Comparisons of Phosphorothioate and Phosphate Monoester Transfer Reactions: Activation Parameters, Solvent Effects, and the Effect of Metal Ions

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Abstract: The thermodynamics, pH dependency, solvent effects, and the effect of divalent metal ions have been examined for the hydrolysis reactions of the phosphorothioate monoester *p*-nitrophenyl phosphorothioate (pNPPT) and compared with those of the corresponding phosphate monoester, *p*-nitrophenyl phosphate (pNPP). The pH dependency for pNPPT hydrolysis mirrors that for typical phosphate monoesters, with the monoanion being the most reactive species. The ratio of the rate constants for pNPPT hydrolysis to that for pNPP is 1380 for the monoanion reactions and 12.6 for the dianion reactions at 39 °C. The free energy of activation for hydrolysis of the pNPPT dianion in water is 27.9 kcal/mol versus 29.5 kcal/mol for pNPP; for the monoanion reactions the values are 22.2 kcal/mol for pNPPT and 26.8 kcal/mol for pNPP. The free energies of solvation for pNPPT and pNPP are within 0.1 kcal/mol of each other despite the poorer hydrogen bonding ability of sulfur versus oxygen. This minimal difference in ground-state energies thus does not account for the difference in activation energies, which must therefore arise from transition state effects. The more favorable $\Delta G^{\ddagger}$ for pNPPT dianion hydrolysis versus pNPP is entropic in origin; the enthalpic barrier is greater in the pNPPT reaction but is more than offset by a more favorable entropy of activation that arises from the fully dissociative mechanism followed by pNPPT. By contrast, the more favorable $\Delta G^{\ddagger}$ for hydrolysis of the pNPPT monoanion compared to the pNPP monoanion is enthalpic in origin. The hydrolysis rate of the pNPPT dianion increases by nearly 10^7-fold as DMSO content is changed from 0 to 95%. The rate acceleration is due to a lower enthalpy of activation in the mixed solvent. In contrast, the rate of hydrolysis of the monoanion of pNPPT in aqueous DMSO remains almost unchanged from the aqueous value; the enthalpic barrier in 95% DMSO is decreased but is offset by an increase in the entropic barrier. The kinetic effects on hydrolysis of the pNPPT dianion caused by complexation with magnesium, which coordinates with oxygen in phosphorothioates, and cadmium, which coordinates with sulfur, were found to be similar with each metal, resulting in a small decrease in the rate constant.

Introduction

O-phosphorothioate analogues of phosphate monoesters have historically been used to probe kinetic and stereochemical aspects of phosphoryl transfer. Both uncatalyzed reactions in solution and enzymatic reactions have been carried out, the latter most notably with alkaline phosphatase. The differences observed in alkaline phosphatase activity have been used in attempts to infer mechanistic details of this enzymatic reaction.[1−3] Alternative interpretations of such differences, termed thio effects, in enzymatic reactions have been discussed.[4,5]

While a considerable body of kinetic and thermodynamic data have been collected for the phosphoryl transfer reactions of monoesters, less is known about some aspects of thiophosphoryl transfer. In particular, the activation parameters, the effect of metal ions, and the solvent effects on thiophosphoryl transfer reactions have not been reported. Knowledge of how phosphorothioates differ from phosphates in these aspects is important in interpreting differences in enzymatic reactions in terms of mechanistic effects.

Phosphate monoesters typically react in solution by mechanism A in Figure 1. This is a concerted A_ND_N reaction[6] in which the nucleophile enters and the leaving group departs in a single transition state. This pathway for phosphate monoester dianions is supported by a very small entropy of activation,[7] a large (-1.2) β_{lg},[8] and a small β_{nuc},[7] and the occurrence of inversion of configuration when the phosphoryl group is made chiral.[9] An essential difference that occurs in the phosphoryl transfer from the dianion of *p*-nitrophenyl phosphate (pNPP) to anhydrous *tert*-butyl alcohol, where this acceptor also is the reaction solvent, is the observation of racemization at phosphorus when the reactant was made chiral by the use of oxygen isotopes.[9] This outcome was interpreted to indicate the formation of a free metaphosphate intermediate in the $D_N + A_N$ mechanism B in Figure 1.[9] This outcome was in contrast to that in more

(1) Breslow, R.; Katz, I. *J. Am. Chem. Soc.* **1968**, *90*, 7376−7377.
(2) Chlebowski, J. F.; Coleman, J. E. *J. Biol. Chem.* **1974**, *249*, 7192−7202.
(3) Han, R.; Coleman, J. E. *Biochemistry* **1995**, *34*, 4238−4245.
(4) Hollfelder, F.; Herschlag, D. *Biochemistry* **1995**, *34*, 12255−12264.
(5) Herschlag, D.; Jencks, W. P. *Biochemistry* **1990**, *29*, 5172−5179.
(6) For a description of the IUPAC nomenclature for reaction mechanisms, see: Guthrie, R. D.; Jencks, W. P. *Acc. Chem. Res.* **1989**, *22*, 343−349.
(7) Kirby, A. J.; Jencks, W. P. *J. Am. Chem. Soc.* **1965**, *87*, 3209−3216.
(8) Kirby, A. J.; Varvoglis, A. G. *J. Am. Chem. Soc.* **1967**, *89*, 415−423.
(9) Friedman, J. M.; Freeman, S.; Knowles, J. R. *J. Am. Chem. Soc.* **1988**, *110*, 1268−1275.

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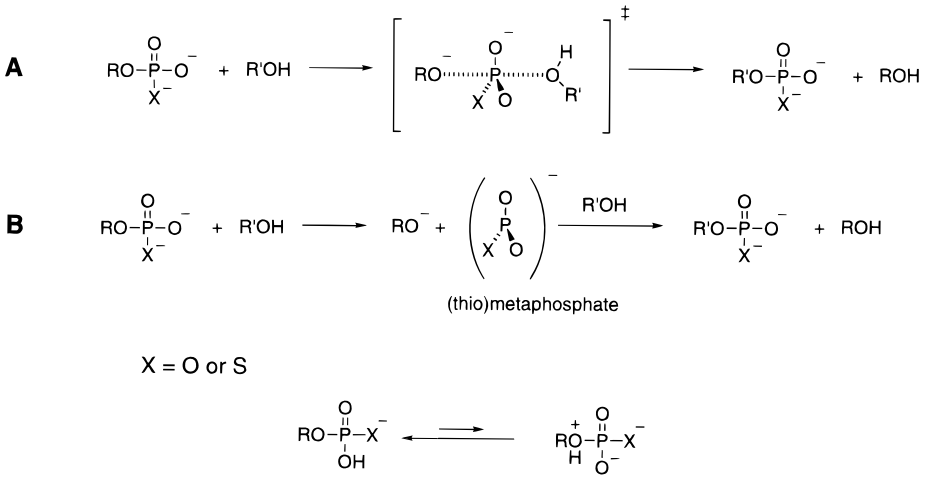
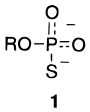


