# Hydrolysis of Phosphotriesters: Determination of Transition States in Parallel Reactions by Heavy-Atom Isotope Effects

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**Abstract:** The remote label method was used to measure primary and secondary $^{18}$O isotope effects in the alkaline hydrolysis of *O,O*-diethylphosphorylcholine iodide (DEPC) and the primary $^{18}$O effect in the alkaline hydrolysis of *O,O*-diethyl-*m*-nitrobenzyl phosphate (DE*m*NBP). Both the leaving group of interest (choline or *m*-nitrobenzyl alcohol) and ethanol can be ejected during hydrolysis due to the similarity of their p*K* values. The heavy-atom isotope effects were measured by isotope ratio mass spectrometry. Parallel reaction and incomplete labeling corrections were made for both systems. DEPC has a primary $^{18}$O isotope effect of 1.041 ± 0.003 and a secondary $^{18}$O isotope effect of 1.033 ± 0.002. The primary $^{18}$O isotope effect for DE*m*NBP was 1.052 ± 0.003. These large effects suggest a highly associative transition state in which the nucleophile approaches very close to the phosphorus atom to eject the leaving group. The large values are also indicative of a large compression, or general movement, on the reaction coordinate.

## Introduction

Phosphoryl transfer is enormously important in biological systems. With the exception of water, adenosine triphosphate (ATP) is easily the most important chemical in the world for animal life. Indeed, ATP supplies the energy for muscular movement, neural activity, biosynthesis, and active transport. As such, the chemistry of phosphomonoesters and phosphodiesters has been studied intensely for many years. In 1955, Westheimer[1] and Bunton[2] independently proposed a metaphosphate intermediate in the hydrolysis of phosphomonoesters. Several years later, Herschlag[3] showed that, while there is considerable metaphosphate-like character in the transition state, the evidence is against formation of a true metaphosphate intermediate in aqueous solution. For phosphomonoesters the kinetics,[1,2,4,5] pH−rate profiles,[6] effect of metal ions,[7] ab initio calculations,[8] stereochemistry,[9] and isotope effects,[10] and reactivity[11] have all been determined at one time or another. Phosphodiesters, the most stable of the phosphate esters, have also been studied extensively. It is widely accepted that these compounds go through $S_N2$ type mechanisms with inversion of configuration with respect to the phosphorus.[12] Indeed most, if not all, of the studies done on monoesters have been used to determine the reaction mechanism of phosphodiester hydrolysis also.[13] Both esters have been studied enzymatically and have added greatly to the understanding of the chemistry that takes place in the active site of the protein.[14]

The chemical hydrolysis of triesters has not been studied as extensively as that of the less ligated phosphoesters, and the principal reason is that there are no naturally occurring phosphotriesters in nature. This is rather strange when one considers that phosphotriesterase activity was discovered in the soil microbe *Pseudomonas diminuta* in 1974. Where it came from and how it came to be are still unknown, but at the time of its discovery activity on the pesticide parathion (*O,O*-diethyl-*O*-*p*-nitrophenyl phosphorothioate) was observed.[15] Since then, phosphotriesterase and the enzymatic hydrolysis of phospho-

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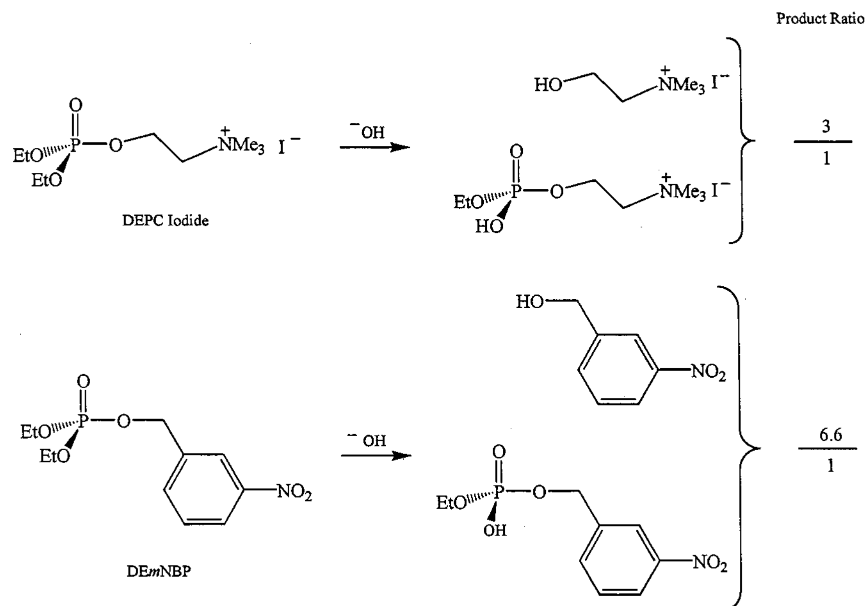
**Scheme 1**[a]




[a] Only the relevant (N-containing) products are shown. Diethyl phosphate and ethanol are also products of both reactions.

triesters have been studied at length.[16] Use of man-made pesticides such as parathion and malathion (*S*-1,2-bis(ethoxycarbonyl)ethyl *O,O*-dimethylphosphorodithioate) is one of the primary reasons why phosphotriesters are coming under closer scrutiny.[16] The mechanism of their chemical breakdown,[17] and the products derived from it, have become quite important from an ecological as well as a business standpoint.

