.23, 1.30, 1.38, to 1.56. The calculated changes in thiophosphate were in the same direction, but smaller: 1.28, 1.29, 1.31, to 1.44.[26] Another study using different computational methods found that the conjugate bases of phosphoric acid and thiophosphoric acid respond oppositely to protonation.[28] Protonation of $H_2PO_4^-$ caused the computed gross atomic charge on phosphorus to change from 1.42 to 1.48, while protonation of $H_2PO_3S^-$ caused a change from 1.01 to 0.85.[28] Perhaps further computational study will reveal which of these tendencies is correct and shed more light on the reason for our NMR results.

Equilibrium Isotope Effect for Complexation of *p*NPPT. In complexes with phosphorothioates, cadmium coordinates virtually completely with sulfur. In zinc complexes with phosphorothioates, about three-fourths of the species in solution are sulfur coordinated, with the remainder coordinated to oxygen.[29] The lack of a significant change in $^{18}O\delta - ^{16}O\delta$ even at the highest Zn^{2+} concentration indicates there is no significant isotope effect. For Cd^{2+} the results were similar, indicating the EIE is negligible (unity within experimental error). For comparison, the reported EIE for Ca^{2+} complexation with *p*NPP is also near unity, $^{18}K = 0.997$.[30] The results indicate that complexation in aqueous solution with either zinc or cadmium does not result in significant changes in bonding within the phosphoryl group. Cobalt(III) complexes with phosphate esters have been found to result in significant EIEs,[31] not unexpected in light of the stronger coordinating properties of this metal ion.

Kinetic Isotope Effects. (A) Hydrolysis of the *p*NPPT Dianion. The kinetic isotope effects in the leaving group in the reaction of the dianion of *p*NPPT are indicative of significant P−O bond fission and nearly a full negative charge on the leaving group in the transition state. These KIEs were measured at 50 °C in contrast to 95 °C for the *p*NPP data. Higher temperature reduces the magnitudes of KIEs, and the difference in the magnitudes of $^{18}k_{\mathrm{bridge}}$ in the reactions of *p*NPP and *p*NPPT is partly due to this effect, but this temperature difference should only account for a small change (compare the $^{18}k_{\mathrm{nonbridge}}$ values for the *p*NPP monoanion at 95 and at 30 °C in Table 3). The larger value for $^{18}k_{\mathrm{bridge}}$ in the *p*NPPT dianion reaction compared to the *p*NPP reaction implies the former reaction has a larger degree of P−O bond fission in the transition state. Evidently this occurs without a significant difference in the amount of charge delocalization into the nitro group in the transition states of the two reactions, as the values of ^{15}k are the same.

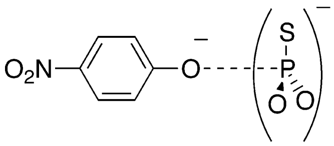

Figure 6. Schematic diagram of the transition-state structure for the hydrolysis of *p*NPPT dianion. P−O bond fission is extensive, and nearly a full negative charge resides on the leaving group.

Inconsistencies in the synchronization of development of charge and its delocalization by resonance have been well-documented.[32] Nitro compounds are particularly susceptible to this phenomenon, and such transition-state imbalances in the deprotonation of nitroalkanes (termed the "nitroalkane anomaly") have been central to the development of the principle of nonperfect synchronization.[32] One example is the deprotonation of arylnitroalkanes ($ArCH_2NO_2$) by hydroxide ion in water. The Hammett ρ value for the deprotonation rate is 1.28, while ρ for the equilibrium is 0.83.[33] This is unusual in that the rate of deprotonation is more sensitive to substituent effects than the equilibrium, implying that in the transition state more charge is felt by the aryl group than in the fully deprotonated ion. The most widely accepted explanation is that the transition state is imbalanced in that delocalization of the negative charge into the nitro group lags considerably behind deprotonation and charge development.

