Biochemical Pharmacology, Vol. 55, pp. 159–168, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00430-9

Combined Effect of Organophosphorus Hydrolase and Oxime on the Reactivation Rate of Diethylphosphoryl-Acetylcholinesterase Conjugates

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ABSTRACT. Reactivation of inhibited acetylcholinesterase (AChE) is essential for rapid recovery after organophosphate (OP) poisoning. However, following administration of an oxime reactivator, such as pralidoxime mesylate (P2S), in patients poisoned with certain diethylphosphorothioate pesticides, no reactivation is observed, presumably due to reinhibition by circulating anti-cholinesterase OPs. Pretreatment alone with organophosphorus hydrolases (OPH) that are capable of rapidly hydrolyzing OPs was demonstrated, in animals, to confer significant protection against OP toxicity. One strategy to augment the potentially therapeutic scope of OPHs is a combined post-exposure treatment consisting of a drug(s) commonly used against OP toxicity and a suitable hydrolase. In this study, we examined the *in vitro* ability of OPH from *Pseudomonas* sp. (OPH*ps*) to prevent reinhibition of P2S-reactivated AChE by excess OPs. The kinetic parameters of the reactivation of a series of diethylphosphoryl-AChE (DEP—AChE) conjugates, obtained by the use of various diethylphosphates, were determined and compared with the rates of reactivation in the presence of OPH*ps*, with and without the OP inhibitors in the reactivation medium. Extrapolation of the *in vitro* results to *in vivo* conditions suggests that an OPH*ps* concentration as low as 1 μg/mL blood would result in a 100-fold decrease in the concentration of circulating anti-AChE pesticides within less than one blood-circulation time, thereby minimizing reinhibition of the reactivated enzyme. Thus, for DEP-based pesticides, the combination of P2S–OPH treatment can significantly improve clinical recovery after OP intoxication. In addition, it is shown here for the first time that an OPH can effectively hydrolyze quaternary ammonium-containing OPs. This indicates that hydrolysis of phosphorylated oximes, toxic side products of oxime treatment, may also be accelerated by OPHs. BIOCHEM PHARMACOL **55**;2:159–168, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. organophosphates; inhibition; reactivation; acetylcholinesterase; phosphotriesterases; detoxification

The toxicity of OP§ pesticides and nerve agents is attributed primarily to the inhibition of AChE (EC 3.1.1.7) at important physiological sites. OPHs (EC 3.1.8.1) from *Pseudomonas* sp. (OPH*ps*) and *Pseudomonas diminuta* MG (OPH*pd*) were shown to confer significant protection against OP toxicity when given alone [1–5], in combination with atropine [6], or in conjunction with a carbamate–atropine treatment [7]. The antidotal efficacy of OPHs depends on the affinity of the OP substrates (K_m), and the catalytic efficiency (k_{cat}) of the enzymic-enhanced hydrolysis (Fig. 1) [1–8]. Calculations based on the results of protection experiments established a correlation between the kinetic constants of the enzymic hydrolysis of OPs and the blood–hydrolase concentration needed to rapidly hydrolyze a variety of potent anti-ChE OPs [1, 2, 4, 5]. These observations, taken with the reported wide range of values of the affinity and the kinetic constants, indicate that in order to confer a meaningful protection with low doses of an OPH against broad types of OPs, more than one kind of enzyme may be required. Pretreatment with ChEs alone has also been shown to protect animals against lethal doses of highly toxic OPs [9–12]. However, the stoichiometry of scavenging by ChEs is limited to a single turnover [11–13]. Thus, OPHs have the advantage of being able to detoxify a large amount of OPs by a relatively small dose of enzyme in less than one blood-circulation time.

One approach to amplify the potentially therapeutic scope of OPHs is a combined treatment consisting of a

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§ *Abbreviations:* AChE, acetylcholinesterase; ChE, cholinesterase; DECP, *O*,*O*-diethyl chlorophosphate; DEFP, *O*,*O*-diethyl flurophosphate; DEP, diethylphosphoryl; DEPQ, 7-(*O*,*O*-diethylphosphinyloxy)-1-methylquinulinium methylsulfate; HuBChE, human serum butyrylcholinesterase; MEPQ, 7-(methylethoxyphosphinyloxy)-1-methylquinulinium iodide; OP, organophosphate, organophosphorus; OPH, organophosphorus hydrolase; OPH*ps*, OPH from *Pseudomonas* sp.; P2S, 2-(hydroximinomethyl)-1-methylpyridinium methansulfonate; 2-PAM, iodide salt of P2S; POX, phosphoryl oxime; phospholine iodide, S-[2-(*N*,*N*,*N*-trimethylammonio)ethyl]-*O*,*O*-diethylphosphorothiolate iodide; rMoAChE, recombinant mouse AChE; and TEPP, O,O,O′,O′-tetraethyl pyrophosphate.

Received 21 April 1997; accepted 2 July 1997.

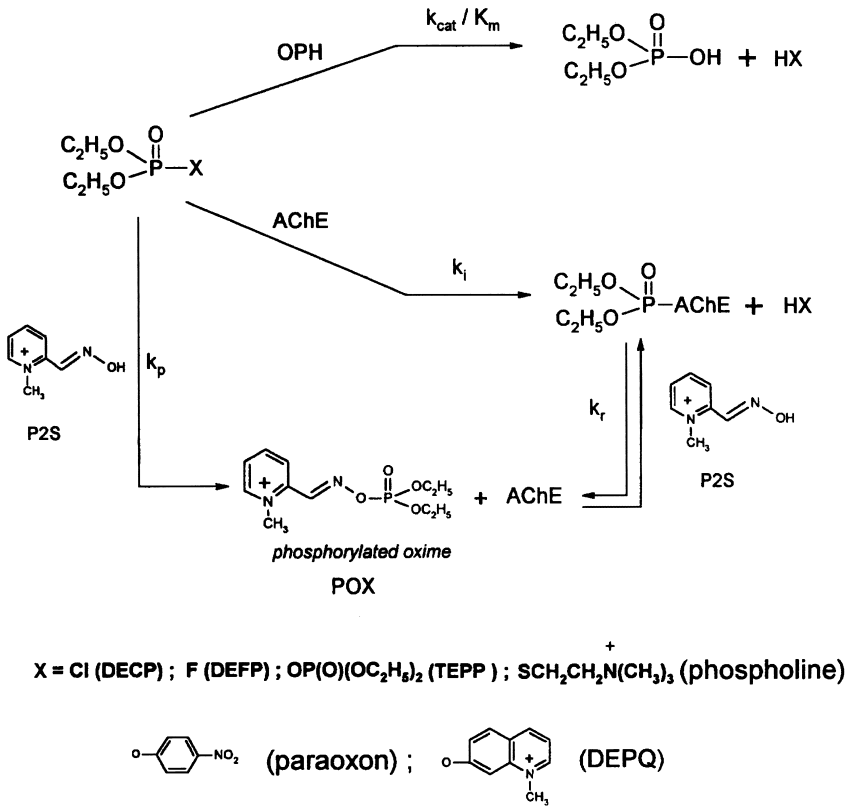

FIG. 1. Chemical pathways of the OPH-catalyzed hydrolysis of diethylphosphates and the inhibition and P2S-induced reactivation of DEP—AChE.

