strong evolutionary relationship (divergent or convergent) in the origin of these enzymes.

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Mechanism and Stereochemical Course at Phosphorus of the Reaction Catalyzed by a Bacterial Phosphotriesterase[†]

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ABSTRACT: The reaction mechanism for the phosphotriesterase from *Pseudomonas diminuta* has been examined. When paraoxon (diethyl 4-nitrophenyl phosphate) is hydrolyzed by this enzyme in oxygen-18-labeled water, the oxygen-18 label is found exclusively in the diethyl phosphate product. The absolute configurations for the (+) and (−) enantiomers of *O*-ethyl phenylphosphonothioic acid have been determined by X-ray diffraction structural determination of the individual crystalline 1-phenylethylamine salts. The (+) enantiomer of the free acid corresponds to the R_P configuration. The R_P enantiomer of *O*-ethyl phenylphosphonothioic acid has been converted to the S_P enantiomer of EPN [*O*-ethyl *O*-(4-nitrophenyl) phenylphosphonothioate]. (S_P)-EPN is hydrolyzed by the phosphotriesterase to the S_P enantiomer of *O*-ethyl phenylphosphonothioic acid. The enzymatic reaction therefore proceeds with inversion of configuration. These results have been interpreted as an indication of a single in-line displacement by an activated water molecule directly at the phosphorus center of the phosphotriester substrate. (R_P)-EPN is not hydrolyzed by the enzyme at an appreciable rate.

A broad variety of organophosphate triesters have been used as pesticides, insecticides, and other biological control agents. First discovered in Germany in the 1930s (Schrader, 1963), some modern organophosphates are considered to be among the safest of all chemical pesticides. However, many compounds known to be mammalian neurotoxins (McEwen & Stephenson, 1979) are also included in this class of compounds. In general, these pesticides are considered to be chemically reactive materials that do not persist in the soil for long periods of time. The lack of persistence in the soil has been attributed to their susceptibility to microbial hydrolysis (Munnecke, 1981). The microbial degradation of organophosphates such as paraoxon (diethyl 4-nitrophenyl phosphate) or the related phosphorothioate (e.g., parathion) has been defined in several

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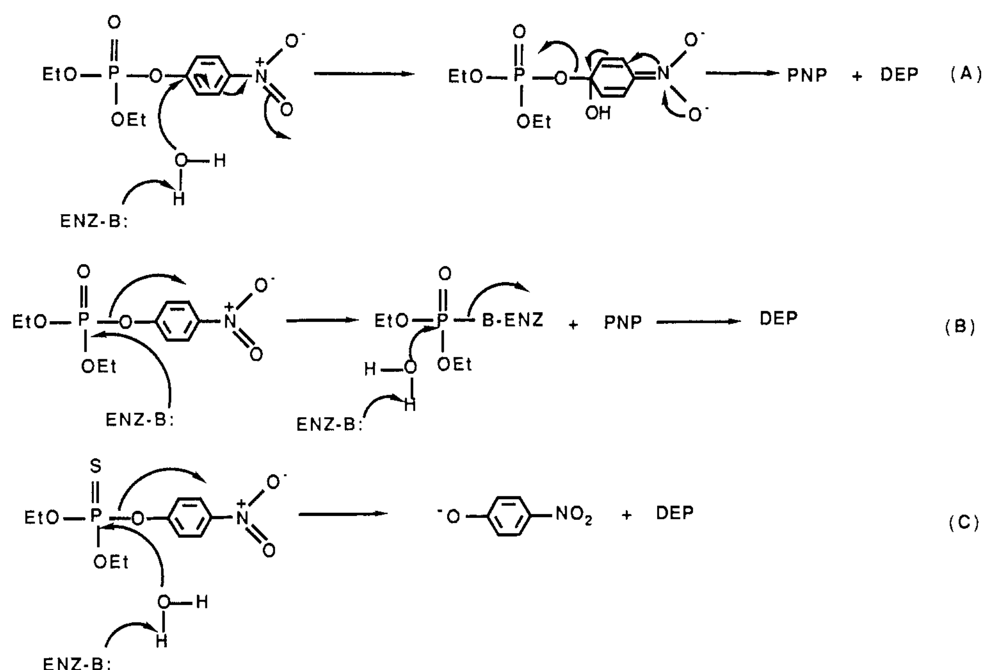
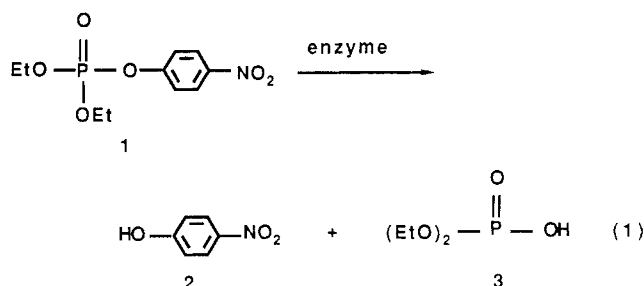
Scheme I



microbial systems (Munnecke & Hsieh, 1976).

