MECHANISM OF ENYZMATIC PHOSPHOTRIESTER HYDROLYSIS

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Introduction, There are numerous reports from various sources of crude enzyme systems that appear to be capable of hydrolyzing organophosphotriesters (1-8). Unfortunately, none of these enzymes has ever thoroughly been characterized or purified to homogeneity. However, we have recently succeeded in the first isolation and characterization of a phosphotriesterase from *Pseudomonas diminuta* and this enzyme is the focus of this presentation.

The phosphotriesterase from *Pseudomonas diminuta* catalyzes the hydrolysis of typical phosphotriesters such as paraoxon to diethyl phosphate and p-nitrophenol as illustrated in Figure 1. The primary interest in enzyme catalyzed phosphotriesterase

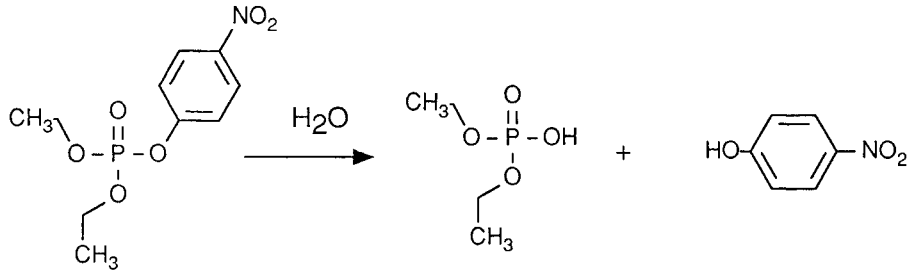

Figure 1. Hydrolysis of Paraoxon by the Phosphotriesterase.

hydrolysis stems from several experimental observations and results. For example, over 1000 organophosphate pesticides have been synthesized and approximately 3 x 10^7 kg of these toxic materials are consumed by the agricultural community in the U.S. annually (9). The consequence of this heavy usage is illustrated by the estimated 800 deaths per year and the thousands of reported pesticide poisonings attributed to exposure with these compounds (12). The lethal dose of paraoxon is 0.5 mg/kg. Recently, it has been shown

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that a wide spectrum of these organophosphate pesticides are hydrolyzed by the enzyme from *Pseudomonas diminuta* (10). Many of those tested are among the more important and heavily utilized in the agricultural industry (11). The catalytic capability of the phosphotriesterase provides a promising method for detoxification of these pesticides.

Several chemical warfare weapons, namely sarin and soman, are also hydrolyzed by the phosphotriesterase at substantial rates. The lethal dose for these nerve toxins are about 0.01 mg/kg and the potential application of this enzyme is quite obvious and significantly important. The current estimated and reported stockpiles of sarin and soman are at least 10^4 tons in the United States. The Soviets are believed to have an equally large arsenal of these types of weapons (13).

In addition to the more practical aspects, it is interesting to note that no naturally occurring phosphotriester is known to exist. The first reported synthesis of a phosphotriester was in the 1937 by Schrader (14). The question thus arises concerning the function and the evolution of this enzyme. Is there a "natural" substrate for this enzyme or has the enzyme evolved in a very short period of time specifically for the hydrolysis of agricultural phosphotriesters?

Cloning, Overexpression, and Isolation. The capability of microorganisms to enzymatically hydrolyze phosphotriesters was observed in experiments performed by Munnecke and Hsieh with the soil microbe, *Pseudomonas diminuta* (15). Earlier work had also demonstrated that *Flavobacterium sp.* was able to degrade diazinon and parathion (16). Both *Pseudomonas diminuta* and *Flavobacterium sp.* (ATCC 27551) exhibited constitutive hydrolytic activity against parathion and the enzyme activity was later found to be associated with a plasmid borne gene in both organisms (17). The gene from each organism was isolated to a 1.3 kb *Pst*I restriction fragment and has been sequenced by two laboratories (18,19). The gene sequenced by the Serdar group at the Amgen Corporation indicates that the enzyme is synthesized as a proenzyme consisting of 365 amino acids. Apparently, 29 amino acids at the amino terminal end are subsequently removed in processing resulting in a protein with a molecular weight of about 36 kDa. It is presently believed that these 29 amino acids have the characteristics of a signal peptide. The predicted amino acid composition, based on the nucleotide sequence, is consistent with the observed amino acid content of the purified enzyme (10). Overexpression of the enzyme activity has been achieved using recombinant plasmids incorporated into *E. coli* vectors (17,19). The enzyme activity has also been significantly overexpressed using a baculovirus expression system developed in the laboratories of Summers (20). In this system, the gene expression is under the control of the polyhedron promoter. Cultured cells from *Spodoptera frugiperda* (sf9 cells) are infected with a recombinant baculovirus carrying the phosphotriesterase gene. Expression of the gene follows the infection of the cultured sf9 cells. Both systems provide an adequate source of enzyme and have been utilized to obtain significant amounts of homogeneous enzyme for biochemical studies.

