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# HYDROLYSIS OF TETRISO BY AN ENZYME DERIVED FROM *PSEUDOMONAS DIMINUTA* AS A MODEL FOR THE DETOXICATION OF *O*-ETHYL *S*-(2-DIISOPROPYLAMINOETHYL) METHYLPHOSPHONOTHIOLATE (VX)

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**Abstract**—An enzyme termed organophosphorus hydrolase (OPH), derived from *Pseudomonas diminuta*, had been found previously to hydrolyze the powerful acetylcholinesterase (AChE) inhibitor *O*-ethyl *S*-(2-diisopropylaminoethyl) methylphosphonothiolate (VX). This enzyme has now been shown to be correlated with the loss of AChE inhibitory potency (detoxication). OPH also hydrolyzed and detoxified the VX analogue, *O*,*O*-diisopropyl *S*-(2-diisopropylaminoethyl) phosphorothiolate (Tetriso), also a potent AChE inhibitor, about five times faster than VX. The $K_m$ for the hydrolysis of the P—S bond of Tetriso was $6.7 \times 10^{-3}$ M. OPH also hydrolyzed diisopropylphosphorofluoridate (DFP) 50–60 times faster than Tetriso, and 1,2,2-trimethylpropyl methylphosphonofluoridate (Soman) about seven times faster than Tetriso. DFP was a non-competitive inhibitor of Tetriso hydrolysis, $K_i = 8.7 \times 10^{-4}$ M. The DFP hydrolysis product, diisopropyl phosphate, was a competitive inhibitor, $K_i = 2.3 \times 10^{-4}$ M. The rate of detoxication of Tetriso compared with the rate of hydrolysis suggests that OPH may not be totally specific for P—S bond cleavage. OPH was inhibited completely by $1.5 \times 10^{-4}$ M 8-hydroxyquinoline-5-sulfonate or 1,10-phenanthroline, both transition element chelators, but inhibited only partially by EDTA, a much more potent chelator.

*Key words:* acetylcholinesterase; detoxication; organophosphorus hydrolase (OPH); *Pseudomonas diminuta*; Tetriso; VX

The enzymatic hydrolysis, and thus detoxication, of organophosphorus acid anhydrides, commonly termed "nerve gases" (often P—F compounds), is now well documented [1–3]. The best known of these compounds, although itself not considered a nerve gas, is DFP¶. A second generation of these potent and highly toxic AChE inhibitors includes phosphoro- and phosphonothiolo compounds typified by the military agent VX [4]. Until now, it has been generally known but seldom documented that these compounds are not readily biodegraded. Even the few reports that have appeared [5, 6] showed extremely low rates and did not reveal a potential for large-scale degradation. Recently, for the first time, this has changed dramatically [7]. We now present further data on the enzymatic hydrolysis, and thus detoxication, of VX, and more especially of a VX analogue, Tetriso [8], which may prove more useful in exploring the degradation of VX, and of other structurally related toxic compounds.

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¶ Abbreviations and trivial names: DFP, diisopropylphosphorofluoridate; AChE, acetylcholinesterase; VX, *O*-ethyl *S*-(2-diisopropylaminoethyl) methylphosphonothiolate; Tetriso, *O*,*O*-diisopropyl *S*-(2-diisopropylaminoethyl) phosphorothiolate; Soman, 1,2,2-trimethylpropyl methylphosphonofluoridate; OPH, organophosphorus hydrolase; PIPES, piperazine-*N*,*N*′-bis (2-ethanesulfonic acid); DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); OPAA, organophosphorus acid anhydrolase; 8-OHQ-5-SA, 8-hydroxyquinoline-5-sulfonate; 1,10-$\phi$, 1,10-phenanthroline; and Sarin, isopropyl methylphosphonofluoridate.

## MATERIALS AND METHODS

*Chemicals.* Tetriso was originally synthesized for neurophysiological experiments, but has proven useful (even while producing generally negative results) in a search for detoxication pathways. Over the past 25 years, the compound has been resynthesized several times by the original method [8], with consistent yields and properties.

VX is not readily available, but has been synthesized by one of us [4] by published methods [9] with funds from the National Institutes of Health. Small quantities in dilute aqueous solution (approximately 4 mL, 1 mM) had been held at −80° in sealed double-walled containers, and have now been used only by one of us (F.C.G.H.) under safety conditions described in detail elsewhere [2]. The small remaining amount of VX solution has now been destroyed under strong alkaline conditions.

