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Malathion as a Model for the Enzymatic Hydrolysis of the Neurotoxic Agent, VX

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The powerful acetylcholinlesterase (AChE) inhibitors of the general structure $R_1R_2P(=O)$-F, otten termed "nerve gases", have been known for half a century to be enzymatically hydrolyzed (Mazur, 1946) and thus detoxified (Hoskin, 1990). A second generation of these neurotoxir agents of the general structure $R_1R_2P(=O)$-S-R_3, including O-ethyl S-(2-disopropylaminoethyl) methylphosphonothiolate (VX), has recently been shown to he cleaved at the P-S linkage by an enzyme from *Pseudomonas diminuta* (Hoskin et al., 1995). In this same report, the hydrolysis of a VX analogue; O,O-diisopropyl S-(2-diisopropylaminoethyl) phosphorothiolate (Tetriso) by the same enzyme source was examined in greater detail. Tetriso is also not generally available, and its synthesis (Hoskin et al., 1969) is not a trivial undertaking. Yet another analogue. this time of low mammalian toxicity (Aldridge et al., 1979), O,O-dimethyl S-(2-diethylsuccinyl) phosphorothionate (malathion), is readily available. To complete the analogy to Tetriso, and in turn to VX, it is necessary to show that the enzyme that hydrolyzes these two also cleaves the P-S bond of malathion, detoxifies malathion, and does not cleave the carboxylester bonds of malathion.

To accomplish these three aims of the present research, malathion was subjected to the VX- and Tetriso-hydrolyzing enzyme derived from *Pseudomonas diminuta* and termed OPH (Dave et al., 1993; Hoskin et al., 1995). The reaction system was assayed by the "direct" Ellman reaction to detect cleavage of the P-S bond (Hoskin et al.; 1995): by the more usual original Ellman reaction (Ellman et al., 1961) to reveal loss of AChE inhibitory potency (for brevity termed detoxication); and by gas chromatography (GC) for the possible presence of ethanol, related to the carboxlyester bonds of malathion.

MATERIALS AND METHODS

Malathion was obtained from ChemService, West Chester, Pennsylvania 19831, USA. All other reagents were from Sigma Chemical Co., or similarly reliable sources.

The aqueous solubility of malathion is limited to about 4×10^{-4} M and the AChE inhibitory potency (I_{50} in the older literature) is also in that range (Holmstedt, 1963). These properties necessitated some modifications of the methods previously

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used (Hoskin et al., 1995). Prior to using malathion, OPH was again tested on Tetriso. In a spectrophotometer cuvette there was combined, in a final volume of 3.4 mL, Tetriso at 4 X 10^{-4} *M*, 0.4 mg 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 20 m*M* Pipes buffer, all at pH 7.0. The reference cuvette contained buffer only. After several minutes of zero readings at 412 nm, 0.05 mL OPH solution (containing 10-15 µg enzyme) was added. There followed a linear increase in absorbancy for a period of 20 min. Malathion was then used in place of Tetriso, and the full scale of the spectrophotometer was changed from 1 absorbancy unit to 0.2. The increase in absorbancy was again linear over the 20 min period. In one experiment boiled OPH was used. A molar extinction coefficient of 13,600 (Ellman, 1959) permitted the conversion of spectrophotometer readings to amounts of substrate hydrolyzed. The results are presented in Table 1; in brief, they show that the OPH enzyme splits the P-S bond of malathion.

Table 1. Hydrolysis of two organophosphates (OPs) by organophosphorus hydrolase (OPH) from *Pseudomonas diminuta* determined by direct Ellman reaction.

Conditions	Absorbancy change at 412 nm in 20 min	Amount of OP hydrolyzed, μmoles mL^{-1} min^{-1}
Malathion, 4 X 10^{-4} *M*		
+ 0.1 mL OPH	0.057	0.007
+ 0.15 mL OPH	0.084	0.007
+ 0.15 mL boiled OPH	0	0
Tetriso, 4 X 10^{-4} *M*		
+ 0.05 mL OPH	0.351	0.09

To measure the loss of AChE inhibitory potency (detoxication in our terminology), the malathion systems were again assembled but without DTNB. After 36 hr at room temperature (estimated from the 20 min results as likely to cause more than 50% P-S bond hydrolysis with OPH), aliquots from each vessel were combined with AChE, and exactly 30 min later AChE activity was determined as previously described (Ellman et al., 1961; Hoskin, 1990). The results are presented in Table 2, and show that incubation of malathion with the OPH enzyme completely detoxifies the malathion.

To examine the possibility of hydrolysis of one or both of the carboxylester bonds of malathion by OPH, an event that would also detoxify the malathion (Mounter, 1963), 1 µl, samples of each of the 36 hr incubates, with and without OPH, and of freshly prepared malathion solutions were subjected to GC analysis. Since 1 µL of 4 X 10^{-4} *M* malathion is capable of producing 0.03-0.04 µg ethanol on complete carboxylester hydrolysis, the GC analyzer was standardized with 1 µL samples containing 0.018,0.034, and 0.06 µg ethanol in water, in Pipes buffer, and in Pipes buffer with malathion, The results are illustrated in Fig. 1. This composite set of recordings was chosen as best for reproduction, but all of the analyses gave exactly the same finding, namely, that OPH does not hydrolyze the carboxylester bonds of malathion.

