Entropy and Enthalpy Contributions to Solvent Effects on Phosphate Monoester Solvolysis. The Importance of Entropy Effects in the Dissociative Transition State

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The solvolysis reactions of a series of aryl phosphates in *tert*-butyl alcohol and in *tert*-amyl alcohol have been examined. The dianion of *p*-nitrophenyl phosphate reacts 7500- and 8750-fold faster in these solvents, respectively, than the corresponding aqueous reactions. The monoanion reacts 14- and 16-fold slower respectively in *tert*-butyl alcohol and in *tert*-amyl alcohol. Analysis of the activation parameters shows that the rate enhancement for the dianion is due solely to entropic factors, while the slower reaction of the monoanion is due to increased enthalpy of activation. The significantly more positive entropy of activation for the solvolysis of *p*-nitrophenyl phosphate dianion in *tert*-butyl alcohol supports the original proposal that racemization at phosphorus in this reaction is caused by a switch to a $D_N + A_N$ mechanism, rather than subsequently proposed mechanisms which avoid the formation of metaphosphate. Rate enhancements of similar magnitudes are seen for the dianion reactions of all of the aryl phosphates examined; the slope of a plot of the rate constants for solvolysis versus the aqueous pK_a of the leaving phenols has a slope of −1.1, within experimental error of the value for the aqueous reaction. However, in the reactions in *tert*-amyl alcohol, *para*-substituted and *meta*-substituted aryl phosphates fall on separate but parallel lines with *para*-substituted compounds reacting faster than *meta*-substituted reactants with leaving groups of similar pK_a. The pK_a values for a series of *para*- and *meta*-substituted phenols in *tert*-butyl alcohol and in *tert*-amyl alcohol were determined and were found to have a linear relationship with the aqueous pK_a values, with no distinction between *para* and *meta* substitution. Thus the different Brønsted behavior of *para*- and *meta*-substituted aryl phosphates in these solvents is not due to differential solvent-induced perturbations of the pK_a values of the leaving groups. The mechanistic implications of these results and their relevance to enzymatic phosphoryl transfer are discussed.

Introduction

The chemistry of phosphoryl transfer from phosphate monoesters and diesters is essential and ubiquitous in biological systems. A considerable amount of work over the past decades has been devoted to gaining an understanding of the mechanisms of these reactions in solution and to understanding the mechanisms of enzyme-catalyzed phosphoryl transfer. Of particular interest is whether the mechanistic pathways and the transition states involved in the enzymatic reactions differ from uncatalyzed phosphoryl transfer reactions in solution.

Three distinct reaction mechanisms, have been observed for various types of phosphate esters under different conditions, which are illustrated diagrammatically in Figure 1.[1,2] Two are two-step mechanisms; A is an S_N1 ($D_N + A_N$ in the IUPAC nomenclature[3]) type of mechanism in which a metaphosphate intermediate is formed in the rate-determining step; this highly reactive species is then attacked by a nucleophile in a subsequent rapid step. Mechanism B is addition−elimination ($A_N + D_N$) and involves nucleophilic attack in the first step to form a pentacoordinate phosphorane intermediate. This intermediate may or may not pseudorotate before expelling a leaving group in a second step. Mechanistic possibility C is a concerted A_ND_N reaction in which the nucleophile enters and the leaving group departs in a single step, with no intermediate.

The primary factor that influences which mechanism is followed is the alkylation state of the phosphate. Monoesters are generally observed to follow, depending on conditions, either a concerted mechanism with a highly dissociative transition state (in nucleophilic solvents) or, in very nonnucleophilic solvents such as *tert*-butyl alcohol, a $D_N + A_N$ mechanism with a metaphosphate intermediate. Diesters and triesters follow successively more associative mechanisms, either concerted ones if the leaving group is good (i.e., an aryloxy group), with more nucleophilic participation in the transition state, or fully associative mechanisms via phosphorane intermediates.

Mechanism C for the aqueous hydrolysis of phosphate monoester dianions is supported by a very small entropy of activation,[4] a large (−1.2) β_{lg}[5] and a small β_{nuc},[4] and the occurrence of inversion of configuration when the phosphoryl group is made chiral.[6] The reaction of the

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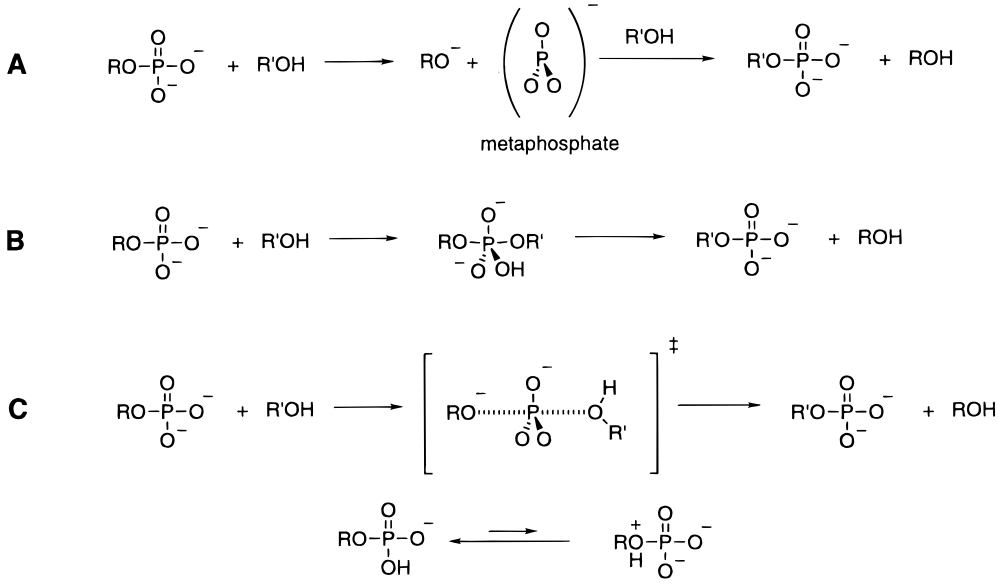