Figure 1. Pathways observed for phosphoryl transfer from monoester dianions (top) and monoanions (bottom). A is the concerted A_ND_N mechanism with a dissociative transition state with the bond to the leaving group largely broken and a small degree of bond formation with the nucleophile. B is a stepwise $D_N + A_N$ mechanism proceeding by way of a (thio)metaphosphate intermediate. At the bottom is a mechanism for reaction of the monoanionic species; proton transfer may occur in a preequilibrium step as shown before nucleophilic attack, or proton transfer can occur simultaneous with leaving group departure, depending upon the basicity of the leaving group.

nucleophilic solvents such as methanol, where the product had inverted configuration consistent with nucleophilic participation in the transition state of the concerted mechanism A.[10] The observation of racemization in the *tert*-butyl alcohol reaction could be due to a bimolecular displacement of the nitrophenolate by *tert*-butyl alcohol followed by a succession of displacements of the bridge-protonated *tert*-butyl phosphate prior to the deprotonation step forming the stable product. However, the finding of a significantly more positive entropy of activation for the *tert*-butyl alcohol reaction ($\Delta S^{\ddagger} = +24.5$ eu)[11] compared to the aqueous reaction ($\Delta S^{\ddagger} = +3.5$ eu)[7] is more consistent with a switch to the fully dissociative $D_N + A_N$ mechanism in *tert*-butyl alcohol.

The reactions of phosphate monoanions are believed to proceed by a mechanism in which the proton is transferred to the leaving group either in a preequilibrium step as shown in Figure 1 or, for less basic leaving groups, simultaneously with P−O bond cleavage.[8] This aqueous reaction also shows a very small entropy of activation,[8] and the reaction of pNPP in methanol proceeds with inversion of configuration at phosphorus.[10]

Experimental[12] and theoretical[13] evidence regarding the bonding in phosphorothioates indicates that structure **1** is the



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most accurate representation of the charge distribution and bonding, with greater negative charge and less double bond character on the sulfur atom than each of the two nonbridge oxygens. A study of the hydrolysis reactions of O-aryl phosphorothioate dianions using linear free-energy relationships found a value for $\beta_{\text{leaving group}}$ of -1.1[4] which suggests that the transition states resemble those for reactions of the aryl phosphate ester counterparts, which exhibit a value of -1.2 for this parameter.[8]

In contrast to stereochemical results with chiral phosphate monoesters, stereochemical studies with the dianion and the monoanion of chiral *p*-nitrophenyl [O,^{18}O^{16}]phosphorothioate show that ethanolysis[14,15] and hydrolysis[16,17] both proceed with a large degree of racemization, indicating the formation of free thiometaphosphate as an intermediate. The observation of a large volume of activation for the hydrolysis of the dianion of 2,4-dinitrophenyl thiophosphate, in contrast to the near zero value measured with the corresponding phosphate ester, also is indicative of a difference in mechanism and is consistent with formation of a thiometaphosphate intermediate.[17] These results indicate that phosphorothioates typically react via mechanism B in Figure 1. This is an S_N1 ($D_N + A_N$) type of mechanism in which a (thio)metaphosphate intermediate is formed in the rate-determining step; this highly reactive species is then attacked by a nucleophile in a subsequent rapid step.

Thus in contrast to phosphoryl transfer, where a metaphosphate intermediate forms only in the absence of a competent potential phosphoryl acceptor, thiophosphate transfer in solution routinely occurs via a thiometaphosphate intermediate. The notion that thiometaphosphate possesses greater stability than metaphosphate is supported by the fact that while the latter has been directly detected only in the gas phase[18,19] trithiometaphosphate has been isolated as the tetraphenylarsonium salt.[20]

With this information as background, the aim of this study was to discover further information about the chemistry of phosphorothioate monoesters compared with phosphate counterparts. So-called "thio effects", the ratio of reaction rates of corresponding phosphate and phosphorothioates, have been used to infer mechanistic information for the enzymatic catalysis of

(10) Buchwald, S. L.; Friedman, J. M.; Knowles, J. R. *J. Am. Chem. Soc.* **1984**, *106*, 4911−4916.
(11) Hoff, R. H.; Hengge, A. C. *J. Org. Chem.* **1998**, *63*, 6680−6688.
(12) Iyengar, R.; Eckstein, F.; Frey, P. A. *J. Am. Chem. Soc.* **1984**, *106*, 8309−8310.
(13) Liang, C.; Allen, L. C. *J. Am. Chem. Soc.* **1987**, *109*, 6449−6453.
(14) Cullis, P. M.; Iagrossi, A. *J. Am. Chem. Soc.* **1986**, *108*, 7870−7871.
(15) Cullis, P. M.; Misra, R.; Wilkins, D. J. *J. Chem. Soc. Chem. Commun.* **1987**, 1594−1596.
(16) Domanico, P.; Mizrahi, V.; Benkovic, S. J. In *Mechanisms of enzymatic reactions: stereochemistry*; Frey, P. A., Ed.; Elsevier: New York, 1986; pp 127−137.
(17) Burgess, J.; Blundell, N.; Cullis, P. M.; Hubbard, C. D.; Misra, R. *J. Am. Chem. Soc.* **1988**, *110*, 7900−7901.
(18) Meyerson, S.; Harvan, D. J.; Hass, J. R.; Ramirez, F.; Maracek, J. *J. Am. Chem. Soc.* **1984**, *106*, 6877−6883.
(19) Henchman, M.; Viggiano, A. A.; Paulson, J. F.; Freedman, A.; Wormhoudt, J. *J. Am. Chem. Soc.* **1985**, *107*, 1453−1455.
(20) Roesky, H. W.; Ahlrichs, R.; Brode, S. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 82−83.

phosphoryl transfer. Thus we were particularly interested in examining properties of thiophosphate chemistry with relevance to enzymatic reactions. In this study we report the pH dependency of the aqueous hydrolysis and the activation parameters for the reactions of the dianion and monoanion of *p*-nitrophenyl phosphorothioate (pNPPT), and the comparative free energies of solvation of pNPPT and the pNPP dianions. We have also examined the effect of added DMSO on the rates and activation parameters of the reactions of pNPPT and compared the results with a prior analogous study of the phosphate ester. This added cosolvent accelerates the hydrolysis of phosphate monoesters by an effect that has been implicated to be associated with enzymatic catalysis. We also report the mechanistic and kinetic effects of divalent metal ions on the aqueous hydrolysis reaction of the pNPPT dianion, using both an oxygen- and a sulfur-preferring metal ion.