Phosphotriesterase (organophosphorus acid anhydrase; parathion hydrolase), an enzyme found in certain native soil bacteria (*Pseudomonas diminuta* and *Flavobacterium* sp.), has been shown to degrade organophosphates such as paraoxon and parathion (Munnecke, 1976). These enzymes have been shown to be encoded on plasmid-borne genes (*opd*) in both bacteria (Serdar & Gibson, 1985) and constitutively expressed as membrane-associated complexes (Brown, 1980; McDaniel, 1985). This degradation occurs by hydrolysis as shown in eq 1. In this reaction diethyl 4-nitrophenyl phosphate (paraoxon, **1**) is hydrolyzed to produce 4-nitrophenol (**2**) and diethyl phosphate (**3**).

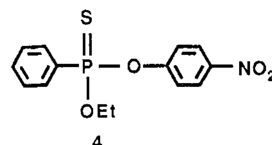


The mechanism of hydrolysis may be viewed as proceeding by one of three possible routes (Scheme I). The first of these three mechanisms (A) is essentially a nucleophilic aromatic substitution. The reaction would entail nucleophilic attack by water on the C-1 carbon of the phenyl ring. The resulting intermediate would be stabilized by the resonance capability of the nitro group at C-4. Subsequent C–O bond cleavage would yield the two products.

The second and third mechanisms may be described as variant S_N2 mechanisms that differ by the presence of a covalent enzyme–product intermediate (mechanism B). In this mechanism there are two in-line displacement reactions at the phosphorus center. The first would occur by a nucleophilic attack at the phosphorus atom by the enzyme. This would be followed by expulsion of 4-nitrophenol to produce the covalent enzyme–product complex. Subsequent direct nucleophilic attack by water at the phosphorus would yield diethyl phosphate and the free enzyme. This mechanism would proceed via two in-line steps, with independent inversions of configuration; thus, the overall stereochemical course of the reaction would be a retention of configuration at phosphorus (Knowles, 1980).

The third mechanism (C) involves a single displacement reaction. In this mechanism a base at the active site of the enzyme directly activates a water molecule. This activated water molecule then would attack the phosphorus atom, yielding diethyl phosphate and 4-nitrophenol. Due to the single in-line step in this mechanism, the reaction would be expected to result in an overall inversion of configuration at phosphorus (Knowles, 1980).

These three mechanisms can be experimentally distinguished from each other. Mechanism A would predict that if the reaction is conducted in the presence of oxygen-18-labeled water, the label would appear in the phenol. In contrast, the alternative mechanisms would predict that the oxygen-18 would be found exclusively in the diethyl phosphate. Mechanisms B and C can be distinguished from each other by determining the stereochemical course of the reaction at the phosphorus center. Mechanisms A and B would predict net retention while mechanism C would predict net *inversion* of configuration. We have used a chiral substrate [EPN[1] (**4**)]



to demonstrate that the phosphotriesterase from *P. diminuta* catalyzes the hydrolysis with inversion of configuration and thus only mechanism C would be appropriate.

Materials and Methods

Materials. Tryptone and yeast extract were obtained from Difco Laboratories. Dicylcohexylamine was distilled from barium oxide prior to use. All other solvents and reagents were

[1] Abbreviations: EPN, *O*-ethyl *O*-(4-nitrophenyl) phenylphosphonothioate; DMSO, dimethyl sulfoxide.

obtained and used directly from either Sigma or Aldrich.

Methods. Proton and carbon-13 NMR spectra were obtained on a Varian XL-200E spectrometer. Phosphorus-31 spectra were obtained on Varian XL-200 and XL-400 spectrometers. Specific rotations were obtained by using a Perkin-Elmer 241 polarimeter. Melting points were determined by using a Fisher-Johns melting point apparatus.

X-ray Structure Determination. The intensity data for the X-ray structure determination of the chiral salts of *O*-ethyl phenylphosphonothioic acid were collected on a Nicolet R3m diffractometer, maintained by the X-ray crystallography laboratory at Texas A&M University, employing the ω scanning technique. A total of 1214 unique reflections ($F \geq 3.0\sigma F$) were employed by full-matrix least-squares refinement on F to refine 163 parameters to convergence. Final R = 0.118, wR = 0.117, and s = 1.83. The chirality of the phosphorus centers were determined by comparison of those to the known chirality of the ammonium cations. Two unique cation–anion pairs and one molecule of ethyl acetate crystallized in the noncentrosymmetric space group $P4_12_12$ [$C_{16}H_{22}NO_2PS\cdot{}^1/_2(C_4H_8O_2)$, M_r 367.4, tetragonal, a = 13.758 (4) Å, c = 41.06 (1) Å, V = 7766 Å^3, μ = 2.11 cm^{-1}].