Figure 1. The dissociative (A) and the associative (B) mechanistic extremes, and the concerted (C) pathway for phosphoryl transfer. The concerted pathway is drawn to indicate a dissociative transition state, which is typical for phosphate monoester reactions. At the bottom is the proposed mechanism for the first step in phosphoryl transfers of monoanions; for less basic -OR groups such as phenols, the proton transfer to the leaving group may be concerted with P−O bond cleavage.[4]

monoanion is also believed to proceed by a dissociative mechanism, with the proton transferred to the leaving group either in a preequilibrium step as shown in Figure 1 or, for less basic OR groups, simultaneously with P−O bond cleavage.[5] This aqueous reaction also shows a very small entropy of activation,[5] and the reaction of pNPP in methanol proceeds with inversion of configuration at phosphorus.[7]

Very large rate accelerations are observed in enzyme-catalyzed reactions of phosphate monoesters. Apart from the direct contribution from catalytic groups, the microenvironment in the active site experienced by the substrate is one possible source of the catalytic efficiency. One technique which has been used to address the effects of microenvironment on phosphoryl transfer reactions is solvent effects on the rate and mechanism of the reaction. Several effects have been noted to arise in the aqueous reactions of phosphate monoesters from the addition of organic cosolvents, or of carrying out reaction in anhydrous solutions of alternative phosphoryl acceptors. Abell and Kirby found that added DMSO or HMPA accelerated the aqueous hydrolysis of pNPP dianion by up to 10^6- to 10^7-fold, by effects that were attributed to decreased hydrogen bonding of the solvent to the anionic nonbridge oxygen atoms.[8] Knowles et al. found that the stereochemical outcome of phosphoryl transfer from pNPP, made chiral by the use of oxygen isotopes, changes from inversion in protic solvents such as methanol to racemization in *tert*-butyl alcohol.[6] No kinetic studies were reported, but the reported approximate half-life of the reaction in *tert*-butyl alcohol was significantly faster than for the aqueous reaction at the same temperature.

To better understand the origins of these rate accelerations we have conducted kinetic and thermodynamic studies of the reactions of a series of aryl phosphate monoesters in anhydrous *tert*-butyl alcohol and in *tert*-amyl alcohol and compared these reactions to the aqueous ones. This report presents the results of those studies.

Experimental Section

General. Solvents were obtained from commercial sources, and all distillations were done under anhydrous nitrogen. *tert*-Butyl alcohol was distilled from NaH, *tert*-amyl alcohol (*t*-AOH) was distilled from Mg, pyridine was distilled from CaH_2, and acetone was dried over molecular sieves and distilled. Other solvents were used as received. Tetraphenylarsonium chloride and sodium tetraphenylborate were obtained from commercial sources. Tetraphenylarsonium chloride was recrystallized from acetone, while tetraphenylborate was recrystallized from acetone/toluene.[9]

Synthesis of Aryl Phosphates. Substituted phenyl phosphates were synthesized by the method of Bourne and Williams.[10] The identities of the products were confirmed by ^{1}H and ^{31}P NMR analysis. The phosphate monoesters were converted to the free acid forms by cation exchange with Dowex-50X8-100 cation-exchange resin in the proton form followed by drying in vacuo. However, with *para*-substituted halogenated phenyl phosphates, cation exchange was accompanied by significant hydrolysis. With these monoesters the bis-cyclohexylammonium salts in water were made 1 N in HCl and then extracted three times with diethyl ether. The ether layers were dried over $MgSO_4$ and filtered, and solvent was removed under vacuum. This yielded the pure acid form of the phosphate monoester.

Synthesis and Purification of Tetrabutylammonium and Tetraphenylarsonium Salts. The synthesis of tetrabutylammonium tetrabutylborate is described elsewhere in detail.[11] The product was confirmed by NMR analysis, elemental analysis, and melting point.

Tetraphenylarsonium tetraphenylborate was synthesized by combining aqueous solutions of tetraphenylarsonium chloride and sodium tetraphenylborate. An aqueous solution was made of each of these compounds, which when combined produced a gelatinous suspension of tetraphenylarsonium tetraphenyl-

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borate. This product was filtered, dried in vacuo, and recrystallized from nitromethane. ^{1}H NMR analysis confirmed the desired product.

Tetraphenylarsonium chloride is noted in the literature for problems resulting from the presence of water of hydration.[9] It was necessary to obtain a water-free tetraphenylarsonium halide for evaluation of its solubility in nonaqueous solvent. Therefore, the anion was exchanged by metathesis in water with sodium iodide, the iodide salt of the tetraphenylarsonium ion being much less soluble in water than the chloride salt. The tetraphenylarsonium iodide was then recrystallized from dry acetone.

Kinetics Methods. The acid form of the substituted phenyl phosphate monoester was dissolved in dried, freshly distilled *t*-AOH in a dried, screw-top test tube. Typical solution concentrations were 6−20 mM. This solution was brought to the appropriate temperature, and a stoichiometric amount of tetrabutylammonium hydroxide (Aldrich 1.0 M solution in methanol, stored under nitrogen) was added. To study the dianion reactions, a slight excess of 2 equiv of base was added, while to study the monoanion, only 1 equiv was added.

Over time, aliquots were removed and added to measured portions of a solution of 0.1 N NaOH. The reaction rates for phosphate monoesters in basic aqueous solution are many times slower than the rates in *t*-AOH, so this transfer effectively stopped the reaction. This solution was analyzed spectrophotometrically to determine the concentration of free phenol. The plot of absorbance versus time over the first 1−5% of reaction was analyzed by assuming first-order kinetics using the initial rates method. To determine initial substrate concentration, an aliquot of the reaction mixture was subjected to complete hydrolysis by alkaline phosphatase in pH 9.0 Tris buffer, 100 mM, containing 1 mM $ZnCl_2$ and $MgCl_2$, and the final absorbance of this solution was determined.

