Dumas, D. P., Caldwell, S. R., Wild, J. R., & Raushel, F. M. (1989) *J. Biol. Chem. 264*, 19659-19665.
Dumas, D. P., Durst, D., Landis, W. G., Raushel, F. M., & Wild, J. R. (1990) *Arch. Biochem. Biophys. 277*, 155-159.
Dunford, H. B., & Hewson, W. D. (1977) *Biochemistry 16*, 2949-2956.
Eto, M. (1974) in *Organophosphorus Pesticides; Organic and Biological Chemistry*, pp 158-192, CRC Press, Cleveland, OH.
Fersht, A. (1985) in *Enzyme Structure and Mechanisms*, 2nd ed., pp 148-149, W. H. Freeman and Co., New York.
Hardy, L. W., & Kirsch, J. F. (1984) *Biochemistry 23*, 1282-1287.
Harper, L. L., McDaniel, C. S., Miller, C. E., & Wild, J. R. (1988) *Appl. Environ. Microbiol. 54*, 2586-2589.
Hill, T. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4918-4922.
Jencks, W. P. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 1-11.
Jencks, W. P., & Carriuolo, J. (1961) *J. Am. Chem. Soc. 83*, 1743-1750.
Jencks, W. P., & Gilchrist, M. (1968) *J. Am. Chem. Soc. 90*, 2622-2637.
Khan, S. A., & Kirby, A. J. (1970) *J. Chem. Soc. B*, 1172-1182.
Knowles, J. R. and Albery, W. J. (1977) *Acc. Chem. Res. 10*, 105-111.
Laveglia, J., & Dahm, P. A. (1977) *Annu. Rev. Entomol. 22*, 483-513.
Lewis, V. E., Donarski, W. J., Wild, J. R., & Raushel, F. M. (1988) *Biochemistry 27*, 1591-1597.
Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431-441.
Munnecke, D. M., & Hsieh, D. P. H. (1974) *Appl. Microbiol. 28*, 212-217.
Pocker, Y., & Janjic, N. (1987) *Biochemistry 26*, 2597-2506.
Schmitz, K. S., & Schurr, J. M. (1972) *J. Phys. Chem. 76*, 534-545.
Schrader, G. (1963) *Die Entwicklung neur Insektizider Phosphasaure-Ester*, 3rd Edition, Verlag Chemie, Weinheim.
Serdar, C. M., Gibson, D. T., Munnecke, D. M., & Lancaster, J. H. (1982) *Appl. Environ. Microbiol. 44*, 246-249.
Sheldrick, G. M. (1988) *Direct Methods* SHELXS, SHELXTL-PLUS Institüt für Anorganishe Chemie der Universtät, Tammanstasse 4, Göttingen, FRG.
Wycoff, H. W., Doscher, M., Tsernglou, D., Inagami. T., Johnson, L. N., Hardman, K. D., Allewell, N. M., Kelly, D. M., & Richards, F. M. (1967) *J. Mol. Biol. 27*, 563-578.

Transition-State Structures for Enzymatic and Alkaline Phosphotriester Hydrolysis†

Steven R. Caldwell‡ and Frank M. Raushel*,‡,§
Departments of Chemistry and Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843
Paul M. Weiss and W. W. Cleland*
Institute for Enzyme Research, University of Wisconsin—Madison, Madison, Wisconsin 53705
Received January 8, 1991; Revised Manuscript Received May 6, 1991

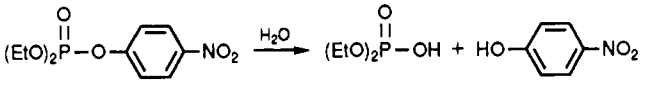
ABSTRACT: The primary and secondary ^{18}O isotope effects for the alkaline (KOH) and enzymatic (phosphotriesterase) hydrolysis of two phosphotriesters, *O,O*-diethyl *p*-nitrophenyl phosphate (I) and *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate (II), are consistent with an associative mechanism with significant changes in bond order to both the phosphoryl and phenolic leaving group oxygens in the transition state. The synthesis of [^{15}N, *phosphoryl*-^{18}O]-, [^{15}N, *phenolic*-^{18}O]-, and [^{15}N]-*O,O*-diethyl *p*-nitrophenyl phosphate and *O,O*-diethyl *O*-(4-carbamoylphenyl)phosphate is described. The primary and secondary ^{18}O isotope effects for the alkaline hydrolysis of compound I are 1.0060 and 1.0063 ± 0.0001, whereas for compound II they are 1.027 ± 0.002 and 1.025 ± 0.002, respectively. These isotope effects are consistent with the rate-limiting addition of hydroxide and provide evidence for a S_N2-like transition state with the absence of a stable phosphorane intermediate. For the enzymatic hydrolysis of compound I, the primary and secondary ^{18}O isotope effects are very small, 1.0020 and 1.0021 ± 0.0004, respectively, and indicate that the chemical step in the enzymatic mechanism is not rate-limiting. The ^{18}O isotope effects for the enzymatic hydrolysis of compound II are 1.036 ± 0.001 and 1.0181 ± 0.0007, respectively, and are comparable in magnitude to the isotope effects for alkaline hydrolysis, suggesting that the chemical step is rate-limiting. The relative magnitude of the primary ^{18}O isotope effects for the alkaline and enzymatic hydrolysis of compound II reflect a transition state that is more progressed for the enzymatic reaction.

The mechanism of phosphoryl transfer has been studied in detail because of the importance of this reaction for biological systems. Extensive characterization of phosphoryl transfer from phosphate monoesters and diesters in enzymatic processes has been made in terms of general chemical mechanisms (Bruice & Benkovic, 1966; Benkovic & Schray, 1973; Westheimer, 1981). However, the current limited understanding of chemical phosphotriester hydrolysis has not been applied to enzymatic hydrolytic reactions because unlike phosphate monoesters and diesters, which serve as specific substrates for well-characterized enzymes, a naturally oc-

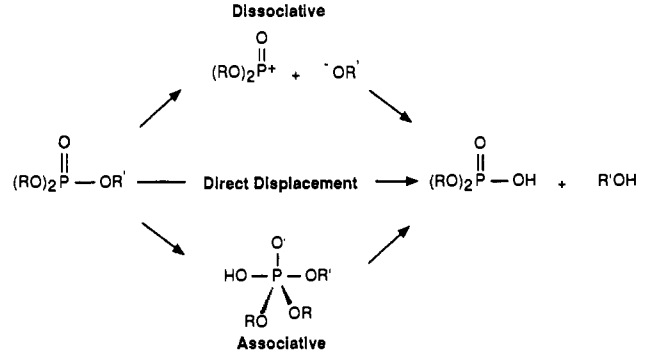
† This work was supported in part by the Army Research Office (DAALO3-90-G-0045), the Texas Advanced Technology Program, and the National Institutes of Health (GM 18938).
‡ Department of Biochemistry and Biophysics.
§ Department of Chemistry.