In previous studies of triester hydrolysis, we used the remote label method to determine the isotope effects for diethyl phosphotriesters with reasonably good leaving groups (*p*-nitrophenol, p*K* 7.0, and *p*-carbamoylphenol, p*K* 8.6). These compounds underwent hydrolysis at basic pH with expulsion of the phenol group only. The transition-state structures for the reactions were probed by measuring the $^{18}$O isotope effects for the breaking of the P$-$O$_{phenol}$ bond (the primary isotope effect), which depends on the extent of bond cleavage in the transition state, and the secondary $^{18}$O isotope effect, which is determined by the change in bond order between the phosphorus and the phosphoryl oxygen.[18]

In the present work, we have synthesized diethyl phosphotriesters with leaving group p*K* values that are much larger (choline, p*K* 13.9,[19] and *m*-nitrobenzyl alcohol, p*K* 14.9[20]) in order to compare the transition state of the hydrolysis of a substrate with a good leaving group versus a poor one. As a consequence of the p*K* values being relatively close to that of ethanol (p*K* 16.0[19]), the hydrolysis of diethylphosporylcholine iodide (DEPC) and diethyl-*m*-nitrobenzyl phosphate (DE*m*NBP) results in the ejection of some ethanol as well as choline from DEPC, and *m*-nitrobenzyl alcohol (*m*-NBA) from DE*m*NBP. These are thus parallel reactions (Scheme 1).

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## Data Analysis for a Simple System Using Nitrogen Isotope Effects

For a reaction that involves a one-step process with no side or competing reactions, determining isotope effects in systems containing a single N atom is straightforward by the internal competition method.

$$A \xrightarrow{k_1} P \qquad (1)$$

We will assume that a C$-$N bond is broken in the rate-limiting step of the reaction and that after bond scission the portion with the N atom will be the isolated product. No labeled syntheses are necessary, and changes in the natural abundance ratio of $^{15}$N/$^{14}$N in the compound will be sufficient to determine the isotope effect of the reaction. The reaction is run from 10 to 70% completion, and the amount of initial substrate that reacts, the fraction of reaction *f*, is determined accurately (NMR, UV$-$vis, etc.). The product and residual substrate are separated, purified, and converted to $N_2$ gas (combustion, Kjeldahl acid digestion/hypobromite oxidation, etc.). Each gas sample is analyzed individually by IRMS to determine its isotopic ratio compared to a known standard (we are using N as an example here, but the equation is valid for any element):

$$\delta = 1000[(^{15}N_{sample}/^{14}N_{sample})/(^{15}N_{standard}/^{14}N_{standard}) - 1] \qquad (2)$$

A change of 1$\delta$ is equal to a change of 0.1% of the $^{15}$N/$^{14}$N ratio. To determine the isotope effect, the samples are converted to an *R* value defined as

$$R = (\delta_{sample}/1000) + 1 \qquad (3)$$

*R* values for the reaction product, $R_p$, and residual substrate, $R_s$, are used in the following equations to arrive at the isotope effect (IE).

$$IE_{prod} = \ln(1 - f)/\ln(1 - fR_p/R_o) \quad (4)$$

$$IE_{sub} = \ln(1 - f)/\ln[(1 - f)(R_s/R_o)] \quad (5)$$

In these equations, $R_o$ is the isotopic ratio of the starting material (found by direct combustion or complete reaction of the starting material). The isotope effect can also be found by using a comparison of the product and residual substrate $R$ values by eq 6.

$$IE_{sub/prod} = \ln(1 - f)/\ln\{(1 - f)/[1 - f + (fR_p/R_s)]\} \quad (6)$$

If the starting material is pure and the separation process is good, the isotope effect should be easy to determine and should be quite reproducible when varying the fraction of reaction.

## Remote Label Method

The remote label method is used to measure primary and secondary isotope effects in systems where the atom that is actually responsible for the effect is extremely difficult to isolate and study. Oxygen is one such atom because in $CO_2$ the O of interest exchanges with the O of water. Even a slight water contamination can lead to $^{18}$O washout and useless isotopic results. Since the approach to studying hydrolysis reactions of phosphotriesters is to measure primary $^{18}$O isotope effects in the leaving groups and secondary isotope effects in the nonbridging phosphoryl oxygen, the easiest way to follow these reactions is with a remote label.[21] Remote labeling allows the determination of isotope effects by using a substrate labeled in two positions, the site of chemical interest and another position which can be isolated and measured isotopically. In these studies, the leaving group of the triester is synthesized with $^{18}$O at the point of bond breaking and $^{15}$N at the remote site on the molecule. A larger amount of the compound is synthesized with $^{16}$O at the point of bond breaking[22] and $^{14}$N (i.e., nitrogen from which the 0.37% natural abundance of $^{15}$N has been removed) at the remote site. The $^{18}$O,$^{15}$N molecule is mixed with the $^{14}$N one in the natural abundance ratio. The experimental isotope effect with this material is the product of the $^{18}$O primary isotope effect and any effect from $^{15}$N in the remote position. The $^{15}$N effect alone is determined by using natural abundance substrate, and the ratio of the two isotope effects gives the $^{18}$O one. Determination of the secondary $^{18}$O isotope effect is done by the same method, using doubly labeled substrate which has $^{18}$O at the phosphoryl O and an $^{15}$N label in the remote position.

If the syntheses yielded isotopically pure compounds, the determination of the isotope effects would occur as described above. However, syntheses almost never result in completely labeled compounds. Therefore, the observed isotope effects must be corrected for the amount of label in each position by using the following equations:

$$\text{IE per atom of label} = \{(T^{1/i} - 1)/[1 - T^{1/i}(1 - y)/i]\} + 1 \quad (7)$$

where $T$ is defined as

$$T = (P/R)/[1 - Q(P/R - 1)] \quad (8)$$

and $P$ is the the isotope effect with remote labeled substrate, $R$ is the isotope effect in the remote label position with natural abundance material, $i$ is the the number of discriminating atoms, $y$ is the the fraction of heavy discriminating label in doubly labeled material, and $Q$ is the degree to which light material in the remote labeled mixture is depleted below natural abundance, defined as

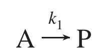
$$Q = (1 - b)z/bx \quad (9)$$

where $b$ is the the fraction of doubly labeled material in the mixture (determined by IRMS), $z$ is the the fraction of heavy label present in the remote label position of light material, and $x$ is the the fraction of heavy label in the remote label position of the doubly labeled material.

