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Fundamentals of Phosphate Transfer

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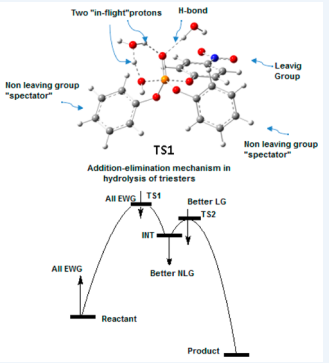
CONSPECTUS: Historically, the chemistry of phosphate transfer—a class of reactions fundamental to the chemistry of Life—has been discussed almost exclusively in terms of the nucleophile and the leaving group. Reactivity always depends significantly on both factors; but recent results for reactions of phosphate triesters have shown that it can also depend strongly on the nature of the nonleaving or "spectator" groups. The extreme stabilities of fully ionised mono- and dialkyl phosphate esters can be seen as extensions of the same effect, with one or two triester OR groups replaced by O^-. Our chosen lead reaction is hydrolysis−phosphate transfer to water: because water is the medium in which biological chemistry takes place; because the half-life of a system in water is an accepted basic index of stability; and because the typical mechanisms of hydrolysis, with solvent H_2O providing specific molecules to act as nucleophiles and as general acids or bases, are models for reactions involving better nucleophiles and stronger general species catalysts. Not least those available in enzyme active sites.



Alkyl monoester dianions compete with alkyl diester monoanions for the slowest estimated rates of spontaneous hydrolysis. High stability at physiological pH is a vital factor in the biological roles of organic phosphates, but a significant limitation for experimental investigations. Almost all kinetic measurements of phosphate transfer reactions involving mono- and diesters have been followed by UV−visible spectroscopy using activated systems, conveniently compounds with good leaving groups. (A "good leaving group" OR* is electron-withdrawing, and can be displaced to generate an anion R^*O^- in water near pH 7.) Reactivities at normal temperatures of P−O-alkyl derivatives—better models for typical biological substrates—have typically had to be estimated: by extended extrapolation from linear free energy relationships, or from rate measurements at high temperatures.

Calculation is free from these limitations, able to handle very slow reactions as readily as very fast ones, and capable of predicting rate constants with levels of accuracy acceptable to the experimentalist. We present an updated overview of phosphate transfer, with particular reference to the mechanisms of the reactions of alkyl derivatives and triesters. The intention is to present a holistic (not comprehensive!) overview of the reactivity of typical phosphate esters, in terms familiar to the working chemist, at a level sufficient to support informed predictions of reactivity for structures of interest.

1. INTRODUCTION

The properties of phosphate esters and the "convenience" of phosphate transfer in water are fundamental reasons "why nature chose phosphate".[1,2] Excellent comprehensive reviews of basic phosphate transfer chemistry are available,[3,4] and we start with brief summaries of our current understanding of the process for triesters, diesters, and monoesters (Scheme 1).

2. TRIESTERS

Phosphate triesters differ in several respects from the more familiar mono- and diesters. As polar but uncharged molecules, only the lower trialkyl derivatives are soluble in water, and before the introduction of organophosphorus agrochemicals triesters had no significant presence in the biosphere. Their chemistry has become of intense interest because of the need to render harmless stockpiles of some organophosphorus biocides—which include nerve gases as well as herbicides and pesticides.[5] And for the way nature has responded to the same chemical challenge by evolving bacterial phosphotriesterase enzymes.[4] The objective in both cases is to engineer an efficient phosphate transfer to water, to convert the active triester agent to an unreactive diester (Scheme 2).

Evidence is accumulating that the hydrolysis reactions of simple phosphate triesters, in addition to those involving five-membered rings,[6] can involve phosphorane intermediates (INT in Scheme 2), comparable with the tetrahedral addition intermediates familiar from the acyl transfer chemistry of carboxylic acid derivatives. A phosphorane INT, and TS close to it in energy, are expected to be stabilized by electron-withdrawing equatorial substituents OR. (And thus destabilized by OH and especially O^- groups: so that diester and monoester anions are hydrolyzed without formation of intermediates.) Intermediate lifetime is also sharply reduced by the presence of a good (electron-withdrawing) leaving group OR* in an apical position. There are thus conflicting demands on the substituents: the best leaving group in an intermediate INT formed from a mixed triester takes up an apical position (OR* in Scheme 2), and is destabilizing; but additional good leaving

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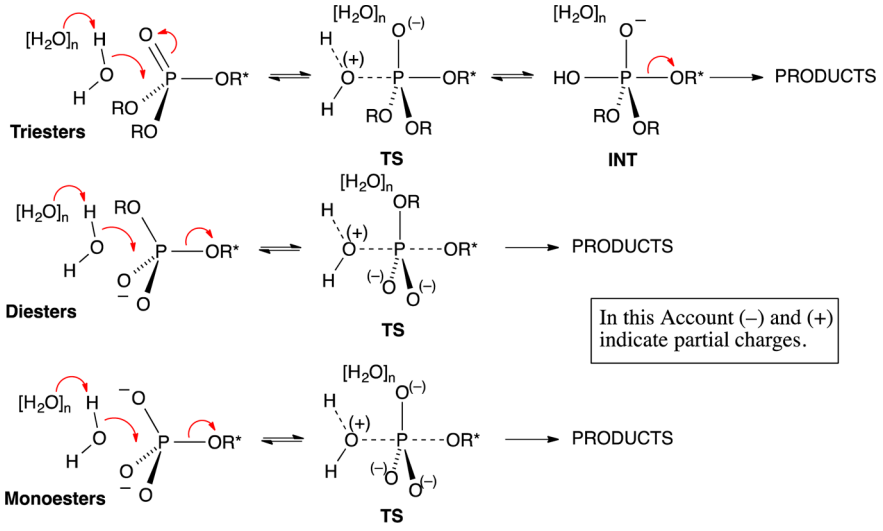
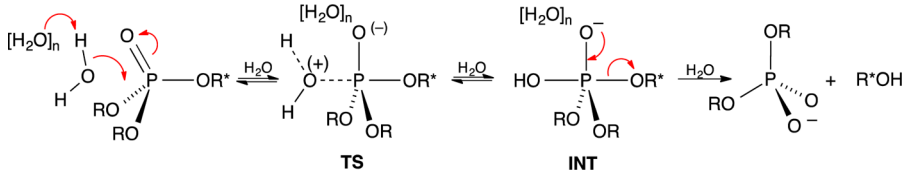
Scheme 1. Mechanisms for Phosphate Transfer to Water from the Major Ionic Forms of Phosphate Esters Present at Physiological pH



Scheme 2. Spontaneous Hydrolysis of a Phosphate Triester Involves Water as Both Nucleophile and General Base[a]



[a]The reaction can be catalyzed by better nucleophiles, by stronger general bases, or by both.

groups must occupy stabilizing, equatorial positions. (Small [4–7] ring formation constrains pairs of connected OR groups to an apical-equatorial relationship, with a nominal 90° internal O–P–O angle.[7]) Good leaving groups have a common effect—of raising the energy—on the ground state (Figure 1). Thus, reactivity depends significantly on both leaving and nonleaving groups.