For each different substituted phenol, a spectroscopic study was performed comparing free phenolate to the corresponding phenyl phosphate. For each, a wavelength was selected for kinetic analysis such that no correction for the presence of the aryl phosphate was required.

pK_a Determinations. The pK_as in *tert*-butyl alcohol and *t*-AOH of the phenols listed in this study were evaluated using the method of Marple and Fritz[12] with an H-cell custom-made for this work. A drawing of this cell is included in the Supporting Information. The description below refers to the procedure using *t*-AOH; the procedure used for *tert*-butyl alcohol was identical, with *tert*-butyl alcohol replacing *t*-AOH in both reference and titration cells. The reference was an Accumet saturated calomel electrode with a cracked bead junction. This junction extended into a saturated aqueous KCl solution. Above this solution was *t*-AOH, also saturated in KCl, which was in contact with a frit. On the other side of this frit was a solution of *t*-AOH, saturated in tetrabutylammonium bromide. This solution was in contact with a frit which joined the reference assembly to the titration cell. In the titration cell, a glass pH indicating electrode connected to a pH meter was used to monitor pH and electrode potential as titrations were performed at 25 °C under nitrogen. Before use of the cell for any data collection the response of the cell was tested by titration with *p*-toluenesulfonic acid monohydrate. Over a range of 5 pH units, the response of the cell was found to be 57 ± 1 mV/pH. Phenols were dissolved in *tert*-amyl or *tert*-butyl alcohol and titrated with tetrabutylammonium hydroxide (Acros Organics, 0.1 N solution in toluene/methanol) that was normalized against a standard HCl solution. The mass of phenol used in each titration experiment was equivalent to about 0.2 mmol, to allow a titration volume of about 2 mL.

Between experiments, the H-cell and reference electrode annular container were cleaned with solvent and distilled water and oven dried. The dried glassware was assembled and filled, beginning with the reference cell solutions. Once solutions were at the proper level, the reference electrode was sealed into this assembly under nitrogen. Dried *t*-AOH was added to the other side of the cell under a blanket of nitrogen, and the glass electrode and titration buret were introduced, with the tip of the titration buret immersed in the solution. Phenol was added to the cell, with constant stirring, and the cell was allowed to stabilize for at least 10 min. Once the cell voltage was stable, titration was started. Titrations were carried at least to the neutralization point, the data were plotted, and the pK_a was calculated by graphic interpolation of the half-neutralization point. Experiments in *t*-AOH were performed at least in duplicate. No effort was made to evaluate junction potentials in this cell, as these measurements are intended only for comparison to each other. The stability and reproducibility of the junction potentials was indicated by the reproducibility of the titration results.

One manifestation of the method in this experiment is noted at the beginning of each titration. When the titration is in its early stages, the phenol is predominantly present in the protonated form. Since this is the only electrolyte in the solution where the indicating electrode is located and the titrant is not in significant concentration yet, this solution has almost no charge carriers and the impedance is very high. The very low current flow produces a regular oscillation in the voltage reading of about ±2 mV, most likely due to the stirring of the solution. As the titration proceeds, phenol dissociates and electrolyte concentration rises, corresponding to the disappearance of this oscillation.

Solubility Experiments. Experiments were performed to evaluate the solubilities of tetrabutylammonium tetrabutylborate, tetraphenylarsonium tetraphenylborate, tetraphenylarsonium iodide, sodium tetraphenylborate, and sodium iodide in *t*-AOH and water. We were unable to reliably quantify the very slight solubilities of the solutes tetrabutylammonium tetrabutylborate and tetraphenylarsonium tetraphenylborate, and others have reported similar problems with direct measurement of these solubilities.[13] For the more soluble salts, a series of tubes were prepared with 5.0 mL of freshly dried *t*-AOH and sufficient mass of dried solute to exceed saturation concentration. Saturation concentrations were estimated on the basis of preliminary experiments of a similar nature. One set of solutions was stirred at the experimental temperature representing solutions approaching saturation from undersaturation conditions, while another set, representing solutions approaching saturation from super-saturation, was stirred at elevated temperature for a period of time and then allowed to cool to the experimental temperature. Samples were analyzed over time until apparent equilibrium concentrations were reached. Analysis was spectrophotometric (Ph_4AsI at 265 nm, $NaBPh_4$ at 225 nm) for all but NaI, which was quantified by an Ag/Cr colorimetric titration.[14] Some inconsistencies in data were observed, and others have reported the decomposition of the tetraphenyl solutes in alcohols and in water.[9,13,15,16] Due to these problems, the solubility of tetraphenylarsonium tetraphenylborate was not determined directly in either water or *t*-AOH.

Partitioning Experiments. Phenyl phosphate was prepared in the bis-cyclohexylammonium salt form as described above. Conversion of this compound to the tetrabutylammonium form using cation-exchange resin resulted in incomplete exchange. Therefore the potassium salt was prepared by treating a 10 mM solution of the free acid form with KOH to pH 9, followed by cation exchange using Dowex 50X8-100 resin

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Table 1. Rates and Activation Parameters for Reactions of *p*-Nitrophenylphosphate Dianion and Monoanion

	aqueous	*tert*-butyl alchol	*tert*-amyl alchol
	A. Dianion		
k at 39 C, s^{-1}	1.6×10^{-8}	1.2×10^{-4}	1.4×10^{-4}
$k_{solv}/k_{aqueous}$	1	7500	8750
$\Delta H^{\ddagger}$, kcal/mol	30.6	31.6 ± 1.0	30.9 ± 0.3
$\Delta S^{\ddagger}$, eu	+3.5	$+24.5 \pm 0.8$	$+23.0 \pm 0.3$
$\Delta G^{\ddagger}$, kcal/mol	29.5	24.0 ± 1	23.7 ± 0.3
source	ref 3	this work	this work
	B. Monoanion		
k at 39 C, s^{-1}	1.1×10^{-6}	7.7×10^{-8}	6.81×10^{-8}
$k_{solv}/k_{aqueous}$	1	0.07	0.06
$\Delta H^{\ddagger}$, kcal/mol	25.4	37.1 ± 0.9	32.6 ± 1.0
$\Delta S^{\ddagger}$, eu	−4.5	$+27.7 \pm 0.7$	$+13.4 \pm 0.8$
$\Delta G^{\ddagger}$, kcal/mol	26.8	28.5 ± 0.9	28.4 ± 1.0
source	ref 4	this work	this work

in the tetrabutylammonium form. This yielded a clean solution of the bis-tetrabutylammonium salt of phenyl phosphate (8 mM).

Partitioning experiments were performed with the 8 mM aqueous solution described above at 4 °C in a coldroom. This solution and the alcohol were brought to the experimental temperature, and 5.00 mL of the aqueous solution and 5.00 mL of *t*-AOH were introduced into screw-top tubes with PTFE-lined caps. The tubes were placed on a shaker for a measured period of time. To sample the solutions, tubes were removed from the mixers and centrifuged to speed separation of the layers. All equipment was maintained at the experimental temperature, and all handling of samples was performed in a coldroom. Aliquots were removed by pipet from both the aqueous and organic layers and introduced into tubes containing premeasured mixtures for spectrophotometric quantification. These mixtures were prepared such that, after addition of the experimental sample, each tube contained 2.0 mL of 50% ethanol 0.1 N in NaOH, 0.5 mL of water, and 0.5 mL of *t*-AOH. The concentration of phenyl phosphate was determined at 267 nm. In addition, samples were scanned for detection of phenolate ion at 289 nm, which would indicate hydrolysis of the phenyl phosphate.