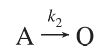
In a simple system of this type, the apparent isotope effects for $R_s$ and $R_p$ are determined by using IRMS and then corrected for incomplete isotopic substitution by using eqs 7−9. Hydrolysis reactions of labeled material leading to the extrusion of only the remote labeled group are good examples of this process. Reactions such as these are the hydrolysis of phosphomonoesters (monoanionic and dianionic), where the system has only one leaving group, and phosphotriesters, where the leaving group of interest has a p$K$ value much lower than those of the other two ester substituents.

## Parallel Reactions

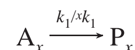
When the rates of hydrolysis for two different groups of a phosphotriester are similar, the system is known as a parallel reaction. The schemes for the hydrolysis of the diethylphosphorylcholine (DEPC) and diethyl *m*-nitrobenzyl phosphate (DE*m*NBP) point out the dual nature of these reactions. In both cases the labeled substituent is not the only possible leaving group. Here, the p$K$'s of the possible leaving group substituents are relatively close to one another (ethanol 16.0,[19] choline 13.9,[19] *m*-nitrobenzyl alcohol 14.9[20]), and ethanol can be extruded as well as choline in the case of DEPC and *m*-nitrobenzyl alcohol in the case of DE*m*NBP. Isotope effects for such a cleavage may be as isotopically sensitive as for the ligand of interest (choline and *m*-NBA).[23] Here, the kinetic scheme becomes more complex, as shown below using the hydrolysis of DEPC as an example.



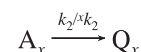
P is ethyl choline phosphodiester after the release of ethanol upon hydrolysis of DEPC.



Q is choline released upon hydrolysis of DEPC.



The rate constant for the heavy labeled compound for the release of ethanol upon hydrolysis of DEPC is reduced by $^{x}k_1$.



The rate constant for the heavy labeled compound for the release of choline upon hydrolysis of DEPC is reduced by $^{x}k_2$ ($x$ = double $^{18}$O,$^{15}$N or single $^{15}$N label). The definitions of the $R$ values for the hydrolysis of DEPC are more complex than for a simple system.

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(22) Oxygen containing the natural abundance of $^{17}$O and $^{18}$O. The small amounts of the heavier atoms have no effect on the measured isotope effects.
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$$R_p = Q_x/Q$$

(the $^{15}N/^{14}N$ ratio in the extruded choline at a known fraction of reaction, $f$)

$$R_s = (Q_{x\infty} - Q_x)/(Q_\infty - Q)$$

(the $^{15}N/^{14}N$ ratio in unreacted DEPC at a known fraction of reaction, $f$)

$$R_\infty = (Q_{x\infty}/Q_\infty)$$

(the $^{15}N/^{14}N$ ratio in choline after complete hydrolysis of the triester)

$$R_{p\infty} = P_{x\infty}/P_\infty$$

(the $^{15}N/^{14}N$ ratio in the phosphodiester after complete hydrolysis of the triester)

The integrated equations describing the concentrations of the various molecules at a known fraction of reaction, or at infinite time are

$$A = A_o\,e^{-(k_1+k_2)t} \qquad A_x = A_{xo}\,e^{-k't}$$

where

$$k' = k_1/{}^xk_1 + k_2/{}^xk_2$$

$$Q = k_2A_o(1 - e^{-(k_1+k_2)t})/(k_1 + k_2)$$
$$Q_x = k_2A_{xo}(1 - e^{-k't})/{}^xk_2k'$$

$$P = k_1A_o(1 - e^{-(k_1+k_2)t})/(k_1 + k_2)$$
$$P_x = k_1A_{xo}(1 - e^{-k't})/{}^xk_1k'$$

Using the experimentally determined isotope ratios, an apparent isotope effect can be calculated starting with the following equations:

$$^xk_{app} = \ln(1 - f)/\ln(1 - fR_p/R_\infty) = \ln(1 - f)/\ln[(1 - f)(R_s/R_\infty)] \quad (10)$$

$$^xk_{app} = {}^xk_2(1 + k_1/k_2)/(1 + k_1{}^xk_2/(k_2{}^xk_1)) \quad (11)$$

When the ratio of $k_1/k_2$ is known, the above isotope effect can be used along with the ratio of ${}^xk_2/{}^xk_1$ to determine ${}^xk_1$ and ${}^xk_2$. The ratio of the isotope effects ${}^xk_2/{}^xk_1$ is equal to $R_{p\infty}/R_\infty$.

This methodology was used by Rawlings to determine heavy-atom isotope effects on reaction of Co(III)-bound *p*-nitrophenyl phosphate.[23] However, for the DEPC and DE*m*NBP systems, the apparent $R$ values for the ethyl choline phosphodiester and ethyl *m*-nitrobenzyl phosphodiesters could not be accurately determined since the samples were too small. Instead, an assumption was made that the value of the isotope effect for the loss of ethanol, ${}^{18,15}k_1$, was equal to unity. This assumption is probably valid, since no appreciable bonding changes occur in the $P-O_{choline}$ bond when ethanol is the leaving group. The apparent isotope effect (from the IRMS) is equal to

$$k_{app} = {}^{18,15}k_2(k_1 + k_2)/[k_2 + k_1({}^{18,15}k_2/{}^{18,15}k_1)]$$

and if it is assumed that ${}^{18,15}k_1 = 1.000$, then

$$k_{app} = {}^{18,15}k_2(k_1 + k_2)/(k_2 + k_1{}^{18,15}k_2)$$

and

$$^{18,15}k_2 = k_{app}k_2/(k_1 + k_2 - k_1k_{app}) \quad (12)$$

## Results

**Synthesis of Labeled and Depleted Phosphotriesters.** The synthetic routes for the $^{18}O$,$^{15}N$-labeled and the $^{14}N$ phosphotriesters are shown in Schemes 2−4, and the details of the syntheses are given in Supporting Information. The $^1H$, $^{13}C$, and $^{31}P$ NMR spectra of the three separate labeled DEPC compounds and the two labeled DE*m*NBP compounds matched those of natural abundance counterparts.