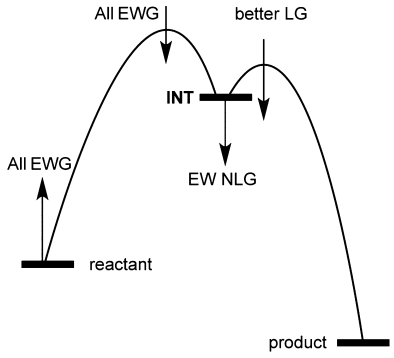


Figure 1. Effects of leaving group capability on the reactivity of phosphate triesters.

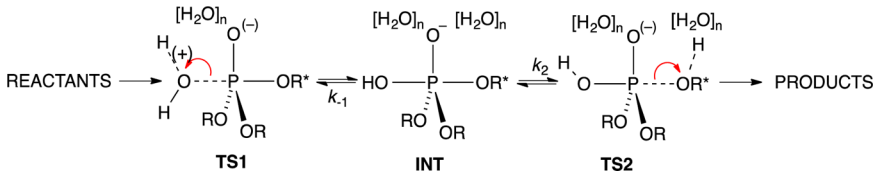
The involvement of an intermediate means two possible rate-determining steps, involving its formation or its breakdown to products. These competing processes are simply compared, in terms of the departure from the intermediate (INT, Scheme 3) of a hydrogen-bonded hydroxide (k_{-1}) vs the loss of the R*O$^-$ anion (k_2). In most cases, hydroxide is expected to be the poorer leaving group, and thus, the formation of the phosphorane rate-determining (as shown in Figure 1).

Recent calculations[8] (discussed below) suggest that the intermediate is unlikely to have a significant lifetime, at least when a leaving group as good as 4-nitrophenolate is attached, so that TS1 can be compared directly with TS for the concerted hydrolysis of structurally related diesters.

3. DIESTERS

Diesters (Scheme 4) are among the least reactive phosphate esters, and with the pK_a's of dialkyl phosphoric acids $(RO)_2P(O)OH$ typically between 1 and 2 exist as the monoanion across almost the complete pH region. The negative charge, shared between two equivalent oxygens, makes the phosphorus center substantially less electrophilic than that of triesters. And with no mobile proton to trigger

Scheme 3



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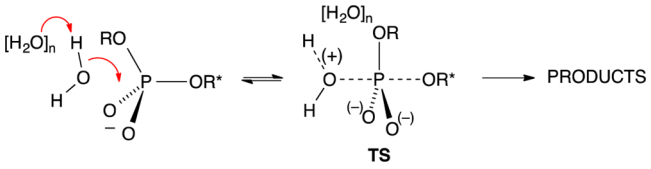
Scheme 4



special reactivity (cf. the reactions of monoester monoanions, below), there is no reactive alternative ionic form near neutrality.

Phosphate transfer involving diesters (Scheme 4) is thus typically a very slow process, in the absence of one or more special structural features: which include five-membered (P–O–R–R*O) rings[9] and neighboring nucleophiles[10] as well as very good leaving groups. In the absence of these factors, mechanisms are generally understood to involve concerted $S_N2(P)$ processes, with no addition intermediates of significant lifetime.

The primary difference from triesters is the negative charge. In the trigonal bipyramidal transition state TS (Scheme 4), the two (equatorial) P–$O^{(-)}$ share more than a single negative charge, and an addition intermediate (cf. INT in Figure 1) would carry a negative charge on each oxygen. In practice, these charges are decisively delocalized via the $n_O-\sigma^*_{P-OR}$ interactions that define the transition state. The third group in the equatorial plane is the neutral nonleaving group OR, the poorer of the two possible leaving groups for an unsymmetrical diester. Changing the nonleaving group pK_a has minimal effect on the rates of hydrolysis of diesters with good leaving groups,[11] in competition with two negatively charged oxygens. Suggesting that the change from triester to diester anion could be treated simply as the ultimate change of nonleaving group: as illustrated by the relationship shown in Figure 2.

Figure 2 extrapolates a published linear free energy relationship showing how the rates of spontaneous hydrolysis of a series of phosphate triesters with a common, 4-nitrophenolate, leaving group depend on the sum of the pK_a's of the nonleaving groups HOR.[12] The point for the hydrolysis of the related bis-4-nitrophenyl diester BPNPP (1.1×10^{-11} s^{-1}, derived from high temperature measurements[13]) falls on the extrapolated line if we use a pK_{NLG} for the P–O^- oxygen (i.e., for the ionization of HO^- to O^{2-}) of 31: which is of a reasonable expected order of magnitude for this unmeasurable quantity.[14]

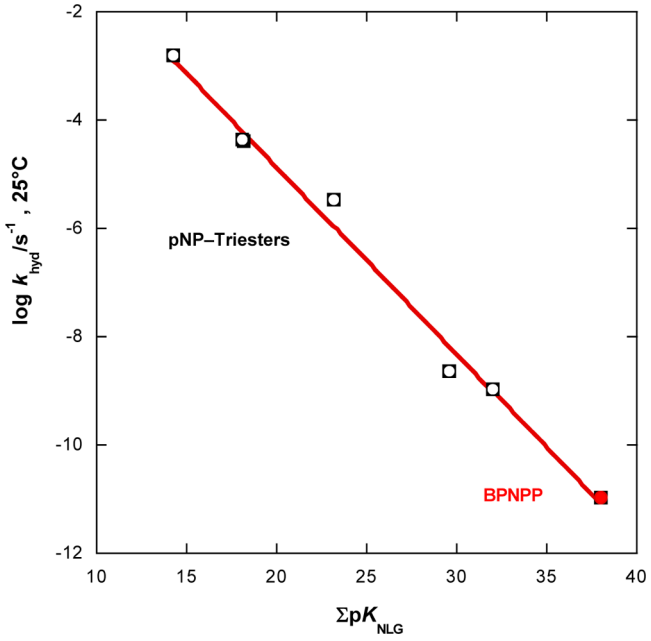


Figure 2. Dependence of the rates of spontaneous hydrolysis of 4-nitrophenyl triesters and one diester on the sum of the pK_a's of the nonleaving groups ROH (Scheme 3, see the text).