Purification of Phosphotriesterase. The phosphotriesterase from *P. diminuta* is a membrane-associated enzyme capable of hydrolyzing organophosphotriesters in intact whole cells (Chiang et al., 1985). Sonic disruption or explosive decombustion of cells in a French pressure cell resulted in 95% of the enzymatic activity being located in the particulate fraction of the cell (removed by 10000*g* centrifugation). Most of the activity could be removed as a nonparticulate fraction by treating whole cells with 1 M NaCl and 1% Triton X-100. Partially purified phosphotriesterase was obtained in the following manner. Five liters of *P. diminuta* was grown in a rich broth at 25 °C under aerobic conditions. The broth contained 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter. The cells were harvested at late log phase and resuspended in 50 mM triethanolamine buffer (5 mL/g of cell paste) at pH 9.0. The buffer contained 1 M NaCl and 1% Triton X-100. The whole cells were removed by centrifugation (10000*g* for 10 min). The suspension was sonicated (by using a Heat Systems–Ultrasonics Model 225R) 5 times in 20-s pulses at a high power setting. After centrifugation (using a Sorvall centrifuge, Model RC-5B) at 30000*g* for 60 min, the supernatant liquid was decanted and stored at 4 °C until needed.

Synthesis of Racemic O-Ethyl Phenylphosphonothioic Acid. This molecule was synthesized by a modification of the method of Steurbaut et al. (1975). A three-necked, 50-mL round-bottomed flask was charged with 10 mL of benzene and 6.53 g (4.8 mL, 30.9 mmol) of phenylphosphonothioic dichloride. The flask was fitted with a thermometer and placed under argon. The solution was then heated to 40 °C. When this temperature had been reached, a solution containing 1.5 g (1.99 mL, 34.0 mmol) of ethanol in 5.96 mL (34.0 mmol) of triethylamine was added in a dropwise manner. When the triethylamine solution had been added, the reaction mixture was heated to 60 °C and stirred at this temperature for 2 h. The reaction was quenched by addition of 10 mL of ice water. The two phases were separated, and the aqueous phase was extracted once with benzene. The organic phases were then combined and placed in a 100-mL round-bottomed flask. Sodium hydroxide (30 mL of a 5 N solution) was added, and the two-phase mixture was stirred at room temperature overnight. The two phases of the mixture were then separated, and the aqueous phase was acidified to a pH <2 with 50% H_2SO_4. The crude phosphonothioic acid was isolated by extraction with ether. The ether layers were combined, dried over magnesium sulfate, and concentrated. The crude acid was then purified, according to the method of Nomeir and Dauterman (1979). The crude phosphonothioic acid was dissolved in 20 mL of a 1:1 solution of ether and petroleum ether. This solution was stirred at room temperature while a solution of 5.96 mL (30.9 mmol) of dicyclohexylamine, in 5 mL of the ether/petroleum ether solution, was added in a dropwise manner. During the addition a white precipitate formed. This two-phase mixture was stirred at room temperature for 0.5 h and then filtered. The dicyclohexylammonium salt was recrystallized from ethyl acetate to give 7.61 g (64%) of a white crystalline solid that melted at 137–141 °C (lit. 139–143 °C). The acid was stored as the dicyclohexylammonium salt and was liberated in situ as needed [^{1}H NMR (200 MHz, DMSO-d_6) 1.15 (t, 3 H, OCH_2CH_3), 1.0–2.1 (m, 22 H, cyclohexyl protons), 3.6–3.9 (m, 2 H, OCH_2CH_3), 7.25–7.4 (m, 3 H, aromatic protons), 7.6–7.85 (m, 2 H, aromatic protons), 8.9 ppm (br s, 2 H, acidic protons)].

Synthesis of (R)-1-Phenylethylammonium (S_P)-O-Ethyl Phenylphosphonothioate. Dicyclohexylammonium *O*-ethyl phenylphosphonothioate (3.0 g, 7.83 mmol) was suspended in 25 mL of ether. This mixture was stirred at room temperature while 20 mL of a 1 N solution of NaOH was added. This three-phase mixture was stirred at room temperature until all of the dicyclohexylammonium salt had been consumed, and then the reaction mixture was stirred for an additional $^1/_2$ h. The two phases were then separated, and the aqueous phase was extracted once with ether. The aqueous phase was then cooled to 0 °C and acidified to a pH <2 with 50% sulfuric acid. The acidic aqueous solution was extracted twice with 20 mL of ether, and the ether layers were combined, washed twice with water, dried over magnesium sulfate, and concentrated. The liberated phosphonothioic acid was dissolved in 20 mL of a 1:1 solution of ether and petroleum ether. This colorless solution was stirred at room temperature while a solution of $^1/_2$ equiv of (*R*)-1-phenylethylamine (0.54 mL, 3.91 mmol) in 2 mL of the ether/petroleum ether solution was added in a dropwise fashion. This solution was stirred at room temperature for 24 h, during which time a white precipitate formed. The reaction mixture was filtered and the supernatant liquid set aside for later use (see below). The chiral nonracemic salt was collected and purified by recrystallization from ethyl acetate to yield 0.406 g (36%). The melting point range was found to be 117–121 °C [^{1}H NMR (200 MHz, $CDCl_3$) 1.09 (t, 3 H, OCH_2CH_3), 1.47 (d, J = 8 Hz, 3 H, CH_3), 3.43–3.66 (m, 1 H, diastereotopic proton of methylene group), 3.72–3.92 (m, 1 H, diastereotopic proton of methylene group), 4.06 (q, 1 H, benzylic methine proton), 7.1–7.4 (m, 8 H, aromatic protons), 7.55–7.71 (dd, J = 1.8 and 16 Hz, aromatic protons ortho to the carbon attached to phosphorus), 8.75 ppm (br s, acidic protons); ^{13}C NMR (50 MHz, $CDCl_3$) 16.09 (d, J = 7 Hz, OCH_2CH_3), 21.59 (CH_3), 51.44 (benzylic methine), 61.73 (d, J = 5Hz, OCH_2CH_3), 126.96–140.55 ppm (12 lines, aromatic carbons)].