**Hydrolysis of Phosphotriesters in $^{18}O/^{16}O$ $H_2O$.** Although most evidence indicates that the nucleophilic attack of the hydroxyl takes place at the P center of a phosphotriester,[18,24,25] it is not impossible that some could be taking place at the methylenic carbons attached to the bridging oxygens of the system.

Since isotope effect studies have not been done on the hydrolysis of compounds with high p$K$ values, it is important to verify that the mechanism of attack is the same as for compounds with good leaving groups. The hydrolysis of both triesters was followed by $^{13}C$ NMR in an alkaline solution consisting of $H_2{}^{18}O/H_2{}^{16}O$ (30/70). Attack at the $-CH_2-$ group by $^{18}OH^-$ would result in a ~0.04 ppm upfield shift for that carbon in the $^{13}C$ NMR spectrum of a product.

When DEPC and DE*m*NBP were subjected to these conditions and the $^{13}C$ spectrum of the $H_2{}^{18}O/H_2{}^{16}O$ reaction mixture was compared to that in 100% $H_2{}^{16}O$, no difference was seen in the chemical shifts of interest in either the released ethanol, choline, or *m*-nitrobenzyl alcohol (DEPC 66.29, ethyl $CH_2$ of ethanol; 66.86, cholyl $CH_2$ of the choline; DE*m*NBP 65.79, ethyl $CH_2$ of ethanol; 68.88, $CH_2$ of the *m*-nitrobenzyl alcohol).

$^{31}P$ NMR was then used to determine the whereabouts of the $^{18}O$ atom. High-resolution NMR of the hydrolyzed samples clearly showed the upfield shift of the $P-{}^{18}O$ in the diester products (DECP $P-{}^{16}O$ diethyl phosphate, 0.62 ppm; $P-{}^{18}O$ diethyl phosphate, 0.59 ppm; $P-{}^{16}O$ ethyl choline phosphate, −0.27 ppm; $P-{}^{18}O$ ethyl choline phosphate, −0.30 ppm; DE*m*NBP $P-{}^{16}O$ diethyl phosphate, 0.48 ppm; $P-{}^{18}O$ diethyl phosphate, 0.45 ppm; $P-{}^{16}O$ ethyl *m*-nitrobenzyl phosphate, 0.22 ppm; $P-{}^{18}O$ ethyl *m*-nitrobenzyl phosphate, 0.19 ppm). When the overlapping peaks were deconvoluted and the areas were calculated, the ratio of the $P-{}^{16}O$ peaks to the $P-{}^{18}O$ peaks was 69%/31% (±3%). The lack of change in any of the $^{13}C$ NMR shifts coupled with the 0.03 ppm upfield shifts of the $P-{}^{18}O$ diesters and the correct 30/70 ratio of the $P-{}^{18}O$/$P-{}^{16}O$ areas in the $^{31}P$ NMR shows conclusively that the hydrolysis of the two systems involves attack of the hydroxyl group at the phosphorus center.

**Isotope Effects.** The primary and secondary effects for the alkaline hydrolysis of DEPC and the primary effect for the alkaline hydrolysis of DE*m*NBP were determined by using the remote label method.[21] The observed isotope effects were determined from comparative isotopic analysis of the residual substrate versus the products of the hydrolysis (choline and ethyl choline phosphodiester, or *m*-nitrobenzyl alcohol and ethyl-*m*-nitrobenzyl phosphodiester), which were collected and combusted as one product. The hydrolyses were run to 30−70% completion ($f$ values of 0.3−0.7). The observed $^{18}O$ isotope

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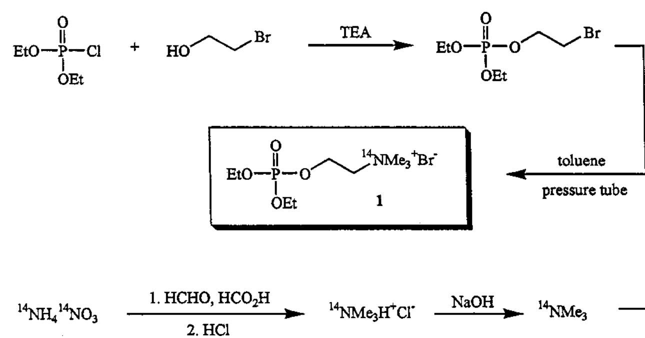
(25) Cleland, W. W.; Hengge, A. C. *FASEB J.* **1995**, *9*, 1585.

**Scheme 2**




effects were calculated by using eqs 4−6 and were corrected for incomplete label incorporation by using eqs 7 and 8. The correction for parallel reactions was calculated assuming ${}^{18,15}k_1 = 1$ and using eq 12, where $k_1/k_2$ was the ratio of diester to choline or *m*-nitrobenzyl alcohol.

The corrected primary isotope effects for DEPC and DE*m*NBP were 1.041 ± 0.003 and 1.052 ± 0.002, respectively. The secondary isotope effect for DEPC was 1.033 ± 0.002. The secondary isotope effect for DE*m*NBP was not determined. The isotope effects for these compounds are compared to those for phosphotriesters with good leaving groups in Table 1.