0006-2960/91/0430-7444$02.50/0

Scheme I



Scheme II



curring phosphotriester has remained unidentified. Nonetheless, proteins from various sources exist that are capable of hydrolyzing these phosphotriesters (Dumas, 1989, and references cited therein). Recently, a phosphotriesterase has been isolated from a soil microbe, *Pseudomonas diminuta*, which exhibits a broad substrate specificity for the hydrolysis of phosphotriesters (Dumas et al., 1989). The chemical and kinetic mechanisms of this enzyme have been recently determined (Caldwell et al., 1991a), and interest is now directed at refining the enzymatic reaction mechanism.

Phosphotriester hydrolysis occurs with phosphorus–oxygen bond cleavage as illustrated with the reaction of paraoxon (Scheme I). There are potentially two limiting reaction schemes for the hydrolysis of phosphotriesters (Knowles, 1980). The *dissociative* mechanism is characterized by the initial expulsion of the leaving group to generate an electropositive phosphorus intermediate that is then susceptible to nucleophilic attack. In contrast, a fully *associative* mechanism can be depicted as an *addition–elimination* reaction where the attack of the nucleophile leads to a pentavalent phosphorane intermediate followed by the collapse of this structure to products. However, the formation of an intermediate is not absolutely required and a *direct-displacement* mechanism with a single S_N2-like transition state is a distinct possibility. These extreme mechanisms are graphically depicted in Scheme II.

For phosphotriester hydrolysis, there is currently no experimental support for a dissociative mechanism. Unlike the anionic phosphomonoesters, where a metaphosphate intermediate is quite likely (Herschlag & Jencks, 1987), there are no negative charges on the phosphoryl oxygens to stabilize the developing positive charge on the phosphorus during the formation of the putative metaphosphate-like intermediate. Moreover, the entropy of activation for the hydrolysis of phosphotriesters is higher (25 eu) relative to that observed for the reactions of phosphomonoesters, which is near zero eu, and the reaction rate of phosphotriesters is dependent on the pK_a of the nucleophile ($\beta_{nuc} = 0.3$–0.6) (Benkovic & Schray, 1973; Knowles, 1980). Therefore, the mechanism for phosphotriester hydrolysis occurs via some form of the associative pathway.

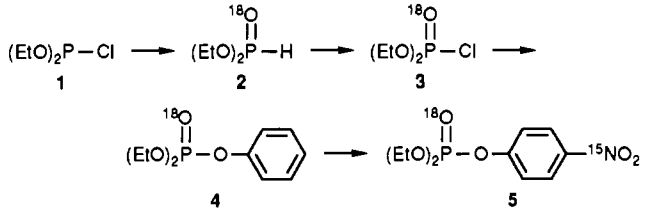
The distinction between the addition–elimination and direct-displacement pathways (or the various hybrids) is reflected by the relative change in the bond order to the leaving group and phosphoryl oxygen during the transition state. The addition–elimination pathway is characterized by a change in the bond order to the phosphoryl oxygen that completely precedes bond cleavage to the leaving group, whereas in the direct-displacement mechanism, the addition of the nucleophile is concerted with the bond cleavage of the leaving group. In a hybrid mechanism there is a simultaneous, but not necessarily synchronous, decrease in bonding to both the phosphoryl oxygen and the leaving group upon addition of the nucleophile. The relative magnitude of the bond order changes in the transition state at these two atoms is variable and dependent on the identity of the attacking nucleophile and the ability of the leaving group to depart. The structure of the transition state for phosphotriester hydrolysis can be probed by measuring the effect of oxygen-18 substitution on the alkaline and enzymatic kinetic rate constants. The magnitude of the primary oxygen-18 effect will depend on the extent of bond cleavage to the leaving group while the secondary oxygen-18 isotope effect will be determined by the change in the bond order to the phosphoryl oxygen. In this report we have utilized primary and oxygen-18 isotope effects with slow and fast substrates to elucidate the transition-state structure for alkaline and enzymatic phosphotriester hydrolysis. A preliminary communication of some of these isotope effects has appeared (Caldwell et al., 1991b).

Materials and Methods

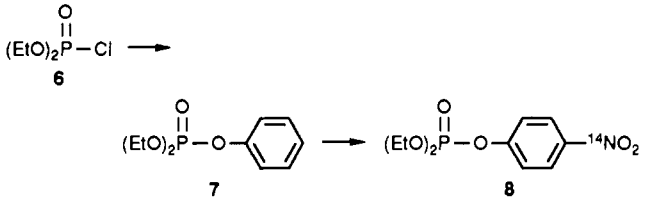
General. The phosphotriesterase used in these experiments was isolated from *Escherichia coli* carrying the cloned gene on a plasmid as previously described (Dumas, 1989). The enzyme preparation was purified to a specific activity of 3200 μmol/(min·mg). The enzymatic activity was measured by monitoring the appearance of *p*-nitrophenol produced by the hydrolysis of 1.0 mM paraoxon at pH 9.0. All spectrophotometric determinations were made at 25 °C with use of a Gilford Model 260 spectrophotometer. Melting points were determined with use of a Fischer Johns melting point apparatus. Chemicals were purchased from Aldrich Chemical Co., Sigma Chemical Co., or Fisher Scientific. All isotopically labeled starting materials were purchased from Cambridge Isotope Laboratories except ammonium [^{14}N]nitrate, which was obtained from Monsanto Research Corp. Solvents were distilled prior to use for the isotope experiments. Unlabeled paraoxon was purchased from Sigma Chemical Co. and purified by organic extraction (dichloromethane) with successive washings with 10 mM pyrophosphate buffer at pH 9.0. Unlabeled *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate was synthesized as previously described (Caldwell et al., 1991a).

