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The Thermodynamics of Phosphate versus Phosphorothioate Ester Hydrolysis

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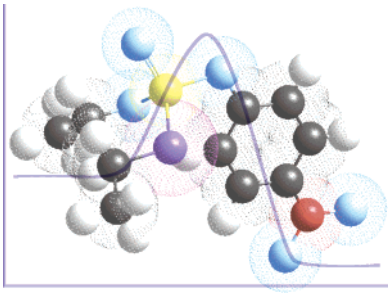

Phosphorothioate esters are phosphate esters in which one of the nonbridging oxygen atoms has been replaced by sulfur. In the comparative hydrolysis reactions of phosphorothioate and phosphate esters, the sulfur substitution accelerates the rates of the monoesters while slowing the rates of diesters and of triesters. Previously measured enthalpies and entropies of activation for the hydrolysis reactions of the monoesters, *p*-nitrophenyl phosphate and *p*-nitrophenyl phosphorothioate, were compared to the activation parameters measured herein for the diesters, ethyl *p*-nitrophenyl phosphate and ethyl *p*-nitrophenyl phosphorothioate, and the triesters, diethyl *p*-nitrophenyl phosphate and diethyl *p*-nitrophenyl phosphorothioate. A consistent trend of a greater $\Delta H^{\ddagger}$ for the phosphorothioate analogue was found in all three classes of ester. In the monoester case, a more positive $\Delta S^{\ddagger}$ arising from a mechanistic difference ($D_N + A_N$ for the phosphorothioate versus $A_N D_N$ for the phosphate) compensates, resulting in a lower $\Delta G^{\ddagger}$ for the phosphorothioate monoester. Spectroscopic investigations indicate there is no significant difference in bond order to the leaving group in phosphates, as compared to their phosphorothioate analogues, ruling this out as a contribution to the consistently higher enthalpies of activation.

Introduction

Phosphorothioates are analogues of phosphate esters in which a sulfur atom is present in place of one of the nonbridging oxygen atoms. A change in the rate of hydrolysis has been observed to accompany this sulfur substitution, leading to a phenomenon known as the "thio effect". The thio effect is reported as k_O/k_S, the ratio of the rate of phosphoryl transfer to that for thiophosphoryl transfer. This sulfur substitution significantly increases the rates of hydrolysis of phosphomonoester dianions, leading to an inverse thio effect ($k_O/k_S < 1$). However, similar substitution has the opposite effect on the hydrolysis rates of diesters and triesters (Table 1). As a result of this trend, the magnitudes and directions of thio effects have been used to examine mechanistic details of enzymatic phosphoryl transfer. An understanding of the origin of the opposite thio effect for monoesters versus diesters and triesters is needed to properly interpret such data.

TABLE 1. Correlation of Thio Effects for Alkaline Hydrolysis Reactions of Phosphate and Phosphorothioate Esters[1,2]

phosphate ester	range of thio effects (k_O/k_S)
triester	10−160
diester	4−11
monoester	0.1−0.3

Linear free energy relationships and kinetic isotope effects have identified the mechanisms and yielded information regarding the transition states of the reactions of phosphate and phosphorothioate monoesters, diesters, and triesters. Monoesters undergo hydrolysis via loose transition states in which bond fission to the leaving group is extensive, and nucleophilic participation in the rate-limiting step is either small (in the case of phosphates, where the mechanism is $A_N D_N$) or absent (in the case of phosphorothioates, where the mechanism is $D_N + A_N$). Acyclic diesters and triesters with good (i.e., aryl)

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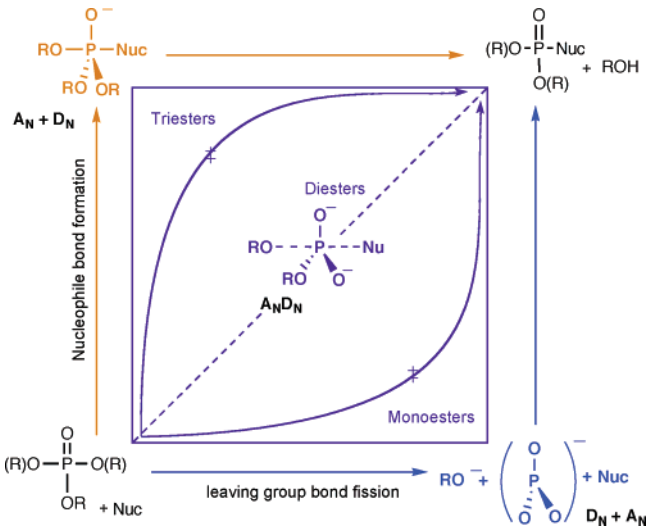

FIGURE 1. More−O'Ferrall−Jencks diagram depicting the general location of transition states of uncatalyzed phosphoryl transfer reactions of phosphomonoesters, diesters, and triesters. In general, the transition states become tighter as the alkylation state of the ester increases.

leaving groups undergo hydrolysis by concerted reactions via transition states with greater nucleophilic participation. In reactions of diesters, nucleophilic attack and leaving group departure are more synchronous, in a concerted, A_ND_N mechanism. Triester reactions exhibit tight, phosphorane-like transition states with nucleophilic attack ahead of leaving group departure. This is shown pictorially on the More−O'Ferrall−Jencks diagram in Figure 1.