commonly used drug against OP toxicity and a suitable OP hydrolase. The accepted approach to prevent and alleviate OP toxicity is based on pretreatment with pyridostigmine, a carbamate capable of partial and temporary masking of AChE, followed by administration of a cholinergic antagonist, such as atropine, and an oxime reactivator of the inhibited enzyme [14]. Combined treatment with a reactivator and OPH offers a promising approach with dual therapeutic action. First, it has been shown that the continuous and slow release of anti-ChE metabolites of pesticides, such as parathion and related phosphorothioates, into the blood renders standard therapy management quite difficult [15]. Willems *et al.* [15] demonstrated the dependence of the therapeutic effect of the oxime P2S (pralidoxime mesylate) on the persistence of anti-ChEs in the blood of patients intoxicated with OP pesticides. Reactivation was not observed when parathion and methyl parathion blood levels exceeded 30 ng/mL. Second, one of the drawbacks of oxime therapy is the possible accumulation of POXs, both inside and outside the active site gorge. POXs are substantially more toxic than the parent OPs (Fig. 1) [16–19]. The origin of the POX is 2-fold: (i) direct reaction between the oxime and OPs (k_p, Fig. 1); and (ii) a possible intermediate formed during the reactivation of phosphorylated ChEs. Therapy with OPH may benefit from substantial reduction in the concentration of these toxic by-products.

Several reactions can occur simultaneously among OPs, ChEs, oxime reactivators, and an OP hydrolase (Fig. 1). These reactions, together with the activity of endogenous scavengers of OPs, produce potential complications in the interpretation of the results from *in vivo* experiments. We therefore embarked first on the *in vitro* characterization of the efficacy of an oxime–OPH combination. The kinetic parameters of P2S-induced reactivation of *O,O*-diethylphosphorylated AChE (DEP—AChE), obtained by using a variety of *O,O*-diethylphosphates, were determined and compared with the rates of reactivation of the same conjugates in the presence of OPH*ps*, with and without the OP inhibitors in the reactivation medium. Extrapolation of the *in vitro* results to *in vivo* conditions suggests that the proposed combination can significantly improve clinical recovery after intoxication with certain OPs. We also present for the first time evidence that an OPH can effectively hydrolyze potent ChE inhibitors that contain a quaternary ammonium moiety. The observation that charged OPs are substrates of an OP hydrolase indicates that the detoxification of the structurally related POX may also be accelerated by OPHs.

MATERIALS AND METHODS

Materials

MEPQ [20], DEPQ [21], and TEPP [22] were prepared as described elsewhere [20–22]. DEFP was prepared by fluorination of DECP as described by Saunders and Stacey [23]. DECP was obtained from the Aldrich Chemical Co. and distilled before use. *O,O*-Diethyl *p*-nitrophenyl phosphate (paraoxon) was purchased from the Sigma Chemical Co. and used as obtained. The structure and the purity (>96%)

of all OPs were confirmed by ^{1}H NMR and ^{31}P NMR spectroscopy and gas chromatography. TEPP contained less than 5% O,O,O-triethyl phosphate. Phospholine iodide was obtained from Ayerst Laboratories. P2S and its iodide salt, 2-PAM, were purchased from Sigma.

OPH from *Pseudomonas* sp. was purified as previously described [24]. Briefly, the enzyme was obtained by precipitation of cell-free extract with ammonium sulfate (50% saturation) and fractionated on DEAE-cellulose and Sephadex G-100 columns. These purification steps resulted in a single protein band with a molecular mass of 35 kDa, as analyzed by SDS–PAGE [24]. OPH*ps* was stored at −20° in 50 mM Tris-50% glycerol buffer containing 1% Triton X-100, pH 8.0. Enzyme was diluted 100- to 1000-fold into the reactivation medium. Purified wild-type rMoAChE (~14 nmol/mg) was donated by Prof. P. Taylor and Dr. Z. Radic from the Department of Pharmacology at the University of California, La Jolla. HuBChE (~12 nmol/mg) was purified by procainamide-Sepharose 4B gel affinity chromatography [25]. Active-site concentrations of rMoAChE and HuBChE were determined by titrations with both MEPQ [12, 20] and DEPQ [21].

Enzyme Assays

The release of *p*-nitrophenol (400 nm) from 1 mM paraoxon was used to measure OPH activity in 50 mM phosphate buffer, pH 8.0, at 25°. The specific activity was approximately 1000 units/mg. OPH-catalyzed hydrolysis of phospholine iodide was monitored by using Ellman's reagent, 5,5′-dithio-bis-2 nitrobenzoic acid (DTNB), as previously described [26]. The activity of AChE and HuBChE was determined by the method of Ellman *et al.* [27], using 0.5 mM acetylthiocholine iodide and butyrylthiocholine iodide, respectively. Assays were carried out in 50 mM phosphate buffer, pH 8.0, at 25°.

Preparation of DEP—AChE and DEP-HuBChE Conjugates

Two to ten microliters of a concentrated solution of paraoxon, DEFP, and TEPP (2 μM each, in isooctane) was added by stirring into 3 mL AChE (1–3 nM) in 10 mM Tris, containing 0.05% BSA, pH 7.8, at 25°. DEPQ and phospholine were diluted from a stock solution in deionized water to a final concentration of 3 and 10 nM, respectively. DECP-inhibited enzyme was obtained by dropwise addition of 10 mM DECP in isooctane over a period of 20 min (total of 10 μL). The decrease in enzymic activity was monitored until inhibition was almost total. To remove traces of unreacted OPs, the inhibited enzyme was dialyzed two times against 2 L of 10 mM Tris, pH 7.8, for 48 hr at 5°.

A stock solution of approximately 50 μM HuBChE in 10 mM Tris, pH 7.8, containing 0.05% BSA was incubated for 15 min with 0.4 mM paraoxon. To ascertain removal of excess paraoxon, 1 mL of the inhibited enzyme was dialyzed six times against 2 L of 10 mM Tris, pH 7.8, over 4 days at 5°.

