Pergamon

Biochemical Pharmacology, Vol. 47, No. 2, pp. 347–357, 1994.
Elsevier Science Ltd
Printed in Great Britain
0006-2952/94 $6.00 + 0.00

AMPLIFICATION OF THE EFFECTIVENESS OF ACETYLCHOLINESTERASE FOR DETOXIFICATION OF ORGANOPHOSPHORUS COMPOUNDS BY BIS-QUATERNARY OXIMES*

GERMAN R. CARANTO,† KIRK H. WAIBEL,† JACOB M. ASHER,† ROBERTA W. LARRISON,† KAREN M. BRECHT,‡ MICHAEL B. SCHUTZ,‡ LILY RAVEH,§ YACOV ASHANI,§ ALAN D. WOLFE,† DONALD M. MAXWELL‡ and BHUPENDRA P. DOCTOR†‖

†Division of Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20307-5100; ‡Pharmacology and Drug Assessment Divisions, United States Army Medical Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5425; and §Israel Institute for Biological Research, Ness-Ziona, Israel

(*Received* 26 *March* 1993; *accepted* 30 *August* 1993)

Abstract—Pretreatment of rhesus monkeys with fetal bovine serum acetylcholinesterase (FBS AChE) provides complete protection against 5 LD_{50} of organophosphate (OP) without any signs of toxicity or performance decrements as measured by serial probe recognition tests or primate equilibrium platform performance (Maxwell *et al.*, *Toxicol Appl Pharmacol* **115**: 44–49, 1992; Wolfe *et al.*, *Toxicol Appl Pharmacol* **117**: 189–193, 1992). Although such use of enzyme as a single pretreatment drug for OP toxicity is sufficient to provide complete protection, a relatively large (stoichiometric) amount of enzyme was required *in vivo* to neutralize OP. To improve the efficacy of cholinesterases as pretreatment drugs, we have developed an approach in which the catalytic activity of OP-inhibited FBS AChE was rapidly and continuously restored, thus detoxifying the OP and minimizing enzyme aging by having sufficient amounts of appropriate oxime present. The efficacy of FBS AChE to detoxify several OPs was amplified by addition of bis-quaternary oximes, particularly 1-(2-hydroxyiminomethyl-1-pyridinium)-1-(4-carboxyaminopyridinium)-dimethyl ether hydrochloride (HI-6). When mice were pretreated with sufficient amounts of FBS AChE and HI-6 and challenged with repeated doses of *O*-isopropyl methylphosphonofluoridate (sarin), the OP was continuously detoxified so long as the molar concentration of the sarin dose was less than the molar concentration of AChE in circulation. The *in vitro* experiments showed that the stoichiometry of sarin:FBS AChE was higher than 3200:1 and *in vivo* stoichiometry with mice was as high as 57:1. Addition of HI-6 to FBS AChE as a pretreatment drug amplified the efficacy of enzyme as a scavenger of nerve agents.

Present treatment for poisoning by OP¶ consists of a combination of drugs such as carbamates (e.g. pyridostigmine), an anti-muscarinic (e.g. atropine), and reactivators (e.g. pralidoxime chloride) administered in post-exposure modalities. Although this drug regimen is effective in protecting experimental animals against lethality by OP poisoning, it is not effective in preventing convulsions, performance deficits, or permanent brain damage [1–8]. To alleviate these post-exposure symptoms, the use of ChEs as a pretreatment drug was tested successfully in animals [9–13] including non-human primates [14–17] for the sequestration of highly toxic OP anti-ChEs before they reach their physiological targets. For example, pretreatment of rhesus monkeys with FBS AChE [15, 16] or horse serum BChE [16, 17] protected them against a challenge of up to 5 LD_{50} of *O*-pinacolyl methylphosphonofluoridate (soman), a highly toxic OP. These monkeys pretreated with FBS AChE were devoid of any behavioral incapacitation after a soman challenge, as measured by the serial probe recognition task [15, 17] or the primate equilibrium platform performance task [16]. *In vivo* and *in vitro* titration of ChEs with a variety of OPs produced a 1:1 stoichiometry between OP-inhibited enzymes and the cumulative dose of the toxic nerve agent. These results substantiated the hypothesis that exogenously administered ChEs can effectively

* A portion of this report was presented at the 36th Oholo Conference on "Multidisciplinary Approaches to Cholinesterase Functions" held 6–10 April 1992, in Eilat, Israel, and at the 2nd International Meeting on "Esterases" held 20–24 April 1992, in Salsomaggiore, Italy.

‖ Corresponding author. Tel. (202) 576-3001; FAX (202) 576-1304.