The quantitative effects of varying both leaving and nonleaving groups on the hydrolytic reactivities of all three classes of phosphate esters are summarized in Table 1.

4. MONOESTERS

Monoesters (MP) present the simplest case, differing in the case of the dianions, the predominant form at physiological pH, only in the leaving group. The three equivalent "nonleaving group" oxygens of the $PO_3^=$ group play important, well-established roles in the reactions of monoester dianions. Three P–$O^{(-)}$ bonds make alkyl derivatives $MP^=$ (Scheme 5) the most stable phosphate esters.[15] $O^{(-)}$ is also a potential electron donor, and given a very good leaving group OR* (Scheme 1) the triple $n_O-\sigma^*_{P-OR*}$ interactions available in all conformations of the dianion can lead to P–OR* cleavage under relatively mild conditions, with concurrent transfer of the PO_3^- group to an available nucleophile (Scheme 5).[16] For reactions in solution, the nucleophile must be present in the encounter complex, as discussed below: but the reaction is primarily dissociative, driven by (and thus strongly dependent on) the stability of the leaving group R^*O^-.

The same effect dominates the chemistry of monoester monoanions MP^-; which with a typical second pK_a of 6–7 are present in significant amounts at pHs below neutrality, and the major ionic form near pH 4. The MP^- monoanion (Scheme 6) could in principle react like a diester, that is, very slowly (rates of diester hydrolysis reach a *minimum* near pH 4): but the mobile proton makes available a much faster escape route, taking advantage of the pathway of Scheme 5 via the unfavorable pre-equilibrium (K of ca. 10^{-11})[17] of Scheme 6. This converts OR* into a very good alcohol leaving group, R*OH, thus accounting for the characteristic maximum near pH 4 in pH–rate profiles for the hydrolysis of monoesters.[18]

An instructive recent example is the hydrolysis of mono-2-pyridyl phosphate MPP (Scheme 7), which also shows a rate maximum near pH 4 in its pH–rate profile.[19] The pyridine nitrogen of the leaving group of MPP^- (pK_a 2.73) is more basic than the bridging oxygen of a monoalkyl phosphate (Scheme 6), but although N-protonation lowers the effective pK_a of the leaving group by only some 10 units, the observed cleavage is still over 10^5 times faster than expected for the standard mechanism of Scheme 6. Reactions of other nucleophiles with the $MPP^{+=}$ form are also readily identified and characterized.[19]

The triple $n_O-\sigma^*_{P-OR*}$ electron-donation interaction of Scheme 5 provides the major nucleophilic "push" for the displacement of the leaving group, to the extent that for a very good leaving group reactivity depends little, or even not at all, on the basicity of the nucleophile (Table 1).[20] However, the nucleophile must be present to act as the acceptor in the (unmistakably second order) phosphate transfer process. The simplest explanation for the limiting case, with a very good leaving group and no measurable involvement of the nucleophile in bond-breaking, is a preassociation-concerted reaction (Scheme 8).[21] A recent calculation concludes that

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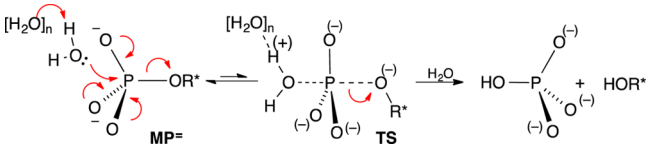
Table 1. Phosphate transfer: how reactivity depends on nucleophile, leaving group and non-leaving group

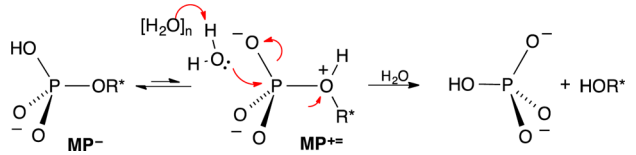
	dependence on			
system	nucleophile[a]	nonleaving group	leaving group[a]	mechanism
monoester dianions	minimal β_{nuc} 0.15 ± 0.15	N/A	maximal β_{LG} −1.0 ± 0.2	dissociative concerted
diester anions	moderately high β_{nuc} 0.39 ± 0.10	minimal: see also below, section 9	high β_{LG} −0.95 ± 0.21	associative concerted
triesters	moderately high β_{nuc} 0.50 ± 0.21	moderately high β_{NLG} −0.39 ± 0.03[b]	high to moderate β_{LG} −0.50 ± 0.22	concerted/stepwise

[a]Beta values from Lassila et al.[3] [b]Per NLG: for 4-nitrophenolate LG at 25 °C.[8]

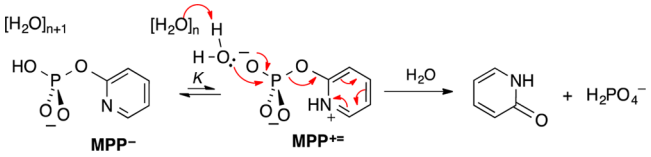
Scheme 5. Hydrolysis, Like Other $S_N2(P)$ Reactions of Monoester Dianions, Involves "Dissociative" Transition States TS with Weak, Extended Bonding to Both Incoming Nucleophile and Leaving Group



Scheme 6. Cleavage of $MP^{+=}$ Can Be up to 10^{20} Times Faster than That of $MP^{=}$



Scheme 7



metaphosphate is too short-lived in water to exist as a full (solvent-equilibrated) intermediate:[22] though it has been identified in the gas phase,[23] and may be involved in phosphate transfer reactions in some organic solvents.[3,4]

5. ELECTROSTATIC EFFECTS ON PHOSPHATE TRANSFER

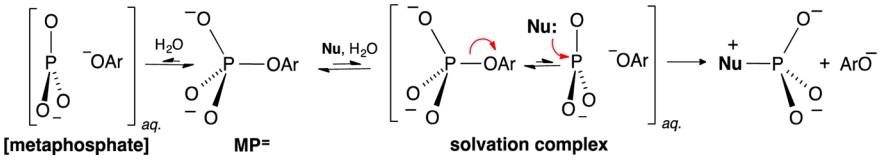
This picture also explains why highly reactive α-nucleophiles show little enhanced reactivity toward (reactive) phosphate monoester dianions.[24] And why enzyme-catalyzed phosphate transfers involving monoesters, by phosphatases, mutases, and so forth, involve strict inversion of configuration, as expected for an in-line $S_N2(P)$ process.[4] Some enzyme-catalyzed reactions certainly involve phosphate transfer to oxyanion nucleophiles; though phosphate monoester dianions are not attacked by anions in vitro. Amines readily displace aryloxide anions from the dianions of phosphate esters with good leaving groups (Scheme 8), but fluoride and oxyanions show no readily measurable reaction at the phosphorus centers of 4-nitrophenyl or 2,4-dinitrophenyl phosphate dianions.[20,25] Hence the conclusion: "the two negative charges provide a considerable electrostatic barrier toward reactions with nucleophilic reagents which have a negative charge on the attacking atom."[25]