Synthesis of (S)-1-Phenylethylammonium (R_P)-O-Ethyl Phenylphosphonothioate. The mother liquor from the previous reaction [containing approximately 3.91 mmol of (R_P)-*O*-ethyl phosphonothioic acid] was concentrated and redissolved in 10 mL of ether. Any unreacted (*R*)-1-phenylethylamine was removed by treatment of the mother liquor with an excess (10 mmol) of a 1 N NaOH solution. This two-phase mixture was stirred for 10 minutes at room temperature. The layers were

allowed to separate, and the aqueous phase was extracted once with ether, cooled to 0 °C, and acidified to a pH <2 using 50% sulfuric acid. The resulting solution was extracted twice with ether. The ether extracts were combined, washed twice with water, dried over magnesium sulfate, and concentrated. The phosphonothioic acid was then dissolved in 20 mL of a 1:1 solution of ether and petroleum ether. This colorless solution was stirred at room temperature while a solution of 1 equiv of (*S*)-1-phenylethylamine (0.504 mL, 3.91 mmol, in 2 mL of the ether/petroleum ether solution) was added in a dropwise fashion. This solution was stirred at room temperature for 24 h, during which time a white precipitate formed. The precipitate was removed by filtration and recrystallized from ethyl acetate to yield 0.851 g (67%). The melting point range was found to be 117–120 °C [^{1}H NMR (200 MHz, $CDCl_3$) 1.09 (t, 3 H, OCH_2CH_3), 1.47 (d, J = 8 Hz, 3 H, CH_3), 3.43–3.66 (m, 1 H, diastereotopic proton of methylene group), 3.72–3.92 (m, 1 H, diastereotopic proton of methylene group), 4.06 (q, 1 H, benzylic methine proton); 7.1–7.4 (m, 8 H, aromatic protons), 7.55–7.71 (dd, J = 1.8 and 16 Hz, aromatic protons ortho to the carbon attached to phosphorus), 8.75 ppm (br s, acidic protons)].

Synthesis of (R_P)- *and* (S_P)-*EPN*. Both (R_P)- and (S_P)-EPN were made from their respective salt precursors in a two-step process as follows. The chiral nonracemic amine salts (0.2 g, 0.619 mmol) were suspended in 1-mL volumes of ether in 5-mL round-bottomed flasks. These suspensions were cooled to 0 °C, and 0.619 mL of a 1 N NaOH solution was added in a dropwise manner. The reaction mixtures were stirred at 0 °C until the amine salts had been consumed. The reaction mixtures were then diluted with 20 mL of a 1:1 mixture of ether and water. The layers were separated and the aqueous phases extracted once with ether. The aqueous phases were then recooled to 0 °C, acidified to a pH <2 with 50% sulfuric acid, and extracted twice with 10 mL of ether. The ether layers were combined, washed twice with water, dried over magnesium sulfate, and concentrated to yield 0.090 g (0.44 mmol, 72%) of the crude acid. These materials were used without further purification. An oven-dried, argon-purged, 5-mL round-bottomed flask was charged with 0.092 g (0.44 mmol) of PCl_5 and 0.5 mL of CCl_4. Each suspension was placed under argon and cooled to −15 °C. The crude phosphonothioic acid was dissolved in 0.5 mL of CCl_4 and added to the PCl_5 suspension in a dropwise manner. During this addition, care was taken to maintain the temperature at −15 °C. The reaction mixture was stirred at −15 °C for an additional $^1/_2$ h and then filtered. The solution was concentrated, and the residue was dissolved in 0.5 mL of dimethoxyethane (DME). This solution was then added, dropwise at room temperature, to a stirred suspension of 0.072 g (0.44 mmol) of sodium 4-nitrophenoxide in DME. This yellow suspension was stirred, under argon, at room temperature for 24 h. The reaction mixture was concentrated, and the residue was dissolved in 10 mL of $CHCl_3$. This yellow solution was washed 4 times with 10 mL of a 5% $NaHCO_3$ solution, and it was washed twice with 10 mL of water. The organic phase was dried over magnesium sulfate, filtered, and chromatographed on Florisil. The chiral EPN isomers were eluted with 10% ethyl acetate in hexanes to give 0.059 g (41%) [^{1}H NMR (200 MHz, $CDCl_3$) 1.4 (t, 3 H, OCH_2CH_3), 4.2–4.4 (m, 2 H, OCH_2CH_3), 7.25 (dd, J = 1 and 8 Hz, 2 H, protons at the 2- and 6-positions of the 4-nitrophenoxy group), 7.45–7.7 (m, 3 H, aromatic protons), 7.95–8.1 (m, 2 H, aromatic protons), 8.2 ppm (d, J = 8 Hz, protons at the 3- and 5-positions of the 4-nitrophenoxy group)].