Results

Kinetics of Reactions in *tert*-Butyl Alcohol and *tert*-Amyl Alcohol. Values of the first-order rate constants for the reactions of aryl phosphate dianions and monoanions were determined by spectrophotometrically following the liberation of the free phenols or phenolate anions. The rate constants for the reactions of the monoanion and the dianion of *p*-nitrophenyl phosphate in both *tert*-butyl alcohol and in *tert*-amyl alcohol gave good linear Eyring plots (Supporting Information) which were used to calculate the enthalpies and entropies of activation, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. The dianion of pNPP was found to undergo phosphoryl transfer to solvent approximately 9000-fold faster in *tert*-amyl alcohol than in water, while the monoanion reacted about 16-fold more slowly in *tert*-amyl alcohol. The rate and activation parameter data together with literature values for the aqueous reactions are shown in Tables 1a and 1b.

The kinetic data for the reactions of the dianions of a series of aryl phosphates in *tert*-amyl alcohol were used to construct the Brønsted plot in Figure 2. The rates for *meta*-substituted and for *para*-substituted compounds fall on separate but parallel lines with a slope of -1.1 ± 0.1.

Phenolic pK_a Values in *tert*-Amyl Alcohol and *tert*-Butyl Alcohol. The pK_a values for the substituted phenols were measured in the anhydrous alcohols as described in the Experimental Section. The values are

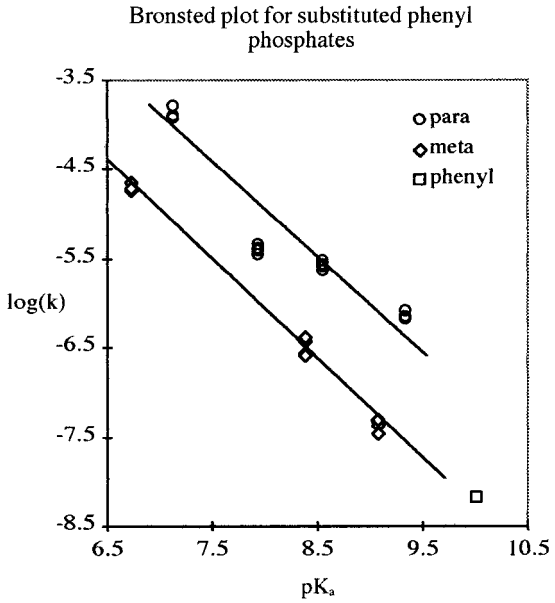

Figure 2. Brønsted plot for solvolysis of substituted aryl phosphates in *tert*-amyl alcohol: circles represent *para*-substituted aryl phosphates, diamonds represent *meta*-substituted aryl phosphates. The slope of the best-fit line for *para*-substituted compounds is -1.14 ± 0.12 and that for *meta*-substituted compounds is -1.11 ± 0.02. The phenyl phosphate rate constant was used for the slope calculations for both groups.

Table 2. pK_a Values for Substituted Phenols in Aqueous Solution, in *tert*-Butyl Alcohol and in *tert*-Amyl Alcohol

	pK_a		
substituent	aqueous[a]	*tert*-amyl alcohol[b]	*tert*-butyl alcohol[b]
p-nitro	7.14	10.0	10.57 (10.48[24])
p-cyano	7.95	11.83	12.5
p-amido	8.56[30]	13.95	
p-bromo	9.34	15.00	15.35
p-phenyl	9.51	15.75	
H	10.00	16.20	16.82 (16.64[24])
3,5-dinitro	6.73[31]	9.37	9.98
m-nitro	8.39	12.97	13.84
m-chloro	9.02	14.06	

[a] Aqueous values are from the *CRC Handbook of Biochemistry and Molecular Biology*, Physical Chemical Data, Part 1 unless otherwise specified. [b] Values in *tert*-butyl alcohol and *tert*-amyl alcohol are from this work unless otherwise specified.

tabulated in Table 2 and are plotted against their literature aqueous pK_a values in Figure 3. The wider range of pK_a values for the phenols in the nonaqueous solvent as compared to the range observed in water is consistent with previous observations.[17]

Solvent Partitioning Studies. Partitioning of the phosphate dianion between water and *t*-AOH was studied with the bis-tetrabutylammonium salt of phenyl phosphate. Phenyl phosphate was chosen due to its relatively slow rate of hydrolysis for the dianion in *t*-AOH. The experiments were performed at 4 °C in order to further minimize hydrolysis. The free energy of transfer from water to *t*-AOH is calculated by the equation

$$\Delta G_{tr}(\text{water}/t\text{-AOH})(\text{bis-tetrabutylammonium phenyl phosphate}) = RT \ln(P) \quad [18]$$

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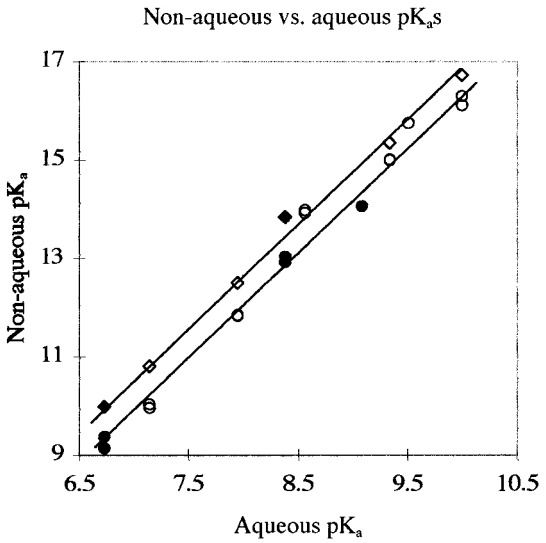

Figure 3. Comparison of aqueous and nonaqueous pK_as of the substituted phenols from Table 2: substituted phenolic pK_a in *tert*-butyl alcohol are represented by diamonds, pK_as in *tert*-amyl alcohol are represented by circles; filled shapes represent *meta*-substituted phenols, open shapes represent *para*-substituted phenols.

where P is the ratio of solubilities in water and *t*-AOH. The value for P was found to be 9.4 ± 0.1. The free energy of transfer is calculated to be 1.24 ± 0.01 kcal/mol. This represents a destabilization of the ground state of the bis-tetrabutylammonium phenyl phosphate in changing solvent from water to *t*-AOH.