This broad generalization remains sound, though the effect is not insurmountable (there are no zeros in kinetics!). The single negative charge shared by the two $O^{(-)}$ of a phosphate diester anion is clearly not an insuperable barrier, as evidenced by the relatively rapid alkaline hydrolysis of diesters. But anions do react with model diesters some 100 times more slowly than do neutral amines of similar basicities, consistent with some electrostatic effect.[26]

For monoester dianions, the work of Herschlag defines the "bottom line".[27] The strongly phosphorophilic fluoride anion reacts at about the same rate as a molecule of water with the terminal $PO_3^{=}$ group of the ATP tetraanion; and with the 4-nitrophenyl phosphate dianion, which gives a few percent of fluorophosphate on hydrolysis in the presence of 1−3 M KF at 95 °C.[27] This is consistent with the involvement of either an indiscriminately reactive electrophilic species (metaphosphate monoanion, Scheme 8) or an $S_N2(P)$ reaction with fluoride (pK_a 3.1), reacting by coincidence at a rate similar to that of (less basic, but neutral) water. Since simple phosphate transfers in water are known to involve consistent inversion at P, the latter explanation is perhaps more likely—presumably with a small electrostatic effect disfavoring attack by fluoride. Herschlag estimates a maximum 50-fold electrostatic effect on rate at high ionic strength in water.[28]

Significantly, the electrostatic effect disappears when $PO_3^{=}$ is attached to a positively charged group, as in a phosphoramidate $R_3N^+-PO_3^{=}$.[28] So enzyme-catalyzed phosphate transfer reactions involving phosphoryl-imidazolium intermediates, for example, are exempt. So also is general acid catalyzed phosphate transfer, when the general acid involved is cationic.[29] Thus, the neighboring COOH group of the salicyl phosphate monoanion (**1**) is a highly effective catalyst for the hydrolysis of the phosphate group (Scheme 9, Nu: = H_2O:), and for phosphate transfer to other, better nucleophiles than water, but not to anions.[29] By contrast, the dimethylammonium group of

Scheme 8. Metaphosphate Is Too Short-Lived in Water to Exist as a Significant Intermediate



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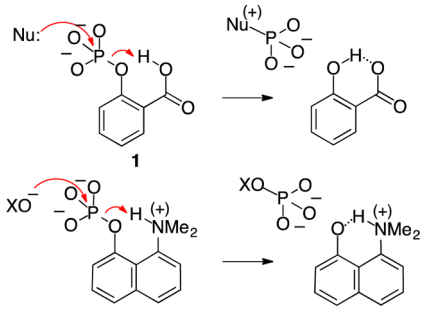
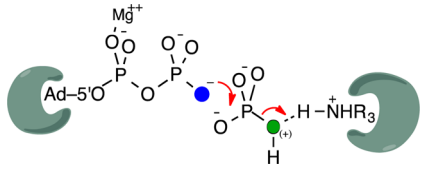
Scheme 9



2, also a highly effective general acid for the hydrolysis of the phosphate ester group and for the attack of other, better nucleophiles, does not discriminate between neutral (amine) and oxyanion nucleophiles.[29]

This could be key to the mechanism of arguably the most important phosphate transfer in biology, the ATP synthase reaction. This enzyme—an extraordinary "machine" composed of over 30 different proteins—produces ATP in the human body from ADP and inorganic phosphate in multikilogram amounts.[30] Though the detailed mechanism is unknown, ^{18}O labeling experiments show that the terminal bridge oxygen in the ATP formed comes from ADP, not from P_i. The clear message from the model reactions is that the simplest plausible mechanism involving inorganic phosphate as the phosphorylating agent demands (at least!) a cationic general acid in the active site (Scheme 10).

Scheme 10. Simplest Plausible Mechanism for the ATP Synthase Reaction



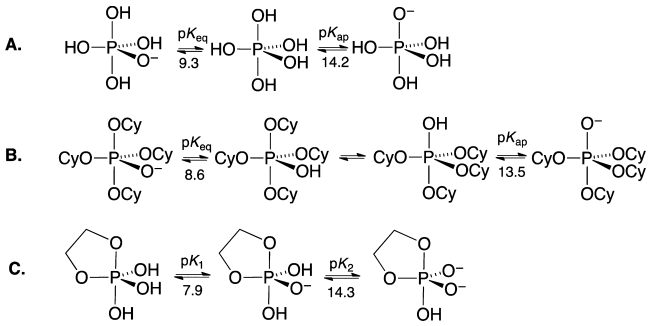
To summarize: nucleophilic anions react fastest with (neutral, most electrophilic) triesters, more slowly with diester anions, and scarcely detectably with monoester dianions. Neutral amines, on the other hand, are relatively more reactive toward diester anions than triesters, and react readily with $PO_3^=$ derivatives. It is clear that electrostatic effects are involved, though not at which stage (or stages) of the $S_N2(P)$ process they might operate. The nucleophile and a cationic leaving group, and especially a cationic general acid, are at opposite ends of the reaction coordinate (cf. Scheme 10), ruling out direct interactions. There would appear to be more scope for electrostatic effects in the reversible formation of the solvation complex (Scheme 8).

6. CALCULATION: PHOSPHORANES

The intermediates and transition states relevant to phosphate transfer are pentavalent phosphorus systems. For hydrolysis, these are formally pentaoxyphosphoranes, which make a convenient starting point because simple $P(OR)_5$ can be synthesized. They are typically thermally stable, though readily hydrolyzed in the presence of water, and especially in acid or base, and stabilized by small (particularly 5-membered) rings linking apical and equatorial substituents. In fact, 115 of the 117 pentaoxyphosphorane structures in the Cambridge Crystallographic Database (v. 5.34) have one or more such rings.