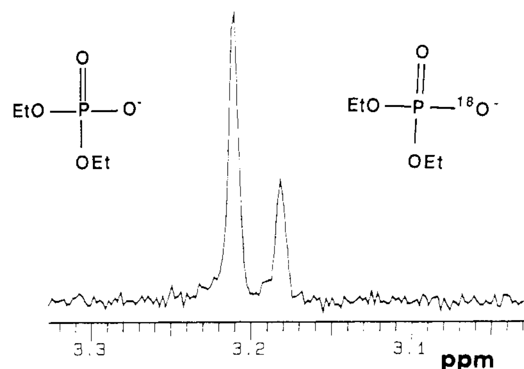


FIGURE 1: Phosphorus-31 NMR of diethyl phosphate and oxygen-18-labeled diethyl phosphate. Paraoxon was hydrolyzed by the triesterase in the presence of 30% oxygen-18-labeled water. The upfield resonance is due to the diethyl phosphate containing 1 atom of oxygen-18.

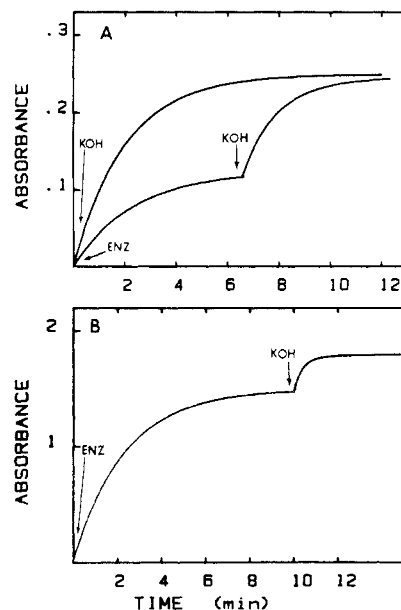
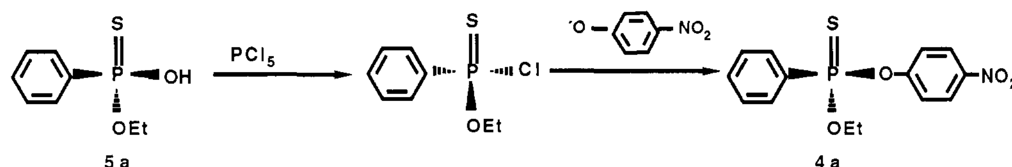


FIGURE 2: (A) Time course for the hydrolysis of racemic EPN (0.014 mM) by the enzyme and 1 N KOH. (B) Hydrolysis of (S_P)-EPN (0.10 mM) by the enzyme.

Preparation of (S_P)-*EPN Hydrolysis Products for NMR Analysis. Method A*: *Enzymatic Hydrolysis.* (S_P)-EPN (25 mg, 0.077 mmol) was dissolved in 5 mL of methanol. This solution was diluted to provide 100 mL of a 35% (v:v) methanol/water solution. This solution was buffered to pH 10.0 by using 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer. Phosphotriesterase (0.5 mL) was added, and the reaction mixture was stirred continuously at room temperature. The reaction was monitored by removing aliquots and measuring the absorbance at 400 nm until the reaction was complete. When the reaction was complete, the methanol was removed in vacuo. The residual aqueous solution was cooled to 0 °C and then acidified to a pH <2 with 50% sulfuric acid. This acidic solution was extracted twice with diethyl ether. The ether extractions were combined, washed twice with water, dried over $MgSO_4$, filtered, and concentrated. The residue was diluted with 5 mL of a 1:1 ether/petroleum ether solution. This solution was stirred at room temperature while a 3-fold molar excess of (*S*)-1-phenylethylamine, in 1 mL of a 1:1 ether/petroleum ether solution, was added dropwise. The solution turned yellow immediately and was stirred at room temperature for 24 h. At the end of this time, the solution was concentrated. The residue was dissolved in 3 mL of $CDCl_3$ and analyzed directly by ^{31}P NMR.

Scheme II



Method B: KOH Hydrolysis. This hydrolysis was performed in accordance with the method of Dudman and Benkovic (1977). (S_P)-EPN (25 mg) was dissolved in 2 mL of acetonitrile, and an excess (1 mL) of 1 N KOH was added. The resulting yellow solution was stirred at room temperature for 1 h. The acetonitrile was removed in vacuo, and the remaining aqueous solution was acidified to a pH <2 with sulfuric acid. The acidic solution was then extracted twice with ether. The ether phases were combined, washed twice with water, dried over magnesium sulfate, filtered, and concentrated. The residue was then dissolved in 2 mL of a 1:1 solution of ether and petroleum ether. A 3-fold excess of (*S*)-1-phenylethylamine was added, and the reaction was stirred at room temperature for 24 h. The solution was concentrated, and the residue was dissolved in 3 mL of $CDCl_3$ and analyzed directly by ^{31}P NMR.