DFP was obtained from the Sigma Chemical Co. (St. Louis, MO), and has been used routinely by us. Soman was synthesized and used by one of us (F.C.G.H.) in a manner and under safety conditions described many times (see Ref. 2 for summary and methods).

*Enzymes and enzyme measurements.* The purification of the enzyme OPH* derived from *Pseudomonas diminuta*, the genetic and biochemical manipulations involved, and indeed the first report of its ability to hydrolyze VX, are all the work of one of us (J.R.W.), together with colleagues [3, 7]. The hydrolytic rate for VX was reported as ". . . . small but measurable . . ."†, and detoxication had not yet been determined. This latter measurement, and especially both the hydrolysis and detoxication of the VX analogue Tetriso constitute a major part of the present paper.

The degradation of VX or Tetriso was measured in two ways. One was by the loss of AChE inhibitory potency (detoxication) by the original Ellman reaction [10], termed by us the "indirect" Ellman method. The second was by the production of the hydrolysis product diisopropylaminoethanethiol for both VX and Tetriso, by what we term the "direct" Ellman reaction. The indirect method was carried out exactly as has been described for Soman [2], based on the original publication [10], with slight modifications to accommodate VX in place of Soman. A second order rate constant for the inhibition of AChE by VX was found to be $3.2 \times 10^7\,L \cdot mol^{-1} \cdot min^{-1}$, comparing well with values previously reported [4, 11]. Based on this rate constant, we determined loss or lack of loss of AChE inhibitory potency (and hence loss or lack of loss of VX) in the presence of OPH, in the presence of boiled OPH, and in the absence of OPH.

The direct Ellman method is illustrated with Tetriso as a substrate. Two spectrophotometer cuvettes contained, each, 2.45 mL PIPES buffer (20 mM, pH 7.0), 0.70 mL of 0.01 M Tetriso (in PIPES, pH adjusted to 7.0), and 0.20 mL DTNB (19.8 mg DTNB, 7.5 mg $NaHCO_3$, 10 mL of 0.1 M phosphate, pH 7.0). After about 5 min of recording at 412 nm, the sample cuvette was removed from the spectrophotometer, 0.05 mL OPH was added (a single preparation was used throughout, 0.2 to 0.3 mg enzyme per mL, $Co^{2+}$ form), the cuvette was inverted several times, and the recording was continued. In this particular instance, the absorbancy reading went from 0.000 to −0.003 during the first 5 min, and was essentially linear from 0.052 to 0.743 from min 6 to min 16. With a molar extinction coefficient of 13,600 [12], spectrophotometer readings were converted to micromoles Tetriso hydrolyzed per unit time and quantity of enzyme.

Table 1. Detoxication of VX and Tetriso by an enzyme (OPH) derived from *Pseudomonas diminuta*

| OPH (mL) | VX* (% detoxication) | Tetriso* (% detoxication) |
|---|---|---|
| >0.1 | 100 | |
| 0.1 | 98 | 96 |
| 0.1† | 0 | |
| 0.02 | 0 | 37 |

* Substrate (i.e. VX or Tetriso) concentration, $1.5 \times 10^{-4}$ M; final volume, 1.0 mL; enzyme (OPH) volume is for a single preparation containing 0.2 to 0.3 mg enzyme per mL, $Co^{2+}$ form; pH 7.0; 24–26°.

† Boiled for 15 min.

## RESULTS

When VX was incubated with OPH (final volume, 1.0 mL; VX at $1.5 \times 10^{-4}$ M; 0.1 mL OPH; pH 7.0; 24–26°), there was a 98% loss of AChE inhibitory potency, i.e. detoxication of VX, as measured by the indirect Ellman method, in 4 hr, the only time sampled after the zero hour determination. Higher levels of OPH, used initially, resulted in essentially complete detoxication. This wording is chosen since, had we made smaller and smaller dilutions from the initial $1.5 \times 10^{-4}$ M, some remaining VX might have been found, for example, instead of 2%, perhaps 0.2%, 0.02%, and so on. When the level of OPH was reduced from 0.1 to 0.02 mL, no detoxication was found. When OPH that had been held in a boiling water bath for 15 min was used, no detoxication was found.