Why there should be a difference in the synchronization of charge development and delocalization between the reaction of the phosphorothioate compared with the phosphate ester is uncertain. This may arise from a difference in the electrostatic description of the phosphoryl group compared with thiophosphoryl. In both transition states the leaving group resembles *p*-nitrophenolate ion and is in proximity to the negatively charged (thio)phosphoryl group. The extent to which charge is delocalized, and presumably the synchronization with P−O bond fission, may be influenced by electrostatic interactions between these moieties.

Nonetheless, the $^{18}k_{\mathrm{bridge}}$ and ^{15}k data together suggest that a similar and substantial negative charge is delocalized in the leaving groups of both reactions, even though P−O bond fission is slightly greater in the reaction of *p*NPPT. Stereochemical results and other data indicate that reactions of phosphorothioate dianions such as *p*NPPT are stepwise $D_N + A_N$ mechanisms in which a thiometaphosphate intermediate forms,[34] while phosphate esters react by concerted mechanisms.[35] The loose transition state implied by the isotope effects is shown schematically in Figure 6.

The $^{18}k_{\mathrm{nonbridge}}$ data reported here for phosphorothioate esters differ from previous data for phosphate esters in two respects. The value for $^{18}k_{\mathrm{nonbridge}}$ is normal, not inverse, for the loose transition state of the *p*NPPT reaction, and it changes toward the inverse direction as the transition state becomes tighter in the diester reaction. This trend is logical if bending modes are

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the dominant contributor to $^{18}k_{\mathrm{nonbridge}}$ for phosphorothioate esters, in contrast to the situation with phosphates. A likely reason for the greater importance of bending modes in phosphorothioates is the larger van der Waals radius of sulfur.

(B) Monoanion Hydrolysis. In reactions of the monoanions of phosphate and phosphorothioate monoesters a proton is transferred from the phosphoryl group to the leaving group in the transition state. Protonation of *p*-nitrophenolate results in an inverse ^{18}O isotope effect of 0.985.[36] A transition state in which P−O bond cleavage and proton transfer are both far advanced would be expected to show an observed $^{18}k_{\mathrm{bridge}}$ that is approximately the product of 0.985 and the 1.0237 value for $^{18}k_{\mathrm{bridge}}$ observed for the dianion hydrolysis, which would be 1.008. The observed result of 1.0091 is close to this value. The magnitude of ^{15}k is also significantly reduced relative to the dianion reaction, consistent with nearly full charge neutralization of the leaving group by protonation.

The normal value of $^{18}k_{\mathrm{nonbridge}}$ primarily reflects the normal isotope effect for deprotonation. If the thiophosphoryl group resembles thiometaphosphate in the monoanion reaction as it does in the dianion one, then the observed $^{18}k_{\mathrm{nonbridge}}$ should be close to the product of the equilibrium isotope effect for deprotonation (1.015) and $^{18}k_{\mathrm{nonbridge}}$ for the hydrolysis of *p*NPPT dianion (1.0135), or 1.0287. The observed $^{18}k_{\mathrm{nonbridge}}$of 1.0221 is relatively close but smaller. The lower value may be due to incomplete proton transfer from the thiophosphoryl group in the transition state. The small but measurable ^{15}k in this reaction also indicates that proton transfer to the leaving group, while far advanced, is not complete.

(C) Alkaline Hydrolysis of the EtO*p*NPPT Diester and the (MeO)$_2$*p*NPPT Triester. LFER data suggest that phosphorothioate diesters react by a mechanism with a tighter transition state than monoesters. The leaving group isotope effects $^{18}k_{\mathrm{bridge}}$ and ^{15}k in the diester and triester reactions are smaller than in the hydrolysis of *p*NPPT^{2-}, confirming the notion that leaving group bond fission is less advanced. A comparison with previous data for phosphate diesters with the same leaving group (Table 3) reveals that the EtO*p*NPPT diester $^{18}k_{\mathrm{bridge}}$ lies below this range. This suggests that leaving group bond fission is less advanced in the phosphorothioate diester reaction.