Synthesis of [^{15}*N,phosphoryl-*^{18}*O*]*Paraoxon* (**5**). The formation of **5** was conducted by the condensation of ^{18}O-labeled diethyl chlorophosphate (**3**) with phenol. The synthesis of [^{18}O]diethyl chlorophosphate (**3**) was performed according to a procedure of Reynolds et al. (1983). In nitrogen atmosphere, 7 mL of triethylamine was mixed with 1.0 mL of [^{18}O]H_2O (98%) and cooled to 0 °C when 7 mL of diethyl chlorophosphite (**1**) was added dropwise. After the mixture stood overnight at room temperature, 25 mL of anhydrous diethyl ether was added and then the mixture was filtered and concentrated. Immediately, the resulting oil (**2**) was cooled to 0 °C and 12.8 g of carbon tetrachloride was added, followed by 0.41 g of triethylamine. After stirring at room temperature for 3 h, the mixture was filtered and concentrated. Fractional distillation of the oily residue gave 3.9 g (46% overall yield) of the desired product (**3**) (54–57 °C, 1 mmHg). The ^{31}P NMR spectrum indicated an 80% isotopic content at the oxygen-18 label. The diethyl chlorophosphate (**3**) (1.72 g) was added dropwise to a cooled solution (0 °C) of phenol (0.94 g) dissolved in diethyl ether followed by the addition of a 10% excess of 20% sodium hydroxide. The reaction mixture was stirred for 1 h and then extracted with diethyl ether. The combined organic layers were washed with 10% sodium hy-

droxide (2 × 10 mL), dried over magnesium sulfate, filtered, and concentrated to give 1.6 g (68% yield) of a clear oil (**4**). The oil was treated with 1.5 mL of concentrated sulfuric acid at 0 °C, and then 0.82 g of ammonium [^{15}N]nitrate (99%), dissolved in 1.5 mL of sulfuric acid, was added dropwise. After 30 min, the reaction was quenched with ice and extracted with diethyl ether, and the combined organic layers were dried over magnesium sulfate, filtered, and concentrated. Purification by chromatography on florisil (ethyl acetate/hexane 1:4) gave 1.5 g (80% yield) of the desired product (**5**). The isotopic content at the phosphoryl oxygen label was found to be 79% by ^{31}P NMR spectroscopy.

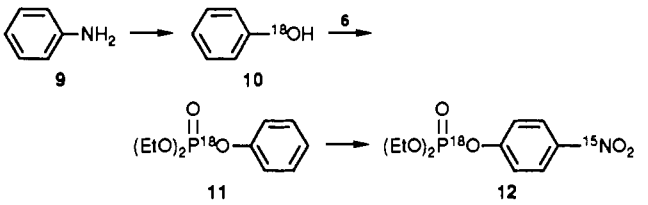


Synthesis of [^{14}N]*Paraoxon* (**8**). The synthesis of the [^{14}N]paraoxon(^{15}N-depleted) (**8**) was accomplished as described for compound **5** with 3.44 g of diethyl chlorophosphate (DECP) (**6**) and 1.88 g of phenol to give 2.9 g of the condensation adduct (**7**). Nitration was conducted with 1.0 g of ammonium [^{14}N]nitrate (99.99%) and purification by chromatography on florisil (ethyl acetate/hexane 1:4) gave 2.1 g (87% yield) of a clear oil (**8**).

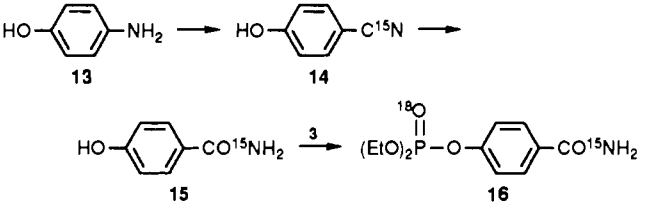


Synthesis of [^{15}N,*phenolic-*^{18}O]*Paraoxon* (**12**). The procedure leading to the synthesis of **12** involves the condensation of [^{18}O]phenol (**10**) and DECP (**6**). The synthesis of [^{18}O]-phenol (**10**) was conducted according to a modified procedure of Pinchas et al. (1965). An acidic ethanol solution (25 mL, 16% w/v HCL) was cooled to 0 °C, and 3.5 g of freshly distilled aniline (**9**) in 7.5 mL of ethanol was added dropwise while the temperature was maintained below 4 °C. To this was added dropwise 4.8 g of isoamyl nitrate, and then 100 mL of diethyl ether was poured into the reaction mixture to give a white slurry. The diazonium salt was collected by vacuum filtration and transferred to a stirred solution containing 15 mL of diethyl ether and 3 mL of [^{18}O]H_2O (98%). It was refluxed at 50 °C until nitrogen evolution had ceased. The layers were separated, and the aqueous layer was extracted with 2 × 25 mL of diethyl ether. The combined organic layers were dried over magnesium sulfate, filtered, and concentrated. Fractional distillation of the oily residue gave 1.4 g (39% yield) of a clear oil (**10**). Mass spectral analysis of compound **10** indicated an ^{18}O isotopic content of 89%. The condensation of 0.6 g [^{18}O]phenol (**10**) with diethyl chlorophosphate (**6**) as described in the synthesis of compound **5** gave the adduct, **11**. Nitration of **11** with ammonium [^{15}N]nitrate (99%) gave compound **12**, which was purified by chromatography on florisil (ethyl acetate/hexane 1:4) to give 0.4 g (80%) yield. The ^{31}P NMR and mass spectra indicated an ^{18}O isotopic content of 87 and 89% at the indicated position, respectively.