Of particular interest for phosphate transfer reactions are the pK_a's of hydroxyphosphorane OH groups. The best estimates use calculation, or the dependence of reactivity on bond length. There are good linear relationships between the length of a given type of C−OX bond and the pK_a of the leaving group (HOX).[31] Thus, the lengths of the Cy−OP bonds of pentacyclohexyloxyphosphorane depend predictably on the pK_a's of the corresponding P−OH groups. The lengths of the (shorter) apical and (longer) equatorial C−OP bonds correspond to pK_a values of 13.5 ± 1.5 and 8.62 ± 1.87, respectively, for the apical and equatorial OH groups of the two hydroxyphosphoranes $(CyO)_4P$−OH (Scheme 11B).[32] Apical OH groups are expected to be more weakly acidic because apical P−OH bonds are significantly longer than equatorial.

Scheme 11. Three Different Estimates for pK_a's of Hydroxyphosphoranes without Electron-Withdrawing Substituents



The complementary (density function based ab initio molecular dynamics) calculation[32] was for the parent, pentahydroxyphosphorane (Scheme 11A): at 300 K in a cubic cell of side 9.86 Å, containing also 29 molecules of water. $(HO)_5P$ and its equatorial monoanion were found to be stable under the conditions and on the time scale (5 ps) of the simulation: the apical monoanion relaxing to the equatorial form by pseudorotation, as expected. The dianion was found to be strongly basic, reverting to the monoanion by removing a proton from a coordinated water molecule. The calculation gave values of 9.8 and 14.2 for the pK_a's of the equatorial and apical P−OH groups, respectively.[32]

These figures are consistent also with an independent calculation of the first and second pK_a's of the pentacovalent dihydroxyphosphorane involved in the hydrolysis of ethylene phosphate (Scheme 11C).[33] Density functional theory derived gas-phase protonation energies were corrected for solvation by continuum dielectric methods, and the results normalized using phosphoric acid and dimethyl phosphate as models.[33] The pK_a's estimated from the three very different approaches are in remarkably good agreement for equatorial OH groups, and fall within the (wide) range of estimates obtained indirectly from experiment.[33]

A pK_a in the region of 8 for a hydroxyphosphorane bearing only O−alkyl or OH groups provides a basis for discussing phosphate transfer mechanisms of phosphate esters under biological conditions. The thermodynamically more stable form at pH 7 is tentatively identified as the neutral phosphorane, but the key conclusion is to expect both neutral OH form and monoanion to be present in significant amounts at physio-

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logical pHs. Phosphorane dianions are understood to have at best a borderline existence toward P−O cleavage under these conditions and thus to be viable as transition states, but not as intermediates;[4] but given a significant lifetime they could extract a proton from water.

7. CALCULATION: REACTIONS

Calculation can be particularly useful for investigations of seriously unreactive alkyl mono- and diesters, and for reactions of di- and triesters where stepwise mechanisms are possible. There is substantial calculational literature on the hydrolysis reactions of monoesters and related systems with a terminal $-OPO_3^=$ group,[1] and a major ongoing discussion on the practical aspects of an appropriate calculational environment. This can range for a hydrolysis reaction from the (relatively) simple gas phase to the obvious (but computationally challenging and seriously expensive) liquid water. Most practical is often a continuum solvation model,[34] specifically parametrized for water. In a given situation, the choice will depend on the resources available, but also on the substrate, and the reaction, of interest. For phosphate transfer, the substrate can range from a polyanion like ATP to a neutral and relatively hydrophobic triester.

A fundamental question for hydrolysis reactions is how many discrete waters are required to optimize a calculation using a continuum model. Water is a reactant, so one molecule is a minimum, but a single molecule of water in an artificial "aqueous" environment feels unnatural (a fish out of water?). An informed guess might ask for a minimum of three, perhaps depending on the charge on the substrate. A monoester dianion, with three negatively charged centers and multiple H-bonding requirements at the reactant stage as well as throughout the reaction process, may be more demanding.[35] Our recent work on the hydrolysis mechanisms of triesters addresses these questions directly,[36] and has recently been extended to diester hydrolysis.[14]

We use the same basic calculational methodology and parameters throughout: B3LYP and M06 levels of theory, basis sets 6-31+G(d) and 6-311++G(d,p), and the GAUSSIAN 09 package implemented in Linux operating systems.[12] Energy minima and activated complexes are identified in each case, and their structures characterized by frequency calculations at 1 atm and 298.15 K, giving access to thermodynamic quantities. All calculations are performed in the implicit presence of solvent.[37] Activated complexes (TS) are obtained by the quadratic synchronous transit (QST) protocol, and their structures identified by their single imaginary frequencies. Intrinsic reaction coordinates (IRC) are computed to confirm reaction paths. Separate calculations are run for reactions with varying numbers (1−4) of discrete water molecules present.

8. CALCULATION: TRIESTER HYDROLYSIS

Our recent kinetic investigation of the hydrolysis of aryl triesters showed that, for a given leaving group OAr, reactivity depends strongly on the nature of the "spectator" or nonleaving groups (OR in Scheme 3, above):[36] increasing the electron-withdrawing ability of the two OR groups accelerates the reaction by many orders of magnitude. Recent calculations on the phosphotriesterase-catalyzed hydrolysis of pesticides methyl and ethyl paraoxon (dialkyl 4-nitrophenyl phosphates)[38,39] had made a case for a stepwise hydrolysis mechanism, summarized in Scheme 3. Classical stepwise vs concerted mechanisms are unlikely to be distinguished by experiment, but calculations for the same series of compounds, in the presence of varying numbers of discrete water molecules, extend the scope of the discussion significantly.

Preliminary calculations on the diphenyl triester **6a** (Scheme 12) using the PCM Solvation Model and a single molecule of

Scheme 12

6a Ar = C_6H_5 **6c** Ar = $C_6H_4SO_3^-$ **7** R = CH_2CF_3
6b Ar = C_6H_4Cl **6d** Ar = $C_6H_4NO_2$ **8** R = CH_3

water at 25 °C showed a clear preference for a stepwise over a concerted $S_N2(P)$ mechanism, but a free energy of activation far greater than observed. Different levels of theory and basis sets produced no improvement, but adding a second and third discrete water molecule to the calculation gave much better agreement, marginally reduced by adding a fourth (Table 2). Subsequent calculations on the stepwise mechanism (Scheme 3) used three molecules of water.[12]

Attempts to calculate a concerted $S_N2(P)$ TS gave a pentacovalent intermediate. Transition states for the formation and breakdown of intermediates were verified by IRC calculations. The IRC for triester **6a** (Figure 3) shows a moderately stable intermediate with well-defined participation of water molecules in its formation and cleavage, consistent with proton inventory measurements and the substantial solvent deuterium isotope effect previously reported.[8]

Using this TS as a model, potential energy surfaces were evaluated for the hydrolysis of all six triesters of Scheme 12. The activation parameters shown in Table 2 were obtained from stationary points characterizing reactant van der Waals complexes, transition states, and intermediates.