Phosphotriesterase was first purified from *Spodoptera frugiperda* (sf9) cells which were infected with the recombinant baculovirus (10). The procedure employed four steps after the cells were sonicated and the typical results are shown in Table 1. The purification resulted in a 1500 fold purification of a homogeneous protein with a specific activity of 3200 units/mg for paraoxon hydrolysis. This scheme has allowed a very simple and straightforward purification of the phosphotriesterase.

Table 1. Purification of Phosphotriesterase.

STEP	UNITS	UNITS/MG	PURIFICATION
SUPERNATANT	5400	1.6	1
GREEN-A	7300	130	80
PHENYL SEPHAROSE	7900	1100	703
G-75	4100	3700	2400

Properties of the Purified Phosphotriesterase. The molecular weight of the purified protein was calculated at 39,000 gram/mole based on an estimate from a SDS-PAGE (10). This value was also confirmed using high resolution gel filtration chromatography. This value is very close to a predicted molecular weight of 37,826 based on the DNA sequence and suggests that the enzyme exists as a monomer in solution. Atomic adsorption analysis revealed the association of one zinc atom per molecule of phosphotriesterase (10). The kinetic parameters were measured for the homogeneous enzyme and a value of 2100 s^{-1} was obtained for V_{max}, while the value for V/K was 4 x 10^7 $M^{-1}s^{-1}$ using paraoxon as a substrate. These rates are quite substantial and compare quite favorably with the V_{max} (4.3 x 10^3 s^{-1}) and V/K (2.4 x 10^8 $M^{-1}s^{-1}$) values for triose phosphate isomerase (21). The estimated rate enhancement produced by this enzyme relative to the rate of hydrolysis in water at pH 7.0 is 2.8 x 10^{11} (10).

Mechanism of Action. The enzymatic hydrolysis of paraoxon gives two products as shown in Figure 1. From a mechanistic viewpoint, the products can be obtained by a minimum of three different pathways. These limiting mechanisms are illustrated in Figure 2. First, the attack could occur by a nucleophilic substitution at the carbon atom of the phenyl ring with the formation of an tetrahedral intermediate followed by C-O bond cleavage to give the products. Secondly, the attack might occur at the phosphorus atom by the direct attack of an active site nucleophile to give a covalent enzyme intermediate and p-nitrophenol. This would follow with an attack by water at the phosphorus atom to give the second product diethyl phosphate. Lastly, in an S_N2 like process the reaction could

proceed by the general base catalyzed attack of a water molecule directly at the phosphorus center with P-O bond cleavage to give two products in a single step.

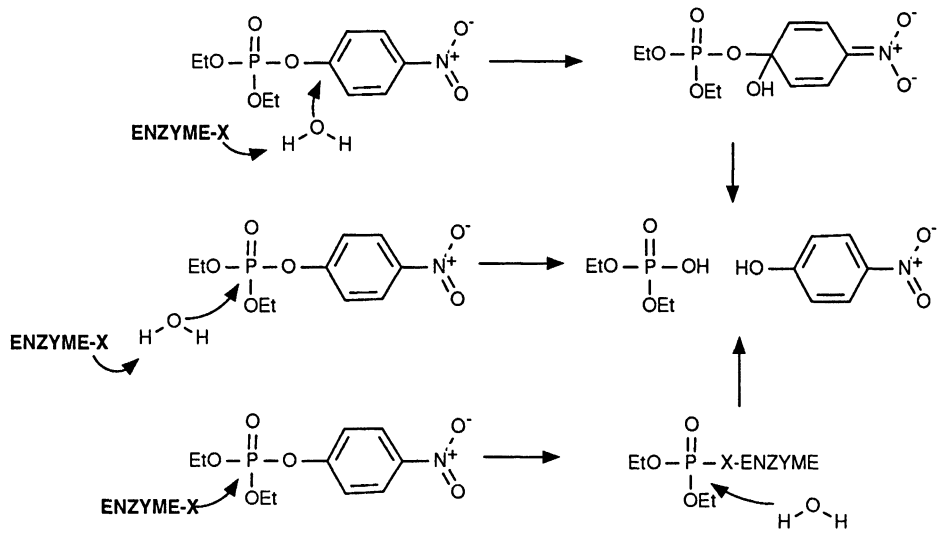


Figure 2. Possible Mechanisms for Phosphotriester Hydrolysis.