Synthesis of [^{15}N,*phosphoryl-*^{18}O]*-O,O-Diethyl O-(4-Carbamoylphenyl) Phosphate* (**16**). Compound **16** was formed

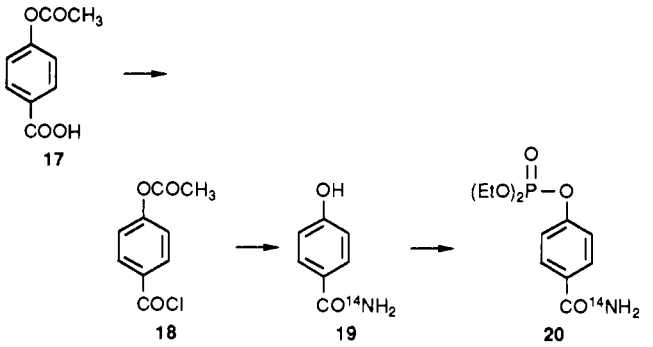


by the condensation of [^{15}N]-4-hydroxybenzamide (**15**) and compound **3**. [^{15}N]-4-Hydroxybenzamide (**15**) was synthesized according to a modified procedure of Pichat et al. (1955), where 0.27 g of 4-aminophenol (**13**) was converted to 0.25 g of 4-cyanophenol (**14**) by use of 1.0 g of potassium [^{15}N]-cyanide (99%). The resulting yellow oil was dissolved in 8 mL of ethanol, and then 8 mL of 6 N sodium hydroxide was added. Oxidation using 6 mL of 30% hydrogen peroxide under alkaline conditions (Noller, 1943) gave the desired product (**15**). Extraction with ethyl acetate removed the organic product from the reaction mixture, and the combined organic layers were dried over magnesium sulfate, filtered, and concentrated. Purification was accomplished by chromatography on florisil (ethyl acetate) to give 0.11 g (32% overall) of the [^{15}N]-4-hydroxybenzamide (**15**). Compound **16** was synthesized by the condensation of 0.17 g of [*phosphoryl-*^{18}O]-diethyl chlorophosphate (**3**) with 0.11 g of [^{15}N]-4-hydroxybenzamide (**15**) as previously described for the synthesis of *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate (Caldwell et al., 1991a) and was purified by chromatography on florisil (ethyl acetate/hexane) to give 0.11 g (52% yield) of a white crystalline solid (mp 99–101 °C). The ^{31}P NMR and mass spectra indicated an ^{18}O isotopic content of 80 and 84% at the phosphoryl oxygen, respectively.

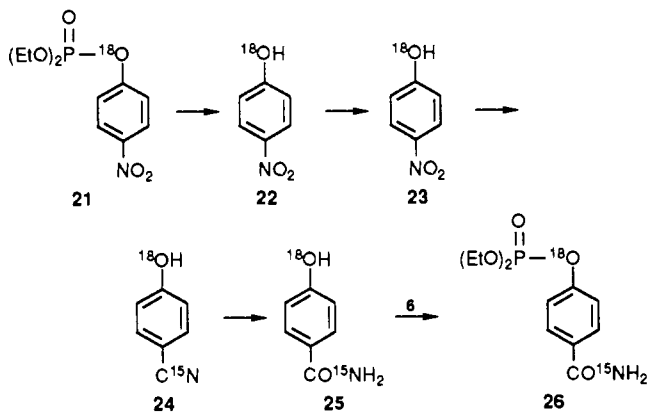


Synthesis of [^{14}N]*-O-Diethyl O-(4-Carbamoylphenyl) Phosphate* (**20**). Compound **20** was synthesized by the condensation of [^{14}N]-4-hydroxybenzamide and DECP (**6**). The synthesis of [^{14}N]-4-hydroxybenzamide was accomplished in the following manner. To 10 g of 4-acetoxybenzoic acid (**17**) in 200 mL of dichloromethane was added 7 g of oxalyl choride and 3 drops of *N,N*-dimethylformamide. After stirring for 2 h, the solvent was removed under vacuum to give the acid chloride (**18**). This was immediately dissolved in 100 mL of dichloromethane and cooled to 0 °C. While stirring, [^{14}N]-ammonia gas (99.99%) was introduced into the reaction mixture via an acetone/dry ice condenser. Following the addition, the reaction was allowed to sit overnight and then 150 mL of water was added and the pH was adjusted to 4.0. The aqueous mixture was extracted with ethyl acetate, and the combined organic layers were dried over magnesium sulfate, filtered, and concentrated to give 6.4 g (85% yield) of a white crystalline solid (mp 157–160 °C). The isolated phenol (**19**) was then condensed with 9.7 g of diethyl chlorophosphate as described (Caldwell et al., 1991a) to give 3.2 g (23% yield) of a white crystalline product (**20**) (mp 99–100 °C).

Synthesis of [^{15}N,*phenolic-*^{18}O]*-O,O-Diethyl O-(4-Carbamoylphenyl) Phosphate* (**26**). The procedure leading to the synthesis of compound **26** involved the condensation of [^{15}N,^{18}O]-4-hydroxybenzamide (**25**) and DECP (**6**). The



synthesis of [^{15}N,^{18}O]-4-hydroxybenzamide (**25**) was performed by starting with 0.56 g of [^{18}O]phenol. [^{18}O]Paraoxon (**21**) was synthesized as described above in the synthesis of **12** (except the nitration was conducted with unlabeled ammonium nitrate) and was then hydrolyzed enzymatically to completion with the addition of phosphotriesterase. The oxygen-18-labeled *p*-nitrophenol product was isolated by the organic extraction from an acidic medium to give 0.55 g of **22**. This compound was dissolved in 20 mL of absolute ethanol and reduced to [^{18}O]-4-aminophenol (**23**) with use of 0.12 g of PtO under a hydrogen atmosphere (55 psi). After 30 min the mixture was filtered and the solvent removed to give 0.42 g (68% yield) of the [^{18}O]-4-aminophenol (**23**). With the use of 1.0 g of potassium [^{15}N]cyanide (99%) compound **23** was converted to [^{15}N]-4-cyanophenol (**24**) as previously described for **14**. Oxidation with hydrogen peroxide under alkaline conditions resulted in the synthesis of 0.4 g (52% yield) of **25** (mp 159–163 °C) (see synthesis of **10**). Compound **26** was synthesized by the condensation of 0.22 g of DECP (**6**) with 0.16 g of [^{15}N,^{18}O]-4-hydroxybenzamide (**25**) as described in the similar synthesis of *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate (Caldwell et al., 1991a). Purification by chromatography on florisil (ethyl acetate) afforded 40 mg of a pure product **26** (mp 99–101 °C). The ^{31}P NMR and mass spectra indicated an ^{18}O isotopic content at the phenolic oxygen of 90 and 92%, respectively.