Single Ion Solvation Studies of the Aryl Phosphate Dianion. To isolate the thermodynamics of the solvation of single ions, extrathermodynamic assumptions are used. These assumptions are based on the observation that when both the cation and anion of an ion pair are large, structurally similar, and radially symmetric, the charge is essentially shielded from the solvent, and solvent interactions are with a dispersed charge density on the outer surface of the ion. Therefore, solvent interactions with both ions are essentially equal, and any thermodynamic parameters of solvation for the ion pair can be divided equally between the ions. In some applications of this extrathermodynamic assumption, investigators have used compounds with somewhat dissimilar structures, for example the use of triisoamylbutylammonium tetraphenylborate.[19] If the extrathermodynamic assumption can be used to equate the thermodynamic contributions of these two ions, then it is reasonable for us to assume that the free energy of transfer from water to *t*-AOH for the tetraphenylarsonium ion is a good approximation for that of the tetrabutylammonium ion.

The activities of the solutes used in this study were assumed to be close enough to unity to be disregarded, given the dilute concentrations at saturation and the qualitative nature of the conclusions that are drawn. The free energies of solvation can then be approximated from the solubility data using the equation $\Delta G_s = -RT \ln(K_{sp})$.

The solubilities of tetrabutylammonium tetrabutylborate and salts of its ions were investigated in an attempt to either directly measure the solubility of

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Ph_4AsI ---> $Ph_4As^+ + I^-$

$NaBPh_4$ ---> $Na^+ + BPh_4^-$

$Na^+ + I^-$ ---> NaI

Ph_4AsBPh_4 --> $Ph_4As^+ + BPh_4^-$

Solute	Solubility (M)	ΔG_s (kcal/mol)
Ph_4AsI	3.31×10^{-5} (1)	12.2
$NaBPh_4$	8.0×10^{-4} (2)	8.4
NaI	2.23×10^{-1} (1)	1.8
Ph_4AsBPh_4		18.8 (calculated from cycle above)

Figure 4. The thermodynamic cycle used to determine the free energy of solvation of Ph_4AsBPh_4 in *tert*-amyl alcohol, with the concentrations at saturation at 25 °C.

tetrabutylammonium tetrabutylborate or to solve for its solubility through a thermodynamic cycle. These efforts were hindered by the extremely low solubility of tetrabutylammonium tetrabutylborate and the high solubility of some of the salts in *t*-AOH which precluded using a thermodynamic cycle to obtain its free energy of solvation indirectly. Due to these difficulties, we decided to use a similar approach with tetraphenylarsonium tetraphenylborate, a more frequently used and widely accepted subject of studies involving the extrathermodynamic assumption.

Since the direct determination of the concentration of Ph_4AsBPh_4 in *t*-AOH was not reliable, saturation concentrations of Ph_4AsI, $NaBPh_4$, and NaI in *t*-AOH were measured at 25 °C for use in the thermodynamic cycle shown in Figure 4. Solubilities and free energies of solvation in *t*-AOH are given in Figure 4. From this result, applying the extrathermodynamic assumption the free energy of solvation of the Ph_4As^+ ion in *t*-AOH = 9.4 kcal/mol.

The next step in this process is to compare the ΔG_s(*t*-AOH) to the ΔG_s(water) to calculate the free energy of transfer of the species from water to *t*-AOH. The solubility of Ph_4AsBPh_4 in water is very low, and in our hands direct attempts to determine the equilibrium concentration again proved to be unreliable. The high solubilities of NaI and $NaBPh_4$ in water mean that activities cannot be assumed to be approximately equal to concentration, and so the thermodynamic cycle approach cannot be used. A literature value for ΔG_s(water) of Ph_4AsBPh_4 which is derived from indirect methods is 23.5 kcal/mol[9] which leads to ΔG_s(water)(Ph_4As^+) = 11.8 kcal/mol.

This allows calculation of the free energy of transfer for the Ph_4As^+ ion from water to *t*-AOH according to the equation

$$\Delta G_{tr}(\text{water}/t\text{-AOH})(Ph_4As^+) = \Delta G_s(t\text{-AOH})(Ph_4As^+) - \Delta G_s(\text{water})(Ph_4As^+)$$

Using our experimental results and the literature value for ΔG_s(water)(Ph_4AsBPh_4), we obtain a ΔG_{tr}(water/*t*-AOH)(Ph_4As^+) of −2.4 kcal/mol. To a first approximation this result is reasonable, given the free energies of transfer from water to various alcohols for the Ph_4As^+ ion as reported by Marcus:[19] −5.8 kcal/mol for methanol, −5.1 kcal/mol for ethanol, and −6.0 kcal/mol for *n*-propanol and −3.5 kcal/mol for the BPh_4^- ion in 2-propanol.

The experimental cycle to derive the ΔG for transfer of the phenyl phosphate dianion can be completed by measuring the partitioning of a bis-tetraphenylarsonium-substituted phenyl phosphate between water and in *t*-AOH. This will allow calculation of the free energy of transfer for the compound, from which the contributions of the tetraphenylarsonium ions can be subtracted. Unfortunately, we were unable to isolate the pure bis-tetraphenylarsonium salt of phenyl phosphate. As an approximation, using the extrathermodynamic assumption that the free energies of solvation for tetraphenylarsonium and for tetrabutylammonium are similar, we can estimate this quantity as follows:

$$\Delta G_{tr}(\text{water}/t\text{-AOH})(\text{phenyl phosphate}) = \Delta G_{tr}(\text{water}/t\text{-AOH})((n\text{-Bu}_4\text{N}^+)_2 \text{ phenyl phosphate}) - 2\Delta G_{tr}(\text{water}/t\text{-AOH})(\text{Ph}_4\text{As}^+)$$

$$\Delta G_{tr}(\text{water/tAOH})(\text{phenyl phosphate}) = 1.24 \text{ kcal/mol} - 2(-2.4 \text{ kcal/mol}) = 6 \text{ kcal/mol}$$

This demonstrates a significant destabilization of the phenyl phosphate dianion in *t*-AOH relative to water, presumably arising from less efficient solvation by *t*-AOH.