The mechanisms for P-O versus C-O bond cleavage were distinguished by conducting the enzymatic hydrolysis in oxygen-18 water and subsequent analysis by phosphorus-31 NMR spectroscopy (22). The analysis showed a 0.02 ppm upfield chemical shift in the phosphorus NMR spectrum of the diethylphosphate product which was expected with the incorporation of an oxygen-18 in the product, diethyl phosphate and not p-nitrophenol. These results are consistent only with the attack of the water at the phosphorus center and subsequent P-O bond cleavage, clearly eliminating the nucleophilic aromatic substitution mechanism. To distinguish between the covalent enzyme intermediate and the direct displacement mechanism required the determination of the net stereochemical outcome at the phosphorus center. The formation of a covalent phosphoenzyme intermediate suggests a two step mechanism, and thus, retention of configuration at the phosphorus. The single in-line displacement mechanism would consist of a stereochemical inversion at the phosphorus atom. Since paraoxon and diethyl phosphate are prochiral molecules the stereochemical assignments could not be accomplished with these compounds. Instead, the stereochemical analysis was performed by utilizing a chiral substrate, [O-ethyl O-(4-nitrophenyl)] phenyl phosphonothioate (EPN) (22). Each stereoisomer was independently synthesized from optically pure phenylphosphonothioic acid. It was found by phosphorus-31 NMR spectroscopy that only the (Sp) isomer of EPN was hydrolyzed by the enzyme to give only the (Sp) isomer of the product O-ethyl phenyl phosphonothioc acid as shown in Figure 3. Therefore, hydrolysis of EPN by the phosphotriesterase occurs with complete inversion of stereochemistry at the

phosphorus center. These results eliminated the possible formation of an covalent phosphoenzyme intermediate and thus supported a single in-line displacement process at the phosphorus center with an activated water molecule.

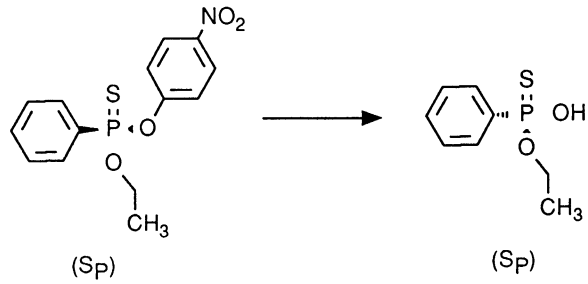


Figure 3. Hydrolysis of Chiral EPN.

Active Site Residues. It was observed from the pH rate profiles of the kinetic parameters, V_{max} and V/K, that a single unprotonated group was involved in catalysis (23). As shown in Figure 4, the V_{max} profile indicates that a species with a pK_a of 6.1 must be unprotonated when paraoxon is a substrate. Since paraoxon does not ionize in this pH range, these results are consistent with general base catalysis in the activation of the attacking water molecule. The identification of the active site base was attempted by measuring the pH dependence for paraoxon hydrolysis as a function of temperature and by the effect of organic solvent on the pK_a values (24). The results from the effect of temperature on the kinetic pK_a gave the thermodynamic values of 7.9 kcal/mole and -1.4 entropy units for enthalpy and entropy of ionization, respectively. These values are most consistent with the titration of a histidine residue (25). The effect of organic solvent suggested a cationic acid, also consistent with a histidine residue as the active site base.

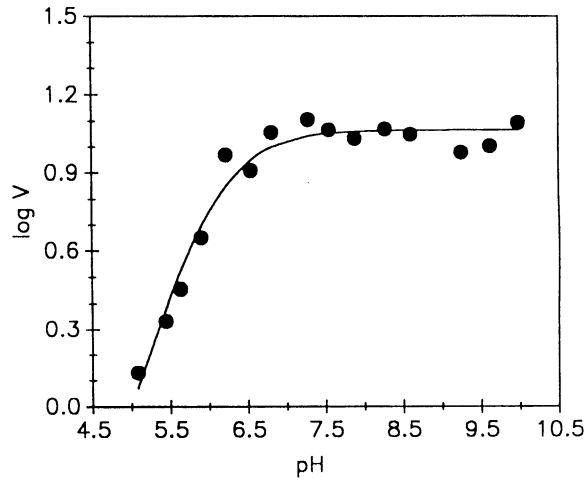


Figure 4. pH Rate Profile for Hydrolysis of Paraoxon.