When Tetriso was incubated in exactly the same way, but with smaller subsequent dilutions to reflect the smaller second order rate constant ($1.76 \times 10^6\,L \cdot mol^{-1} \cdot min^{-1}$), 0.1 mL OPH caused 96% detoxication, and 0.02 mL OPH, 37% detoxication.

These results are summarized in Table 1.

Both Tetriso and VX were incubated with OPH in the direct Ellman reaction. For Tetriso, 14 determinations spanned the concentration range $7 \times 10^{-4}$ M to $10^{-2}$ M. The results were processed by a computer program and are presented in Fig. 1. The computed Y-axis intercept and the slope give a $K_m$ of $6.7 \times 10^{-3}$ M. For reasons to be presented shortly, DFP and the DFP hydrolysis product diisopropyl phosphate were tested for their effects on OPH with Tetriso as substrate. These results can also be seen in Fig. 1. The processed data demonstrated that DFP is a non-competitive inhibitor, with a $K_i$ of $8.7 \times 10^{-4}$ M, and that diisopropyl phosphate is a competitive inhibitor, with $K_i$ of $2.3 \times 10^{-4}$ M. The $K_m$ and $K_i$ values, and even the nature of the inhibition, are subject to some uncertainty since, of necessity, most of the data were obtained at high substrate concentrations and reaction rates.

* The recommendation for numbering will probably be for either EC 3.1.8.1 or EC 3.1.8.2. Lack of evidence about a natural substrate continues to be an impediment.

† Lai K, Dave KI, Wild JR, Szafraniec LL, Beaudry WT and Harvey SP, Enzymatic decontamination of organophosphorus chemical agents by genetic and biochemical manipulation of OP-hydrolase (OPH). In: *Scientific Conference on Chemical Defense Research* (U.S. Army Edgewood RD&E Center, Aberdeen Proving Ground, MD 21010), Abstr. 109, 1993.

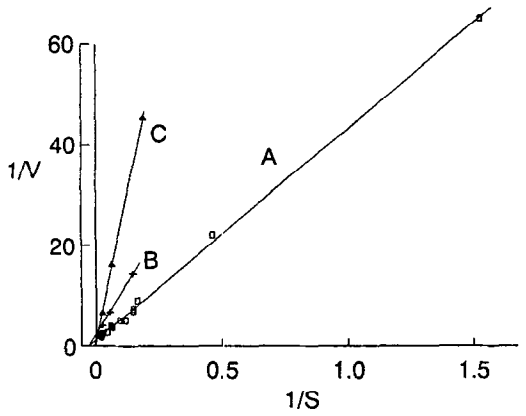


Fig. 1. Reciprocal plot of the hydrolysis of Tetriso by an enzyme (OPH) derived from *Pseudomonas diminuta*. On the abscissa, $0.5 = 2 \times 10^{-4}$ M Tetriso; on the ordinate, 20 = 0.05 μmol Tetriso hydrolyzed per min and per mL of OPH containing 0.2 to 0.3 mg enzyme, $Co^{2+}$ form; pH 7.0; 24–26°. A (□), Tetriso only; B (+), Tetriso in the presence of $1.18 \times 10^{-4}$ M DFP; C (▲), Tetriso in the presence of $1.18 \times 10^{-4}$ M diisopropyl phosphate. $K_m = 6.7 \times 10^{-3}$ M. $K_i$ (DFP; non-competitive) $= 8.7 \times 10^{-4}$ M. $K_i$ (diisopropyl phosphate; competitive) $= 2.3 \times 10^{-4}$ M. These values are calculated from computer-generated slopes and Y-axis intercepts.

Table 2. Hydrolysis* of VX compared with that of Tetriso by an enzyme (OPH) derived from *Pseudomonas diminuta*

| Substrate | Concentration (M) | Rate of hydrolysis ($\mu mol \cdot min^{-1} \cdot mL^{-1}$) |
|---|---|---|
| VX | $2.4 \times 10^{-4}$ | 0.005 |
| Tetriso | $2.4 \times 10^{-4}$ | 0.0325 |
| VX | $9 \times 10^{-5}$ | 0.0025 |
| Tetriso | $9 \times 10^{-5}$ | 0.01 |

* By "direct" Ellman as described in the text; the rate of hydrolysis is expressed per mL of a single enzyme (OPH) preparation containing 0.2 to 0.3 mg enzyme per mL, $Co^{2+}$ form; pH 7.0; 24–26°.