Discussion

Phosphate monoesters exhibit bell-shaped pH−rate profiles with an optimum pH of around 4, with a lower, pH-independent rate at higher pH values, indicating that the monoanionic species is more reactive than the dianion. Nevertheless, the less reactive dianion is typically the substrate for phosphatases, and these enzymes exhibit catalytic efficiencies (defined as the ratio of k_{cat} to the uncatalyzed rate constant) of 10^{10} or more. The large rate accelerations provided by phosphatases have led to proposals that, while dissociative transition states occur in uncatalyzed reactions, enzymatic phosphoryl transfer from monoesters may proceed by a more associative mechanism. Since all known phosphatases bear positively charged residues and/or metal ions at their active sites, it is postulated that this charge may electrostatically function in the same way as alkylation does in causing a mechanistic shift; that is, by neutralizing the negative charge on the substrate and enhancing the electrophilicity of the phosphorus atom toward attack. This notion is intuitively attractive since phosphate triesters are considerably more reactive than their monoester counterparts. Another motivating factor has been a perceived difficulty in rationalizing how an enzyme can stabilize a dissociative transition state.[20] Methods by which an enzyme could stabilize a dissociative phosphoryl transfer have been considered.[21]

This descriptive term can be misleading however; the aqueous hydrolysis reference reaction for phosphatases, while having a dissociative transition state, is nonetheless A_ND_N. Entropy effects are therefore still important, and overcoming entropic barriers is a potential source of catalytic efficiency. Despite this, entropic contributions have been omitted from many discussions of the catalytic efficiencies of phosphatases. A discussion follows of entropic and enthalpic effects in the rate enhancements observed in *tert*-butyl alcohol and in *t*-AOH.

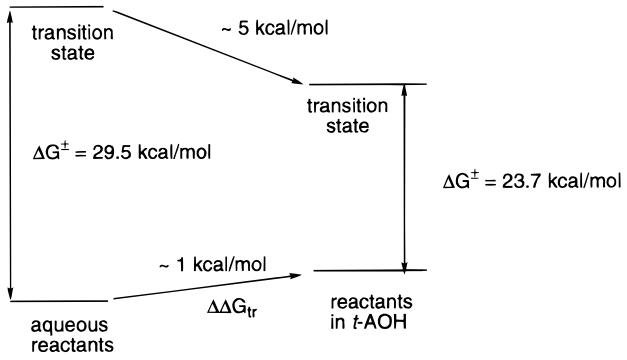

Figure 5. Diagram showing the effect of changing the solvent from water to *tert*-amyl alcohol on the free energy of activation and the contributions of ground state versus transition state effects.

The Dianion Reaction. Our work reported in this study was motivated by an interest in analyzing how the microenvironment can affect the kinetics and mechanism of phosphoryl transfer reactions. An essential difference in the phosphoryl transfer from the dianion of pNPP to anhydrous *tert*-butyl alcohol, where this acceptor also is the reaction solvent, is the observation of racemization at phosphorus when the reactant *p*-nitrophenyl phosphate was made chiral by the use of oxygen isotopes.[6] This outcome was interpreted to indicate the formation of a free metaphosphate intermediate. This outcome was in contrast to the observation in more nucleophilic solvents such as methanol, where the product had inverted configuration consistent with nucleophilic participation in the transition state of a concerted mechanism.[7] Because we were interested in performing solvent partitioning studies in order to determine differences in solvation free energies, we also examined the reaction in *tert*-amyl alcohol, which is structurally very similar but is much less miscible with water. Since this alcohol is, if anything, even more hindered as a nucleophile than *tert*-butyl alcohol, we assume that the stereochemical course of the phosphoryl transfer reaction in these two alcohols is the same. The very similar kinetics and activation parameters measured for the two reactions supports this assumption.

The reduced activation barrier in the tertiary alcohols can occur by raising the free energy of (destabilizing) the reactant, by lowering the free energy of the transition state, or a combination of both. The portion of the more favorable entropy of activation from reduced solvent reorganization could arise from less effective solvation (increased free energy) of the substrate or from stabilization (reduced free energy) of the transition state, relative to the aqueous reaction. To determine the contribution of differences in the free energies of solvation (ΔG_s) of the reactant, we compared the free energies of the tetrabutylammonium salt of *p*-nitrophenyl phosphate in water and in *tert*-amyl alcohol. The difference in the solvation free energies in the two solvents, shown as ΔG_{tr}(water/*t*-AOH) in Figure 5, represents the free energy of transfer of the substrate from water to the alcohol. The solvent partitioning experiments thus indicate that the overall reduction in $\Delta G^{\ddagger}$ of 5.8 kcal/mol in the dianion reaction can be attributed to approximately 1 kcal/mol of ground state destabilization, which presumably reflects

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less effective solvation of the substrate in *tert*-amyl alcohol, with the balance being a transition state effect.