Thio effects have been used in attempts to discern whether enzymatic phosphoryl transfer is associative or dissociative, although the risks associated with such interpretations of thio effects have been pointed out.[3] A number of proposals have been made to explain reactivity differences between phosphates and their thiophosphate analogues, such as the comparative electron-donating ability of oxygen versus sulfur,[4,5] the greater electrophilicity of phosphorus in phosphoryl versus thiophosphoryl compounds,[6,7] differences in polarization of P=O and P=S bonds,[8] and structural differences between the two ester types, including interatomic distances and atomic radii.[9] However, none of these provides an explanation for the thio effects observed across all three classes of ester.

TABLE 2. Activation Parameters for Hydrolysis Reactions of Phosphate and Phosphorothioate Monoesters, Diesters, and Triesters at 312 K

	$\Delta G^{\ddagger}$ (kcal/mol)	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (eu)
Monoester Dianions[a]			
*p*NPP[10]	29.5	30.6	+3.5
*p*NPPT[11]	27.9 ± 1.0	37.0 ±1.0	+29 ± 3
Diesters[b]			
EtO-*p*NPP	27.4 ± 0.5	14.91 ± 0.06	−36.6 ± 0.1
EtO-*p*NPPT	27.9 ± 0.4	18.5 ± 0.1	−26.9 ± 0.3
Triesters[b]			
paraoxon	20.0 ± 0.3	12.69 ± 0.03	−24.81 ± 0.05
parathion	21.5 ± 0.2	16.43 ± 0.08	−17.3 ± 0.1

[a] Previously reported data. [b] This work.

In this work we report the activation parameters for the hydrolysis reactions of the P=O and P=S analogues of a monoester, diester, and triester, to ascertain the thermodynamic contributions to the differences in activation energies. This information should provide further understanding of the fundamental causes of the observed thio effects. We have also carried out spectroscopic investigations via ^{31}P NMR and FTIR in order to determine whether sulfur substitution in a nonbridging position has any effect on the scissile P−O bond.

Results

The activation parameters were calculated from Eyring plots for the alkaline hydrolysis of the diester and triester phosphate and phosphorothioate esters, giving the values in Table 2. Data for the monoester hydrolysis of *p*NPP[10] and *p*NPPT[11] are from previous work.

The ^{18}O-induced isotope effect on the ^{31}P chemical shifts of the monoesters and triesters were obtained from the NMR of labeled versions of the four compounds, containing 41% ^{18}O incorporation in the bridging position. As an example of these spectra, Figure 2 shows the ^{31}P NMR spectrum of the monoester *p*NPPT. Table 3 shows the isotope shift data obtained for the phosphate and phosphorothioate analogues of the monoester and triester.

The frequencies of the infrared stretching bands of the P−O(nitrophenyl) bond in the monoesters and triesters were measured as described in Experimental Section and are shown in Table 4.

Discussion

The activation parameters reveal that for all three classes of phosphoesters (monoester, diester, and triester) hydrolysis of the thiophosphate is accompanied by a higher enthalpy of activation ($\Delta H^{\ddagger}$), but a more favorable entropy of activation ($\Delta S^{\ddagger}$), than the corresponding phosphate ester. In the diester and triester reactions, the enthalpic contribution to the overall barrier is larger than the $T\Delta S^{\ddagger}$ term, resulting in slower reaction rates for the phosphorothioate analogues. However, in the monoester case, a significantly more positive (favorable) $\Delta S^{\ddagger}$ for the phosphorothioate ester overcomes the larger $\Delta H^{\ddagger}$, result-