## Discussion

It is known that phosphotriesters undergo hydrolysis with associative transition states[26] and considerable shortening of the reaction coordinate.[25] This may seem intuitive until it is realized that, in some reactions with dissociative transition states, the reaction coordinates are also compressed. An example is hydrolysis of phosphomonoesters, where the reactants start at the van der Waals contact distance and the entering and leaving groups do not move during the reaction. If the reaction coordinate is slightly shortened and the phosphoryl oxygen remains motionless (as possibly occurs in enzyme mechanisms), the axial bond order will be ~0.15, illustrating reaction coordinate compression in a dissociative mechanism.[25]

In the case of a triester with a low p*K* value leaving group, the axial bond order is much higher. In diethyl-*p*-nitrophenyl phosphate, the p*K*'s of the possible leaving group groups are 7.0 (*p*-nitrophenol) and 16.0 (ethanol). Here, a parallel reaction is not a possibility, and the hydrolysis will lead to the ejection of only the *p*-nitrophenol due to the wide discrepancy in the p*K* values. By assuming that the total bond order of the P remains at 5 and that the bond order for the EtO− groups remains at 1, the secondary isotope effect can be used to estimate the sum of the axial bond orders. The calculated isotope effect for reducing the phosphoryl bond order from 2 to 1 is 4%.[27] The secondary isotope effect of 0.63% for the nitrophenolic triester gives a bond order of 1.85 for the P=O component. The total axial bond order sum can then be estimated to be ~1.15 (5 − 1.85 − 2). While much larger than for phosphomonoesters, this still translates to only a slightly associative transition state.[25]

Using the same assumptions for the diethyl-*p*-carbamoylphenyl phosphate (secondary isotope effect 2.5%), the bond order of P=O is ~1.375, and the sum of the axial bond orders becomes ~1.625. The sum of the axial bond orders has increased by 40% when the leaving group p*K* value is increased by 1.6 units.

When the estimates are done for DEPC and DE*m*NBP (assuming the same secondary isotope effect for both compounds), the P=O bond order is 1.175 and the sum of the axial bond orders is 1.825, an increase of 12% from the diethyl-*p*-carbamoylphenyl phosphate. This does not seem like a large increase in the axial bond order for a difference in p*K* value of 5.6 units. However, it may be that the axial bond order is as large as possible in these systems. It may only be possible to see a phosphoryl bond order change from 2 to 1 in a cyclic phosphotriester when a true phosphorane intermediate is formed prior to pseudorotation.[25,28] Even though phosphorane intermediates have been hypothesized in acyclic phosphotriester hydrolysis, the evidence has shown that, for acyclic phosphotriesters at least, these mechanisms are in-line displacements without phosphorane intermediates.[25]

With high p*K* leaving groups, the transition state presumably is approximately symmetrical, and the attacking nucleophile must get in much closer to the phosphorus center to eject the leaving group. The Hammond postulate supports this conclusion, since the incoming nucleophile and the leaving group have roughly the same p*K* values (14.9−16.0), so that the transition state will be symmetrical, or at least much more symmetrical than in the diethyl-*p*-nitrophenyl phosphate or the diethyl-*p*-carbamoylphenyl phosphate systems.[29] The secondary isotope effect values show a noticeable change in the bond order of the phosphoryl group which can only be attributed to the hydroxyl group moving in very close to the phosphorus center to dislodge the leaving group. The fact that the leaving groups are unable to stabilize themselves as ions (lack of resonance forms) or to be protonated in the transition state suggests that the nucleophile would need to move in much closer than van der Waals contact distance in order to displace the leaving group. A comparison of hypothesized energy diagrams for diethyl phosphotriesters with either *p*-nitrophenol or *m*-nitrobenzyl alcohol as leaving groups is shown in Figure 1. In the hydrolysis of diethyl-*p*-nitrophenyl phosphate, the ability of the leaving group to stabilize itself allows the phenol to move away from the phosphorus early in the reaction due to a slight association with the nucleophile. Conversely, in the DE*m*NBP reaction, the nucleophile and leaving group are both closer to the phosphorus center, and the transition state is nearly symmetrical and centered on the reaction coordinate.

The size of the primary isotope effects in these systems also points to much more movement on the reaction coordinate than in the phenolic systems. For phosphomonoester hydrolysis with dissociative transition states, the major primary isotope effect comes from the zero point energy difference between ground and transition states. With the low (8−15%) bond order along the reaction path between phosphorus and entering and leaving groups, this accounts for up to 2% of the observed isotope effect. By contrast, the imaginary frequency factor is small because the coupling of the two stretches in off-diagonal position of the force field matrix is small because of the low bond orders.