The change to a two-step mechanism in the tertiary alcohols offers one explanation for the faster rate, since the entropy of activation should be more favorable for a reaction involving rate-limiting unimolecular dissociation than for one in which nucleophilic participation by a second molecule occurs. This is revealed in the more favorable $\Delta S^{\ddagger}$ for the reactions in *tert*-butyl alcohol and *tert*-amyl alcohol compared to that of the aqueous reaction. Another possible energetic source for the enhanced rate is the weaker hydrogen-bonding ability of these solvents compared to that of water. The driving force for the dissociative reaction (whether in a fully two-step dissociative mechanism or in a concerted mechanism with a dissociative transition state) can be rationalized as coming from the internal electron donation of the negative charges on the nonbridge oxygen atoms. Indeed, the loss of these charges by alkylation deactivates this pathway and leads to mechanisms with greater nucleophilic participation. If these charges are less involved in stabilizing interactions such as hydrogen bonds with the solvent, they ought to be more capable of acting as internal nucleophiles in expelling the leaving group. This effect has been postulated to explain the acceleration of the hydrolysis of *p*-nitrophenyl phosphate dianion in aqueous DMSO or HMPA.[8] In the reactions examined in this study in *tert*-butyl alcohol and *tert*-amyl alcohol, the tetra-*N*-butylammonium counterion is also unable to interact effectively with these nonbridge oxygen atoms, due to the steric shielding of the positive charge by the bulky *n*-butyl groups. The single ion ΔG_{tr} of 6 kcal/mol measured for transfer of the phenyl phosphate dianion from water to *t*-AOH confirms that this ion is considerably less solvated in the latter solvent. An increased hydrolysis rate due to the loss of stabilizing hydrogen-bonding interactions with the solvent should be analogous to the rate accelerations observed in the alkaline hydrolysis of esters caused by added aprotic cosolvents; the enthalpy of activation for the saponification of ethyl acetate and ethyl benzoate are reduced by 4 and 6 kcal/mol, respectively, in aqueous DMSO versus aqueous ethanol.[22] The increased rates and lower $\Delta H^{\ddagger}$ were attributed to decreased solvation of hydroxide ion. While these are bimolecular reactions and the phosphoryl transfers in the tertiary alcohols are unimolecular, the rate-limiting steps share the features of an anionic species providing the driving force for the reaction, with negative charge becoming more dispersed in the transition state. In the ester saponification reactions a larger destabilization of the reactants relative to the transition state resulted in a significant net reduction in $\Delta H^{\ddagger}$.[22] Thus the expectation is reasonable that rate enhancement caused by desolvation of the dianionic phosphate reactant should show up as a decrease in $\Delta H^{\ddagger}$.

Surprisingly, the activation parameters (Table 1a) for the reaction of the dianion of pNPP reveal that the rate enhancements for the reaction in *tert*-butyl alcohol and in *tert*-amyl alcohol are due entirely to a more favorable entropy of activation. The activation enthalpy is unchanged within experimental error from the value in the aqueous reaction. This indicates that the loss of stabilizing hydrogen-bonding interactions with the nonbridge oxygens may not be a major factor in the rate accelerations in these alcohols and that any such destabilization is evidently felt equally in both the ground state and transition state. A possible alternative explanation for the similar enthalpies of activation is a coincidental compensation for the loss of a stabilizing bonding interaction in the transition state with the nucleophile (present in the aqueous concerted reaction but absent in the stepwise reaction in *t*-AOH) by a more favorable $\Delta H^{\ddagger}$ for the unimolecular dissociation in *t*-AOH compared with the same hypothetical process in water. Given a similar degree of bond cleavage to the leaving group in the transition state, the unimolecular dissociation should have a higher $\Delta H^{\ddagger}$ than the concerted reaction since in the latter case the enthalpic cost of bond cleavage is partly offset by formation of the new bond. Though bond formation to the nucleophile in the transition state is small in the aqueous reaction, there should be some enthalpic effect in lowering $\Delta H^{\ddagger}$ which will not be present in the reaction in *t*-AOH. It is possible that solvation differences lower the enthalpic barrier for unimolecular dissociation in *t*-AOH by an amount similar to the lost bonding interactions, leaving an unchanged overall $\Delta H^{\ddagger}$. Though perhaps unlikely, this possibility cannot be ruled out.

There are two sources for the more favorable entropy of activation. One is the aforementioned change from a bimolecular concerted reaction in water to a mechanism with rate-limiting unimolecular dissociation. Even though bonding between the phosphorus atom and the nucleophile is thought to be small in the transition state of the aqueous reaction, a solvent molecule must still be recruited to assume an apical position in the trigonal bipyramidal transition state, with an associated loss of entropy. In addition, water is a more highly structured solvent than the tertiary alcohols. Solvent reorganization must occur in the transition state, driven by the change in geometry and in charge distribution. The overall charge of the transition state is the same as that of the ground state; however, since the size of the structure increases and charge is dispersed, a larger solvation shell is required in the transition state with an accompanying loss in entropy. This solvent effect should be more pronounced in the more highly structured solvent water than in the tertiary alcohols. The latter solvents are not without structure, however, and so the potential entropic acceleration obtainable from eliminating solvent reorganization is probably not fully realized in these solvents.

Although a nucleophilic role is eliminated in the rate-determining step in the reactions in *tert*-butyl alcohol and *tert*-amyl alcohol, isotope effect data[23] indicate that this occurs with no change in the transition state structure of the pNPP reactant. The ^{18}O isotope effects in the nonbridging oxygen atoms and in the bridging oxygen atom and the ^{15}N isotope effects for the reaction of *p*-nitrophenyl phosphate in *tert*-butyl alcohol are very similar to those for the aqueous reaction.[23] That the transition state structure within the reactant is unchanged in the $D_N + A_N$ reaction compared with the A_ND_N one is not surprising in view of the very small degree of bond order to the nucleophile in the transition state of the aqueous reaction. Evidently the loss of this interaction is not accompanied by significant changes in the

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degree of bond cleavage to the leaving group or in the transition state structure of the phosphoryl group. Thus the entropic accelerations observed do not result from making the reaction mechanism more dissociative with respect to the degree of transition state bond cleavage to the leaving group, but arise solely from eliminating the need for nucleophilic participation and from possible contributions from solvent reorganization.

A fair question is whether the rate enhancement observed with pNPP also occurs with less activated phosphate esters. The rates were therefore measured for the phosphoryl transfer reaction with a range of substituted phenyl phosphates, and the results were plotted versus the aqueous pK_a values for the leaving groups in Figure 2. Although the rates for *meta*- and *para*-substituted aryl phosphates fall on separate but parallel lines, the Brønsted slopes of -1.1 are very similar to the Brønsted slope of -1.2[5] reported for the aqueous reaction. Thus similar rate enhancements are seen with all of the aryl phosphates tested.

The occurrence of separate lines for *meta*- and *para*-substituted aryl phosphates contrasts with the aqueous reactions where the hydrolysis rates of *meta*- and *para*-substituted phenyl phosphates fall on the same line in the analogous Brønsted plot, both for the reaction of the dianion and for that of the monoanion.[5] We initially hypothesized that the reason might be differential changes in the pK_a values of *meta*-substituted phenols compared to *para*-substituted ones in *tert*-amyl alcohol. If this were the case then if the rate constants were plotted against the true pK_a values in the reaction solvent, the *meta*- and *para*-substituted compounds should fall on the same line as they do in the aqueous reaction.