(1) Domanico, P.; Mizrahi, V.; Benkovic, S. J. In *Mechanisms of Enzymatic Reactions: Stereochemistry*; Frey, P. A., Ed.; Elsevier: New York, 1986; pp 127−137.
(2) Herschlag, D. *J. Am. Chem. Soc.* **1994**, *116*, 11631−11635.
(3) Holtz, K. M.; Catrina, I. E.; Hengge, A. C.; Kantrowitz, E. R. *Biochemistry* **2000**, *39*, 9451−9458.
(4) Williams, A.; Douglas, K. T. *J. Chem. Soc., Perkin Trans. 2* **1975**, 1010−1016.
(5) Zhang, Y.-L.; Hollfelder, F.; Gordon, S. J.; Chen, L.; Keng, Y.-F.; Wu, L.; Herschlag, D.; Zhang, Z.-Y. *Biochemistry* **1999**, *38*, 12111−12123.
(6) Ketelaar, J. A. A.; Gresmann, H. R.; Koopmans, K. *Rec. Trav. Chim. Pays-Bas* **1952**, *71*, 1253−1258.
(7) Neimysheva, A. A.; Savchik, V. I.; Ermolaeva, M. V.; Knunyants, I. L. *Bull. Acad. Sci. USSR. Div. Chem. Sci. Engl. Transl.* **1968**, 2104−2108.
(8) Chlebowski, J. F.; Coleman, J. E. *J. Biol. Chem.* **1974**, *249*, 7192−7202.
(9) Teichmann, H.; Hilgetag, G. *Angew. Chem., Int. Ed. Engl.* **1967**, *6*, 1013−1023.
(10) Kirby, A. J.; Jencks, W. P. *J. Am. Chem. Soc.* **1965**, *87*, 3209−3216.
(11) Catrina, I. E.; Hengge, A. C. *J. Am. Chem. Soc.* **1999**, *121*, 2156−2163.

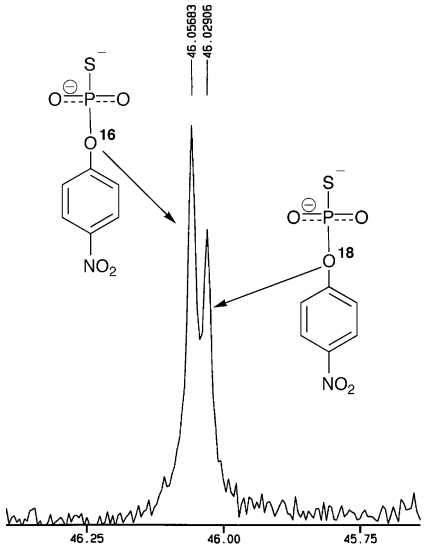

FIGURE 2. ^{31}P NMR spectrum of 41% [^{18}O]-labeled *p*NPPT showing the ^{18}O-induced perturbation to the ^{31}P chemical shift.

TABLE 3. Isotope-Induced ^{31}P NMR Shifts ($\Delta\delta_P$) for Monoesters *p*-NPP and *p*-NPPT and Triesters Paraoxon and Parathion

compound	$\Delta\delta_P = (\delta^{16}O - \delta^{18}O)$ (ppb)
*p*NPP	20.2
*p*NPPT	27.8
paraoxon (diethyl *p*-NPP)	15.8
parathion (diethyl *p*-NPPT)	20.4

TABLE 4. Stretching Frequency of the P−(O)R Bond in Monoesters and Triesters

compound	P−O(R) stretch (cm^{-1})
*p*NPP	730
*p*NPPT	725
paraoxon (diethyl *p*-NPP)	785, 764, 737
parathion (diethyl *p*-NPPT)	788, 770, 749

ing in a faster hydrolysis and a thio effect of less than 1. The significantly more positive $\Delta S^{\ddagger}$ in the phosphorothioate monoester reaction results from a mechanistic difference. Phosphorothioate monoesters undergo hydrolysis via a thiometaphosphate intermediate,[1,12] making the rate-limiting step a unimolecular dissociation, reflected in the significantly more positive entropy of activation. In contrast, the oxygen analogue reacts by a loose transition state with little, but measurable, nucleophilic participation.

Several reasons have been proposed to explain why sulfur substitution should facilitate this mechanistic change from a loose transition state in a concerted reaction to a completely dissociative mechanism. One proposal is that the sulfur atom is more able to donate negative charge, thus facilitating leaving group expulsion.[5] However, replacing oxygen by sulfur in the alkaline hydrolysis of phenyl esters of phenylphosphonamidates changes the mechanism in the opposite direction (from *E*1cB to addition−elimination), a change that has been rationalized on the *poorer* electron-donating ability of sulfur.[4] The lower electronegativity, but greater polarizability of sulfur, relative to oxygen, complicates any rationalization of the differential effects of sulfur substitution on the rates of these compounds to a single atomic property.