Reactivation of DEP—AChE and DEP-HuBChE

The pH of the inhibited enzymes was readjusted to 7.8 prior to the initiation of the reactivation. Stock solutions of 1–50 mM P2S or 2-PAM in deionized water adjusted to pH 7.8 were diluted into the inhibited enzyme that was preincubated at 29°, to a final concentration of 0.01 to 0.5 mM. The rate of reactivation was followed at 29° by diluting the reactivation medium 150 times in the assay cuvette. Nonspecific hydrolysis of the substrate was subtracted. Reactivation was practically completed within 6 hr for DEP-AChE and 16 hr for DEP-HuBChE. The reactivation in the presence of OPH, with or without OPs (at 1–3 μM), was carried out as described above. The OP was first added to the DEP—AChE in the reactivation medium, followed by simultaneous dilution of stock solutions of oxime and OPH, both in 10 mM Tris, pH 7.8.

Hydrolysis of DEPQ and MEPQ by OPH

The equilibrium and kinetic constants of OPH*ps*-induced hydrolysis of DEPQ were determined by measuring the initial velocity of the release of 7-hydroxyquinulinium cation (7-HQ) at 406 nm, in 50 mM phosphate buffer, pH 8.0, at 25° [21]. The same approach was used to monitor the hydrolysis of OPH*ps*-catalyzed hydrolysis of MEPQ [20].

Determination of the Inhibition Rate Constants of rMoAChE

Standard stock solutions of OPs (0.1 to 50 μM) were freshly prepared in deionized water and diluted at time zero into AChE solution (0.2 to 2 nM) in 10 mM Tris, pH 7.8, 29°. The final concentration of the inhibitors was sufficiently high to establish pseudo-first-order reaction conditions. At selected time intervals, aliquots were diluted into the Ellman's mixture for measurements of residual activity. Data for the time–course of the inhibition were analyzed by nonlinear regression analysis using a monoexponential decay equation.

^{31} NMR Spectrometry of TEPP

Spectra were recorded with a GN 300WB NMR instrument (General Electric) at 121.65 MHz (^{31}P) for 14 mM TEPP in 0.1 M Tris, pH 7.8, at room temperature. A D_2O ampule immersed in the tested solution served for field frequency lock. Spectral data were accumulated at 30° pulse width, 8 kHz spectral width, and 2-sec pulse delay between consecutive scans. One hundred transients were accumulated for each run. ^{31}P NMR chemical shifts were assigned to external 1% trimethyl phosphate in C_6D_6 that was set to 0 ppm.

TABLE 1. Biochemical parameters of the reactivation of $(C_2H_5O)_2P(O)X$-inhibited AChE by 2-(hydroximinomethyl)-1-methylpyridinium cation*

OP	X	K_{OX}† (mM)	k'_{max}† (min^{-1})	k_r† (M^{-1} min^{-1})
DECP	Cl	0.142 (±0.017)	0.222 (±0.006)	1563
DEFP	F	0.206 (±0.056)	0.250 (±0.036)	1213
Paraoxon	*p*-Nitrophenoxy	0.157 (±0.038)	0.211 (±0.020)	1344
TEPP	$OP(O)(C_2H_5)_2$	0.136 (±0.038)	0.216 (±0.024)	1588
Phospholine§	$SCH_2CH_2N^+(CH_3)_3$	0.254 (±0.051)	0.278 (±0.027)	1094
DEPQ‖	7-Oxyquinulinium-1-methyl	0.189 (±0.030)	0.263 (±0.034)	1391

* Obtained by use of either P2S or 2-PAM (10 mM Tris, pH 7.8, 29°).
† Obtained from nonlinear regression analysis in accordance with Equation 1. Figures in parentheses are SEM (N = 5–13).
‡ $k_r = k'_{max}/K_{OX}$.
§ Iodide salt.
‖ Methylsulfate salt.

RESULTS

Reactivation of Diethylphosphoryl-AChE Conjugates

The main goal of this study was to define the capacity of OPH*ps* to prevent reinhibition of P2S-reactivated enzyme by a variety of $(C_2H_5O)_2P(O)X$ (Fig. 1; Table 1). In several cases, the inhibited enzyme was spiked with a different diethylphosphate than the OP that was used to obtain the DEP—AChE conjugate. Thus, it was important first to ascertain that all DEP—AChEs display the same reactivation characteristics. The data of the time–course of the reactivation were best fitted by a single exponential kinetic equation (upper curve, Fig. 2A). The homogenous class of reactivatable conjugates is attributed to the extensive dialysis that removes the excess of unreacted inhibitors, and to the low molar concentration of the inhibited enzyme in the reactivation medium (1–3 nM) that minimizes reinhibition by POX. Results from a recent study [28] showed a slight deviation from monoexponential kinetics of the reactivation of DEP—AChE that was obtained by the use of paraoxon. In addition, earlier investigations with bovine AChE demonstrated significant deviations from first-order reactivation profiles of DEP—AChE [16, 29]. These discrepancies are likely to arise from differences in either the experimental protocols, the purity of the enzyme used, or the source of the AChEs.

Reactivation depends on the affinity of the oxime to the inhibited enzyme (K_{ox}) and the subsequent nucleophilic displacement (k'_{max}), as described below in Scheme 1:

$$\text{DEP-AChE} + \text{OX} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [\text{DEP-AChE][OX}] \xrightarrow{k'_{max}} \text{AChE} + \text{DEP-OX}$$

SCHEME 1. Oxime-induced reactivation.

The relationship between the apparent first-order reactivation constant (k_{obs}) at oxime concentration [OX], and the reactivation parameters K_{ox} and k'_{max} are given in Equation 1:

$$k_{obs} = k'_{max}(1 + K_{ox}/[OX])^{-1} \quad (1)$$

Assuming that the dissociation of the complex [DEP-AChE][OX] (k_{-1}) is appreciably faster than k'_{max}, K_{ox} in