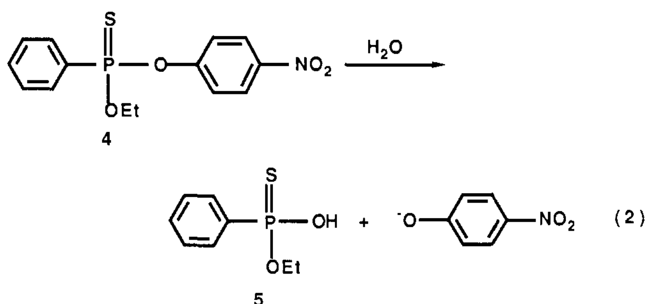
Kinetics for Enzymatic Hydrolysis of Paraoxon and (S_P)-EPN. The kinetic constants V_{max} and V/K were determined for (S_P)-EPN and paraoxon. Owing to the low solubility of EPN in water, both samples were analyzed in 33% methanol in water. The measurements for both compounds were monitored at 400 nm on a Gilford 260 UV/vis spectrometer using a Cole-Parmer Model 8373-10 strip chart recorder. The assays were run at pH 10.0 with 50 mM CAPS buffer at 25 °C. A constant temperature was maintained by using a Neslab refrigerated circulating water bath, Model RTE-9.

Results and Discussion

Mechanism of the Bacterial Phosphotriesterase Reaction. The three limiting mechanisms for the enzymatic hydrolysis of activated phosphotriesters such as paraoxon are indicated in Scheme I. Mechanism A involves the cleavage of the carbon–oxygen bond and can therefore be distinguished from mechanisms B and C. If the hydrolysis reaction is conducted in oxygen-18-labeled water, mechanism A would predict that the label would appear in the 4-nitrophenol, whereas mechanisms B and C would predict that the label would appear only in the diethyl phosphate. These three mechanisms can also be differentiated by a determination of the stereochemical course of the reaction at the phosphorus center. Mechanism A and B would predict retention of configuration while mechanism C would predict inversion of configuration.

Carbon–Oxygen versus Phosphorus–Oxygen Bond Cleavage. A sample of paraoxon was hydrolyzed enzymatically in a mixture of ^{16}O- and ^{18}O-labeled water. The initial oxygen-18 content was 30%. The results of this enzymatic hydrolysis reaction were analyzed by examination of the ^{31}P NMR spectrum of the diethyl phosphate product. Nucleophilic attack, by water, at C-1 of the phenyl ring (mechanism A) would lead to production of diethyl phosphate containing only oxygen-16. On the other hand, nucleophilic attack by water directly at the phosphorus center would result in a mixture of labeled and unlabeled diethyl phosphate. Since ^{18}O has a slight shielding effect on phosphorus, the ^{31}P NMR spectrum of this hydrolysis product would consist of two lines, in about a 2.3:1 ratio (Cohn & Hu, 1978). The smaller of these two lines would be expected to be slightly upfield relative to the more intense line. Examination of Figure 1 shows that there are indeed two resonances for the diethyl phosphate. The two lines are separated by 0.023 ppm and are in the expected ratio of 2.3:1. This experiment clearly indicates that the hydrolysis reaction occurs with phosphorus–oxygen bond cleavage. Mechanism A can thus be eliminated from further consideration.

Stereochemistry of Hydrolysis Reaction. In order to distinguish between mechanisms B and C of Scheme I, the stereochemical course at phosphorus must be determined. In order to address this issue, two related problems must first be solved. First, a chiral substrate of known configuration at the phosphorus center must be synthesized and subsequently hydrolyzed by the enzyme. Second, a method needs to be developed to determine the chirality of the hydrolysis product. Both of these problems have been solved with the use of the commercial insecticide EPN (**4**), a potential chiral substrate for the bacterial phosphotriesterase reaction. This compound was utilized because both enantiomers could be synthesized separately with high enantiomeric excess according to previously published procedures (Nomeir & Dauterman, 1979). However, the absolute configurations of the isomers were not known when these experiments were initiated. Moreover, the product of the hydrolytic reaction, *O*-ethyl phenylphosphonothioic acid, would also be chiral because it has four different substituents attached to be phosphorus center (see eq 2). Therefore, we would not have to utilize oxygen-18 and/or oxygen-17 isotopes to induce chirality in the product.



Hydrolysis of Racemic EPN (**4**). Prior to using EPN (**4**) to determine the mechanism for triester hydrolysis by the phosphotriesterase, it was necessary to assess the ability of the enzyme to hydrolyze one or both enantiomers of EPN. Figure 2A shows a comparison of EPN hydrolysis by the enzyme versus hydrolysis using KOH. This figure shows that enzymatic hydrolysis consumes only about 55% of the total EPN. KOH was added to complete the hydrolysis of the remaining EPN. Thus, it was determined that only one enantiomer of EPN is a substrate of the bacterial phosphotriesterase, though at this point the identity of that enantiomer was unknown.