Primary and Secondary Oxygen-18 Isotope Effects. The oxygen-18 isotope effects were determined by the remote isotope label method (O'Leary & Marlier, 1979), which requires the isotope mass ratio in the nitrogen of the remote labeled compound to be very near natural abundance (0.365% ^{15}N/^{14}N). This was accomplished by mixing the [^{15}N,^{18}O]-labeled compound with the ^{14}N-labeled compound (^{15}N-depleted), such that the final mixture attained the natural abundance ratio. This results in a mixture of isotopically labeled compounds where every molecule that possesses an ^{18}O atom also has a ^{15}N label.

Alkaline hydrolysis was performed with 0.1 N potassium hydroxide at 25 °C. Enzymatic hydrolysis was conducted in 20 mM pyrophosphate buffer at pH 8.1. After approximately 50% hydrolysis of 200 μmol of material in 100 mL, the reaction was quenched by lowering the pH to 3 or 4 with concentrated HCl for alkaline hydrolysis or 10 mM dithiothreitol for the enzymatic reaction (K_i = 8.84 μM; Dumas et al., 1989). With enzymatic hydrolysis, the aqueous solutions were passed through an Amicon ultrafiltration apparatus (PM-10 filter) to remove the enzyme and then the pH was carefully adjusted to 9.1 in the case of paraoxon and 10.3 in the case of *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate. The samples were extracted with dichloromethane, dried over magnesium sulfate, filtered, and concentrated to dryness in order to quantitatively separate the residual substrate from the phenolic product. The residual paraoxon was then completely hydrolyzed to *p*-nitrophenol with 0.1 N potassium hydroxide. The aqueous mixture containing *p*-nitrophenol was acidified to pH 3.0 and extracted with dichloromethane until all of the phenol was removed. The aqueous solution containing the product 4-hydroxybenzamide was concentrated to 1 mL in vacuo.

The lone atom of nitrogen in either the residual substrate or phenolic product was isolated in the form of ammonium sulfate according to the procedure developed by Weiss (1990). This procedure involves Kjeldahl digestion and alkaline steam distillation. Oxidation of the ammonia to diatomic nitrogen was accomplished with sodium hypobromite. With paraoxon, the nitro group was first reduced quantitatively to the amine with sodium thiosulfate and sulfuric acid. The isolation of the diatomic nitrogen and the apparatus necessary have been described in detail elsewhere (Weiss, 1990). The mass ratios of the nitrogen were determined with a Finnigan MAT 251 dual-inlet isotope ratio mass spectrometer.

Nomenclature. The isotope effects on the kinetic parameters for the enzymatic reaction are denoted by the nomenclature of Northrop (1977) where $^{18}(V/K)$ represents the ^{18}O isotope effect on V/K. Similarly, ^{18}k represents the ^{18}O isotope effect on an individual chemical step.

Data Processing. Equation 1 was used to calculate the observed isotope effect [$^{15,18}(V/K)$] from the isotopic ratio of ^{15}N/^{14}N in the phenolic product, R_p, after some known extent of hydrolysis, f, and from the isotopic ratio of ^{15}N/^{14}N in the phenolic product, R_o, after 100% hydrolysis.

$$^{15,18}(V/K) = \log (1 - f) / \log (1 - fR_p/R_o) \quad (1)$$

Equation 2 was used to calculate the observed isotope effect from the ^{15}N/^{14}N ratio in the residual triester, R_s, after the partial reaction, and from R_o.

$$^{15,18}(V/K) = \log (1 - f) / \log [(1 - f)(R_s/R_o)] \quad (2)$$

In the remote label method for measuring small heavy atom isotope effects one uses a mixture of a double-labeled material and a depleted material (O'Leary & Marlier, 1979). The double-labeled material has an isotopic label both in the heavy atom remote position and in the discriminating position (the position where one wishes to know the isotope effect). The depleted material has a depleted content of the heavy atom in the remote labeled position but otherwise natural abundance content in other positions, including the discriminating one. The two species are mixed to restore the natural abundance level of the heavy atom in the remote label position. Discrimination between the two species is then caused by the product of the isotope effects in the remote and discrimination positions. As a control, one measures the isotope effect in the remote labeled position with use of natural abundance material. The general equation for calculating the isotope effect

Table I: Primary and Secondary Isotope Effects for the Chemical and Enzyme-Catalyzed Hydrolysis of Paraoxon (I) and *O,O*-Diethyl *O*-(4-Carbamoylphenyl) Phosphate (II)

compound	isotope effect	P^a	n^d	^{18}k
I	1°-chemical[b]	1.0058	1	1.0060
I	1°-enzymatic[b]	1.0017	2	1.0020 ± 0.0004
I	2°-chemical[b]	1.0056	2	1.0063 ± 0.0001
I	2°-enzymatic[b]	1.0016	2	1.0021 ± 0.0004
II	1°-chemical[b,c]	1.023	6	1.027 ± 0.002
II	1°-enzymatic[c]	1.031	3	1.036 ± 0.002
II	2°-chemical[b,c]	1.020	5	1.025 ± 0.002
II	2°-enzymatic[c]	1.0144	3	1.0181 ± 0.0007

[a] These values are the observed isotope effect and are really $^{15,18}(V/K)$ (enzymatic) or $^{15,18}k$ (chemical) values. [b] These values were calculated from eq 1. [c] These values were calculated from eq 2. [d] n equals the number of determinations of each value. The average value was calculated along with the standard error for each type of experiment.