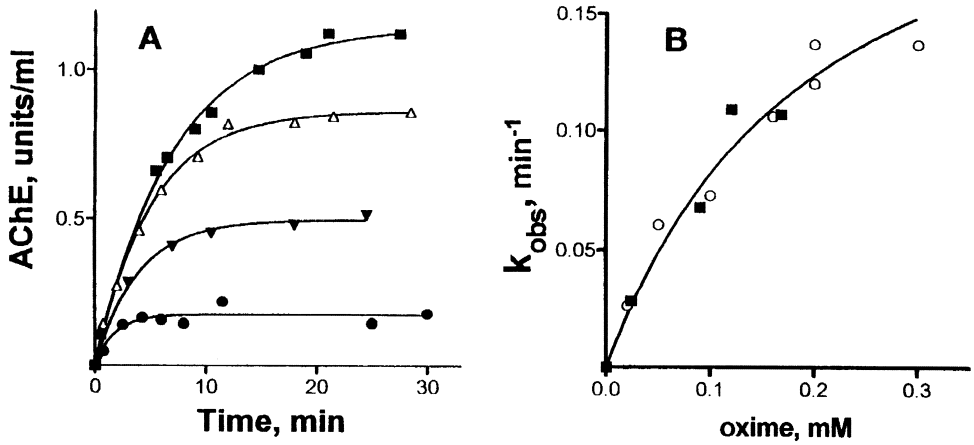

FIG. 2. Reactivation of paraoxon-inhibited AChE. (A) Time–course of reactivation of paraoxon-inhibited AChE by 0.2 mM P2S in the presence of increasing concentrations of paraoxon. Key: (■) no inhibitor; (△) 0.068 μM; (▼) 0.4 μM; and (●) 2.0 μM. (B) Plot of k_{obs} versus [oxime] for reactivation of paraoxon-inhibited AChE by P2S (○) and its iodide salt, 2-PAM (■).

Equation 1 is approximated by the dissociation constant of the complex [DEP—AChE][OX] (k_{-1}/k_{+1}). The bimolecular rate constant of the reactivation, k_r, is the ratio k'_{max}/K_{ox}. The constants K_{ox} and k'_{max} were resolved by nonlinear regression analysis of the plots of k_{obs} versus [OX] as illustrated for paraoxon-inhibited AChE (Fig. 2B), and are summarized in Table 1. As expected, P2S and 2-PAM, which consist of the same cation nucleophile, gave the same results. Table 1 shows that similar K_{ox} and k'_{obs} control the overall rate of reactivation of DEP—AChE conjugates, suggesting that all the diethylphosphates used produced the same reactivatable enzyme, irrespective of the leaving group X. This observation permitted the use of a variety of diethylphosphates listed in Table 1, to spike any of the DEP—AChE conjugates, because the reinhibited enzyme is expected to undergo reactivation at the same rate as the starting DEP—AChE.

Reactivation of DEP—AChE in the Presence of $(C_2H_5O)_2P(O)X$

Reactivation of 1–3 nM DEP—AChE that was spiked with inhibitory concentrations of diethylphosphates proceeded to a level that differed appreciably from that obtained in the absence of inhibitor. This is illustrated in Fig. 2A for paraoxon-inhibited AChE that was spiked with increasing concentrations of paraoxon. Similar results were obtained for DEP—AChE conjugates obtained from DEPC and TEPP, and spiked with paraoxon (not shown). It should be noted that for initial concentrations of 1–3 nM DEP—AChE, it is assumed that possible interference of POX (Fig. 1) is negligible.

The data points of the reactivation time–course in the presence of inhibitor were best fitted to a monoexponential equation, in accord with a first-order opposing reaction. Thus, the rate of approach to equilibrium (i.e. steady-state), derived for Scheme 2, is given by Equation 2:

$$\text{DEP-AChE} \underset{k'_i(+\text{ inhibitor})}{\overset{k'_r(+\text{ oxime})}{\rightleftharpoons}} \text{AChE}$$

SCHEME 2. Kinetic scheme of opposing reactivation–inhibition reactions.

$$k'_{obs} = k'_r + k'_i = t^{-1} \ln [E_{eq}/(E_{eq} - E_t)] \quad (2)$$

and after rearrangement

$$E_t/E_{eq} = 1 - e^{-k'_{obs}t} \quad (3)$$

The constants k'_r and k'_i are the apparent first-order reactivation and inhibition rate constants, respectively. E_{eq} and E_t are the activity of the free enzyme at equilibrium and at time t, respectively. The rate constant k'_{obs} was obtained by nonlinear regression analysis of the plot of E_t/E_{eq} versus t, and compared with the predicted value, which is the sum of $k'_r + k'_i$. The constant k'_r was calculated from Equation 1. Calculation of k'_i was based on the following consideration: P2S is a quaternary ligand that binds to AChE [28]. This reversible complexation is expected to slow the inhibition. The fraction of the enzyme that is unbound to the oxime is $(1 + [OX]/K'_{ox})^{-1}$. Thus, k'_i was corrected as follows:

$$k'_i = k_i[I](1 + [OX]/K'_{ox})^{-1} \quad (4)$$

where k_i and K'_{ox} were set at 1.65×10^6 M^{-1} min^{-1} and 0.05 mM, respectively [28]. [OX] was at 0.2 mM. Results are summarized in Table 2. The close agreement between the observed and calculated rate constants supports the kinetic assumptions that underlie the mechanism of the approach to steady-state in the presence of inhibitor in the reactivation medium.

TABLE 2. Rate constants of the approach to steady-state of DEP—AChE reactivation in the presence of paraoxon and P2S at 0.2 mM

Paraoxon (μM)	k'_{obs},* (min^{-1})	$k'_r + k'_i$† (min^{-1})	Max. reactivation (units/mL)
0	0.131		1.10
0.068	0.197	0.152	0.86
0.17	0.210	0.187	0.65
0.40	0.244	0.233	0.51
2.0	0.670	0.791	0.17

* Observed reactivation rate constants. Calculated from Equation 3.

† Predicted reactivation rate constants. $k'_r = 0.131$ min^{-1}. k'_i (corrected) was calculated according to Equation 4.

Effect of OPHps on the Reactivation of DEP—AChE in the Presence of $(C_2H_5O)_2P(O)X$

OPH*ps* alone did not affect the rate of reactivation of DEP—AChE by P2S, even when the concentration of the enzyme was increased to 4 μg/mL. However, OPH*ps* significantly augmented, in a concentration-dependent manner, the recovery of enzyme activity of DEP—AChE conjugates that were spiked with paraoxon, DEFP, TEPP, and DEPQ (Fig. 3, A–D). This was attributed to the rapid hydrolysis of the OPs. The amount of OPH*ps* required to decrease the concentration of 2 μM DEPQ to a level that did not affect AChE activity ($k_i = 7.46 \times 10^8$ M^{-1} min^{-1}; Table 3) was greater than the amount of OPH*ps* needed to hydrolyze 2 μM of the less potent inhibitors, paraoxon ($k_i = 1.65 \times 10^6$ M^{-1} min^{-1}) and DEFP ($k_i = 3.82 \times 10^5$ M^{-1} min^{-1}), at least 10- and 2.5-fold, respectively. Our calculations show (Table 3) that k_{cat}/K_m was sufficient to decrease 1000-fold the initial concentration of 2 μM paraoxon within 0.18 min. The apparent rate constant of inhibition of AChE by 2 nM paraoxon (0.0033 min^{-1}; $T_{1/2}$ = 209 min) was very low compared with the rate of reactivation by 0.2 mM P2S (0.131 min^{-1}; $T_{1/2}$ = 5.3 min). A similar reduction in the concentration of DEPQ