The calculations also provide a direct, quantitative comparison (Table 2) of the forward and reverse reactions of the phosphorane intermediate Int, which show that the initial addition step is cleanly rate determining for the dialkyl esters **7** and **8**; the second step affects the rate significantly only for the tris-4-nitrophenyl ester **6d**.[12]

9. CALCULATION: DIESTER HYDROLYSIS

The hydrolysis of diesters, even with good leaving groups, is very slow even at 100 °C,[40] and correlations based on rates at 25 °C are practical *only* with calculated data. The free energies of activation for hydrolysis calculated for the series of phosphate diesters **9** (Scheme 13) in the presence of two or three molecules of water (Table 2) are in good agreement with (the one available) experiment.[14] The second molecule of water in the transition state for the favored $S_N2(P)$ mechanism is involved as a general base (Scheme 13), sharing the developing positive charge, while the third stabilizes the TS by hydrogen bonding.

There is a significant difference in β_{NLG} between the value (−0.03 ± 0.001) measured for the hydrolysis of aryl 2,4-dinitrophenyl ester anions at 100 °C[40] and that (0.16 ± 0.08) based on the data calculated, using three discrete waters, for the hydrolysis of *p*-nitrophenyl diesters **9** (Scheme 13). This identifies the methyl diester **9** (R = Me) as the most reactive of the series (albeit far too slow to measure at 25 °C).

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Table 2. Activation free Energies Calculated for the Hydrolysis of Diaryl and Dialkyl 4-Nitrophenyl Phosphate Triesters (Scheme 12) with Three Water Molecules at 25 °C[8]

	compd					
	6a	**6b**	**6c**	**6d**	**7**	**8**
$\Delta G^{\ddagger}$, kcal/mol (calcd 3 H_2O)	25.2	24.7	23.5	22.3	26.2	30.0
$\Delta G^{\ddagger}$, kcal/mol (expt[11])	24.8	24.0	23.6	21.9	24.8	29.6
rate ratio k_{-1}/k_2 (Scheme 11)	0.02	0.03	0.12	0.91	5×10^{-4}	10^{-6}

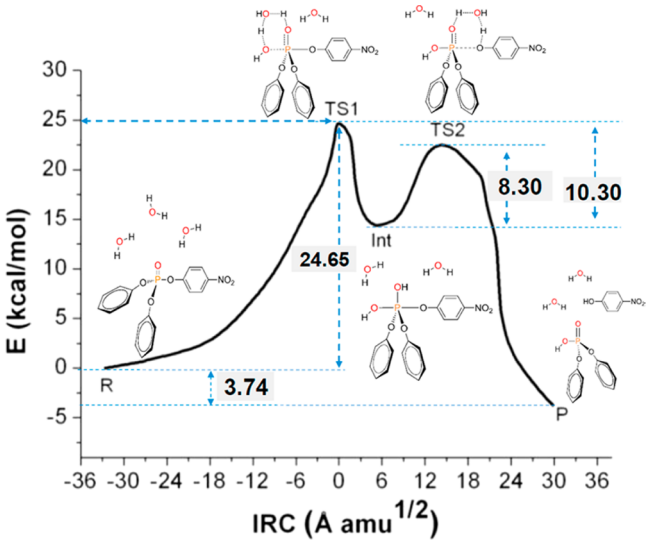
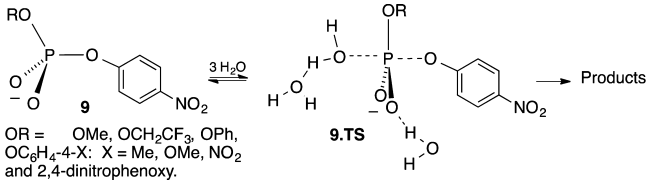


Figure 3. Intrinsic reaction coordinate for the hydrolysis of triester **6a** in the presence of three discrete waters. (Step size 0.1 bohr amu$^{1/2}$.)

Scheme 13



The most significant differences between the rate-determining transition states for the spontaneous hydrolysis of the 4-nitrophenyl diesters **9** and triesters (**6**–**8**) are the extents of bonding to the nucleophilic water and the leaving group (Table 3). For the (much faster) triester reaction closely similar P···O distances of 1.78 ± 0.01 Å to both characterize the compact TS expected for the formation of the phosphorane intermediate.[12] The substrate P−OAr and P═O bonds all lengthen in TS1 as the pentacovalency develops, before shortening slightly in the phosphorane intermediate.

Bond breaking to the leaving group is much further advanced in the $S_N2(P)$ TS for the diester reactions, with P···OAr at 2.30 Å almost 40% longer than in the reactant. The H_2O···P distance to the nucleophilic water is also long, at 2.02 Å, and the major contribution to the departure of the leaving group must come from $n_O-\sigma^*_{P-OR^*}$ overlap with the two negatively charged equatorial oxygens. The two P−$O^{(-)}$ bonds are indeed slightly shorter in the pentacovalent TS than in the tetrahedral reactant. As is the P−OR bond to the nonleaving group, which scarcely changes in length from reactant to TS, and is indeed little more than a spectator. [Phosphodiester hydrolysis at low pH involves the neutral $(RO)_2P(O)OH$ form, and should favor the triester mechanism, with a stable pentaoxyphosphorane intermediate on the reaction pathway (unpublished work with B. S. Souza).]

10. CALCULATION: MONOESTER HYDROLYSIS

Recent calculational work on monoester hydrolysis[35] compares the dianions of 4-nitrophenyl phosphate (pNPP$^{=}$, measurable) and methyl phosphate (impossible). The hydrolysis of methyl phosphate is dominated by the reaction of the monoanion even in 1 M KOH,[41] so the hydrolysis mechanisms of alkyl monoester dianions can be of theoretical interest only. The authors address directly the question of the optimum number of discrete waters required for the hydrolysis of a dianion in a calculational continuum environment: their results do not (quite) converge even with eight additional waters, perhaps because dianion substrates have more complex solvation requirements than di- or triesters.

The calculated interatomic P−O distances for substrate and TS (Table 3) are relatively insensitive to the number of waters present. The results are consistent with the familiar dissociative TS for pNPP$^{=}$, with P···O distances to both nucleophile and leaving group being even longer than those for the diester reaction, for both reactant and TS. This results from the triple $n_O-\sigma^*_{P\text{-}OR^*}$ interactions available to the dianion, which support P−OR* cleavage under relatively mild conditions, given a sufficiently good leaving group. An alkoxide R^*O^- is *not* a good enough leaving group, and a key question is how phosphate transfer from effectively inert alkyl monoester dianions can be catalyzed at biological rates by enzymes.