in the discrimination position (for a single heavy atom) is given by

$$^{18}k = [(T^{1/i} - 1)/(1 - T^{1/i}(1 - y)/i)] + 1 \quad (3)$$

where

$$T = (P/R) \; / \; [1 - Q(P/R - 1)] \quad (4)$$

In eqs 3 and 4, P = observed isotope effect with remote label calculated from eqs 1 and 2 and summarized in Table I [$^{15,18}(V/K)$ in this case]; R = isotope effect in remote label position with natural abundance material and also calculated with use of eqs 1 or 2 [$^{15}(V/K)$ in this case] = 1.0007 ± 0.0001 for the alkaline hydrolysis of paraoxon (I) (Hengge & Cleland, 1990) and = 1.0 for the alkaline hydrolysis of *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate (II) and the enzymatic hydrolysis of I and II; i = number of discriminating atoms = 1 in this case; $Q = (1 - b)z/bx \approx z/b$, or the degree to which light material in remote labeled mixture is depleted below natural abundance; b = fraction of double-labeled material in the remote labeled mixture (approximately natural abundance) = fraction of [^{15}N, ^{18}O]-containing phosphotriester in the final mixture in this case = 0.003851 for primary (I), 0.003602 for secondary (I), 0.003630 for primary (II), and 0.003629 for secondary (II); z = fraction of heavy label present in remote labeled position of light material used for remote label mixture = the fraction of ^{15}N in the ^{14}N phosphotriesters in this case = 0.0001 for both I and II; x = fraction of heavy label in remote label position of double-labeled material used for mixing to give the approximate natural abundance = the fraction of ^{15}N in the [^{15}N,^{18}O]-labeled phosphotriesters in this case = 0.99 for both I and II; and y = fraction of heavy discriminating label in double-labeled material = fraction of ^{18}O in the [^{15}N,^{18}O]-labeled phosphotriesters in this case, and where applicable, an average of the ^{18}O isotope content determined from ^{31}P NMR and mass spectrometry was used, = 0.88 for primary (I), 0.80 for secondary (I), 0.89 for primary (II), and 0.82 for secondary (II).

Results

The primary and secondary ^{18}O isotope effects for the chemical and enzymatic hydrolysis of paraoxon and *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate were determined with use of the remote isotope label method. The observed ^{18}O isotope effects calculated from eq 1 or 2 were corrected for incomplete label incorporation using eq 3 and are presented in Table I. The observed isotope effects were determined from the comparative isotopic analysis between either residual phosphotriester, the phenol product, or both after approximately 50% reaction and the initial phosphotriester. The primary and secondary ^{18}O isotope effects for the alkaline hydrolysis of paraoxon are 1.0060 and 1.0063 ± 0.0001, respectively, where only one determination was made for the primary ^{18}O isotope effect. The alkaline hydrolysis of *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate resulted in larger primary and secondary ^{18}O isotope effects of 1.027 ± 0.002 and 1.025 ± 0.002, respectively. The enzymatic hydrolysis of paraoxon gave primary and secondary ^{18}O isotope effects of 1.0020 and 1.0021 ± 0.0004, respectively, where only one determination was made for the primary ^{18}O isotope effect. The enzymatic hydrolysis of *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate gave primary and secondary ^{18}O effects of 1.036 ± 0.002 and 1.018 ± 0.0007, respectively. The contribution of a ^{15}N isotope effect on chemical or enzymatic hydrolysis of these compounds was determined by using the natural abundance material.

Discussion

The primary and secondary ^{18}O isotope effects have been determined for the alkaline and enzymatic hydrolysis of phosphotriester with use of the remote isotope labeling method in an effort to further refine the mechanism and transition-state structure for each reaction. This method has previously been successfully applied to both nonenyzmatic and enzymatic systems (O'Leary, 1977, 1980; O'Leary & Marlier, 1979; Cleland, 1990; Weiss, 1991). However, its use is limited to isotopic measurements with compounds possessing either a nitrogen atom or an isolable CO_2 unit as the remote label. Two different multilabeled compounds, paraoxon and *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate, were synthesized and employed as isotopic probes for the transition-state structure in phosphotriester hydrolysis. These molecules were selected because the alkaline and enzymatic hydrolysis rates of these phosphotriesters differ due to the variance in the pK_a values for the phenolic leaving groups. Furthermore, the rate-limiting steps are distinctly different during the enzymatic hydrolysis of these compounds since the V/K value for paraoxon is governed by the rate of diffusion to the active site of the enzyme, whereas the hydrolysis of *O,O*-diethyl *O*-(4-carbamoylphenyl)phosphate is limited almost entirely by the bond-breaking step (Caldwell et al., 1991a). The ratio of the values for V_{max} and V_{max}/K_m for the enzymatic hydrolysis of paraoxon and *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate are 30 and 48, respectively (Caldwell et al., 1991a).

Isotope Effects on Alkaline Hydrolysis. The primary and secondary ^{18}O isotope effects for alkaline and enzymatic hydrolysis of paraoxon and *O,O*-diethyl *O*-(4-carbamoylphenyl)phosphate are presented in Table I. The ^{18}O secondary isotope effect of 1.0063 for the alkaline hydrolysis of paraoxon indicates that a measurable change in the bond order to the phosphoryl oxygen has occurred in the transition state. The theoretical maximum ^{18}O secondary isotope effect of 1.04 (Cleland, 1986), calculated for the transformation from an initial bond order of 2 to a bond order of unity, suggests a bond order of about 1.85 to the phosphoryl oxygen in the transition state for paraoxon hydrolysis.

The magnitude of the ^{15}N secondary and ^{18}O primary isotope effects suggests that there is also a measurable amount of phenolic oxygen bond cleavage or loosening in the transition state. A normal ^{15}N secondary isotope effect is observed during the hydrolysis of paraoxon (Hengge & Cleland, 1990) because the [^{14}N]-*p*-nitrophenol is slightly more acidic than the ^{15}N-labeled material. This difference in acidity (and reaction rate) is due to the fact that upon ionization of *p*-nitrophenol (or during phosphorus–oxygen bond cleavage) the

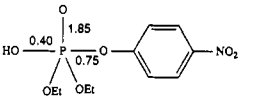
charge is delocalized into the nitro group in the form of a quinoid resonance structure. Although the net bond order to the nitrogen does not change, the resulting carbon–nitrogen double bond is less stiff than the nitrogen–oxygen double bond it replaces and thus the ^{14}N-labeled material is favored relative to ^{15}N. The ^{15}N secondary isotope effect of 1.0007 measured by Hengge and Cleland (1990) is approximately 25% of the ^{15}N isotope effect of 1.0028 seen for the hydrolysis of the dianion of *p*-nitrophenyl phosphate, which relates to complete bond cleavage. These values are thus consistent with a phosphoryl oxygen bond order of about 0.75 in the transition state.

The primary ^{18}O isotope effect of 1.006 is also consistent with this amount of phosphorus–phenolic oxygen bond cleavage in the transition state. If it is assumed that the magnitude of the primary ^{18}O isotope effect is directly proportional to the extent of bond cleavage (assuming the absence of the imaginary frequency factor during the reaction coordinate motion), then for a 25% bond cleavage the maximum calculated ^{18}O primary isotope effect for complete bond cleavage would be about 1.024 for this reaction. This value is reasonably close to the measured equilibrium ^{18}O isotope effect of 1.018 for the deprotonation of *p*-nitrophenol (Rosenberg, 1977).