Experimental Section

Materials. Reagents and solvents were commercial products and were used as received unless otherwise noted. Thiophosphoryl chloride was distilled under nitrogen before use. Pyridine was distilled from calcium hydride.

Synthesis. The bis(cyclohexylammonium) salt of *p*-nitrophenyl phosphate was prepared and purified by recrystallization from 95% ethanol by using the method of Bourne and Williams.[21] pNPPT was prepared as the bis(cyclohexylammonium) salt by the same method and was purified by washing with cold distilled water in lieu of recrystallization, which resulted in substantial hydrolysis of the phosphorothioate ester. Both products were characterized by proton and by phosphorus NMR spectroscopy. The pNPPT showed no detectable levels of pNPP by NMR.

Kinetics. (a) pH−Rate Profile. First-order rate constants for the aqueous hydrolysis of pNPPT were measured over the pH range from −1 to 15 at 39 °C and $\mu = 1$ M (KCl). The reactant concentration varied from 0.1 to 4 mM. Buffers used were as follows: pH 2, 0.2 M glycine or oxalate; pH 3, 0.2 M formate; pH 4−5, 0.2 M acetate; pH 6−7, 0.2 M MES; pH 7−9, 0.2 M TRIS; pH 10−11, 0.2 M carbonate. Appropriate concentrations of HCl and NaOH were used respectively for pH values below 1 and above 13.

At pH 5 and above the initial rate method was used to determine the rate constants. The release of *p*-nitrophenol was followed by adding aliquots of the reaction mixtures to 0.1 N NaOH and measuring the absorbance of the nitrophenolate anion at 400 nm with an extinction coefficient of 18 300 M^{-1}. Below pH 5 reactions were run to completion (greater than 10 half-lives) and rate constants determined by a fit of the full time course data. The rapid reactions around pH 2 were followed in situ at 330 nm with use of a Cary 1Bio spectrophotometer equipped with a temperature controller.

(b) Thermodynamic Studies. The rate constants for the aqueous hydrolysis of the pNPPT dianion were determined at four different temperatures (30, 50, 60, and 70 °C) at a substrate concentration of 4 mM in 0.2 M CHES buffer at pH 10 and $\mu = 1$ M (KCl). The initial rate method was used. The reactions were followed at 400 nm with a Cary 1Bio spectrophotometer.

The rate constants for the aqueous hydrolysis of pNPPT monoanion reactions were determined at four different temperatures (20, 30, 39, and 50 °C). The reactions were run in 0.2 M glycine buffer at pH = 2, $\mu = 1$ M (KCl) and at 0.1 mM pNPPT concentration. At 20 °C the initial rate method was used; for the other three temperatures the reactions were run until completion (>10 half-lives). All reactions were followed at 330 nm with use of a Cary 1Bio spectrophotometer.

Solubility Experiment. The bis(cyclohexylammonium) salts of pNPPT and of pNPP were used for these experiments. The concentrations at saturation of the two salts in water were measured at four different temperatures. These experiments were performed at temperatures lower than 25 °C to avoid measurable hydrolysis of pNPPT during the time necessary for solubility measurements. The stirred solutions were filtered and assayed at time intervals to determine when saturation had been reached, which was 15 min or longer. For pNPPT the concentrations were measured after total hydrolysis of an aliquot in 0.2 M glycine at pH 2, from total *p*-nitrophenol (pNP) concentration. The pNP released was determined by taking an aliquot in 0.1 N NaOH and measuring the absorbance at 400 nm. The concentrations at saturation for pNPP were similarly measured, with the total hydrolysis carried out with alkaline phosphatase in 0.1 M TRIS buffer at pH 9, 1 mM Zn^{2+} and Mg^{2+}.

Mixed Solvent Rate Studies. The DMSO content of the aqueous solutions was varied from 0 to 95% in kinetic studies of the hydrolysis of the pNPPT, under conditions similar to those previously used to study reactions with pNPP.[22] Both the pNPPT dianion and monoanion rate constants were determined by following the release of *p*-nitrophenolate anion at 400 nm or of protonated *p*-nitrophenol at 335 nm, respectively. The dianion reactions were run at concentrations of 0.02 M NaOH, and the monoanion reactions in solutions containing 0.02 M formate. All reactions were run at 39 °C with the exception of the dianion reaction in 95% DMSO. This reaction at this temperature was too fast for accurate measurement of the rate constant, so its value was extrapolated from a linear Eyring plot constructed from reactions at lower temperatures.

The rate constants for hydrolysis of the dianion and the monoanion in 95% DMSO/water were measured at a range of temperatures to construct Eyring plots, from which the activation parameters were determined as described for the aqueous reactions.

The Effect of Metal Ions. Stock solutions of $MgCl_2$ and $Cd(NO_3)_2$ at 1 M were used in these experiments. The formation of metal−pNPPT complexes was determined from changes in the UV−vis spectrum at constant substrate concentration and the metal concentration was varied (metal was present both in the reference and experiment cells). The stability constant for the complex between pNPPT and Cd^{2+} was determined from the change in λ_{max} as a function of Cd^{2+} concentration by using eqs 1 and 2

$$K_{ML} = [ML]/([M_t] - [ML])([L_t] - [ML]) \quad (1)$$

$$[ML] = [L_t]\{(W_0 - W_1)/(W_2 - W_1)\} \quad (2)$$

where $[M_t]$ and $[L_t]$ are the total concentrations of metal and ligand (in this case pNPPT) in all forms, [ML] is the concentration of metal−ligand complex, W_0 is the observed λ_{max}, and W_1 and W_2 are the λ_{max} values in the absence and at saturating metal concentrations, respectively.