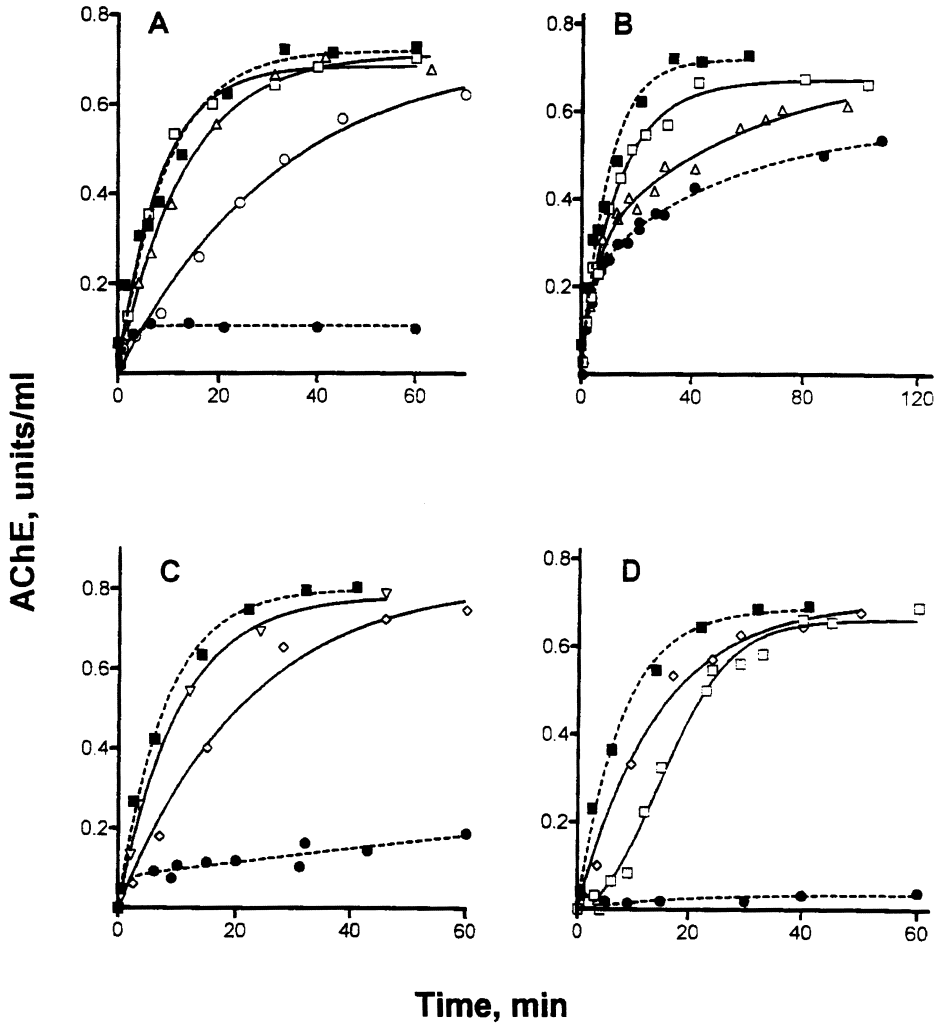

FIG. 3. Time–course of the reactivation of DEP—AChE by 0.2 mM P2S in the presence of 2 μM diethylphosphates and various concentrations of OPH*ps*. *Dotted lines*, controls: (■) no inhibitor, + OPH; and (●) + inhibitor, no OPH*ps*. *Solid lines*, + inhibitor, + OPH*ps* (μg/mL): (○) 0.01; (△) 0.025; (□) 0.1; (◇) 0.25; and (▽) 1.0. (A) paraoxon; (B) DEFP; (C) TEPP; and (D) DEPQ.

would be obtained after 0.79 min. However, the apparent rate constant of inhibition under this condition is 1.5 min^{-1}. Therefore, in order to further decrease the rate of reinhibition of the reactivated AChE, higher concentrations of OPH*ps* were required for this OP than for paraoxon. Further, a 1000-fold decrease in DEFP concentration is expected to occur within 4.65 min. Yet, in view of its relatively small k_i, a 10-fold decrease of 2 μM is sufficient to render the apparent rate constant of the inhibition below 0.08 min^{-1} ($T_{1/2}$ = 8.6 min). This can be completed within 1.5 min. These calculations highlight the quantitative aspects of OPH-enhanced hydrolysis of diethylphosphates by oxime reactivators in the presence of inhibitory concentrations of OPs.

The data in Fig. 3C clearly suggest that TEPP was also hydrolyzed rapidly by OPH*ps*. Since the K_m and k_{cat} of TEPP are unknown, ^{31}P NMR was used to demonstrate its enhanced hydrolysis by the enzyme (Fig. 4). Our results showed that the hydrolysis of TEPP to the corresponding O,O-diethyl phosphoric acid is accelerated in the presence of OPH*ps*, relative to the spontaneous breakdown by water. Signal **3** arose from triethyl phosphate (TEP), a non

TABLE 3. Equilibrium and kinetic constants of diethylphosphates versus OPH*ps* (hydrolysis) and AChE (inhibition)*

	OPH*ps*				
OP	K_m (mM)	$k_{cat} \times 10^4$ (min^{-1})	k_{cat}/K_m (M^{-1} min^{-1})	Time to hydrolyze 2 μM OP† (min)	AChE k_i (M^{-1} min^{-1})
Paraoxon	0.032	4.45	1.39×10^9	0.18	1.65×10^6
DEPQ	0.079	2.38	3.01×10^8	0.79	7.46×10^8
DEFP‡	0.25	1.34	5.2×10^7	4.65	3.82×10^5

* 10 mM Tris, pH 7.8, 29°.

† Calculated by using an approximation of the integrated form of the Michaelis–Menten equation [1]. Figures are estimates of time to decrease 1000-fold the initial concentration of OP, by 1 μg/mL OPH*ps*.

‡ Taken from Ref. 1 (pH 7.4, 37°).