The *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate is hydrolyzed more slowly by hyroxide than is paraoxon. The primary and secondary ^{18}O isotope effects for the alkaline hydrolysis of this compound are larger than those observed for paraoxon hydrolysis as expected for a somewhat later transition state. The secondary ^{18}O isotope effect for the phosphoryl oxygen is 1.025, which indicates a bond order to the phosphoryl oxygen of approximately 1.4 in the transition state. There is no measurable ^{15}N secondary isotope effect since charge delocalization into the carboxamide moiety is unlikely. The primary ^{18}O isotope effect of 1.027 indicates significant bond cleavage of the phosphorus–phenolic oxygen bond has occurred in the transition state and thereby reduces the likelihood of an intermediate in the reaction mechanism. The substantial difference in magnitude between the primary ^{18}O isotope with this compound and that observed for paraoxon hydrolysis is rationalized by a comparison of the leaving groups where *p*-nitrophenol, unlike 4-hydroxybenazmide, possesses a quinoid resonance form due to the nature of the nitro group. On this basis, the observed ^{18}O primary isotope effect for the alkaline hydrolysis of paraoxon will be diminished relative to that observed for *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate. The higher rate of paraoxon hydrolysis would also suggest a transition state with less phosphorus–phenolic oxygen bond cleavage. Therefore, as the leaving group becomes poorer, the transition state occurs later in the associative mechanism where no distinct intermediate is formed.

Kinetic isotope effects, which provide a relative measure of bond breaking in the transition state, can also be related to the β values from a Brønsted analysis. Qualitatively, β values reflect the amount of charge associated with the leaving group in the transition state and (assuming the lack of acid/base catalysis) can provide a measure of bond making and breaking (Fersht, 1985). For phosphotriester hydrolysis, the β values for the leaving group have been reported ranging from −0.35 to −0.44 (Khan & Kirby, 1970; Caldwell et al., 1991a) with a maximum potential β value of −1.2 (Bromilow et al., 1971) for complete group transfer. Therefore, about one-third of the negative charge is associated with the leaving group in the alkaline hydrolysis of phosphotriesters. This approximation is reasonably consistent with the more quantitative analysis

Scheme III



for the phosphorus–phenolic oxygen bond cleavage in the transition state based on the ^{15}N secondary isotope effect. Since no ^{15}N secondary isotope effect was detected during the hydrolysis of *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate, the extent of cleavage at the phosphorus–phenolic oxygen bond in the transition state cannot be determined directly.

From the ^{18}O and ^{15}N isotope effects for alkaline hydrolysis of paraoxon the transition state can be approximated. If it is assumed that the calculated maximum for the secondary ^{18}O isotope effect for the phosphoryl oxygen is correct and that the bond order of the ethyl esters remains 1 (although some electron donation is probable), we can approximate a bond order of about 0.40 for the attacking hydroxide on paraoxon with the total bond order in the transition state of 5 and the total negative charge of 1 as illustrated in Scheme III. These experimental results suggest a transition-state structure where the associative mechanism for the hydrolysis of these phosphotriesters must occur in a concerted, asynchronous fashion. A change in bond order along the axial plane of the transition state caused by the substitution of a different leaving group is modulated by the phosphoryl oxygen. The more associative mechanism is then anticipated with poorer leaving groups and is reflected in larger secondary ^{18}O isotope effects.

Isotope Effects on Enzyme-Catalyzed Hydrolysis. For an enzymatic reaction, the remote isotope labeling method measures the effect of isotopic substitution on the kinetic parameter V/K. The magnitude of this effect is dependent not only on the structure of the transition state for the actual bond-breaking step but also on the relative magnitude of the partitioning ratio of the enzyme–substrate complex. The minimal mechanism for substrate hydrolysis by the phosphotriesterase is given below in Scheme IV, where the k_3

Scheme IV

$$E \underset{k_2}{\overset{k_1 A}{\rightleftharpoons}} EA \xrightarrow{k_3} EPQ \xrightarrow{k_5} E + \text{products}$$

represents the chemical step for irreversible substrate hydrolysis. It can be shown that the relationship of the isotope effect on V/K [$^{18}(V/K)$] to the isotope effect on the bond-breaking step ($^{18}k_3$) is given by the equation

$$\frac{^{18}k - 1}{^{18}(V/K) - 1} = \frac{k_2 + k_3}{k_2} \qquad (5)$$

Therefore, as the ratio of k_3 to k_2 becomes large, the size of the observed isotope effect on V/K is diminished. The calculated values for k_3/k_2 with the paraoxon and *O,O*,-diethyl *O*-(4-carbamoylphenyl) phosphate are 28 and 0.08, respectively (Caldwell et al., 1991a).

The intrinsic isotope effects for the enzymatic hydrolysis of paraoxon are difficult to calculate because of the very small magnitude for the primary and secondary ^{18}O effects of 1.0021 and 1.0020, respectively, and the very large partitioning ratio of k_3/k_2. However, the minimal size of these isotope effects indicates that the chemical step is not rate-limiting in the enzymatic hydrolysis of paraoxon.

The observed primary and secondary isotope effects of V/K for the enzymatic hydrolysis of *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate are 1.036 and 1.018, respectively. These values are significantly larger than those observed for paraoxon hydrolysis because the relative values of k_2 and k_3 are much

more favorable for obtaining intrinsic isotope effects as predicted by eq 5. The intrinsic primary and secondary oxygen-18 isotope effects are calculated from eq 5 to be 1.039 and 1.019, respectively.