The rate constants for pNPPT hydrolysis reactions in the presence of Mg^{2+} and Cd^{2+} were determined by using the initial rate method, assaying for release of *p*-nitrophenol. The reaction mixtures were filtered before absorbance measurement to remove precipitated metal−inorganic phosphorothioate complex, and the reaction solution absorbance was measured at 400 nm in quartz cells. The buffer used was 0.18 M HEPES, pH 8.0 for the reactions with both metal ions ($\mu = 1.1$ with KCl for the reactions with magnesium ion and $\mu = 1$ M with KCl for those with cadmium ion). The concentration of pNPPT was 1 mM, and metal ion concentrations were 0.3 M for magnesium (which was near saturation under these conditions) and 0.05 M for cadmium. The dependence on the hydroxide ion concentration was studied for the hydrolysis reaction in the presence of Mg^{2+}. For this study the rate constants at three pH values were determined; the buffer used was 0.18 M HEPES at pH = 8.0, 8.3, and 8.6, with ionic strength maintained at 1.1 with KCl.

Results

Values of the first-order rate constants for hydrolysis of *p*-nitrophenyl phosphorothioate (pNPPT) were plotted as a function of pH in Figure 2. The data show the bell-shaped pH−

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

(22) Abell, K. W. Y.; Kirby, A. J. *Tetrahedron Lett.* **1986**, *27*, 1085−1088.

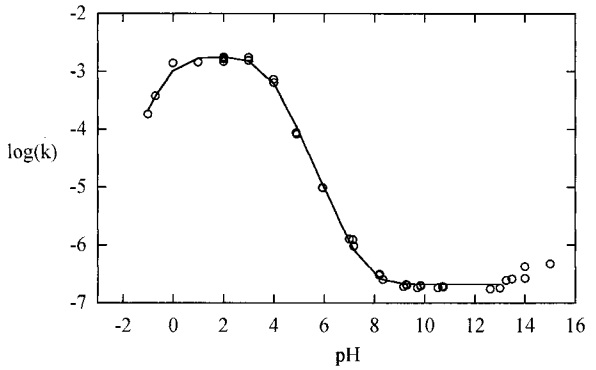

Figure 2. The pH dependence of the first-order rate constants (s^{-1}) for hydrolysis of pNPPT at 39 °C. The line represents the fit to the data by using the equation log $Y = \log[C/(1 + H/K_a + K_b/H)]$ with use of the program BELL of Cleland.[23]

Table 1. Rate Constants for Reactions of *p*-Nitrophenylphosphorothioate (pNPPT) and *p*-Nitrophenyl Phosphate (pNPP) at 39 °C

species	pNPPT	pNPP	k_{pNPPT}/k_{pNPP}
monoanion			
k (s^{-1}) × 10^6	1470[a]	1.07[8]	1380
dianion			
k (s^{-1}) × 10^6	0.195[a]	0.0155[7]	12.6
$k_{monoanion/kdianion}$	7538	69	

[a] Experimental data from this work; see Experimental Section for details. Other data noted with superscripts denote data from the referrences indicated.

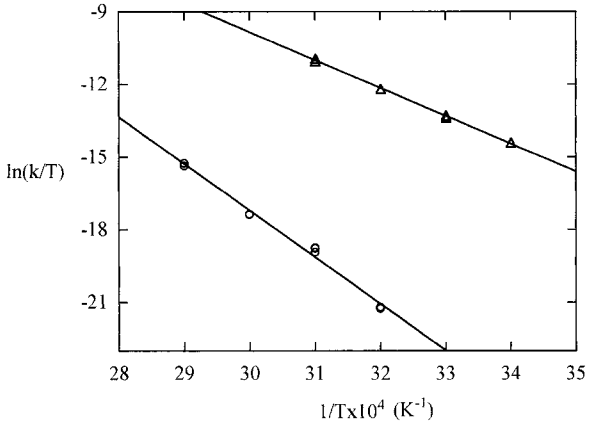

Figure 3. Eyring plots for the aqueous hydrolysis reactions of pNPPT monoanion (Δ) and dianion (○).

rate behavior that is typical of phosphate monoesters. The data were fitted to the equation log $Y = \log[C/(1 + H/K_a + K_b/H)]$ with use of the program BELL of Cleland[23] to give the solid curve shown. The first and second pK_a values were determined to be 3.68 ± 0.08 and −0.10 ± 0.11.

The rate constants for the hydrolysis of the *p*-nitrophenyl phosphorothioate monoanion at pH 2.0 and of the dianion at pH 10.0 were measured at 39 °C and are compared with the literature values for the hydrolysis of *p*-nitrophenyl phosphate in Table 1. The rate constants for *p*-nitrophenyl phosphorothioate hydrolysis were measured as a function of temperature to construct the Eyring plots (Figure 3) which were used to determine the activation parameters. The lines represent the best least-squares linear fit to the data (Sigmaplot, Jandel). The slopes and intercepts were used to calculate the enthalpy and entropy of activation in the two reactions and their standard errors. The

Table 2. Activation Parameters for Hydrolysis of *p*-Nitrophenylphosphorothioate (pNPPT) and *p*-Nitrophenyl Phosphate (pNPP)

	pNPP (aq)	pNPPT (aq)	pNPPT (95% DMSO)
dianion			
$\Delta H^{\ddagger}$, kcal/mol	30.6[7]	37.0 ± 1.0	22.9 ± 0.7
$\Delta S^{\ddagger}$, eu	+3.5[7]	+29 ± 3	+12 ± 3
$\Delta G^{\ddagger}$, kcal/mol	29.5[a]	27.9 ± 1.0[a]	19.1 ± 0.7[a]
monoanion			
$\Delta H^{\ddagger}$, kcal/mol	25.4 [8]	22.0 ± 0.6	18.8 ± 0.3
$\Delta S^{\ddagger}$, eu	−4.5[8]	−1 ± 2	−12 ± 1
$\Delta G^{\ddagger}$, kcal/mol	26.8[a]	22.2 ± 0.6[a]	22.7 ± 0.3[a]

[a] Calculated from $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$ at 39 °C.

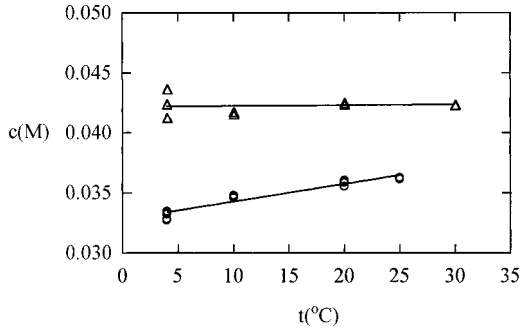

Figure 4. The temperature dependence of the solubility in water of the dicyclohexylammonium salts of *p*-nitrophenyl phosphate (Δ) and *p*-nitrophenyl phosphorothioate (○).

free energies of activation were calculated from $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$ at 39 °C. The free energy of activation $\Delta G^{\ddagger}$ was found to be 27.9 ±1 kcal/mol for the dianion reaction, with $\Delta H^{\ddagger} = 37 \pm 1$ kcal/mol and $\Delta S^{\ddagger} = +29 \pm 3$ eu. For the monoanion reaction, $\Delta G^{\ddagger} = 22.2 \pm 0.6$ kcal/mol with $\Delta H^{\ddagger} = 22.0 \pm 0.6$ and $\Delta S^{\ddagger} = -1 \pm 2$ eu. These values together with the literature values for the activation parameters for the hydrolysis of *p*-nitrophenyl phosphate for comparison are given in Table 2.