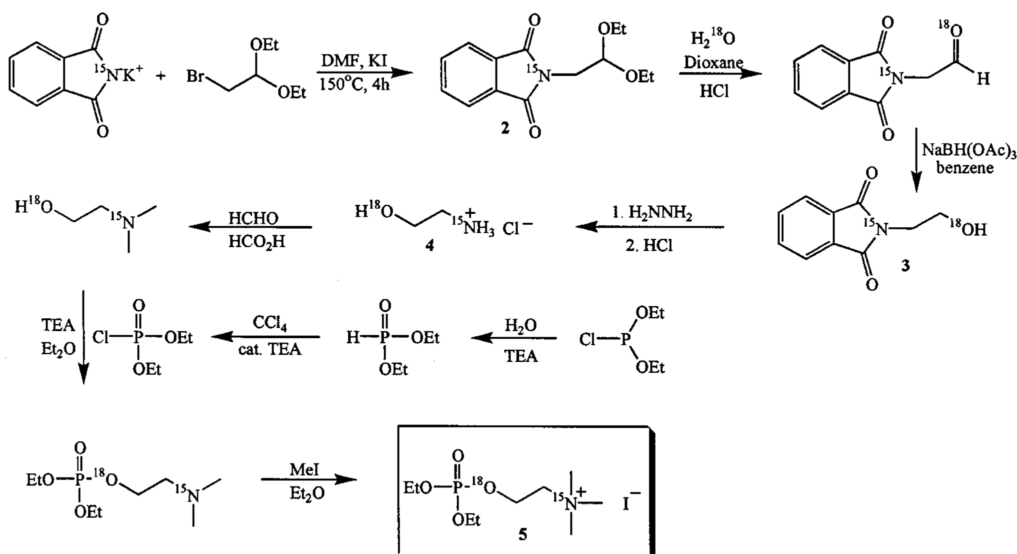
(26) Benkovic, S. J.; Schray, K. J. The Mechanism of Phosphoryl Transfer. In *Transition States of Biochemical Processes*; Candour, R. D., Schowen, R. L., Eds.; Plenum Press: New York, 1978; p 493.

(27) Unpublished calculations by W. W. Cleland, using the BEBOVIB program and force constants that correctly reproduce the Raman frequencies for $PO_4^{3-}$ (Weiss, P. M.; Knight, W. B.; Cleland, W. W. *J. Am. Chem. Soc.* **1986**, *108*, 2761). This value may not be the same in the trigonal bipyramidal transition-state structure of a phosphotriester hydrolysis as in the simple tetrahedral phosphate ion.

(28) Mislow, K. *Acc. Chem. Res.* **1970**, *3*, 321. Musher, J. I. *J. Am. Chem. Soc.* **1972**, *94*, 5662. Westheimer, F. H. *Acc. Chem. Res.* **1968**, *1*, 70. Ugi, I.; Marquarding, D.; Klusacek, H.; Gillespie, P.; Ramirez, F. *Acc. Chem. Res.* **1971**, *4*, 288.

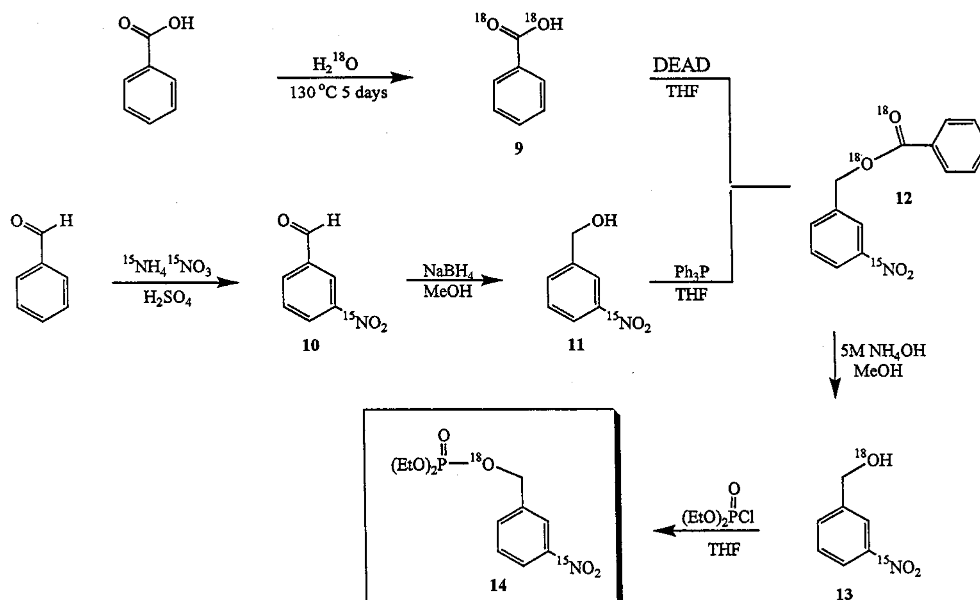
(29) Hammond, G. S. *J. Am. Chem. Soc.* **1955**, *77*, 334. Farcasiu, *J. Chem. Educ.* **1975**, *52*, 76.

**Scheme 3**[a]




[a] The synthesis of **6**, $^{15}$N,(*phosphoryl*)$^{18}$O diethyl choline phosphotriester iodide, was done in the same manner except **2** was hydrolyzed by excess water with natural oxygen isotope abundance. Also diethylchlorophosphine was reacted with $H_2{}^{18}O$ to produce the labeled diethylchlorophosphate used in the reaction with *N*,*N*-dimethylaminoethanol.[31]

**Scheme 4**[a]




[a] The syntheses of $^{14}$N *m*-nitrobenzaldehyde (**7**) and $^{14}$N *m*-nitrobenzyl alcohol (**8**) were done with $^{14}NH_4{}^{14}NO_3$ using the same methodology.

**Table 1.** Kinetic Isotope Effects on the Alkaline Hydrolysis of Diethyl Phosphotriesters

| leaving group | p*K* of leaving group | primary $^{18}$O IE % | secondary $^{18}$O IE % | transition state | ref |
|---|---|---|---|---|---|
| *p*-nitrophenol[a] | 7.0 | 0.6 | $0.63 \pm 0.01$ | slightly associative | 18 |
| *p*-carbamoylphenol[a] | 8.6 | $2.7 \pm 0.2$ | $2.5 \pm 0.2$ | associative | 18 |
| choline iodide[b] | 13.9 | $4.1 \pm 0.3$[c] | $3.3 \pm 0.2$[c] | highly associative | this work |
| *m*-nitrobenzyl alcohol[b] | 14.9 | $5.2 \pm 0.3$[c] | | highly associative | this work |

[a] The phenol is the only leaving group. [b] The leaving groups are accompanied by some ethanol in ratios of 3/1 for choline and 6.6/1 for *m*-nitrobenzyl alcohol. [c] Corrected for incomplete isotopic labeling in parallel reactions; values are the average of six determinations with *f* values between 0.3 and 0.7.