Resolution of (R_P)- and (S_P)-O-Ethyl Phenylphosphonothioic Acid (**5**). In order to synthesize optically pure EPN of known configuration, both enatiomers of *O*-ethyl phenylphosphonothioic acid (**5**) must be isolated and their absolute configurations determined. Racemic **5** was synthesized according to the procedure of Steurbaut et al. (1975) and was

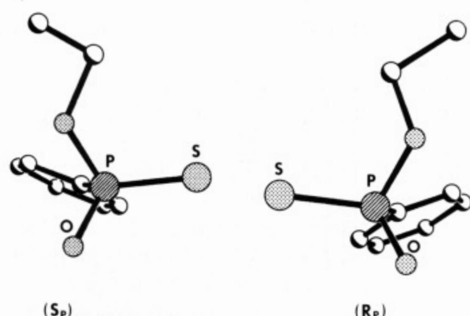


FIGURE 3: X-ray structures of (*R*)- and (*S*)-*O*-ethyl phenylphosphonothioic acid.

purified as the dicyclohexylammonium salt. This salt was treated with hydroxide to release the amine. Acidification of the aqueous phase gave the purified acid, which was treated with $^1/_2$ equiv of (*R*)-1-phenylethylamine. Nomeir and Dauterman (1979) have shown that the *R* amine precipitates preferentially with the (−) enantiomer of **5**, and that the *S* amine precipitates with the (+) enantiomer of **5**. This amine salt was separated, and the residual acid was treated with (*S*)-1-phenylethylamine. In this way **5** was separated into its (+) and (−) enantiomers. However, the absolute configuration of each enantiomer was still unknown.

X-ray Structural Analysis of (R_p)- *and* (S_P)-*O-Ethyl Phenylphosphonothioic Acid.* The absolute configuration of the phosphorus centers for both the (+) and (−) enantiomers of *O*-ethyl phenylphosphonothioic acid was determined by X-ray diffraction analysis of the isolated 1-phenylethylamine salts. An X-ray structure for both enantiomers was determined separately. This analysis clearly shows that the (+) enantiomer is of the R_P configuration and the (−) enantiomer is of the S_P configuration. The structures of both enantiomers are indicated in Figure 3. The atomic coordinates for the S_P isomer have been included with the supplementary material (see paragraph at end of paper regarding supplementary material).

Synthesis of (S_P)-*EPN.* Scheme II shows the two-step procedure used to form (S_P)-EPN (**4a**) from (R_P)-*O*-ethyl phenylphosphonothioic acid (**5a**). Each step in sequences similar to this has been shown to proceed via inversion of configuration at phosphorus (Michalski & Mikolajczyk, 1966). Thus, there is overall retention of configuration in the conversion of the R_P isomer of *O*-ethyl phenylphosphonothioic acid to the S_P isomer of EPN.

Enzymatic Hydrolysis of (S_P)-*EPN.* Both of the enantiomers of EPN were tested as substrates for the bacterial phosphotriesterase. Only the S_P enantiomer was utilized at an appreciable rate by this enzyme. Shown in Figure 2B is the hydrolytic time course when the S_P enantiomer of EPN is used as a substrate. This time course clearly indicates that all of the EPN is hydrolyzed by the enzyme. Kinetic data were obtained for (S_P)-EPN by using paraoxon as a reference standard. Analysis of double-reciprocal plots obtained from initial-velocity studies in 33% methanol/water mixtures shows that the bacterial phosphotriesterase hydrolyzes paraoxon 6.5 times faster than EPN. The K_m values for paraoxon and EPN are 1.3 and 0.02 mM, respectively.

^{31}P *NMR Analysis of the Absolute Configuration of O-Ethyl Phenylphosphonothioic Acid.* Mikolajczyk et al. (1978) have shown that ^{31}P NMR can be used to differentiate between the two enantiomers of chiral phosphorus acids. The technique utilizes a chiral amine to complex the acid and thus forms diastereomeric salts that have different phosphorus chemical shifts. Shown in Figure 4A is the ^{31}P NMR spectrum of a racemic sample of *O*-ethyl phenylphosphonothoic acid when the *S* isomer of 1-phenylethylamine is utilized as the counterion. As expected, two resonances of nearly equal intensity are observed. The difference in chemical shift is 13 Hz. Shown in Figure 4B is the ^{31}P NMR spectrum of the racemic *O*-ethyl phenylphosphonothioic acid after the addition of authentic (R_P)-*O*-ethyl phenylphosphonothioic acid. There is a significant increase in the signal intensity for the upfield resonance. Therefore, it can be concluded that when the (*S*)-1-phenylethylamine is utilized as the chiral shift reagent, the R_P isomer of the *O*-ethyl phenylphosphonothioic acid resonates at higher field than does the S_P isomer.