Table 2. Survey of Chemical Modification Reagents.

Targeted Residue	Reagent	Inactivation Rate (h^{-1})
Cysteine	DTNB	<0.02
Cysteine	MMTS	<0.02
Aspartate, Glutamate	EDC	<0.07
Lysine	Pyridoxal	<0.06
Arginine	Butandione	<0.01
Histidine	Methylene Blue	0.72
Histidine	DEPC	1.92
Cysteine	Iodoacetamide	<0.01

The requirement for an essential histidine residue was confirmed using group specific chemical modification reagents for potential active site nucleophiles (24). The experimental survey is shown in Table 2 and reveals that only those reagents generally specific for histidine residues inactivate the enzyme. Diethyl pyrocarbonate (DEPC), a relatively specific reagent for the reaction with histidine, substantially inactivated the phosphotriesterase activity when only 2.9 of the 7 total histidine residues were modified. Diethyl-p-fluorophenyl phosphate, a competitive inhibitor of paraoxon, completely protected the enzyme from inactivation by DEPC and only two of the three histidines were modified, indicating that only one histidine was associated with the original inactivation event. The negligible effect by the other chemical modification reagents further supports a histidine as the sole species involved in catalysis. The inactivation of the phosphotriesterase by DEPC as a function of pH resulted in a pK_{inact} of 6.1, identical with the value obtained from the kinetic pH rate profile, providing additional evidence. Collectively, these data support a histidine as the active site base involved in catalysis but this evidence does not preclude the involvement of other essential residues such as metal ions.

The specific role of the zinc ion in the active site of phosphotriesterase has yet to be elucidated. A reasonable role for the zinc may involve polarization of the phosphoryl oxygen of the phosphotriester. This scenario would be analogous to carboxypeptidase, a zinc protease (26). Here, the zinc serves in polarizing the carbonyl oxygen, and thus facilitating the attack of a water molecule at the carbonyl carbon. The zinc further aids in stabilizing the developing negative charge on the carbonyl oxygen in the transition state. Another potential role for the zinc atom in phosphotriesterase may involve the stabilization of the phenolic leaving group, thereby assisting in catalysis (27).

Atomic absorption analysis of the holoenzyme detected a single zinc atom per molecule of phosphotriesterase (10). The removal of the zinc atom to form an apoenzyme was accomplished using o-phenanthroline, a metal chelator, and resulted in less than 0.1 zinc atom per enzyme molecule and concommitant loss in enzyme activity. Recent studies have shown that divalent metal ions, especially cobalt(II), when introduced into the growth media yield substantial increases in enzyme activity (19, 28).

Structure of the Transition State. In a determination of the substrate specificity for the phosphotriesterase, several paraoxon analogs were synthesized with varying substituents on the phenyl ring and then kinetically analyzed (23). This analysis revealed a linear structure activity relationship between the pK_a of the phenolic leaving group and the rate of chemical and enzymatic hydrolysis. This relationship can be defined by the Brϕnsted equation, as illustrated in Equation 1

$$\log k_3 = (\beta \times pK_a) + \text{constant} \qquad (1)$$

and provides information concerning the transition state structure for the hydrolysis reaction. For the series of phosphotriesters examined, this relationship was expected to reveal the amount of P-O bond breakage in the transition state. Chemical hydrolysis of the phosphotriesters by KOH gave a linear plot over the entire pK_a range examined with a β value of -0.42, identical with a previous literature report (30). This result is consistent with a P-O bond order of about 1/3 in the transition state. A larger negative β value of -1.2 was obtained when the data for the enzymatic hydrolysis were analyzed (30). These data suggest a later transition state with a substantial amount of the negative charge associated with the leaving group. Enzymatic hydrolysis involves the addition of one substrate and the release of two products. If the simple kinetic model for enzymatic hydrolysis as illustrated in Equation 2 is assumed, then the following rate equations can be derived for the

$$E \underset{k_2}{\overset{k_1 A}{\rightleftharpoons}} EA \xrightarrow{k_3} EPQ \xrightarrow{k_5} E + \text{Products} \qquad (2)$$

kinetic parameters, V_{max} and V_{max}/K_m. With this model as a point of reference,

$$V_{max} = k_3 k_5 / (k_3 + k_5) \qquad (3)$$

$$V/K = k_1 k_3 / (k_2 + k_3) \qquad (4)$$

the Brϕnsted plots obtained for the enzymatic hydrolysis data of several paraoxon analogs can be used to obtain values for k_1 through k_5 (30).