To evaluate the comparative free energies of solvation of *p*-nitrophenyl phosphate and *p*-nitrophenyl phosphorothioate in water, the solubilities of the bis(cyclohexylammonium) salts of both compounds were measured over a range of temperatures as described in the Experimental Section. In dilute solution the activity of a solute is close to unity and the free energy of solvation is directly proportional to solubility. Thus the relative free energies of solvation for these two compounds can be approximated by the ratio of their solubilities. The difference in the free energies of the solvated reactants $\Delta\Delta G_{solvation}$ is then equal to $RT \ln(c_p/c_t)$, where c_p and c_t are the concentrations at saturation of the phosphate and phosphorothioate, respectively.[24] This experiment is a variation of the common method for determining the difference in free energy of solvation of a compound in two different solvents from the relationship $\Delta\Delta G_{solvation} = RT \ln P$, where P is the ratio of the solubility of the compound in the two respective solvents.[24] The solubility data as a function of temperature are presented in Figure 4. Temperatures at or below room temperature were used in this experiment because of the lability of pNPPT and the need to keep hydrolysis below detectable levels during the solubility determinations. The ratio c_p/c_t was close to unity at all temperatures; the value for $\Delta\Delta G_{solvation}$ was found to be approximately 0.1 kcal/mol at 4 °C.

The effect of added DMSO on the aqueous hydrolysis of the monoanion and dianion of pNPPT was examined at 39 °C. The

(23) Cleland, W. W. *Methods Enzymol.* **1979**, *63*, 103−138.

(24) Marcus, Y. *Pure Appl. Chem.* **1983**, *55*, 977−1021.

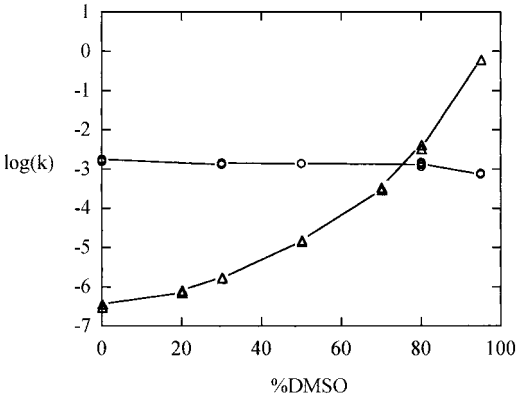

Figure 5. The dependence on the percentage of DMSO of the rate constants for hydrolysis of the pNPPT dianion (Δ) and monoanion (○) at 39 °C.

rate constants were measured for mixtures consisting of from zero to 95% DMSO, and the results plotted in Figure 5. The substrate was kept in the dianion form by maintaining a 20 mM concentration of NaOH in the solvent mixtures. The broad pH-independent range of the dianion hydrolysis gives strong assurance that the substrate is fully deprotonated under these conditions. The monoanion reaction is potentially more problematic in this regard, as the plateau around pH 2 representing the monoanion reaction is small and the organic cosolvent could alter the pK_a values and thus the protonation state of the substrate. This possibility was checked by varying the pH of the formic acid/formate buffer from 2.6 to 2.8. The rate constants were the same within experimental error for hydrolysis in the aqueous DMSO reaction just as was the case in the aqueous reaction, indicating that the kinetics reflect those of the monoanion at the peak of the pH−rate profile.

The dianion reaction was substantially accelerated by added DMSO by up to more than 6 orders of magnitude. The activation parameters were determined for the reactions in 95% DMSO from an Eyring plot (Supporting Information, Figure s-1) as described for the aqueous reactions. For the reaction of the dianion, $\Delta H^{\ddagger} = 22.9 \pm 0.7$ kcal/mol in 95% DMSO, as compared to 37 kcal/mol in the aqueous reaction. The entropy of activation, $\Delta S^{\ddagger} = +12 \pm 3$ eu, compared to the value of $+29 \pm 3$ eu measured for the aqueous reaction.

The rate of the monoanion reaction was affected by added DMSO much less, showing only a slightly decreasing rate with increasing fractions of DMSO. The activation parameters for this reaction in 95% DMSO were also determined: $\Delta H^{\ddagger} = 18.8 \pm 0.3$ kcal/mol in 95% DMSO, versus 22.0 ± 0.6 kcal/mol in the aqueous reaction. The entropy of activation $\Delta S^{\ddagger} = -12 \pm 1$ eu, versus the value of -1 ± 2 eu measured for the aqueous reaction.

Magnesium and cadmium ions each form complexes with phosphorothioates, as they do with phosphates. In their complexes with phosphorothioates Mg^{2+} preferentially complexes with oxygen and Cd^{2+} preferentially binds to sulfur.[25] The formation of the complex between these metal ions and pNPPT results in a shift in λ_{max} to lower wavelength. This shift allows one to follow formation of the metal−pNPPT complex by measuring the change in λ_{max} as a constant concentration of pNPPT is titrated with the metal ion. Figure 6 shows the change in λ_{max} of a solution of pNPPT as it was titrated with cadmium at constant ionic strength. For Cd^{2+} the stability constant for the complex was determined from these data to be 293 M^{-1}

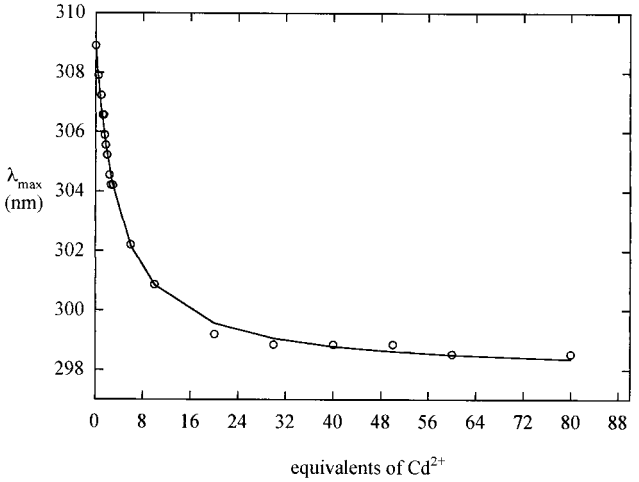

Figure 6. The change in λ_{max} of pNPPT at pH 8.0 in 0.18 M HEPES buffer, $\mu = 1$ (KCl), as a function of cadmium ion concentration. The line represents the best fit of the data with use of eqs 1 and 2.