Because of the limited amount of VX available, the effect of OPH on VX was tested at two concentrations only, $9 \times 10^{-5}$ M and $2.4 \times 10^{-4}$ M. Tetriso was also employed in identical side-by-side experiments. The results are given in Table 2. These experiments consumed most of our supply of VX and, since only two points were obtainable, the data are not suitable for presentation in Fig. 1. Nevertheless, it appears that VX is hydrolyzed at about a fifth the rate of Tetriso. Also, because a direct comparison of the results of Tables 1 and 2 was not considered reliable, separate experiments were undertaken to explore the relationship of hydrolysis to detoxication.

The enzymatic hydrolysis of $10^{-4}$ M Tetriso was followed by the direct Ellman measurement. At a time and absorbance when a fixed percentage of Tetriso had been hydrolyzed, a sample was removed from the cuvette, diluted, and subjected to the indirect measurement. The results are presented in Table 3. Essentially the same experiments were carried out with the hydrolytic/detoxication agency being elevated pH. Because this interferes with the Ellman reaction, some modification was necessary. Solutions of $10^{-4}$ M Tetriso at pH 11.5 to 12 and at pH 7 were sampled at fixed times, readjusted to pH 7 where necessary, and carried through the direct and indirect reactions. The results are also presented in Table 3. Because these reactions were being sampled at predetermined times rather than absorbancies, more determinations were deemed necessary than when OPH was the agent.

Table 3. Hydrolysis and detoxication of Tetriso by two different agencies: enzymatic *versus* elevated pH

| Time (hr) | Agency | Hydrolysis (%) | Detoxication (%) |
|---|---|---|---|
| 0 | * | 0.4 ± 0.2 (6)† | 0‡ |
| 1.25 | Enzymatic | 40 | 56 |
| 1.25 | Enzymatic | 40 | 74 |
| 2 | pH 11.5–12 | 12 ± 0.2 (4) | 35 ± 1 (4) |
| 4 | pH 11.5–12 | 22 ± 1.5 (4) | 60 ± 15 (4) |

* For either agency, these samples were taken just before adding enzyme or elevating pH.

† Values are means ± SD (N), where applicable.

‡ Zero time inhibition was used to calculate the second order rate constant for inhibition of AChE, a necessary component of the "indirect" Ellman method.

Table 4. Hydrolysis of DFP and Soman compared with that of Tetriso by an enzyme (OPH) derived from *Pseudomonas diminuta*

| Substrate | Concentration (M) | Rate of hydrolysis ($\mu mol \cdot min^{-1} \cdot mL^{-1}$) |
|---|---|---|
| DFP* | $3 \times 10^{-3}$ | 32 |
| Soman* | $3 \times 10^{-3}$ | 4 |
| Tetriso† | $3 \times 10^{-3}$ | 0.5 |

* By the $F^-$-sensitive electrode method.

† From Fig. 1.

The higher rate of enzymatic hydrolysis of the phosphoro substrate, Tetriso, than of the phosphono substrate, VX, was reminiscent of a similar property of OPAA purified from squid nerve (formerly termed squid-type DFPase) with respect to DFP and Soman [2]. The rates of hydrolysis of these two substrates by OPH are given in Table 4. The rate of hydrolysis of Tetriso is also included.

Samples of OPH were incubated ice cold for 20 min with the following chelators (see Ref. 2 for background) at $1.5 \times 10^{-4}$ M: EDTA; 8-OHQ-5-SA; and 1,10-$\phi$. The direct Ellman assay was then carried out with Tetriso as substrate in such a manner that the chelators continued to be present at $1.5 \times 10^{-4}$ M. A control determination was also

Table 5. Effects of three chelators on OPH derived from *Pseudomonas diminuta*

| Chelator* | Tetriso† hydrolysis ($\mu$mol·min$^{-1}$·mL$^{-1}$) | Inhibition (%) |
|---|---|---|
| None | 0.51 | 0 |
| EDTA | 0.353, 0.448 | 31, 12 |
| 8-OHQ-5-SA | 0.02 | 96 |
| 1,10-$\phi$ | 0 | 100 |

* Present at $1.5 \times 10^{-4}$ M.
† Present at $2 \times 10^{-3}$ M.

made with no chelator present. The results are shown in Table 5.