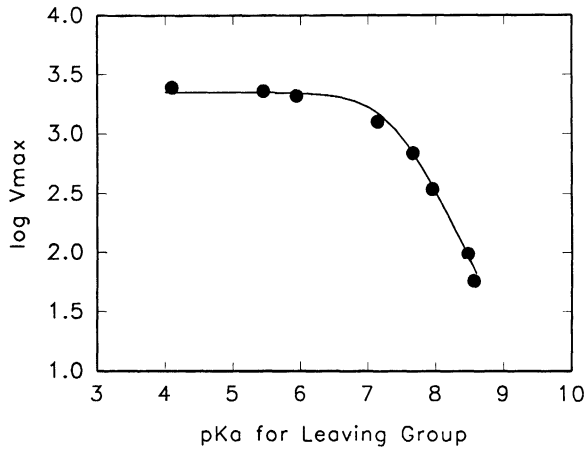


Figure 5. Brϕnsted Plot for log V_{max} Versus the pK_a of Leaving Group.

The Brϕnsted plot for log V_{max} versus the pK_a of the leaving group (Figure 5) is nonlinear for the pK_a range examined and thus there is a change in rate limiting step with these substrates. Thus, for those substrates with leaving group pK_a values ~7, the chemical step (k_3) is apparently rate limiting while the rate of hydrolysis is dictated by the magnitude of k_5 for those substrates with leaving group pK_a values below ~7. A fit of this plot to equation 1 and 3 provides the values for k_5 and β of 2200 s^{-1} and -1.2, respectively. A similar Brϕnsted plot is observed for the V/K data as shown in Figure 6.

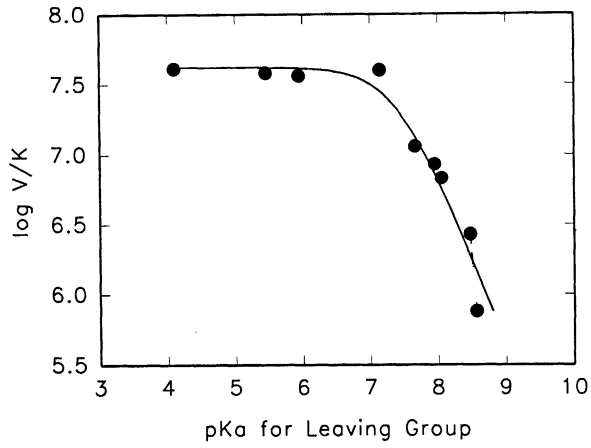


Figure 6. Brϕnsted Plot for log V/K Versus the pK_a of Leaving Group.

A fit of the data in this plot to equations 1 and 4 provides the value of k_1 and k_2 4.1 X 10^7 $M^{-1}s^{-1}$ and 2100 s^{-1}, respectively. Hydrolysis rates for those substrates in the plateau portion of the Brønsted plot are not affected by the pK_a of the leaving group, suggesting that another step in the enzymatic reaction is now more limiting than the chemical step. The nonlinearity of this plot and the relatively large value for k_1 suggests that diffusion of the substrate to active site may be limiting for V/K, since theoretically, second order rates of 10^7 to 10^9 $M^{-1}s^{-1}$ have been suggested for diffusion controlled enzymatic reactions. The measurement of diffusion controlled processes can be determined by varying the viscosity of the medium. In a diffusion limited system the rate of the reaction decreases proportionally with increasing viscosity of the medium. The effect of sucrose on the relative V/K is shown in Figure 7. A limiting slope value of 1.0 indicates a totally diffusion limited system. As predicted, those substrates whose leaving group effects did not affect hydrolysis rates are limited by the encounter of the substrate with the active site. The solid line in this plot is drawn using equation 5 and the calculated values obtained from the fits of the appropriate equations to Figures 5 and 6. The prediction is quite good and confirms the conclusion that for this group of substrates, V/K is limited by the chemical step for those substrates with leaving group pK_a's above ~7 and limited by diffusion for those substrates with leaving group pK_a values below ~7. These data show conclusively that paraoxon is poised such that its rate of enzymatic hydrolysis is limited predominately by diffusion.