TABLE 5. Isotope Shifts for ADP and ATP Isomers,[17,19] Inorganic Phosphate, and Paraoxon[21]

compound	$\Delta\delta_P = \delta^{16}O-\delta^{18}O$ (ppb)	formal bond order
[β,γ-bridging ^{18}O] ATP	16.5	1.0
[α,β-bridging ^{18}O] ADP	16.6	1.0
inorganic phosphate trianion	20.5	1.25
[β-nonbridging ^{18}O] ADP	21.5	1.33
[γ-nonbridging ^{18}O] ATP	22.0	1.33
[β-nonbridging ^{18}O] ATP	28.5	1.5
[nonbridging ^{18}O] paraoxon	40.0	2.0

In the hydrolysis reactions of all three types of esters, regardless of mechanistic differences, bond fission to the leaving group always occurs in the rate-determining step. This raises the question of whether differences in the leaving group P−O bonds might be responsible for the consistently higher enthalpy of activation in the phosphorothioate reactions. Computational and experimental work has shown that sulfur substitution affects the P−O bond order and the charge distribution between nonbridging atoms within the (thio)phosphoryl group.[13,14] If the bridging P−OR bonds were also affected, such that the bond to the leaving group is stronger in the thio analogues, then a higher enthalpy of activation might result. To test this hypothesis, this bond was investigated using NMR and FTIR. A comparison of the magnitudes of the ^{18}O-induced perturbation to the ^{31}P NMR chemical shift ($\Delta\delta_P$) in a labeled monoester and triester was used to investigate this bond, since these ester types give the largest thio effects and were the most amenable to isotopic synthesis.

Such isotope-induced NMR shifts result from the combination of greater nuclear shielding provided by the heavier nucleus, and anharmonicity, manifested in a shortened bond length upon substitution of the heavier isotope. Isotope shift data have been compiled for a large number of compounds, and the cumulative data has led to the observation that, within the same or similar functional groups, the magnitude of the isotope shift is proportional to bond order.[15,16] Published ^{18}O-induced isotope shifts on ^{31}P NMR spectra of phosphate esters also show that the magnitude of this isotope-induced shift ($\Delta\delta_P$) is proportional to the P−O bond order. The magnitude of $\Delta\delta_P$ increases from ~0.016 ppm for P−O single bonds to ~0.041 ppm for double bonds (Table 5 and Figure 3).[17−19] The validity of the correlation is further demonstrated by the linear relationship between the

(12) Burgess, J.; Blundell, N.; Cullis, P. M.; Hubbard, C. D.; Misra, R. *J. Am. Chem. Soc.* **1988**, *110*, 7900−7901.
(13) Frey, P. A.; Sammons, D. *Science* **1985**, *228*, 541−545.
(14) Liang, C.; Allen, L. C. *J. Am. Chem. Soc.* **1987**, *109*, 6449−6453.
(15) Risley, J. M.; Van Etten, R. L. In *Isotope Effects in NMR Spectroscopy*; Diehl, P. F. E., Guenther, H., Kosfeld, R., Seelig, J., Eds.; Springer-Verlag: Berlin, 1990; Vol. NMR 22, pp 81−168.
(16) Jameson, C. J. In *Encyclopedia of Nuclear Magnetic REsonance*; Grant, D. M., Harris, R. K., Eds.; John Wiley: London, 1995; Vol. 6, pp 2638−2655.
(17) Cohn, M.; Hu, A. *J. Am. Chem. Soc.* **1980**, *102*, 913−916.
(18) Cohn, M.; Hu, A. *Proc. Natl. Acad. Sci.* **1978**, *75*, 200−203.
(19) Lowe, G.; Sproat, B. S. *J. Chem. Soc., Chem. Commun.* **1978**, 565−566.

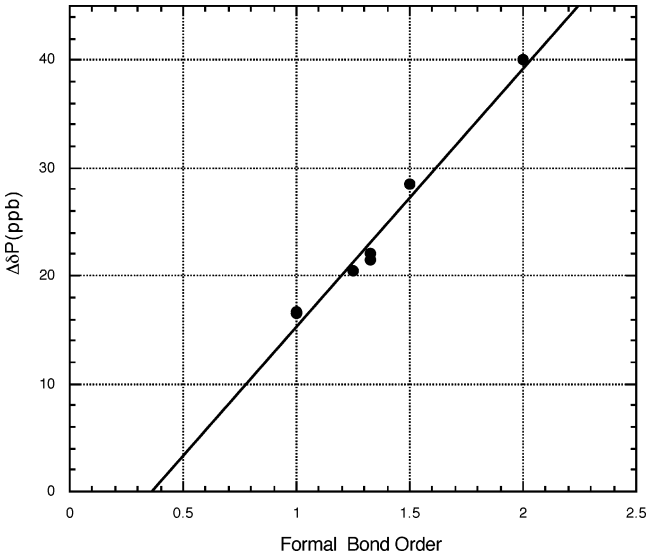

FIGURE 3. Graph of the isotope shifts in Table 5. The line represents a least-squares fit to the data and obeys the relation $\Delta\delta_P = 23.8(\text{bond order}) - 8.4$.

magnitude of $\Delta\delta_P$ and the P−O stretching frequencies in methyl, dimethyl, and trimethyl phosphates.[20]