Many of the results are single observations. For VX, a major reason for this was the limited supply, now exhausted. For all four of the organophosphorus compounds—VX, Tetriso, Soman and DFP—safety was a major consideration since they were employed here at substrate (rather than inhibitor) concentrations. For Tetriso, results given in the tables can usually be found in Fig. 1, which is a collection of fourteen determinations. With every substrate, preliminary experiments were performed that agreed with the findings presented here but were judged unsuitable for presentation. Thus, with DFP and Soman as substrates, the first determinations gave rates too fast to be recorded but in general agreement with those finally presented in Table 4.

## DISCUSSION

An enzyme (OPH) derived from *Pseudomonas diminuta*, already briefly reported to cleave the P—S bond of VX [7], has now been shown to detoxify VX. Tetriso, a close analogue of VX (both structurally and as an AChE inhibitor), was also hydrolyzed and detoxified by OPH. In the $10^{-4}$ M concentration range Tetriso was hydrolyzed about five times faster than VX; the less accurate but more sensitive detoxication measurements confirmed this. The chemical and biological similarities of these two compounds, the more rapid degradation of Tetriso by either method of measurement, and the smaller (although not negligible) hazard of working with Tetriso make this compound a useful research tool in the continuing search for ways to detoxify stockpiles of VX [13].

This *Pseudomonas* enzyme also hydrolyzed DFP and Soman. Several questions arise from this finding. Whether separate designations are justified (OPH and EC 3.1.8.1, or OPAA and EC 3.1.8.2) [3] is the least of these. The rather high $K_m$ values for OPH and Tetriso, as well as all that has been published on the DFP- and Soman-hydrolyzing enzymes [14], and the clearly laboratory origins of all of these compounds, suggest that Tetriso is not the natural substrate for this enzyme. Both VX and Soman are phosphono compounds, a step further removed from most (but not all) of the naturally occurring phosphorus metabolites. The two chiral centers of Soman have resulted in rather wide discrepancies between hydrolytic and detoxication rates (see Chapters 5–9 of Ref. 14). We did not have sufficient VX to permit the exploration of this question, and in addition VX, somewhat like Sarin, has only one chiral center. The OPAAs seem to show little stereoselectivity toward Sarin [15, 16].

The apparently higher rate of detoxication of Tetriso than of P—S bond hydrolysis does not, of course, involve stereoselectivity. The results in the lower half of Table 3 suggest that base-catalyzed hydrolysis attacks any one of the three ester linkages around the P atom of Tetriso with approximately equal probability, the hydrolysis of any one of which destroys the ability to inhibit AChE. Only one of these three attacks produces an Ellman-sensitive product. This may seem incompatible with a report [17] that the P—O hydrolysis product of VX is an AChE inhibitor (and by analogy the P—O hydrolysis product of Tetriso). However, the inhibition constants for VX and this hydrolysis product show that the product is only about 3% as potent as VX itself—a potent inhibitor, but negligible by comparison. Turning again to Table 3, the enzymatic hydrolysis, carried out at pH 7 where Tetriso is normally stable for days, suggests that OPH may also cleave a P—O bond, but at a lower rate than the P—S bond.

The inhibition of OPH hydrolysis of Tetriso by DFP is subject to some ambiguity since, at the level of enzyme used to hydrolyze Tetriso, considerable DFP hydrolysis was simultaneously taking place. The results with diisopropyl phosphate in which the $K_i$ for that compound is about 1/30th the $K_m$ for Tetriso are probably a more accurate reflection of the greater affinity of DFP than of Tetriso for the active site of the OPH. This is in contrast to the squid OPAA in which the $K_m$ for DFP and $K_i$ for diisopropyl phosphate are about equal [18].

Another ambiguity is that, while the transition element chelators 8-OHQ-5-SA and 1,10-$\phi$ cause complete inhibition of OPH, EDTA (which binds the three ions that might be involved—$Co^{2+}$, $Zn^{2+}$ or $Mn^{2+}$—a million times more tenaciously) causes only partial inhibition of the OPH. In contrast, EDTA completely inhibits both the squid OPAA and a *Stearothermophilus* OPAA [2]. One explanation, despite evidence to the contrary [19], is that the OPH is not yet a single pure enzyme.

Despite minor inconsistencies, a mild enzymatic means of hydrolyzing one of the most lethal chemical compounds has now been shown to accomplish detoxication. Further, a less hazardous analogue is now available for the development of optimal conditions for these reactions.

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