Of course there is no single answer, but the principles are relatively clear. The reactivity of the nucleophile can be enhanced in the usual ways, by proximity and by general bases, but not very effectively via the seriously dissociative TS (Scheme 5). The uniquely responsive center of a monoester

Table 3. Calculated Lengths (Å) Compared for Bonds to the Phosphorus Reaction Center in Reactant and TS for the Hydrolysis of 4-Nitrophenyl Tri-, Di-, and Monoester Anions in the Presence of Three Discrete Waters[a]

	triesters (6–8)			diester anions (9)		monoester dianion[b]	
bond	reactant	TS1	INT	reactant	TS	reactant	TS
$H_2O\cdots P$		1.79	1.70		2.02		2.28
P−OAr	1.61	1.77	1.74	1.67	2.30	1.71	2.44
$(P-O^{(-)})_{1,2,3}$	1.49	1.54	1.62	1.51	1.50	1.54	1.50

[a]Values are means ± ca. 0.01 for the group of compounds. [b]See text, below.

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dianion is the leaving group oxygen, as shown by the efficacy of its full or partial protonation. But this depends on $n_O-\sigma^*_{P\text{-}OR^*}$ donation from the $PO_3^=$ group, which is significantly reduced by H-bonding solvation in water,[42] and presumably more so by necessarily stronger active-site interactions. The three basic centers of the $PO_3^=$ group are central to reactant and TS binding: we know that neutralizing one of them has a powerful negative effect on (diester) reactivity. Neutralizing reverses the effect: a dialkyl *p*-nitrophenyl phosphate[36] and the $pNPP^=$ dianion[25] are hydrolyzed at similar rates. And this effect, of neutralization by alkylation, can be enhanced for enzymes catalyzing phosphate transfer from monoesters by readily reversible binding to metal cations.

11. SUMMARY AND OUTLOOK

The "traditional" dependence on activated esters for TS information from experiment has obvious limitations. Calculation allows us to examine details of bond-making and breaking processes relevant to the extraordinarily efficient workings of phosphate transfer enzymes. The driving force derived from negative charges in the equatorial positions of TS and intermediates is evidently fundamental to the mechanisms of these processes.

We look forward to advances—in the near future—in several relevant areas:

- Hydrolysis of electronically excited phosphate esters, which opens the possibility of examining the effects of much better leaving groups.
- Experimental methods available in single molecule spectroscopy, to study the rates of slow reactions.
- Theoretical techniques designed to model the multiple, varying environments of TS in enzyme active sites. And to model better the multiple, varying roles of water in aqueous solution, as reactant and supporting-reagent as well as solvent.

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REFERENCES

(1) Kamerlin, S. C. L.; Sharma, P. K.; Prasad, R. B.; Warshel, A. Why nature really chose phosphate. *Q. Rev. Biophys.* **2013**, *46*, 1–132.
(2) Westheimer, F. H. Why Nature Chose Phosphate. *Science* **1987**, *235*, 1173–1178.
(3) Lassila, J. K.; Zalatan, J. G.; Herschlag, D. Biological Phosphoryl-Transfer Reactions: Understanding Mechanism and Catalysis. *Annu. Rev. Biochem.* **2011**, *80*, 669–702.
(4) Cleland, W. W.; Hengge, A. C. Enzymatic mechanisms of phosphate and sulfate transfer. *Chem. Rev.* **2006**, *106*, 3252–3278.
(5) Kim, K.; Tsay, O. G.; Atwood, D. A.; Churchill, D. G. Destruction and Detection of Chemical Warfare Agents. *Chem. Rev.* **2011**, *111*, 5345–5403.
(6) Timosheva, L. V.; Chandrasekaran, A.; Holmes, R. R. Biologically relevant phosphoranes: Structural characterization of a nucleotidyl phosphorane. *J. Am. Chem. Soc.* **2005**, *127*, 12474–12475.
(7) Kumara Swamy, K. C.; Kumar, N. S. New Features in Pentacoordinate Phosphorus Chemistry. *Acc. Chem. Res.* **2006**, *39*, 324–333.
(8) Kirby, A. J.; Medeiros, M.; Oliveira, P. S. M.; Orth, E. S.; Brandao, T. A. S.; Wanderlind, E. H.; Amer, A.; Williams, N. H.; Nome, F. Activating Water: Important Effects of Non-leaving Groups on the Hydrolysis of Phosphate Triesters. *Chem.—Eur. J.* **2011**, *17*, 14996–15004.
(9) Taira, K.; Fanni, T.; Gorenstein, D. G. Stereoelectronic effects in the hydrolysis of ethyl and methyl ethylene phosphates. *J. Org. Chem.* **1984**, *49*, 4531–4536.
(10) Kirby, A. J.; Hollfelder, F. *From Enzyme Models to Model Enzymes*; Royal Society of Chemistry: Cambridge, 2009.
(11) Kirby, A. J.; Medeiros, M.; Mora, J. R.; Oliveira, P. S. M.; Brandão, T. A. S.; Amer, A.; Williams, N. H.; Nome, F. Intramolecular general base catalysis in the hydrolysis of a phosphate diester. Calculational guidance to a choice of mechanism. *J. Org. Chem.* **2013**, *78*, 1343–1353.
(12) Mora, J. R.; Kirby, A. J.; Nome, F. Theoretical Study of the Importance of the Spectator Groups on the Hydrolysis of Phosphate Triesters. *J. Org. Chem.* **2012**, *77*, 7061–7070.
(13) Chin, J.; Banaszczyk, M.; Jubian, V.; Zou, X. Co(111) Complex Promoted Hydrolysis of Phosphate Diesters. *J. Am. Chem. Soc.* **1989**, *111*, 186–190.
(14) Kirby, A. J.; Souza, B. S.; Nome, F. Structure and Reactivity of Phosphate Diesters. Dependence on the Non-leaving Group. *Can. J. Chem.* **2015**, *93*, 422–427.
(15) Lad, C.; Williams, N. H.; Wolfenden, R. The rate of hydrolysis of phosphomonoester dianions and the exceptional catalytic proficiencies of protein and inositol phosphatases. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 5607–5610.
(16) Ruben, E. A.; Plumley, J. A.; Chapman, M. S.; Evanseck, J. D. Anomeric effect in "High energy" phosphate bonds. Selective destabilization of the scissile bond and modulation of the exothermicity of hydrolysis. *J. Am. Chem. Soc.* **2008**, *130*, 3349–3358.
(17) Kirby, A. J.; Varvoglis, A. G. The Reactivity of Phosphate Esters. Monoester Hydrolysis. *J. Am. Chem. Soc.* **1967**, *89*, 415–423.
(18) Bunton, C. A.; Llewellyn, D. R.; Oldham, K. G.; Vernon, C. A. Hydrolysis of methyl phosphate. *J. Chem. Soc.* **1958**, 3574–3587.
(19) Medeiros, M.; Manfredi, A. M.; Kirby, A. J.; Nome, F. The spontaneous hydrolysis of 2-pyridyl phosphate is a good model for the special mechanism for the hydrolysis of phosphate monoester monoanions. *J. Phys. Org. Chem.* **2013**, *26*, 1044–1047.
(20) Kirby, A. J.; Varvoglis, A. G. The Reactivity of Phosphate Esters: Reactions of Monoesters with Nucleophiles. Reactivity Independent of Basicity in a Bimolecular Substitution Reaction. *J. Chem. Soc. B* **1968**, 135.
(21) Jencks, W. P. When Is an Intermediate Not an Intermediate? Enforced Mechanisms of General Acid-Base Catalyzed, Carbocation, Carbanion, and Ligand Exchange Reaction. *Acc. Chem. Res.* **1980**, *13*, 161–169.