(log $K_a = 2.50 \pm 0.1$). This may be compared with the value reported for the Cd^{2+}−adenosine monophosphorothioate (AMPαS) complex (log $K_a = 4.62$)[26] in which additional interactions between the metal ion and the nucleotide base result in tighter binding, and with the stability constant with ADPαS (log $K_a = 4.95$) [25] where the cadmium ion chelates between the α and β phosphates. The lower pK_a of the phosphoryl group in pNPPT relative to the nucleotides also reduces its stability constant relative to these other compounds. The stability constant for the Cd^{2+}−*p*-nitrophenyl phosphate complex has also been reported (log $K_a = 2.05$).[27]

The complex between Mg^{2+} and pNPPT was too weak to achieve complete complexation even at saturating concentrations of Mg^{2+}, and the stability constant for the complex could not be determined. It can be assumed that its value will be lower than the reported values for Mg^{2+} with pNPP (log $K_a = 1.17$[28] and 1.29[27]) because sulfur substitution for oxygen is known to lower stability constants for metal ion−phosphate complexes with oxygen-preferring metal ions.[25,26]

The effects of each of these metal ions on the rate of hydrolysis of the dianion of pNPPT were measured at 39 °C. The experiments with cadmium were performed at concentrations of the metal ion sufficient to result in full complexation of the pNPPT. Because the complexation of pNPPT with magnesium was too inefficient to achieve saturation, the kinetic experiments with this metal ion were conducted at as high a magnesium concentration as possible under the experimental conditions. Full complexation of pNPPT with Cd ion results in a shift in λ_{max} by about 10 nm; at maximal Mg^{2+} concentrations the λ_{max} of pNPPT solution had shifted by about 2 nm. Assuming that a similar shift results from complexation by the two metals, then only about one-fourth of the pNPPT was complexed by Mg ion in the kinetic experiments. Thus the kinetic consequences of magnesium complexation will be underestimated in these experiments. The rate constants for the two reactions at pH 8.0 were very similar; for the Cd^{2+} reaction $k = 3.02 \times 10^{-7}$ s^{-1} and for the reaction in the presence of Mg^{2+} $k = 2.90 \times 10^{-7}$ s^{-1}. Thus both metal ions were found to result in a small decrease in the rate constant for hydrolysis.

(25) Pecoraro, V. L.; Hermes, J. D.; Cleland, W. W. *Biochemistry* **1984**, *23*, 5262−5271.

(26) Sigel, R. K. O.; Song, B.; Sigel, H. *J. Am. Chem. Soc.* **1997**, *119*, 744−755.

(27) Massoud, S. S.; Sigel, H. *Inorg. Chem.* **1988**, *27*, 1447−1453.

(28) Herschlag, D.; Jencks, W. P. *J. Am. Chem. Soc.* **1987**, *109*, 4665−4674.

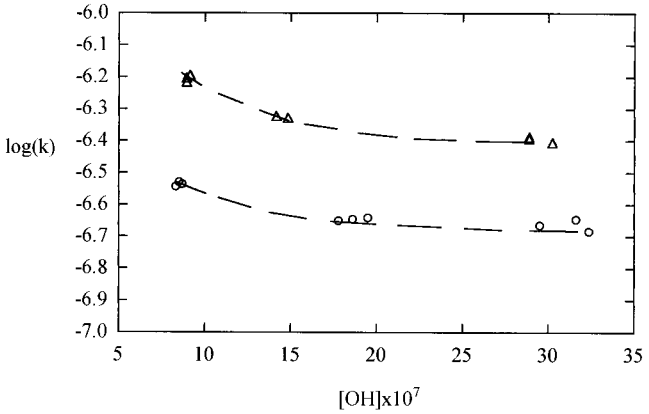

Figure 7. The dependence of the rate constants for hydrolysis of pNPPT on the hydroxide ion concentration in the presence (○) and absence of Mg^{2+} (Δ).

Figure 7 shows there is no measurable effect of hydroxide concentration on the rate hydrolysis of the dianion of pNPPT in the presence of Mg^{2+}. Because of the limitation on the solubility of the metal ion at higher pH the kinetics experiments could not be conducted in a completely pH-independent region of the profile. Significant precipitation at pH values above 8.0 (the lowest point in Figure 7) precluded the analogous experiment with Cd^{2+}.

Discussion

pH−Rate Profile. The aqueous hydrolysis of pNPPT shows a bell-shaped pH−rate profile (Figure 2) like those of phosphate monoesters, which indicates that the monoanion is more reactive than the dianion. However, the difference in rates between the monoanion and dianion is considerably larger for pNPPT than for pNPP. At 39 °C, the ratio of the rate constant for hydrolysis of the monoanion[8] to that of the dianion[7] of *p*-nitrophenyl phosphate is 69, while at the same temperature this ratio for *p*-nitrophenyl phosphorothioate is 7500.

The kinetic effect of sulfur substitution in one of the nonbridging oxygen atoms (termed a "thio effect") for the *p*-nitrophenyl monoester will be pH sensitive due to the differences in the pH−rate profiles of the two species. The ratio of rate constants for pNPPT/pNPP has been reported to equal 48 at 70 °C and pH 8.0, and is 63 at 70 °C and pH 7.0.[1] However, these ratios are misleading because of differences in the populations of the more reactive monoanionic and of the less reactive dianionic forms of the phosphorothioate ester versus the phosphate ester at these pH values. A better way to evaluate the thio effect is to compare the rate constants for the dianions of the two compounds in their respective pH-independent range, and for the monoanionic species by comparing the rate constants at the respective pH optima. Doing this with the values in Table 1 gives ratios for pNPPT/pNPP for the dianion hydrolysis of 12.6 (in agreement with the previously reported value of 12[4]) and for the monoanion hydrolysis of 1380. Thus while both the monoanion and the dianion of pNPPT undergo hydrolysis faster than the corresponding forms of pNPP, the difference is much larger between the monoanion reactions than for the dianion ones.