Substrate Specificity. Although paraoxon remains one of the best substrates, other changes to the paraoxon molecule have been made in an effort to further define the substrate specificity (23). The nonbridging oxygen has been substituted with a sulfur atom with only minimal effects on V_{max}. When changes on the ester portion on the molecule were made, significant effects were observed. Diesters and monoesters are not hydrolyzed by the enzyme, and as the chain length of the ester increases on the phosphotriester both K_m and V_{max} decrease suggesting a hydrophobic binding pocket at the active site.

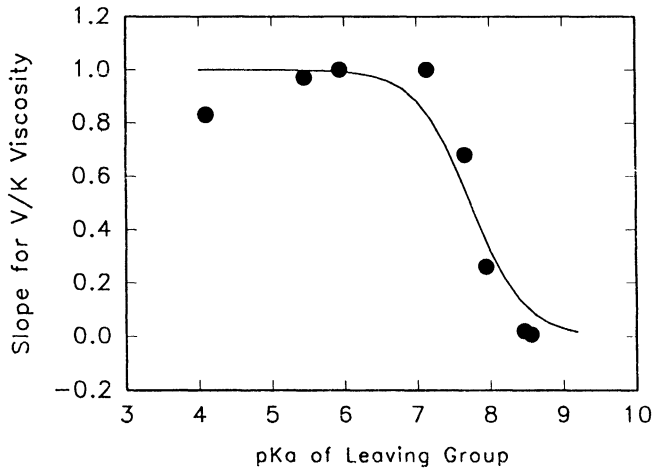
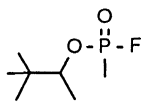
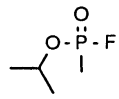


Figure 7. Effect of Solvent Viscosity on V/K.

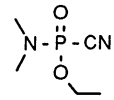
Besides the hydrolysis of the P-O bond, phosphotriesterase can hydrolyze P-F bonds (31). The substrates of interest in this category are some of the chemical warfare agents, sarin, tabun, and soman. The P-F bond of sarin is hydrolyzed 1.5 X 10^6 times faster than the uncatalyzed reaction. Regardless, phosphotriesterase does exhibit the capability to hydrolyze these agents at a rate of 10^6 to 10^7 times greater than the uncatalyzed reaction at pH 7.0 and will perhaps be useful in degrading the enormous stockpiles of these compounds.



SOMAN



SARIN



TABUN

Phosphotriesterase has been shown capable of hydrolyzing commonly used pesticides (10). Since the gene for the enzyme could be incorporated and expressed in sf9 cells from the fall army worm, the possibility of infecting the larval stage of the fall armyworm was examined (24). Production of the enzyme in the baculovirus infected larva was successful up to 90 hours after infection. The time course for production of enzyme activity in the caterpillar shows the greatest expression about 90 hours after infection. In addition to the improved expression, the phosphotriesterase expressing catepillars exhibited a marked resistance to the insecticide, paraoxon. Figure 8 is a graphic representation of the enhanced resistance to paraoxon where the lethal dose increased 280-fold over the dosage for caterpillars not containing the phosphotriesterase gene. This model for the incorporation of the phosphotriesterase gene into an insect may have applications in the industrial sector where insecticidal resistance to commercially important insects such as the silk worm and the honeybee could be advantageous.

Summary. Phosphotriesterase activity can be expressed from a plasmid borne gene in a common soil microorganism, *Pseudomonas diminuta*. The gene for this enzyme has been cloned, sequenced, and overexpressed in bacterial and insect expression systems. Although no naturally occurring substrate for the enzyme has been identified, the phosphotriesterase exhibits a broad substrate specificity, hydrolyzing many organophosphate pesticides and chemical weapons. At the present, paraoxon appears to be the best substrate for the enzyme. From pH dependent studies and chemical modification experiments, there is an active site histidine important for catalysis and a zinc ion, is also required for activity. The size and the pK_a of the phenolic leaving group play an important role in the rate of substrate turnover. The natural role (if any) of this enzyme has not been determined and the hydrolysis of phosphotriesters may only be secondary to some other, but unknown, biological role. However, it is intriguing to speculate that evolutionary pressures due to pesticide exposure in the last fifty years on microorganisms may have

transformed a preexisting protease, phosphatase, or esterase into a highly efficient catalyst for the hydrolysis of phosphotriesters.

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