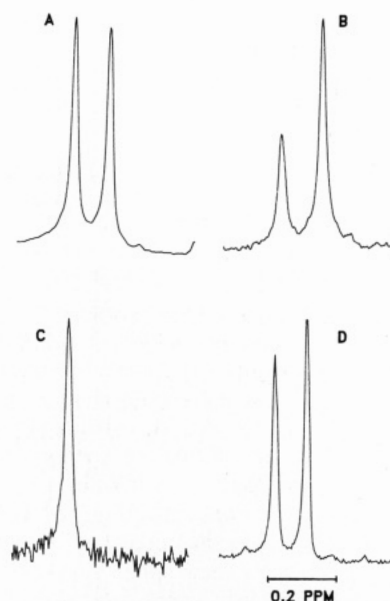
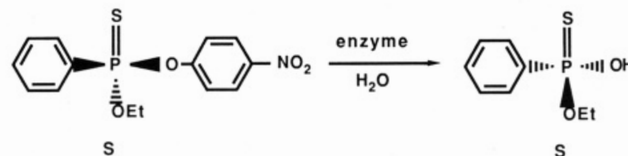


FIGURE 4: Phosphorus-31 NMR evidence for inversion of configuration at phosphorus caused by enzymatic hydrolysis of (S_P)-EPN. (A) Diastereomeric salts formed by interaction of racemic *O*-ethyl phenylphosphonothioic acid and (*S*)-1-phenylethylamine. (B) Mixture of salts from panel A after the addition of authentic (*R*)-*O*-ethyl phenylphosphonothioate. (C) (*S*)-*O*-Ethyl phenylphosphonothioate produced by enzymatic hydrolysis of (S_P)-EPN. (D) Addition of authentic (*R*)-*O*-ethyl phenylphosphonothioate to the sample presented in panel C.

Scheme III



The enzymatic hydrolysis product resulting from using the S_P isomer of EPN as a substrate is shown in Figure 4C. As expected, only a single resonance is observed. When an authentic sample of (R_P)-*O*-ethyl phenylphosphonothioic acid is added to this sample, two resonances are observed (Figure 4D). This result clearly shows that the enzymatic hydrolysis of (S_P)-EPN produces the S_P isomer of the *O*-ethyl phenylphosphonothioic acid. Therefore, it can be conclusively determined that the bacterial phosphotriesterase catalyzes the hydrolysis of EPN with inversion of configuration (see Scheme III). These same protocols were also used to show that the hydroxide-catalyzed reaction of (S_P)-EPN also proceeds with inversion of configuration as expected.

Summary. The phosphotriesterase from *P. diminuta* has been shown to catalyze the hydrolysis of paraoxon with phosphorus–oxygen rather than carbon–oxygen bond cleavage. The hydrolytic reaction has been shown to occur with *inversion*

of configuration at the phosphorus center. These results are consistent with a single in-line displacement by an activated water molecule directly at the phosphorus center.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL AVAILABLE

Atomic coordinates, bond lengths, bond angles, anisotropic displacement parameters, hydrogen atom coordinates, and a table of experimental results for the X-ray structure determination of *O*-ethyl phenylphosphonothioic acid (9 pages). Ordering information is given on any current masthead page.

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Evidence That the Catalytic Differences of Two Structurally Homologous Forms of Cytochrome P-450 Relate to Their Heme Environment

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ABSTRACT: Cytochromes P-450 PB_{3a} and PB_{3b}, which appear to be equivalent to forms *b* and *e* described by Ryan et al. [Ryan, D. E., Thomas, P. E., & Levin, W. (1982) *Arch. Biochem. Biophys. 216*, 272–288], have been shown to share 97% sequence homology [Suwa, Y., Mizukami, Y., Sogawa, K., & Fujii-Kuriyama, Y. (1985) *J. Biol. Chem. 260*, 7980–7984] yet exhibit an intriguing difference in enzymatic activity. Studies to establish the basis for this difference, including a development of the technique of surface-enhanced resonance Raman spectroscopy (SERRS), are reported. Studies on substrate binding, metabolism, and redox properties, as well as SERRS, indicate a significant difference in the heme environment of these two proteins. No significant difference in the interaction of the two proteins with P-450 reductase could be established. However, this interaction appeared sensitive to changes in ionic strength, suggesting ionic interactions are important in the functional coupling of these electron-transport components. A marked variation in the ratio of PB_{3a} to PB_{3b} activity in the metabolism of different substrates, which included a series of structurally similar resorufin analogues, provided further evidence that reductase coupling was not a critical factor. Therefore, the few amino acid differences observed between these proteins indicate sites that may be important in influencing the heme environment of these cytochrome P-450's.

Cytochrome P-450 dependent monooxygenases are a supergene family of proteins that catalyze the oxidation of lipophilic chemicals through the insertion of one atom of molecular oxygen into the substrate (Adesnik & Atchison, 1986; Wolf, 1986). Reactions catalyzed by cytochrome P-450 can be split into various major categories. These are hydroxylation reactions resulting from insertion of oxygen into C–H bonds, epoxidation reactions, and oxidations at nitrogen and sulfur atoms (Wislocki et al., 1980; Wolf, 1982). The products of the latter categories are often electrophilic and bind to DNA, causing mutations, toxicity, and cancer.

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