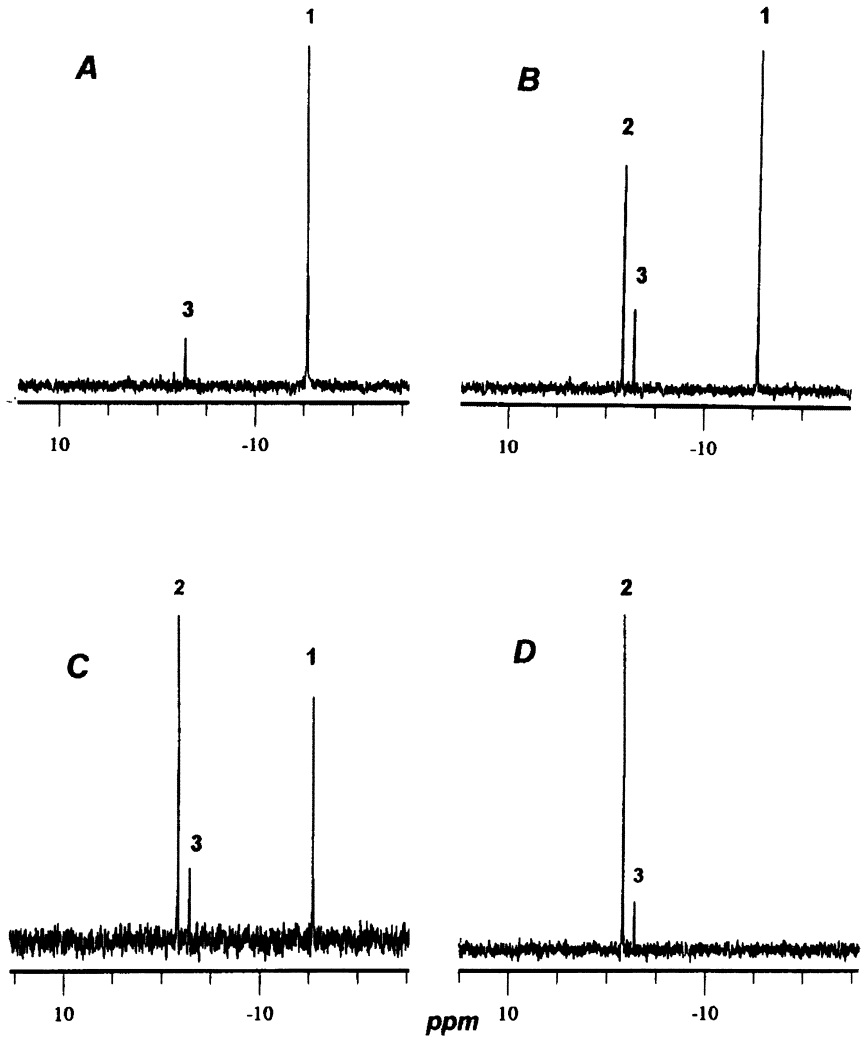

FIG. 4. ^{31}P NMR spectra of 14 mM TEPP in 0.1 M Tris, pH 7.8, with and without 1 μg/mL OPH*ps*. (A) no OPH*ps*, t = 0; (B) no OPH*ps*, t = 120 min; (C) + OPH*ps*, t = 30 min; and (D) + OPH*ps*, t = 85 min. Line assignment: 1, $(C_2H_5O)_2P(O)$-O-$(O)P(OC_2H_5)_2$ (TEPP); 2, $(C_2H_5O)_2P(O)OH$; and 3, $(C_2H_5O)_3P(O)$.

anti-ChE stable impurity in TEPP. TEP was used as an internal standard for monitoring the progression of the hydrolysis of TEPP.

The reactivation time–course in the presence of DEFP and TEPP without OPH*ps* deviated from simple first-order reactivation ↔ inhibition kinetics (see lower dotted curves in Fig. 3, B and C). This was attributed to a parallel spontaneous hydrolysis of both OPs, a fact that precluded approach to a stable equilibrium, as was observed with the stable inhibitor paraoxon (Figs. 2A and 3A).

Based on monitoring changes in DTNB, phospholine was found to be a very poor substrate for OPH*ps*, as would be expected of a phosphorothiolate [30, 31]. No reactivation of DEP—AChE could be detected in the presence of 2 μM phospholine, with as high as 2 μg/mL OPH*ps*, and after a 30-min incubation with 0.2 mM P2S.

Reactivation of DEP-HuBChE in the Presence of OPHps

It has been shown that BChE of human plasma can readily sequester OPs [12, 13]. Since a large quantity of the purified enzyme was available to us, it was used to examine the possibility of reinhibition of a ChE by the putative phosphorylated oxime (POX, Fig. 1), and to determine whether OPH*ps* can counteract the observed POX-related slowed reactivation.

The reactivation time–course was determined in 1 mM P2S. A clear dependence of the rate of reactivation on the initial concentration of DEP-HuBChE was observed (not shown). Thus, 5, 118, and 210 nM DEP-HuBChE were reactivated at 0.058 ± 0.003, 0.022 ± 0.003, and 0.010 ± 0.002 min^{-1}, respectively. The observation that the reactivation depends on the initial concentration of the inhibited enzyme strongly indicates that POX may be accumulated in sufficient quantity to reinhibit HuBChE and slow the overall rate of regeneration of enzyme activity. However, no acceleration of the P2S-induced reactivation was detected in the presence of 1 μg/mL of OPH*ps*. Although OPH*ps* did not accelerate the hydrolysis of the putative POX, we cannot rule out the possibility that it may catalyze the breakdown of POX that is produced outside the catalytic

cavity of ChEs via direct reaction of OPs with oximes (Fig. 1, k_p).

DISCUSSION

In this work we aimed to demonstrate the *in vitro* efficacy of an oxime-OPH combination against the toxicity of the diethylphosphates because a large number of widely used pesticides (e.g. parathion, chlorpyrifos, diazinon, coumaphos) are metabolized to potent AChE inhibitors that eventually produce DEP—AChEs. The results clearly suggest that a combination of a standard oxime reactivator, such as P2S, and a low dose of an OPH can significantly improve clinical recovery after poisoning with diethylphosphate-based pesticides that are characterized by a long persistence of anti-ChE activity in the blood. A concentration as low as 1 μg/mL of OPH*ps* was sufficient to detoxify a 2 μM concentration of potent diethylphosphate inhibitors, and to enable rapid reactivation of the inhibited enzyme within a short period of time.