The extent of phosphorus–phenolic oxygen bond cleavage in the transition state for enzymatic hydrolysis can be estimated from the relative size of the intrinsic primary ^{18}O isotope effect and the β value for the substrate hydrolysis. The β value of −1.8 for the enzymatic reaction is at the extreme of values observed for nonenzymatic reactions. This suggests that a large amount of negative charge is associated with the phenolic leaving group and consequently implies a very late transition state. However, Jencks (1971) has pointed out that the size of the Brønsted β values may be enhanced in enzymatic systems because of the hydrophobic nature of the active site of many enzymes. The size of the maximum primary ^{18}O isotope effect with this leaving group that would be expected for phosphotriester hydrolysis is unknown. However, the ^{18}O equilibrium isotope effects for the deprotonation of water and phenol based on O–H stretching vibrations have been estimated to be approximately 1.039 (Rosenberg, 1977). The observed intrinsic primary isotope effect for *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate hydrolysis is very close to this extreme value, and thus both the Brønsted analysis and the primary oxygen-18 isotope effect for enzymatic hydrolysis of *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate suggest that the phosphorus phenolic bond is largely broken in the transition state.

The secondary isotope effect for *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate hydrolysis is also consistent with a transition state that is very product-like. The calculated intrinsic isotope effect of 1.019 suggests that the bond order has changed from 2 to approximately 1.53 in the transition state. However, it cannot be determined to what extent the interaction of the phosphoryl oxygen with the active site zinc in the transition state will have on the magnitude of the secondary ^{18}O isotope effect.

Summary. It is evident from the primary and secondary ^{18}O isotope effects that the transition-state structures for both alkaline and enzymatic hydrolysis are similar, where the reaction proceeds through an associative-type mechanism without any direct evidence for the formation of a phosphorane intermediate. The ^{18}O isotope effects strongly support the kinetic interpretation for the enzymatic hydrolysis of paraoxon and *O,O*-diethyl *O*-(4-carbamoylphenyl)phosphate. For paraoxon, the magnitude of the observed isotope effects is consistent with a step other than bond cleavage as partially rate-limiting. The similarity between the isotope effects for alkaline and enzymatic hydrolysis of *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate suggests the chemical step as rate-determining in catalysis by the phosphotriesterase. However, the relatively larger primary isotope effects and the larger β value for the enzymatic as compared to alkaline hydrolysis of *O,O*-diethyl *O*-(4-carbamoylphenyl) phosphate suggests that the transition state for the enzymatic reaction is more product-like. The relatively smaller secondary isotope effect for the enzymatic reaction suggests an earlier transition state relative to the alkaline reaction but may be explained by potential interactions of the phosphoryl oxygen with active site residue(s) or the zinc atom.

One theoretical interpretation of phosphoryl transfer predicts that the phosphoryl group in the transition state will have a single negative charge and the bonding to the phosphorus ligands will be adjusted to meet this requirement (Cleland, 1990). Although more systems involving phosphoryl transfer are required to further substantiate this theoretical prediction, the interpretation of the ^{18}O isotope effects for the alkaline and enzymatic phosphotriester hydrolysis are consistent with this conclusion.

Registry No. **1**, 589-57-1; **3**, 134458-27-8; **4**, 134458-28-9; **5**, 134458-29-0; **9**, 62-53-3; **10**, 1739-18-0; **11**, 134458-30-3; **12**, 134458-31-4; **15**, 134458-32-5; **16**, 134458-33-6; **17**, 2345-34-8; **18**, 27914-73-4; **19**, 619-57-8; **20**, 6376-03-0; **25**, 134458-34-7; **26**, 134458-35-8; PhOH, 108-95-2; ^{18}O, 14797-71-8; paraoxon, 311-45-5; phosphotriesterase, 9047-01-2.

References

Benkovic, S. J., & Schray, K. J. (1973) *Enzymes (3rd Ed.)* *8*, 201–238.

Bromilow, R. H., Khan, S. A., & Kirby, A. J. (1971) *J. Chem. Soc. B*, 1091–1097.

Bruice, T. C., & Benkovic, S. J. (1966) in *Bioorganic Mechanisms*, pp 1–103, W. A. Benjamin, Inc., New York.

Caldwell, S. R., Newcomb, J. R., Schlecht, K,. & Raushel, F. M. (1991a) *Biochemistry* (preceding paper in this issue).

Caldwell, S. R., Weiss, P. M., Cleland, W. W., & Raushel, F. M. (1991b) *J. Am. Chem. Soc. 113*, 730–732.

Cleland, W. W. (1990) *FASEB J. 4*, 2899–2905.

Dumas, D. P. (1989) Ph.D. Dissertation, Texas A&M University, College Station, TX.

Dumas, D. P., Caldwell, S. R., Wild, J. R., & Raushel, F. M. (1989) *J. Biol. Chem. 264*, 19659–19665.

Fersht, A. (1985) in *Enzyme Structure and Mechanisms*, 2nd ed., pp 148–149, W. H. Freeman and Co., New York.

Hengge, A. C., & Cleland, W. W. (1990) *J. Am. Chem. Soc. 112*, 7421–7422.

Hershlag, D., & Jencks, W. P. (1987) *J. Am. Chem. Soc. 109*, 4665–4674.

Jencks, W. P. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 1–11.

Khan, S. A., & Kirby, A. J. (1970) *J. Chem. Soc. B*, 1172–1182.

Knowles, J. R. (1980) *Annu. Rev. Biochem. 49*, 877–919.

Noller, C. R. (1943) in *Organic Syntheses Collective* (Blatt, A. H., Ed.) Vol. 2, pp 586–587, John Wiley and Sons, Inc., New York.

Northrop, D. B. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) p 122, University Park Press, Baltimore, MD.

O'Leary, M. H. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) p 233, University Park Press, Baltimore, MD.

O'Leary, M. H. (1980) *Methods Enzymol. 64*, 83–104.

O'Leary, M. H., & Marlier, J. F. (1971) *J. Am. Chem. Soc. 101*, 3300–3306.

Pichas, S., Sadeh, D., & Samuel, D. (1965) *J. Phys. Chem. 69*, 2259–2264.

Pichat, L., Baret, C., & Audinot, M. (1956) *Bull. Soc. Chim.* 151–156.

Reynolds, M. A., Gerlt, J. A., Demou, P. C., Oppenheimer, N. J., & Kenyon, G. L. (1983) *J. Am. Chem. Soc. 105*, 6475–6481.

Rosenberg, S. (1977) Ph.D Dissertation, Department of Chemistry, University of California, Berkeley, CA.

Weiss, P. M. (1991) in *Isotope Effects in Enzymatic Reactions* (Cook, P., Ed.) Chapter 11, CRC Press, Boca Raton, FL, (in press).

Westheimer, F. H. (1981) *Chem. Rev. 81*, 313–326.