The $^{18}k_{\mathrm{nonbridge}}$ obtained for the alkaline hydrolysis reaction of EtO*p*NPPT is smaller compared with that for the monoester *p*NPPT. This supports the hypothesis that bending modes are the dominant contributor to this isotope effect in phosphorothioate esters, in contrast to the situation with phosphate esters. In a more associative transition state the bending modes will contribute to a more inverse isotope effect, analogous to α-deuterium KIEs in situations where carbon hybridization changes from sp^2 to sp^3. In the transition state of the present reaction, the geometry of the phosphorus atom changes from tetrahedral to trigonal-bipyramidal. The small KIEs in the leaving group, in conjunction with the smaller β_{lg} data, indicate less bond fission to the leaving group, confirming the notion that the transition state becomes more crowded in a manner that will compress the bending modes of the nonbridging sulfur and oxygen atoms.

The alkaline hydrolysis of (MeO)$_2$*p*NPPT triester gives values for $^{18}k_{\mathrm{bridge}}$ and ^{15}k that are each smaller than their phosphate triester counterparts. As with the diester results, this suggests that leaving group bond fission in the transition state is less advanced for the phosphorothioate triester, though the difference is not large.

Conclusions

Kinetic isotope effects in the nonbridging oxygen atoms in phosphorothioate esters compared to phosphate esters respond oppositely to hybridization changes at phosphorus. In phosphorothioates the trend is similar to that seen in α-secondary hydrogen isotope effects on atoms undergoing hybridization changes, in which bending modes are the predominant determinants of the isotope effect: normal for changes of the type sp^3 to sp^2 and inverse in the direction sp^2 to sp^3. Changes in π bonding occur in reactions involving the phosphoryl group that are absent in reactions involving hydrogen. Thus, changes in bond order can have a greater impact in the former case, and this is the most likely explanation for the trends observed in α-secondary nonbridge ^{18}O KIEs for phosphate ester reactions. The fact that the opposite trend is seen in the phosphorothioate reactions suggests that the larger size of sulfur causes bending mode changes to predominate.

The EIE for deprotonation of a phosphorothioate monoester is within experimental error of those for phosphate monoesters, indicating that this primary EIE is determined by O−H stretching considerations and is unaffected by bending modes. ^{31}P NMR data reveal differences in the response of the *p*NPPT to protonation compared to the response of phosphate monoesters, suggestive of differences in the effect of protonation on the atomic charges on phosphorus in the two types of compounds. Protonation of the dianion of *p*NPPT is accompanied by an increase in π bonding between phosphorus and sulfur, resulting in lessened P−O bond order involving the nonbridge oxygen atoms in the monoanion compared to the dianion, consistent with computational predictions.

No significant EIEs for metal ion complexation are observed, indicating that in aqueous solution these interactions are weak and do not affect bonding within the thiophosphoryl group.

The kinetic isotope effects in the *p*NPPT dianion reaction suggest slightly greater P−O bond fission than in the reaction of the corresponding phosphate ester. A difference in the synchronization of charge development on the leaving group arising from P−O bond fission and its delocalization was noted in the reactions of the phosphate and phosphorothioate. The *p*NPPT monoanion reaction proceeds with a late transition state in which the proton is largely but not completely transferred from the thiophosphoryl group to the leaving group. The KIEs measured for a phosphorothioate diester and triester suggest that leaving group bond fission is somewhat less advanced than in reactions of their phosphate counterparts.

Acknowledgment. The authors thank the NIH for financial support of this research (Grant GM47297).

Supporting Information Available: Experimental details and the equations used for calculation of the pK_a and EIEs for *p*NPPT, details of procedures used in KIE experiments, and equations used for calculating corrected KIEs (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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