The monoesters and triesters were synthesized as isotopic isomers in which ^{18}O was partially incorporated into the bridging phenolic position, which is the scissile P−O bond. Incorporation of ^{18}O in phosphate esters results in a small upfield shift in the ^{31}P NMR signal. The isotope shift data for both the monoester and triester (Table 3) show a larger $\Delta\delta_P$ for the thio esters. This might be taken as an indication of a stronger P−O bond to the leaving group of the latter compounds, perhaps accounting for the higher enthalpies of activation. However, the phosphate and phosphorothioate functional groups differ, and we considered the possibility that the larger $\Delta\delta_P$ in the phosphorothioates might arise from differences in the relative contributions of nuclear shielding and anharmonicity in the two sets of compounds. To examine this possibility, we compiled published isotope shift values for phosphorothioates and constructed a correlation for these compounds, analogous to the correlation for phosphate esters shown in Figure 3. This compilation of $\Delta\delta_P$ data for a series of phosphorothioates with varying bond orders to the labeled oxygen atom is shown in Table 6 and displayed graphically in Figure 4.

A small but statistically significant difference is noted between the two correlations, indicating that a bond order of approximately unity exhibits a larger isotope shift in phosphorothioates than in phosphates. This raises the possibility that the difference in isotope shifts in Table 3 might arise from a difference in the contributions from anharmonicity between the two functional groups, affirming the caution raised by others[15] that correlations of isotope shifts with bond order must be made within functional groups. An alternate interpretation is that the P−O bond orders in phosphorothioates are indeed higher than the formal bond orders obtained from Lewis structures, and the different correlations result from the formal bond orders not reflecting the true bond orders.

TABLE 6. Literature Values for the ^{18}O-Induced ^{31}P NMR Shift, Used to Construct the Phosphorothioate Calibration Graph in Figure 4

compound	$\Delta\delta_P$ (ppb)	formal bond order
[α,β-bridging ^{18}O]ADPαS	22.0[13,22]	1.0
S-carbamoylethyl-[^{18}O]-phosphate	27.0[13]	1.33
S-methyl-[^{18}O] AMPS	35.0[13,23]	1.5
[α-^{18}O] AMPS	33.0[13,23]	1.5
[α-nonbridging ^{18}O] ADPαS	37.0[13,22]	2.0
endo-uridine-2′3′-cyclic-[^{18}O]-phosphorothioate	41.0[24]	2.0
uridine 2′[^{18}O] phosphorothioate	33.0[24]	1.5
uridine 3′[^{18}O] phosphorothioate	34.0[24]	1.5
[α,β-bridging ^{18}O] ATPβS	21.0[22]	1.0
[β-nonbridging ^{18}O] ATPβS	36.0[22]	2.0
compound **5**[a]	41.0[25]	2.0
compound **4**[b]	19.0[25]	1.0

[a] 1,3,2-Dioxaphosphorinane, 2-hydroxy-5,5-dimethyl-2-sulfide (Registry no. 15762-04-6). [b] 2-[(5,5-Dimethyl-2-oxido-1,3,2-dioxaphosphorinan-2-yl)oxy]-5,5-dimethyl-2-sulfide (Registry no. 55474-16-3).

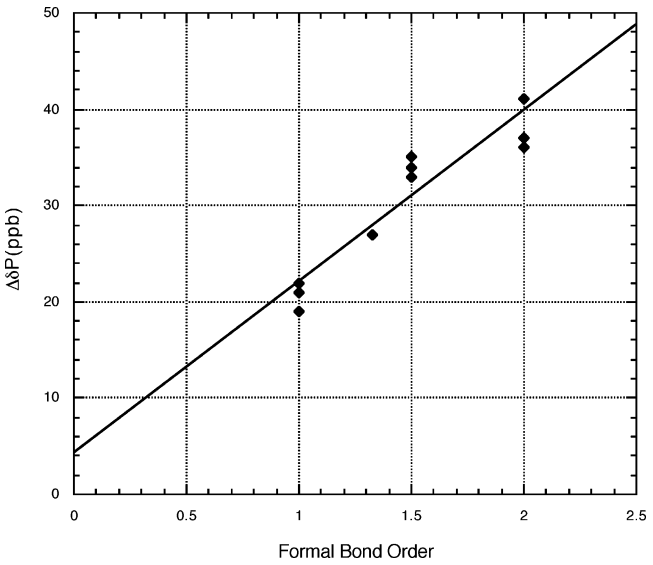

FIGURE 4. Graph of the isotope shifts in Table 6. The line represents a least-squares fit to the data and obeys the relation $\Delta\delta_P = 17.8(\text{bond order}) + 4.4$.

To provide additional evidence of whether the differences in $\Delta\delta_P$ reflect true differences in bond order, the frequencies of the infrared stretching bands of the P−O(nitrophenyl) bond in the monoesters and the triesters were measured. The location of this band in the monoester *p*NPP has previously been assigned to ∼730 cm^{-1}, with a slight dependence on solvent.[26] The IR results are shown in Table 4.