These observations are of particular importance to the reactivation of inhibited AChE *in vivo* because reactivation of DEP—AChE in peripheral tissues is essential for rapid recovery after OP poisoning. Willems *et al.* [15] reported that a clear-cut reactivation of blood ChE by P2S was accompanied by marked improvement in the clinical condition of patients treated for intoxication with commercial pesticides. Assuming similar reactivatability of DEP-inhibited MoAChE and human AChE [32] under physiological conditions, the *in vitro* data offer the following speculation: OPH*ps* rapidly hydrolyzed paraoxon, DEFP, TEPP, and DEPQ so as to render 2 μM inhibitor practically inactive within the time frame of reactivation by 0.2 mM P2S. While 2 μM is a realistic blood concentration of an anti-ChE following diethylphosphorothioate intoxication, the average therapeutic plasma level of P2S in humans is ~0.02 mM [15]. This suggests that in the absence of inhibitory activity the expected $T_{1/2}$ of reactivation of DEP—AChE is approximately 16 min at 37°*, compared with a half-life of 5 min in the presence of 0.2 mM P2S at 29°. It has been reported that in patients intoxicated with parathion, reactivation following administration of P2S could not be observed if the pesticide blood levels exceeded 30 ng/mL (~0.1 μM) [15]. A 100-fold decrease in the concentration of parathion or paraoxon can be effected by a suitable OPH within less than one blood-circulation time. At 0.001 μM paraoxon, reinhibition of AChE should be negligible* compared with the rate of P2S-induced reactivation.

Touvinen *et al.* [5] showed that OPH*pd* hastened the reactivation of paraoxon-inhibited serum ChE in mice, and of paraoxon-inhibited human serum ChE *in vitro*. The same authors further demonstrated an OPH*pd*-enhanced recovery of ChE activity in sarin intoxication [6]. No reactivator was used in these experiments, and conclusions were based on the spontaneous recovery of the inhibited enzymes. It was reasoned that in addition to the rapid hydrolysis of OPs, OPH*pd* seemed to have ChE-reactivating properties [5], and it might hydrolyze a form of bound OP that is released from ChE before it attacks a new AChE molecule [6]. Our data show that OPH*ps*, an enzyme with substrate specificity similar to that of OPH*pd*, did not exhibit detectable enhancement of spontaneous recovery of enzyme activity of either DEP—AChE or DEP-HuBChE. It is proposed that OPH*ps* accelerates oxime-induced reactivation by rapid hydrolysis of excess OPs rather than by breaking the covalent bond that attaches the OP moiety to the active-site serine. This conclusion is consistent with the elucidated 3-D structure of AChE, which implies that the P atom of DEP—AChE is buried inside a deep gorge that would preclude the approach of a bulky protein nucleophile such as OPH [33].

Since the observed increase of the rate of recovery and the enzymic activity of AChE at equilibrium depends on the acceleration of the OP-catalyzed hydrolysis by OPH*ps*, it is possible to generalize the results from the *in vitro* experiments as follows: in most cases the concentration of the circulating OPs in human blood is significantly smaller than the corresponding K_m, and their enzymic hydrolysis by OPH (k_{hyd}) is approximated by a first-order reaction, with $k_{hyd} = (k_{cat}/K_m)[OPH]_0$. We can predict that a blood concentration of 1 μg/mL (≈29 nM) of an even moderately active OPH ($k_{cat}/K_m \approx 1 \times 10^7$ $M^{-1}min^{-1}$) would be sufficient to rapidly detoxify anti-ChE OPs ($k_{hyd} = 0.29$ min^{-1}; $T_{1/2} = 2.4$ min). Thus, together with an oxime reactivator, OPH can preserve AChE levels that are necessary for maintaining normal physiological functions of important sites (e.g. diaphragm), even when anti-ChE metabolites are released continuously into the blood. Obviously, to achieve this therapeutic goal a reasonable plasma concentration of the oxime and OPH will have to be maintained by one of the techniques of controlling drug administration.

A surprising observation was made with DEPQ. OPH*ps* was capable of hydrolyzing this quaternary ammonium-containing OP with a remarkably high k_{cat}/K_m (3×10^8 M^{-1} min^{-1}). MEPQ [20], a charged methylphosphonate homologue of DEPQ, also was hydrolyzed rapidly by OPH (data not shown). The rapid hydrolysis of these OP substrates would suggest that detoxification of the positively charged POX may be enhanced by OPH. However, results of reactivation of a high concentration of DEP-HuBChE in the presence of OPH*ps* did not provide evidence to support this hypothesis, at least not with respect to the POX that is expected to be produced within the active-site gorge during reactivation. Since phosphorylation of P2S can also proceed via a direct reaction of the OPs with the oxime outside the active site gorge, this issue has to be investigated further, under experimental conditions that may allow

* Assuming a 2-fold increase per Δ10°, k_r is estimated at 2.2×10^3 M^{-1} min^{-1} (37°).
* Assuming $k_i = 2.6 \times 10^6$ M^{-1} min^{-1} (37°), $T_{1/2}$ is estimated at 265 min.

meaningful characterization of the OPH-induced hydrolysis of a chemically defined POX.

In vivo experiments with purified organophosphorus hydrolases from bacterial [1–5] and human sources [for literature, see Ref. 34] have resulted in reports showing that treatment solely with low doses of OPHs is capable of providing protection against OP toxicity. Treatment with a combination of OPHs and oxime reactivator offers a promising approach for a substantially improved therapeutic management of poisoning with parathion and related pesticides.

We thank Mrs. Bracha Manisterski for excellent technical assistance, and Prof. P. Taylor and Dr. Z. Radic for donation of purified rMoAChE.