The fit of the pH−rate data yields values of 3.68 ± 0.08 and −0.10 ± 0.11 for the pK_a values of pNPPT. These results are in reasonable agreement with previously reported pK_a values of 3.6 and −0.24.[16] By comparison, the pK_a values for pNPP are 4.96 and +0.30.[21] The pH-independent hydrolysis of the dianion of the phosphorothioate ester between pH 9.5 and 13 mirrors the behavior of the phosphate, as does an increase in the hydrolysis rate at very high pH. In the pNPP reaction this upward curvature has been attributed to a reaction of the dianion with hydroxide ion in a nucleophilic aromatic substitution reaction.[7]

Activation Parameters. Since sulfur is much less able to participate in hydrogen bonding than oxygen, one possible contributor to the greater reactivity (reduced $\Delta G^{\ddagger}$) of pNPPT relative to pNPP might be inferior solvation of the phosphorothioate ester in water. To test this hypothesis, the comparative free energies of solvation of the bis(cyclohexylammonium) salts of pNPPT and pNPP were estimated from a comparison of the solubilities of these two compounds, as described in the Experimental Section. Saturated aqueous solutions of these compounds are dilute (30 to 40 mM); in dilute solution activities are close to unity, and free energies of solvation are proportional to concentration. The difference in solvation between the two compounds was measured at several temperatures and the largest difference, at 4 °C, is only about 100 cal/mol. This is far smaller than the difference in the free energies of activation and indicates that solvation differences in the ground state are at most a minor factor in the rate difference between the dianions of pNPPT and of pNPP.

The breakdown of the activation parameters for the hydrolysis reactions of the monoanion and dianion of pNPPT into enthalpic and entropic contributions (Table 2) and a comparison of these values with those for pNPP hydrolysis reveals some interesting differences. For the dianion reactions, a less favorable enthalpy of activation for the phosphorothioate ester is more than offset by a more favorable entropy of activation. The precise reasons for the larger enthalpic barrier in the reaction of the phosphorothioate ester are not known, but several contributions are possible. The similarity of the values of β_{lg} for the two reactions indicates that the degree of P−O bond fission in the transition states is similar in the two reactions, thus ruling out this as a source of the difference unless the sulfur substitution results in an increase in the bond energy of the P−O bond to the leaving group in the phosphorothioate ester. The higher $\Delta H^{\ddagger}$ for the phosphorothioate reaction may also partly be due to the absence of favorable bonding-formation interactions with the nucleophile that are present in the transition state of the A_ND_N aqueous reaction of pNPP but which are absent in the transition state of the rate-limiting step of the $D_N + A_N$ reaction of pNPPT. The internal energy of bond fission in the concerted reaction of pNPP is the sum of the enthalpic cost of extensive P−O bond cleavage to the leaving group, partially compensated for by the favorable bond formation interaction with the nucleophile. The other interactions which contribute to the enthalpy of the reaction are hydrogen bonding interactions with the solvent. The very similar free energies of solvation by water for pNPP and pNPPT indicate that these compounds are very similarly solvated, making it seem unlikely that differences in solvation contribute significantly to the difference in $\Delta H^{\ddagger}$.

The $D_N + A_N$ mechanism for pNPPT hydrolysis avoids the entropic cost of recruiting a solvent molecule into the transition state of the rate-limiting step, and as a result the reaction of the pNPPT dianion exhibits a much more favorable $\Delta S^{\ddagger}$ of +29 eu compared to +3.5 eu for the reaction of the pNPP dianion. The more favorable entropy term more than outweighs the enthalpic factor, and the net result is a reduction in $\Delta G^{\ddagger}$ by about 1.6 kcal/mol for pNPPT dianion hydrolysis.

In comparing the monoanion reactions, the dominant energetic reason for the faster rate of pNPPT is a lower enthalpy of activation, 22.0 kcal/mol compared with 25.4 kcal/mol for pNPP.

In the monoanion reactions of both compounds the proton is transferred from a nonbridge oxygen atom to the leaving group.[8] For leaving groups less basic than phenol, proton transfer is probably concerted with leaving group departure.[8] Since in the dissociative transition states bond cleavage to the leaving group is considerable, the leaving group pK_a's in the two transition states will be similar. The lower $\Delta H^{\ddagger}$ for the hydrolysis of the monoanion of pNPPT probably reflects the lower pK_a of the thiophosphoryl group compared with that of the corresponding phosphoryl group in the pNPP monoanion, resulting in a lower enthalpic cost for deprotonation. The entropies of activation for the reactions of the pNPPT and pNPP monoanions are fairly similar, but that for the phosphorothioate ester is slightly more favorable, which presumably reflects the fact that the monoanion reaction proceeds largely via a thiometaphosphate mechanism as revealed by stereochemical studies.[29] The entropic advantage of this pathway is considerably less in the monoanion reaction than in the dianion reaction, which suggests that in the monoanion reactions the major entropic problem is assuming the proper geometry for the proton transfer. For geometric reasons this probably occurs via an intervening water molecule that would allow a six-membered transition state, rather than a purely intramolecular process via a four-membered transition state. The combination of enthalpic and entropic factors results in a reduction in $\Delta G^{\ddagger}$ by about 4.6 kcal/mol for the hydrolysis of the monoanion of pNPPT compared with pNPP.

Solvent Effects. The addition of aprotic organic solvents results in large rate accelerations for the aqueous hydrolysis of the dianions of phosphate monoesters. The rate of the aqueous hydrolysis of the dianion of pNPP is increased by 6 to 7 orders of magnitude by added DMSO or HMPA.[22] This acceleration has been attributed to decreased ability of the mixed solvent to participate in stabilizing hydrogen bonding interactions with the anionic nonbridge oxygen atoms of the reactant, and the possible contribution of similar effects to enzymatic catalysis in phosphoryl transfer has been pointed out.[22]

The rate enhancement on the hydrolysis of the dianion of pNPPT found to result from added DMSO closely mirrors the previously reported behavior with pNPP.[22] Figure 5 shows the variation in the rate constants for the reactions of both the monoanion and the dianion of pNPPT with the fraction of DMSO up to 95%. Both the degree of rate enhancement and the overall response to added DMSO found for the dianion of pNPPT are very similar to those for pNPP. The activation parameters for the reaction of the dianion of pNPPT in 95% DMSO reveal the acceleration to be enthalpic in origin: $\Delta H^{\ddagger} = 22.9 \pm 0.7$ kcal/mol, ~14 kcal/mol less than that for the aqueous reaction.