The close similarity of the phosphate-ester stretching vibrations in the phosphate versus phosphorothioate

(20) Lowe, G.; Potter, B. V. L.; Sproat, B. S. *J. Chem. Soc. Chem. Commun.* **1979**, 733−735.
(21) Sorensen-Stowell, K.; Hengge, A. C. *J. Org. Chem.* **2005**, *70*, 4805−4809.
(22) Webb, M. R.; Trentham, D. R. *J. Biol. Chem*. **1980**, *255*, 1775−1778.
(23) Iyengar, R.; Eckstein, F.; Frey, P. A. *J. Am. Chem. Soc.* **1984**, *106*, 8309−8310.
(24) Gerlt, J. A.; Wan, W. H. *Biochemistry* **1979**, *18*, 4630−4638.
(25) Frey, P. A.; Reimschussel, W.; Paneth, P. *J. Am. Chem. Soc.* **1986**, *108*, 1720−1722.
(26) Cheng, H.; Nikolic-Hughes, I.; Wang, J. H.; Deng, H.; O'Brien, P. J.; Wu, L.; Zhang, Z. Y.; Herschlag, D.; Callender, R. *J. Am. Chem. Soc.* **2002**, *124*, 11295−11306.

monoesters and triesters supports the conclusion that there is no significant difference in the P−OR ester bond as a result of sulfur substitution. Using the formulation of Cheng et al.[26] to relate differences in the stretching frequency of this bond to changes in bond length, the ∼5 cm^{-1} difference between the phosphate and phosphorothioate monoesters and the triesters will correspond to differences in bond length of ∼0.004 Å. This suggests that the differences in isotope shift arise from a source other than altered bond orders to the leaving group. We conclude that the effect of sulfur substitution in the nonbridging position on the scissile ester bond is insignificant and is too small to account for the notable differences in $\Delta H^{\ddagger}$.

What, then, could account for the consistently higher values of $\Delta H^{\ddagger}$ in the reactions of phosphorothioates? Differences in the extent of leaving group bond fission in the transition states of phosphates compared to phosphorothioates are not a likely source. Linear free energy relationships and kinetic isotope effects have shown that this parameter is very similar for the respective reactions of the monoesters, and for diesters and triesters, leaving group bond fission is slightly more advanced for reactions of phosphates than phosphorothioates.[27] Moving to a consideration of the (thio)-phosphoryl group, the changes in bonding between phosphorus and the nonbridging atoms during these reactions are diametrically opposite in monoesters than in the other two classes of esters. In monoesters, the reaction coordinate is dominated by leaving group expulsion, and bond order between the nonbridging atoms and phosphorus should increase to maintain constant bond order to phosphorus in the metaphosphate-like transition state. In diesters, and even more so in triesters, nucleophilic attack dominates the reaction coordinate; the transition state resembles a phosphorane, and bond order to the nonbridging atom(s) decreases. This makes it unlikely that bonding changes involving the nonbridging atoms and phosphorus account for the higher $\Delta H^{\ddagger}$ across all three ester classes. A reviewer raised the question of whether the higher barrier might be the result of steric or electrostatic properties of the sulfur atom that slow nucleophilic attack. For this to be the case, an alternative explanation would need to be found for the observations of stereochemical racemization and the significant positive entropy of activation in the hydrolysis of phosphorothioate monoesters, which have been interpreted to imply that unimolecular dissociation to form thiometaphosphate is rate-limiting. At this point, a satisfactory explanation for the trend in the enthalpies of activation remains elusive.

Relevance to Enzymatic Thio Effects. A reversal of the thio effect in monoesters from <1 in uncatalyzed reactions to >1 in enzymatic reactions has been used as an indicator that such reactions (for example, alkaline phosphatase) might proceed by a triester-like transition state.[28−30] The results here suggest a possible alternative explanation. The opposite thio effect in monoester hydrolysis results from a large difference in the entropies of activation. With a preassociated nucleophile in the milieu of the enzymatic active site, the mechanistic difference between the reactions of the phosphate and phosphorothioate monoesters might disappear, minimizing the difference in the entropies of activation. Assuming the relative enthalpies of activation remain similar, then a slower reaction of the phosphorothioate monoester would result without any mechanistic change to a triester type mechanism.