References

1. Ashani Y, Rothschild N, Segall Y, Levanon D and Raveh L, Prophylaxis against organophosphate poisoning by an enzyme hydrolyzing organophosphorus compounds in mice. *Life Sci* **49:** 367–374, 1991.
2. Raveh L, Segall Y, Leader H, Rothschild N, Levanon D, Henis Y and Ashani Y, Protection against tabun toxicity in mice by prophylaxis with an enzyme hydrolyzing organophosphate esters. *Biochem Pharmacol* **44:** 397–400, 1992.
3. Broomfield CA, A purified recombinant organophosphorus acid anhydrase protects mice against soman. *Pharmacol Toxicol* **70:** 65–66, 1992.
4. Kaliste-Korhonen E, Ylitalo P, Hanninen O and Raushel FM, Phosphotriesterase decreases paraoxon toxicity in mice. *Toxicol Appl Pharmacol* **121:** 275–278, 1993.
5. Touvinen K, Kaliste-Korhonen E, Raushel FM and Hanninen O, Phosphotriesterase–A promising candidate for use in detoxification of organophosphates. *Fund Appl Toxicol* **23:** 578–584, 1994.
6. Touvinen K, Kaliste-Korhonen E, Raushel FM and Hanninen O, Protection of organophosphate-inactivated esterases with phosphotriesterase. *Fund Appl Toxicol* **31:** 210–217, 1996.
7. Touvinen K, Kaliste-Korhonen E, Raushel FM and Hanninen O, Eptastigmine-phosphotriesterase combination in DFP intoxication. *Toxicol Appl Pharmacol* **140:** 364–369, 1996.
8. Dumas DP, Durst HD, Landis WG, Raushel FM and Wild JR, Inactivation of organophosphorus nerve agents by the phosphotriesterase from *Pseudomonas diminuta*. *Arch Biochem Biophys* **277:** 155–159, 1990.
9. Ashani Y, Shapira S, Levy D, Wolfe AD, Doctor BP and Raveh L, Butyrylcholinesterase and acetylcholinesterase prophylaxis against soman poisoning in mice. *Biochem Pharmacol* **41:** 37–41, 1991.
10. Broomfield CA, Maxwell DM, Solana RP, Castro CA, Finger AV and Lenz DE, Protection by butyrylcholinesterase against organophosphorus poisoning in nonhuman primates. *J Pharmacol Exp Ther* **259:** 633–638, 1991.
11. Maxwell DM, Castro CA, De La Hoz DM, Gentry MK, Gold MB, Solana RP, Wolfe AD and Doctor BP, Protection of rhesus monkeys against soman and prevention of performance decrement by pretreatment with acetylcholinesterase. *Toxicol Appl Pharmacol* **115:** 44–49, 1992.
12. Raveh L, Grunwald J, Marcus D, Papier Y, Cohen E and Ashani Y, Human butyrylcholinesterase as a general prophylactic antidote for nerve agent toxicity: *In vitro* and *in vivo* quantitative characterization. *Biochem Pharmacol* **45:** 2465–2474, 1993.
13. Raveh L, Grauer E, Grunwald J, Cohen E and Ashani Y, The stoichiometry of protection against soman and VX toxicity in monkeys pretreated with human butyrylcholinesterase. *Toxicol Appl Pharmacol* **145:** 43–53, 1997.
14. Dunn MA and Sidell FR, Progress in medical defense against nerve agents. *J Am Med Soc* **262:** 649–652, 1989.
15. Willems JL, De Bisschop HC, Verstraete AG, Declerck C, Christiaens Y, Vanscheeuwyck P, Buyalert WA, Vugelaers D and Colardyn F, Cholinesterase reactivation in organophosphorus poisoned patients depends on the plasma concentrations of the oxime pralidoxime methylsulphate and of the organophosphate. *Arch Toxicol* **67:** 79–84, 1993.
16. Schoene K, Reactivation of O,O-diethylphosphoryl-acetylcholinesterase, reactivation-rephosphorylation equilibrium. *Biochem Pharmacol* **21:** 163–170, 1972.
17. Schoene K, Phosphonyloxime from soman; *in vitro* formation and reaction with acetylcholineseterase. *Biochem Pharmacol* **22:** 2997–3003, 1973.
18. Lamb JC, Steinberg GM, Solomon S and Hackley BE, Reaction of 4-formyl-1-methylpyridinium iodide oxime with isopropyl methylphosphonofluoridate. *Biochemistry* **4:** 2475–2484, 1965.
19. Harvey B, Scott RP, Sellers DJ and Watts P, *In vitro* studies on the reactivation by oximes of phosphylated acetylcholinesterase–I. On the reactions of P2S with various organophosphates and the properties of the resultant phosphylated oximes. *Biochem Pharmacol* **35:** 737–744, 1986.
20. Levy D and Ashani Y, Synthesis and *in vitro* properties of a powerful quaternary methylphosphonate inhibitor of acetylcholinesterase: A new marker in blood–brain research. *Biochem Pharmacol* **35:** 1079–1085, 1986.
21. Gordon MA, Carpenter DE, Barrett HW and Wilson IB, Determination of the normality of cholinesterase solutions. *Anal Biochem* **85:** 519–527, 1978.
22. Toy ADF, The preparation of tetraethyl pyrophosphate and other tetraalkyl pyrophosphates. *J Am Chem Soc* **70:** 3882–3886, 1948.
23. Saunders BC and Stacey J, Esters containing phosphorus. Part IV. Diisopropyl fluorophosphonate. *J Chem Soc* 695–699, 1948.
24. Dosoretz C, Armon R, Starosvetzky J and Rothschild N, Entrapment of parathion hydrolase from *Pseudomonas* spp. in Sol-Gel glass. *J Sol-Gel Sci Technol* **7:** 7–11, 1996.
25. Grunwald J, Marcus D, Papier Y, Raveh L, Pittel Z and Ashani Y, Large scale purification and long-term stability of human butyrylcholinesterase: A potential bioscavenger drug. *J Biochem Biophys Methods* **34:** 123–135, 1997.
26. Amitai G, Ashani Y, Grunfeld Y, Kalir A and Cohen S, Synthesis and properties of 2-*S*[2′-(*N*,*N*-dialkylamino)ethyl] thio-1,3,2-dioxaphosphorinane 2-oxide and of the corresponding quaternary derivatives as potential nontoxic antiglaucoma agents. *J Med Chem* **19:** 810–813, 1976.
27. Ellman GL, Courtney KD, Andres V Jr and Featherstone RM, A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **1:** 88–95, 1961.
28. Ashani Y, Radic Z, Tsigelny I, Vellom DC, Pickering NA, Quinn DM, Doctor BP and Taylor P, Amino acid residues controlling reactivation of organophosphonyl conjugates of acetylcholinesterase by mono- and bisquaternary oximes. *J Biol Chem* **270:** 6370–6380, 1995.
29. Harvey B, Scott RP, Sellers DJ and Watts P, *In vitro* studies on the reactivation by oximes of phosphylated acetylcholinesterase–II. On the formation of O,O-diethyl phosphorylated AChE and O-ethyl methylphosphonylated AChE and their reactivation by P2S. *Biochem Pharmacol* **35:** 745–751, 1986.
30. Munnecke DM, Enzymatic hydrolysis of organophosphate insecticide, a possible pesticide disposal method. *Appl Environ Microbiol* **32:** 7–13, 1976.

31. Lai K, Stolowich NJ and Wild JR, Characterization of P–S bond hydrolysis in organophosphorothioate pesticides by organophosphorus hydrolase. *Arch Biochem Biophys* **318:** 59–64, 1995.
32. Grosfeld H, Barak D, Ordentlich A, Velan B and Shafferman A, Interactions of oxime reactivator with diethylphosphoryl adducts of human acetylcholinesterase and its mutant derivatives. *Mol Pharmacol* **50:** 639–649, 1996.
33. Sussman JL, Harel M, Frolow F, Oefner C, Goldman A, Toker L and Silman I, Atomic structure of acetylcholinesterase from *Torpedo californica*: A prototypic acetylcholine-binding protein. *Science* **253:** 872–879, 1991.
34. Davies HG, Richter JR, Keifer M, Broomfield CA, Sowalla J and Furlong CE, The effect of the human serum paraoxonase polymorphism is reversed with diazoxon, soman, and sarin. *Nat Genet* **14:** 334–336, 1996.