We measured the pK_a values of the phenols in anhydrous *tert*-butyl alcohol and in *tert*-amyl alcohol using an electrochemical method[24] described in the Experimental Section. The results are given in Table 2. The measured pK_a values in *tert*-amyl alcohol are higher than those in water and cover a larger span compared to the aqueous values. Both of these effects are expected from the results of previous work on nonaqueous pK_a values of phenols.[17] While there is always some uncertainty in the assignment of absolute pK_a values in nonaqueous solvents, insofar as they can be directly compared with the aqueous values, the method can be counted upon to give precise measurements of the relative nonaqueous pK_a values of the phenols. The pK_a values determined in *tert*-amyl alcohol are plotted against the aqueous values in Figure 3, which reveals a linear correlation between the two values. *Meta*- and *para*-substituted phenols fall on the same correlation line, indicating that there is no anomalous perturbation of pK_a values in the alcohol by *meta* versus *para* substituents. Thus the hypothesis that *meta* versus *para* substitution causes a differential perturbation in the leaving group pK_a values is incorrect. We are presently uncertain as to the source of the difference in rates between these two classes of compounds in *tert*-amyl alcohol.

The Monoanion Reaction. The thermodynamic and kinetic data for the monoanion reaction are collected in Table 1b. In contrast to the situation in aqueous solution where the monoanions of phosphate monoesters are the more reactive species, in the tertiary alcohols the monoanion reaction is 2000-fold slower than that of the dianion. The monoanions react more slowly in the alcohols than in aqueous solution, in contrast to the behavior of the dianion. In both alcohols, a more favorable entropy of activation is more than offset by an increased enthalpy of activation.

This increase in $\Delta H^{\ddagger}$ most likely arises from greater difficulty in transferring the proton from a nonbridge oxygen atom of the reactant to the bridging oxygen atom. In the alcohol solvents the pK_a value of the phosphate group will be higher, making proton removal more energetically difficult. In addition, in water this proton transfer could well occur via an intervening water molecule; this would allow a six-membered transition state for the proton transfer process, which is sterically preferable to the four-membered one which would be operative in its absence. The steric bulk of the tertiary alcohols makes them less able to similarly assist in the proton transfer.

Stereochemical studies of the monoanion reaction in *tert*-butyl alcohol have not been carried out. Therefore the more favorable entropies of activation cannot with assurance be ascribed to a change in the molecularity of these reactions, though it is likely. In addition, the reason for the less favorable entropy of activation in the reaction of the monoanion in *tert*-amyl alcohol compared to *tert*-butyl alcohol is uncertain. In both reactions the predominant reason for the slower rates is a higher enthalpic barrier relative to the aqueous reaction.

Significance for Entropic Considerations in Phosphatase Reactions. The results presented here have significance for the enzymatic reactions of phosphatases. Enzymes confer significant entropic advantage relative to uncatalyzed reactions in solution. It has been estimated that 10^8 M is the approximate maximum value for the advantage of an enzymatic reaction compared with a corresponding bimolecular reaction from entropic advantages, in reactions in which the transition state is relatively tight so that a large loss of entropy is required for its formation.[25] The catalytic benefit of induced intramolecularity in a dissociative phosphoryl transfer reaction has previously been pointed out, and a catalytic enhancement of approximately 10^2-fold was attributed to the positioning of a metal-bound hydroxide ion and phosphoryl group by Mg^{2+} ions in a phosphorylated 4-morpholinopyridine/Mg^{2+} complex.[26] In general, however, entropy considerations are often omitted in assessments of the sources for the catalytic efficiencies of phosphatases and in considerations of how these enzymes might catalyze a reaction having a dissociative transition state. This may be because dissociative transition states such as those operative in phosphate monoester reactions may seem like unlikely candidates for potential acceleration from entropic effects.

The term "dissociative", while generally an accurate description of the aqueous reaction, may convey the impression that there is no nucleophilic involvement in the transition state. However, the aqueous reference reactions are concerted, with nucleophilic participation in the transition state; even the weak bond formation to the nucleophile in this reaction nonetheless requires its proper positioning in the trigonal bipyramidal transition state. The removal of the associated entropic barrier must be considered as one source of the enzymatic

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efficiencies of phosphatases. In the ground states of a phosphatase−substrate complex, a nucleophile is already preassociated and the hydration shell has been removed, eliminating the need for solvent reorganization. The rate enhancement observed in these studies of nearly 10^4 is probably a lower limit for the rate enhancement from entropic considerations that are enjoyed by phosphatases. This is not to say that these enzymatic reactions proceed by a metaphosphate intermediate as in the tertiary alcohols. Quite the contrary, with a preassociated nucleophile in place a free metaphosphate intermediate is unlikely. However the entropic advantage enjoyed in the enzymatic reaction is the same as that gained from eliminating the need to recruit a disordered solvent molecule to serve the nucleophilic role in the tertiary alcohol reactions. The factor of 10^4 is a significant fraction of the overall catalytic rate enhancement achieved by some phosphatases.

Mechanistic Conclusions

After the report of racemization in the reaction of *p*-nitrophenyl phosphate in *tert*-butyl alcohol,[6] alternative explanations have been suggested that avoid the formation of metaphosphate. One is the possibility that a bridge-protonated *tert*-butyl phosphate is initially formed which undergoes reversible and rapid phosphoryl transfer with other solvent molecules before loss of the proton which is necessary to form a stable product.[27] It has also been postulated that an associative mechanism occurs, forming a pentacoordinate intermediate which may undergo pseudorotations, leading to racemized product.[28] Both of these alternative explanations are unlikely in view of the significantly more positive entropy of activation in the *tert*-butyl alcohol reaction. If the first alternative mechanism were operative, since nucleophilic participation is still important in the transition state, no significant change in the entropy of activation should be seen. If the phosphorane mechanism were operative, then the entropy of activation should be large and negative, as it is for reactions of phosphate triesters which have such associative transition states. Typical reactions of this type exhibit entropies of activation of −35 eu,[29] dramatically different from the experimental value reported here. The reported results are in best agreement with the originally reported explanation for racemization, namely, a change to a dissociative S_N1-type of mechanism.

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Supporting Information Available: Supporting Information Available: Eyring plots for the reactions of the monoanion and the dianion of *p*-nitrophenyl phosphate in *tert*-butyl alcohol and in *tert*-amyl alcohol and a drawing of the electrochemical cell used in the determinations of the phenolic pK_a values in *tert*-butyl alcohol and in *t*-AOH (3 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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