With phosphotriester hydrolysis with an associative transition state, there is less difference in zero point energies of ground and transition states because of the higher bond orders along the reaction path. Thus, this part of the isotope effect is smaller than with monoesters. The imaginary frequency factor, however, is much larger because of the tight off-diagonal coupling of the stretches resulting from the higher bond order.

We have data for primary isotope effects with *p*-nitrophenol (p*K* 7),[18] *p*-carbamoylphenol (p*K* 8.6),[18] and for choline (p*K* 13.9)[30] and *m*-nitrobenzyl alcohol (p*K* 14.9).[30] The imaginary frequency factor increases with the associative character and

(30) This work.
(31) Reynolds, M. A.; Gerlt, J. A.; Demou, P. C.; Oppenheimer, N. J.; Kenyon, G. L. *J. Am. Chem. Soc.* **1983**, *105*, 6475.

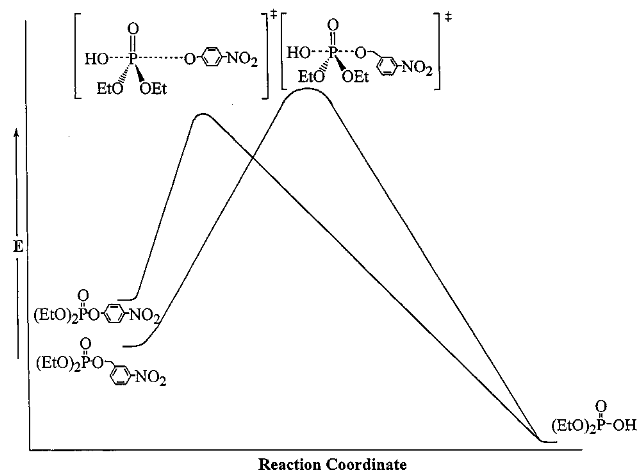



**Figure 1.** Reaction coordinates and transition-state structures for alkaline hydrolysis of phosphotriesters with *p*-nitrophenol or *m*-nitrobenzyl alcohol as leaving groups.

reaches its highest value with the high p*K* leaving groups. The zero point energy difference goes the other way, being maximum for lower p*K*'s. *p*-Nitrophenol is an exception, since electron delocalization into the nitro group in the transition state makes the phenolic oxygen−ring bond have some double bond character.

Thus, the 4% primary O-18 isotope effects we see come from the high imaginary frequency factor, rather than from a zero point energy difference.

## Conclusions

The hydrolysis of phosphotriesters with high p*K* value leaving groups involves parallel reactions. The large primary and secondary $^{18}O$ isotope effects suggest a very associative transition state, and the Hammond postulate suggests that it is fairly symmetrical as well. As the reaction progresses, the nucleophile moves in very close to the P center, and the leaving group is ejected with some difficulty. The large size of the primary isotope effects indicates large compression, or general movement, along the reaction coordinate.

## Experimental Section

**Materials.** Potassium phthalimide ($^{15}N$, 98%+) was obtained from Cambridge Isotope Laboratory. $H_2{}^{18}O$ was obtained from Aldrich (95 atom % $^{18}O$) and Isotec (97 atom % $^{18}O$). $^{15}N$-depleted ammonium nitrate was purchased from Monsanto. $^{15}N$-enriched ammonium nitrate was purchased from Isotec (99.7 atom % $^{15}N$). All of the other chemicals and solvents were commercially available and used without further purification, unless otherwise stated. The syntheses of labeled compounds are shown in Schemes 2−4, and the details are in Supporting Information.

**$^{18}O$ Incorporation in the Hydrolysis of Phosphotriesters. $^{13}C$ NMR and $^{31}P$ NMR Experiments.** The NMR samples were prepared in the same manner and at the same time. In a small vial, 100 mg of DEPC or 100 mg of DE*m*NBP was dissolved in 500 $\mu$L of $CD_3OD$. To this were added 100 $\mu$L of 10 M KOH and 50 $\mu$L of either $H_2{}^{16}O$ or $H_2{}^{18}O$, making the samples 1.5 M in KOH. Four samples were made with each compound, having one sample of 100% $H_2{}^{16}O$, and the other 70% $H_2{}^{16}O$ and 30% $H_2{}^{18}O$. The reaction was allowed to proceed for 1 week, and then the samples were studied by using $^{13}C$ NMR on a Bruker 250 MHz instrument.

The high-resolution $^{31}P$ experiments (162 MHz) were run on a Bruker 400 MHz NMR at ambient temperatures. The spectral width was 1615 Hz, acquisition time 2.53 s, 90° pulse; 16*K* data points; resolution 0.197 Hz/point; $^{1}H$ decoupled; Gaussian multiplication (LB, 0.08 Hz; GB, 0.12). Deconvolution of the peaks and area integration were done with Bruker software.

**Procedure for the Hydrolysis, Isolation of Products, and Determination of $^{15}N/^{14}N$ by Isotope Ratio Mass Spectrometry of Diethylphosphorylcholine Iodide (DEPC).** $^{15}N$,$^{18}O$ DEPC and $^{14}N$-labeled DEPC were mixed to natural abundance (0.37% by IRMS), and this solution was taken to dryness by rotary evaporation. The dry powder was suspended in ~50 mL of anhydrous methyl acetate. To this was added freshly distilled $CH_3CN$ until the solid just dissolved. The solution was refrigerated at −10 °C for 24 h and then the solvent was removed via cannula and $N_2$ pressure. Cold anhydrous methyl acetate (25 mL) was added to the flask via cannula to wash the crystalline product. This wash solution was removed, fresh anhydrous methyl acetate and $CH_3CN$ were added, and the recrystallization process was repeated. The crystals were washed with three 50-mL aliquots of cold methyl acetate and were then dried under vacuum for several hours. NMR studies showed no discernible impurities.