Conclusions

The hydrolysis of the phosphorothioate monoester, diester and triester examined in this study each exhibit a greater enthalpy of activation than their phosphate ester counterpart. The inverse thio effect in the monoester reaction results from a mechanistic difference that results from the sulfur substitution, in which unimolecular dissociation becomes rate-limiting. The accompanying large positive entropy of activation overcomes the increased enthalpy of activation, resulting in a faster hydrolysis rate for the phosphorothioate monoester. In diesters and triesters, the differences in the entropies of activation are more modest and not large enough to offset the increased enthalpic barrier, resulting in slower hydrolysis rates for phosphorothioate diesters and triesters. The consistently greater enthalpic barrier in the reactions of the phosphorothioates does not result from a difference in strength of the scissile ester bond. The source for the trend in enthalpies across the three classes of esters examined herein is not revealed by this study.

Experimental Procedures

Materials. All reagents and solvents were purchased commercially and unless otherwise noted were used as received. Pyridine was distilled from calcium hydride. Tetrahydrofuran (THF) was distilled from sodium. *p*-Nitrophenol is a commercial product that was recrystallized from toluene. *p*-Nitrophenol containing a mixture of ^{16}O and ^{18}O in the phenolic oxygen atom was synthesized by exchange with labeled water under alkaline conditions as previously described.[31] The percentage of ^{18}O incorporation in the *p*-nitrophenol was measured using GC−MS and found to be 41%.

Synthesis. Diethyl *p*-Nitrophenyl Phosphate (Paraoxon). Natural abundance paraoxon was synthesized by adding a solution containing 8.2 mmol of *p*-nitrophenol, 0.8 mmol of dimethyl aminopyridine (DMAP), and 8.2 mmol of triethylamine in 5 mL of THF dropwise to a stirring solution of 8.2 mmol diethyl chlorophosphate in 10 mL of THF. The reaction mixture was allowed to stir under nitrogen in an ice bath for 4 h. The reaction mixture was then quenched with 15 mL of water, extracted with diethyl ether (3 × 25 mL), and dried with magnesium sulfate. The crude product was purified using a flash silica gel column with a 3:1 hexanes/ethyl acetate elution mixture and dried down by rotary evaporation. The product, a light yellow oil, was characterized by NMR and found to be pure.

Diethyl *p*-Nitrophenyl Phosphorothioate (Parathion). Natural abundance parathion was synthesized using the same method as above, but with the appropriate diethyl chlorothiophosphate substitution. Bridging isotopically labeled paraoxon and parathion analogues were also synthesized via the same method using [^{16}O,^{18}O] *p*-nitrophenol in place of natural abundance *p*-nitrophenol.

(27) Catrina, I. E.; Hengge, A. C. *J. Am. Chem. Soc.* **2003**, *125*, 7546−7552.
(28) Breslow, R.; Katz, I. *J. Am. Chem. Soc.* **1968**, *90*, 7376−7377.
(29) Han, R.; Coleman, J. E. *Biochemistry* **1995**, *34*, 4238−4245.
(30) Mushak, P.; Coleman, J. E. *Biochemistry* **1972**, *11*, 201−205.
(31) Hengge, A. C. *J. Am. Chem. Soc.* **1992**, *114*, 2747−2748.

Ethyl *p*-Nitrophenyl Phosphate (EtO-*p*NPP) and Ethyl *p*-Nitrophenyl Phosphorothioate (EtO-*p*NPPT). The diesters EtO-*p*NPP and EtO-*p*NPPT were synthesized as previously described.[27]

***p*-Nitrophenyl Phosphate (*p*NPP) and *p*-Nitrophenyl Phosphorothioate (*p*NPPT).** The bis(cyclohexylammonium) salts of monoesters *p*-NPP[32] and *p*-NPPT[11] were synthesized as previously described. The same method was employed with [^{16}O,^{18}O] *p*-nitrophenol in order to synthesize the bridge-labeled monoesters that were used for spectroscopic investigations.

Kinetics. The activation parameters for the aqueous hydrolysis of the monoanion and dianion of the monoesters *p*-NPP and *p*-NPPT were previously reported.[11] The alkaline hydrolysis of the diesters and triesters followed first-order kinetics with respect to hydroxide concentration (data not shown), consistent with previous studies.[33,34]

Diesters. Reactions were initiated by injection of 500 μL of a 20 mM aqueous stock solution of EtO-*p*NPP into 2 mL of 1.2 N NaOH, giving a final hydroxide concentration of 1 N. Reaction mixtures were placed in sealed Pyrex tubes and were maintained at constant temperatures in a heating block (50, 57, 64, 69, 75, 80, 86, and 92 °C). The progress of the reaction was followed by periodically adding a 100 μL aliquot of the reaction mixture to 3 mL of 0.1 N NaOH and recording the absorbance due to *p*-nitrophenolate production, at 400 nm. Activation parameters were calculated from Eyring plots, using second-order rate constants obtained using the initial rates method.