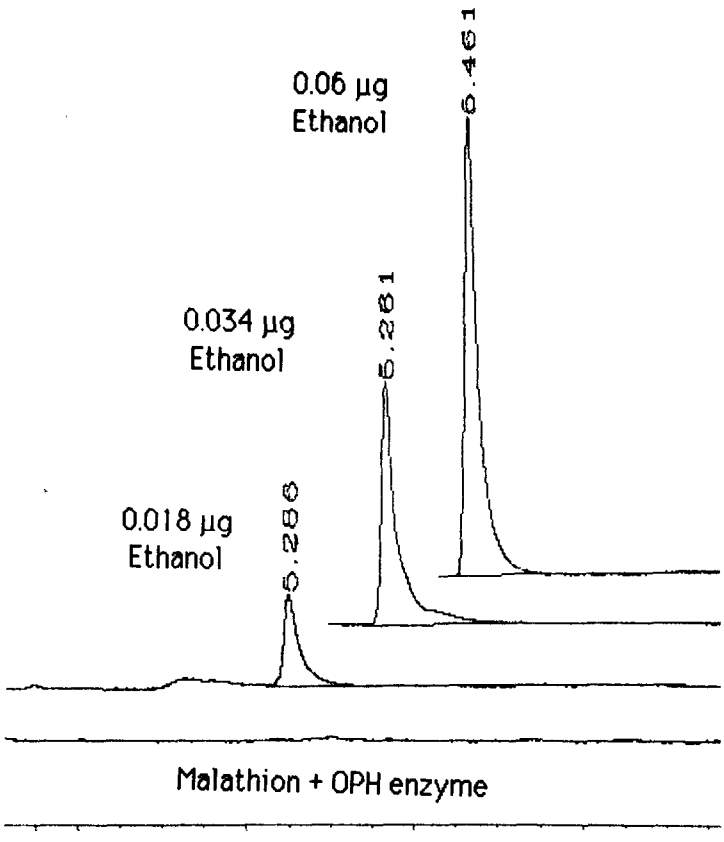

Figure 1. Gas chromatographs of 1 µL buffer (or water) containing varying amounts of ethanol, and of 1 µL of 4 X 10^{-4} *M* malathion after 36 hr incubation with OPH. Times of emergence in minutes are shown above each peak. Traces have been offset approximately equidistantly for clarity.

RESULTS AND DISCUSSION

These three sets of results taken together show that the OPH enzyme derived from *Pseudomonas diminuta* hydrolyzes the P-S bond of malathion. The hydrolysis results in the complete loss of AChE inhibitory potency. The detoxication, as we term this loss, is near the limit of what can be determined because of the low level of AChE inhibition attainable with malathion. This is not an impediment, having previously established with the very much more inhibitory VX and Tetriso that cleavage of the P-S bond results in detoxication (Hoskin et al., 1995). The slight increase in AChE inhibitory potency on 36 hr incubation of malathion with boiled enzyme (Table 2) is probably due to a slight spontaneous conversion of malathion to malaoxon (P=S to P=O), a much more potent inhibitor of AChE (Berkman et al., 1993).This *Pseudomonas* enzyme hydrolyzes malathion at a much lower rate than it hydrolyzes Tetriso - indeed, closer to the rate of hydrolysis of VX (Hoskin et al.,

Table 2. Inhibition of AChE by malathion.

Conditions	Inhibition, %; two experiments	
No malathion	0	0
Freshly prepared malathion	58[1]	62[1]
Malathion + boiled OPH[2], 36 hr	60	75
Malathion + OPH[2], 36 hr	-4[3]	-9[3]

[1]The inhibition of AChE by 4×10^{-4} *M* malathion in 30 min, shown here, gives a 2nd order rate constant of 77 ± 6 M^{-1} min^{-1}, in good agreement with the value that can be calculated from Holmstedt (1963), if reasonable assumptions are made about inhibition time.
[2]Organophosphorus hydrolase from *Pseudomonas diminuta* ; see introductory paragraphs.
[3]Negative values indicate stimulation.

1995). However, such a conclusion is not yet justified because the low water solubility of malathion precludes K_M and V_{max} determinations. Also, the three substrates - VX, Tetriso and malathion - are not well suited for an exploration of structure-activity relationships, displaying as they do the structures C-P(=O)-S- , C-O-P(=O)-S- and C-O-P(=S)-S- , respectively. These compounds are clearly not natural substrates, but the implied question is less demanding of an answer here since microorganisms in genera1 display a wide spectrum of metabolic activities. Furthermore. the *Pseudomonas diminuta* enzyme displays its OPH activity only after a natural processing of the first 29 amino acids (Dave et al., 1993). The same question of a natural substrate for the P-F hydrolyzing enzymes, particularly the "squid type" OPA anhydrase (Hoskin, 1990). also remains unanswered, although the narrow distribution seemed to suggest a physiological role. The *Pseudomonas* OPH is the first enzyme found, after many years of search, that cleaves the P-S bond of these neurotoxic agents. The negative results have not, of course, been published, but include an examination of a wide selection of prokaryotes and eukaryotes, including the squid.

In the search for other enzyme sources to provide a mild means of detoxifying VX stockpiles (National Research Council, 1993), these results suggest **a** relatively safe single step reaction for rapid screening, namely the "direct" Ellman reaction (Hoskin et al., 1995) with malathion as substrate. In addition, the findings suggest a means for bioremediation of sites where VX or malathion residues might remain, for example manufacturing facilities and test areas.

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