The DEPC was dissolved in 10 mL of water. Aliquots of 1 mL (~65 mg of triester) were placed in separate 3-mL vials. To the 1-mL solutions of aqueous DEPC was added a 70% molar equivalent of KOH. The solution was stirred for 24 h, and the reaction was quenched to pH 7 using 0.1 N HCl. Percent completion (*f* value) and product structure were determined by $^{31}P$ and $^{1}H$ NMR. The sample was taken to dryness by rotary evaporation, and ~5 mL of freshly distilled $CH_3CN$ was added. The mixture was allowed to stir for 1 h and then filtered through a 2-mL medium glass frit to remove the KCl, and the solvent was removed by rotary evaporation. The sample was dissolved in ~2 mL of the eluent used in the C18 chromatography (45 mM $K_xH_xPO_4$ {39/1, $KH_2PO_4/K_2HPO_4$}, 27.5% MeOH, 1.5% THF, pH 6.0). The sample solution (~250 $\mu$L, 12−15 mg hydrolysis products) was eluted at 9.9 mL min$^{-1}$ from a Microsorb preparative C18 column (2.2 cm i.d. × 25 cm). The individual product peaks (ethyl choline phosphate and choline) were collected together between 6 and 8 min, and the residual substrate was collected between 14 and 20 min. The individual samples were reduced to dryness and then dissolved in a minimum of $H_2O$ (8−10 mL). A large excess of freshly distilled acetone (100 mL) was added to the filtrate slowly with stirring. A white precipitate ($KP_i$ buffer salt) was formed immediately and was removed by suction filtration through a medium glass frit. The solution was rotary evaporated to dryness, leaving a small amount of precipitate, and the solid was stirred with 25 mL of freshly distilled MeOH. The MeOH solution was suction-filtered through a medium glass frit, and the filtrate was taken to dryness by rotary evaporation. The products were dissolved in freshly distilled methanol and transferred to quartz tubes. Aspirator vacuum was used initially to remove the majority of the methanol, and then the samples were evacuated at ~5 × 10$^{-3}$ Torr to remove any trace of volatile compounds. When the samples were dry, CuO (3−4 g), Cu (~500 mg), ditomaceous earth (~500 mg), and silver (~200 mg) were added, and the tubes were placed under high vacuum (<1 × 10$^{-3}$ Torr) and flame-sealed. The samples were combusted at 775 °C to convert all N products to $N_2$ gas. The samples were distilled on a high-vacuum line, and the $N_2$ was trapped on molecular sieves at −196 °C. The isotope mass ratio was determined on a Finnegan isotope ratio mass spectrometer.

**Procedure for the Hydrolysis, Isolation of Products, and Determination of $^{15}N/^{14}N$ by Isotope Ratio Mass Spectrometry of Diethyl *m*-Nitrobenzyl Phosphate (DE*m*NBP).** $^{15}N$,$^{18}O$ DE*m*NBP and $^{14}N$ DE*m*NBP were mixed to natural abundance (0.37%). The triester was cleaned on Florosil (4:1 ethyl acetate:petroleum ether, 4 cm × 35 cm). DE*m*NBP was dissolved in aqueous methanol (90)/10). Three 1-mL aliquots (~65 mg/mL) were placed in vials with 1 mL of 1.005 M KOH (10% MeOH/90% $H_2O$), and the solutions were stirred from 8 to 48 h. The reactions were quenched to pH 7 using 1 N HCl. Percent completion (*f* value) and product structure were determined from $^{31}P$ and $^{1}H$ NMR. The sample was taken to dryness by rotary evaporation, and ~5 mL of freshly distilled $CH_3CN$ was added. The mixture was allowed to stir for 1 h and then filtered through a 2-mL medium glass frit to remove the KCl, and the solvent was removed by rotary evaporation. The sample was dissolved in ~2 mL of the eluent used in the C18 chromatography (25% i-PrOH, 1.5% THF, 73.5% $H_2O$) and then filtered through a 0.22 $\mu$m syringe filter. The sample solution was

eluted at 9.9 mL min$^{-1}$ from a Microsorb preparative C18 column (2.2 cm i.d. × 25 cm). The *m*-NBA and ethyl *m*-nitrobenzyl phosphate peaks were collected together at 11−14 and 19−22 min, respectively, and residual DE*m*NBP at 26−33 min. The individual samples were reduced to dryness and then dissolved in 12 mL of freshly distilled MeOH and transferred to 25-mL conical flasks. The volume was reduced to ∼2 mL by rotary evaporation, and the samples were transferred to quartz tubes. The solvent was removed by aspirator vacuum, and the samples were then evacuated at $\sim 5 \times 10^{-3}$ Torr for several hours to remove the rest of the MeOH. When the samples were dry, CuO (3−4 g), Cu (∼500 mg), and diatomaceous earth (∼500 mg) were added, and the tubes were placed under high vacuum ($\sim 1 \times 10^{-3}$ Torr) and flame-sealed. The samples were combusted at 775 °C to convert all N products to $N_2$ gas. The samples were distilled on a high-vacuum line and trapped on molecular sieves at −196 °C. The isotope mass ratio was determined on a Finnegan isotope ratio mass spectrometer.

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**Supporting Information Available:** Syntheses and purification of labeled compounds (PDF). This information is available free of charge via the Internet at http://pubs.acs.org.

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