For the thiophosphate diester, reactions were initiated by injection of 200 μL of a 12 mM aqueous stock solution of EtO-*p*NPPT into 1 mL of 1.2 N NaOH. These reactions were heated similarly at seven temperatures (57, 63, 74, 79, 85, 90, and 92 °C). Reaction course was monitored from the absorbance at 400 nm of 50 μL of the reaction mixture added to 1.5 mL of 0.1 N NaOH. Rate constants were also obtained from the initial rates method.

Triesters. Stock solutions (2 mM) of parathion and paraoxon in 1,4-dioxane were prepared. Aliquots of these stock solutions (50 μL) were then added to 3 mL of sodium hydroxide to initiate hydrolysis. Five hydroxide concentrations (0.10, 0.25, 0.50, 0.75, 1.0 M) were used in order to determine second-order rate constants. These reactions were followed to completion (>10 half-lives) with a Cary-1 Bio spectrophotometer at 400 nm to monitor *p*-nitrophenolate liberation. Rate constants for aqueous hydrolysis were calculated for each compound at several temperatures (10, 15, 20, 25, and 30 °C).

Spectroscopic Experiments. All FT-IR spectra were obtained using a Shimadzu FTIR-4800 spectrophotometer. ^{31}P NMR experiments were conducted using a Bruker-400 MHz spectrometer.

Monoesters. Pellets (KBr) were made from oven-dried potassium bromide and approximately 1 mg of either *p*NPP or *p*NPPT, as the cyclohexylammonium salt. The spectrometer was set at a resolution of 2 cm^{-1} for 32 scans between 400 and 4000 wavenumbers. Bridge-labeled analogues (31%-41% ^{18}O incorporation) of each compound were also evaluated as the cyclohexylammonium salts in KBr pellets, to confirm the assignment of the P−(O)R stretching vibration. Incorporation of the heavier ^{18}O atom into the bridging bond causes a slight decrease in the frequency of the stretching vibration. This decrease causes the infrared stretch to appear at a slightly lower wavenumber. The shift enables identification of the specific ester stretch of interest. P−(O)R stretching vibrations for phosphorothioate and phosphate monoesters are, respectively: [*p*-NPPT ($\nu_{\mathrm{P-(O)R\ stretch}}$ = 725 cm^{-1}, $\nu_{\mathrm{P-(^{18}O)R\ stretch}}$ = 712 cm^{-1})], [*p*-NPP ($\nu_{\mathrm{P-(O)R\ stretch}}$ = 730 cm^{-1}, $\nu_{\mathrm{P-(^{18}O)R\ stretch}}$ = 721 cm^{-1})].

Solutions (20 mM) were made of the cyclohexylammonium salt of both monoesters and of their bridge labeled analogues in deuterium oxide and analyzed via ^{31}P NMR to determine the ^{18}O isotope effect on the ^{31}P chemical shift. Spectra were recorded at 161.97 MHz, with an acquisition time of 0.6 s, a sweep width of 8200 Hz, a pulse width of 8 μs, and a line broadening of 0.50 Hz.

Triesters. Solutions of the triesters parathion and paraoxon were made in chloroform. IR spectra of the triesters were obtained using KBr cells. Chloroform solutions of the bridge labeled triesters, with the same isotopic incorporations as the monoesters above, were analyzed similarly. However, due to the complicated nature of the triester spectra between 600 and 800 cm^{-1} for both phosphate and phosphorothioate species, it was more difficult to identify one specific P−(O)R stretch in this area. Therefore, we have included all three stretches that exhibit isotopic shifts in the area in which P−(O)R stretching vibrations are expected. Possible P−(O)R stretching vibrations for phosphorothioate and phosphate triesters are, respectively: [parathion ($\nu_{\mathrm{P-(O)R\ stretch}}$ = 788, 770, and 749 cm^{-1}; $\nu_{\mathrm{P-(^{18}O)R\ stretch}}$ = 779, 758, and 737 cm^{-1})], [paraoxon ($\nu_{\mathrm{P-(O)R\ stretch}}$ = 785, 764, and 737 cm^{-1}; $\nu_{\mathrm{P-(^{18}O)R\ stretch}}$ = 778, 751, and 728 cm^{-1})]. FT-IR spectrometer parameters were identical to those used in the monoester experiments.

^{31}P NMR experiments were performed on 20 mM triester solutions with chloroform as solvent. Acquisition parameters were identical to those employed in the monoester experiments.

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Supporting Information Available: Eyring plots for the alkaline hydrolysis of the phosphate and phosphorothioate diesters and triesters. This material is available free of charge via the Internet at http://pubs.acs.org.

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(32) Bourne, N.; Williams, A. *J. Org. Chem.* **1984**, *49*, 1200−1204.
(33) Khan, S. A.; Kirby, A. J. *J. Chem. Soc. B* **1970**, 1172−1182.
(34) Kirby, A. J.; Younas, M. *J. Chem. Soc. B* **1970**, 510−513.