DOI: 10.1021/acs.accounts.5b00072
Acc. Chem. Res. 2015, 48, 1806–1814

(22) Zhang, L. D.; Xie, D. Q.; Xu, D. G.; Guo, H. Reactivity of metaphosphate and thiometaphosphate in water: A DFT study. *J. Phys. Chem. A* **2005**, *109*, 11295−11303.
(23) Henchman, M.; Viggiano, A. A.; Paulson, J. F. Thermodynamic and Kinetic Properties of the Metaphosphate Anion, PO_3^-, in the Gas Phase. *J. Am. Chem. Soc.* **2005**, *107*, 1453−1457.
(24) Kirby, A. J.; Manfredi, A. M.; Souza, B. S.; Medeiros, M.; Priebe, J. P.; Brandão, T. A. S.; Nome, F. Reactions of alpha-nucleophiles with a model phosphate diester. *ARKIVOC* **2009**, 28−38.
(25) Kirby, A. J.; Jencks, W. P. The Reactivity of Nucleophilic Reagents Toward the *p*-Nitrophenyl Phosphate dianion. *J. Am. Chem. Soc.* **1965**, *87*, 3209−3216.
(26) Kirby, A. J.; Younas, M. The Reactivity of Phosphate Esters. Reactions of Diesters with Nucleophiles. *J. Chem. Soc. B* **1970**, 1165−1172.
(27) Admiraal, S. J.; Herschlag, D. Catalysis of Phosphoryl Transfer from ATP by Amine Nucleophiles. *J. Am. Chem. Soc.* **1999**, *121*, 5837−5845.
(28) Herschlag, D.; Jencks, W. P. Phosphoryl Transfer to Anionic Oxygen Nucleophiles. Nature of the Transition State and Electrostatic Repulsion. *J. Am. Chem. Soc.* **1989**, *111*, 7587−7596.
(29) Kirby, A. J.; Lima, M. F.; da Silva, D.; Nome, F. Nucleophilic attack by oxyanions on a phosphate monoester dianion: The positive effect of a cationic general acid. *J. Am. Chem. Soc.* **2004**, *126*, 1350−1351.
(30) Weber, J. Toward the ATP synthase mechanism. *Nat. Chem. Biol.* **2010**, *6*, 794−795.
(31) Kirby, A. J. Crystallographic Approaches to Transition State Structures. *Adv. Phys. Org. Chem.* **1994**, *29*, 87−183.
(32) Davies, J. E.; Doltsinis, N. L.; Kirby, A. J.; Roussev, C. D.; Sprik, M. Estimating pKa Values for Pentaoxyphosphoranes. *J. Am. Chem. Soc.* **2002**, *124*, 6594−6599.
(33) Lopez, X.; Schaefer, M.; Dejaegere, A.; Karplus, M. Theoretical evaluation of pK(a) in phosphoranes: Implications for phosphate ester hydrolysis. *J. Am. Chem. Soc.* **2002**, *124*, 5010−5018.
(34) Klamt, A.; Mennucci, B.; Tomasi, J.; Barone, V.; Curutchet, C.; Orozco, M.; Luque, F. J. On the Performance of Continuum Solvation Methods. A Comment on "Universal Approaches to Solvation Modeling". *Acc. Chem. Res.* **2009**, *42*, 489−492.
(35) Duarte, F.; Aqvist, J.; Williams, N. H.; Kamerlin, S. C. L. Resolving Apparent Conflicts Between Theoretical and Experimental Models of Phosphate Monoester Hydrolysis. *J. Am. Chem. Soc.* **2015**, *137*, 1081−1093.
(36) Kirby, A. J.; Mora, J. R.; Nome, F. New light on phosphate transfer from triesters. *Biochim. Biophys. Acta, Proteins Proteomics* **2013**, *1834*, 454−463.
(37) Marenich, A. V.; Cramer, C. J.; Truhlar, D. G. Universal Solvation Model Based on Solute Electron Density and on a Continuum Model of the Solvent Defined by the Bulk Dielectric Constant and Atomic Surface Tensions. *J. Phys. Chem. B* **2009**, *113*, 6378−6396.
(38) Zhang, X.; Wu, R.; Song, L.; Lin, Y.; Lin, M.; Cao, Z.; Wu, W.; Mo, Y. *J. Comput. Chem.* **2009**, *30*, 2388.
(39) Chen, S.-L.; Fang, W.-H.; Himo, F. *J. Phys. Chem. B* **2007**, *111*, 1253.
(40) Kirby, A. J.; Younas, M. The Reactivity of Phosphate Esters. Diester Hydrolysis. *J. Chem. Soc. B* **1970**, 510−513.
(41) Schroeder, G. K.; Lad, C.; Wyman, P.; Williams, N. H.; Wolfenden, R. The time required for water attack at the phosphorus atom of simple phosphodiesters and of DNA. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 4052−4055.
(42) Abell, K. W. Y.; Kirby, A. J. Acceleration Of P-O Cleavage Reactions Of Phosphate Monoester Dianions In Dipolar Aprotic Solvents. *Tetrahedron Lett.* **1986**, *27*, 1085−1088.

DOI: 10.1021/acs.accounts.5b00072
Acc. Chem. Res. 2015, 48, 1806−1814