By contrast, the reaction of the monoanion of pNPPT is slowed by added DMSO, as was found[22] to be the case with pNPP. The entropy of activation is less favorable in 95% DMSO, which may reflect a less favorable environment for setting up the hydrogen bonding network for transfer of the proton from the thiophosphoryl group to the leaving group. Unlike in the dianion reaction, this is not offset by a large reduction in the enthalpy of activation. The modest reduction in $\Delta H^{\ddagger}$ from 22.0 to 18.8 kcal/mol is insufficient to offset the less favorable $\Delta S^{\ddagger}$, and thus the net result is a slightly larger $\Delta G^{\ddagger}$ in 95% DMSO versus the aqueous reaction.

Effect of Divalent Metal Ions. The effect of metal ions on the hydrolytic reactions of phosphate monoester dianions is of interest because many phosphatases are metalloenzymes. There is considerable speculation that the complexation of metal ions with the nonbridge oxygen atoms of the phosphate substrate induces a mechanistic shift away from the dissociative reaction seen in solution to an associative transition state with more nucleophilic participation. For example, alkaline phosphatase requires the presence of two Zn^{2+} ions. The fact that this enzyme turns over phosphorothioates much more slowly than phosphate monoesters has been cited as evidence that this enzyme follows a different mechanism and transition state than that operative in the uncatalyzed hydrolysis,[1−3] where this ratio of reactivities is reversed. Alternative interpretations of these results have also been discussed.[4,5]

We compared the effect of divalent metal ions on the aqueous hydrolysis of pNPPT with prior studies of the effects of calcium and magnesium ions on reactions of phosphate monoesters.[28] These metal ions each reduce the reactivity of the reactions of phosphorylated pyridines with pyridine nucleophiles by about 2-fold. The inhibition by the metal ions was attributed to complexation with the nonbridge oxygen atoms, reducing their ability to assist in expelling the leaving group.[28] In contrast to the reaction with pyridine nucleophiles the hydrolysis of the Mg^{2+} complexes of phosphorylated pyridines, where the nucleophile is a chelated $Mg(OH)^{+}$, are 4 to 6 orders of magnitude faster than the second-order rate constants for the reaction of uncomplexed pNPP with water.[5] In that work, the hydrolysis rate of the Mg^{2+} complexes demonstrated a strong dependency on the concentration of hydroxide ion in contrast to the pH-independent hydrolysis of free pNPP. In aqueous hydrolysis reactions of the pNPP dianion, Ca^{2+} increased the rate but Mg^{2+} slowed the rate by about 20%.[28]

In the uncatalyzed aqueous reaction of the pNPPT dianion there is no nucleophilic participation in the first step; however, one might expect the Lewis acidity of the metal ion may influence a mechanistic shift to more resemble the pNPP reaction. Such coordination might disfavor electron donation from the nonbridge electrons in the dissociative reaction, while enhancing the electrophilicity of the phosphorus atom. To examine the effects of divalent metal ions on the aqueous hydrolysis of the dianion of pNPPT we chose to study the effects of added Mg^{2+} and Cd^{2+}. Magnesium ion coordinates to oxygen in complexes with phosphorothioates, while cadmium binds to sulfur.[25,26] The choice of metal ions for this study was limited by the need for significant solubility of the metal in the pH range necessary to study the dianion reaction. Some metal ions which were soluble under initial conditions formed precipitates during the course of the reaction.

Solubility limitations required that the effect of the divalent metal ions on the hydrolysis be examined at pH 8.5 and lower. Both metal ions exhibited modest inhibitory effects. More importantly, no increase in the hydrolysis rate occurs in the presence of Mg^{2+} as the concentration of hydroxide ion increases (Figure 7). The data indicate that these metal ions do not catalyze the reaction of pNPPT with metal-bound hydroxide as occurs with Mg^{2+} complexes of phosphorylated pyridines. The slight decrease in hydrolysis rate of the metal−pNPPT complexes is most likely due to the inhibitory effect on electron donation from the nonbridge oxygen atoms as inferred in the reactions of phosphorylated pyridines. Thus the hydrolysis reactions of the metal ion−pNPPT complexes likely remain fully dissociative $D_N + A_N$ mechanisms as is the case with free pNPPT. The reduction in aqueous hydrolysis rate induced by these metal ions in these solution experiments is much smaller than the 100-fold slower rate of reaction of phosphorothioate monoesters with alkaline phosphatase than with phosphate monoesters, so the

(29) Harnett, S. P.; Lowe, G. *J. Chem. Soc. Chem. Commun.* **1987**, 1416−1418.

slower enzymatic rate is not due simply to the innate effect of metal ion complexation with the thiophosphoryl substrate.

Conclusions

The substitution of sulfur for a nonbridging oxygen atom in the *p*-nitrophenyl phosphate monoester increases the rate of the monoanion hydrolysis reaction (1380-fold) much more than that of the dianion (12.6-fold). Although sulfur forms hydrogen bonds much more weakly than oxygen, the free energies of solvation of the phosphate and phosphorothioate are nearly the same and ground state destabilization is not a significant contributor to the difference in hydrolysis rates of the dianions. The lower activation energy for hydrolysis of the phosphorothioate dianion arises from a more favorable entropy of activation due to the switch to the fully dissociative $D_N + A_N$ mechanism; in contrast, the reaction of the monoanion of the phosphorothioate is faster than that of the phosphate ester due to a lower enthalpic barrier. The dianion reaction is significantly accelerated by increasing fractions of DMSO which causes a lowering of the enthalpic barrier, which is most likely due to disruption of stabilizing hydrogen bonding interactions between the dianionic reactant and the solvent. Similar effects could be one contribution to the catalysis of the reactions of phosphate dianions by phosphatases. In contrast, the monoanion reaction is virtually unaffected by added DMSO. The divalent metal ions Mg^{2+} and Cd^{2+}, which coordinate preferentially with oxygen and with sulfur, respectively, each have small inhibitory effects on the hydrolysis of the dianion. Thus the much slower enzymatic rate of catalysis by alkaline phosphatase of phosphorothioates compared with phosphates is not due simply to the innate effects of metal ion complexation with the thiophosphoryl substrate. One could postulate that in the enzymatic environment this complexation could be considerably stronger, although the significantly greater K_M values for phosphorothioate versus phosphate monoesters do not support stronger coordination of phosphorothioates.

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Supporting Information Available: Eyring plot for the reactions of the monoanion and the dianion of *p*-nitrophenyl phosphorothioate in 95% DMSO−water, and the table of rate constants for aqueous pNPPT hydrolysis with pH (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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