¶ Abbreviations: OP, organophosphate; FBS AChE, fetal bovine serum acetylcholinesterase; HI-6, 1-(2-hydroxyiminomethyl-1-pyridinium)-1-(4-carboxyaminopyridinium)-dimethyl ether hydrochloride; sarin, *O*-isopropyl methylphosphonofluoridate; pralidoxime chloride, 2-[hydroxyiminomethyl]-1-methylpyridinium chloride; ChE, cholinesterase; BChE, butyrylcholinesterase; soman, *O*-pinacolyl methylphosphonofluoridate; MEPQ, 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide; TMB_4, 1,1-trimethylene bis(4-hydroximinomethyl)pyridinium dibromide; tabun, *O*-ethyl *N*,*N*-dimethylphosphoramidocyanidate; VX, *O*-ethyl *S*-2-diisopropylaminoethyl methylphosphonothioate; HLo-7, 1-([[4-(aminocarbonyl)pyridino]-methoxy]-methyl)-2,4-bis[(hydroxyimino)methyl]pyridinium dichloride; and MMB_4, 1,1-methylene-bis-(4-hydroxyiminomethylpyridinium) dibromide.

sequester *in vivo* OPs before they reach their physiological targets.

Although the use of ChEs as a single pretreatment drug for highly potent OPs is sufficient to provide complete protection without the need for post-exposure treatment, its practical use at the present time may be limited. Large quantities of enzymes will be required to provide sufficient protection due to the 1:1 stoichiometry (i.e. a single turnover) between OP and enzyme. One possible approach to increase the scavenging ratio of OP to ChE is to use a combination of enzyme with a specific reactivator drug as a pretreatment for OP exposure.

In general, OP-inhibited AChE can be reactivated rapidly by mono- or bis-quaternary oximes such as pralidoxime chloride and HI-6 so long as it has not undergone aging [18]. For example, exogenously administered FBS AChE inhibited by a powerful anticholinesterase, 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide (MEPQ), was shown to be reactivated in mice by an i.m. injection of 1,1-trimethylene bis(4-hydroxyiminomethylpyridinium) dibromide (TMB_4) [10]. The reactivated FBS AChE was able to protect the mice against exposure to an additional dose of MEPQ. The rate of reactivation of inhibited AChE depends on the source of the enzyme, the specific OP and the oxime used, and its concentration. With irreversible inhibitors such as soman and *O*-isopropyl methylphosphonofluoridate (sarin), the enzyme is inhibited in a relatively short period. Further, in the case of soman, the inhibited enzyme undergoes rapid aging which converts AChE to a non-reactivatable form (see Fig. 1). Therefore, the immediate administration of an appropriate oxime after OP exposure is extremely important. Thus, it was envisaged that the use of a suitable oxime reactivator, as a pretreatment drug combined with the exogenous AChE before exposure to an OP, should regenerate the OP-inhibited enzyme and thereby restore the ability of the scavenger to sequester circulating OP.

We report here results demonstrating that the addition of HI-6 amplified the effectiveness of exogenous FBS AChE to detoxify organophosphate not only *in vitro* but in mice as well. This is possible because the OP-inhibited FBS AChE was reactivated continuously in the presence of HI-6. The selection of HI-6 as the reactivator was based on its demonstrated efficacy in protecting animals against OP poisoning.

MATERIALS AND METHODS

Materials. FBS AChE was purified to electrophoretic homogeneity (98% purity) according to De La Hoz *et al.* [19]. Sarin, soman, *O*-ethyl *N,N*-dimethylphosphoramidocyanidate (tabun) and *O*-ethyl *S*-2-diisopropylaminoethyl methylphosphonothionate (VX) were obtained from the Chemical Research, Development, and Engineering Center (Aberdeen Proving Ground, MD). Acetylthiocholine and bovine serum albumin were purchased from the Sigma Chemical Co. (St. Louis, MO). HI-6, 1-([[4-aminocarbonyl)pyridino] - methoxy] - methyl) - 2,4 - bis[(hydroxyimino)methyl]pyridinium dichloride (HLo-7), 1,1 - methylene - bis(4 - hydroxyimino - methylpyridinium) dibromide (MMB_4), TMB_4 and pralidoxime chloride were obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC. Human plasma was obtained from out-dated human blood provided by the Camp Memorial Blood Center, Fort Knox, KY. Plasma was centrifuged at 105,000 *g* for 1 hr, and aliquots were frozen at −20° until used.

AChE assay. AChE activity was determined by the spectrophotometric method of Ellman *et al.* [20] with 0.5 mM acetylthiocholine as the substrate. The AChE level of mouse blood was determined after at least a 10-fold dilution in distilled water. The assays were conducted in 50 mM phosphate buffer, pH 8.0, at 25°.

Determination of sarin or soman potency. The potency of sarin or soman solutions to be used for the study experiments was determined by (a) titrating with a known amount of FBS AChE, and (b) determination of LD_{50} in mice.

In vitro titration of FBS AChE with OP compounds. Serial dilutions of OP in saline were incubated with 0.1 μM AChE at room temperature for at least 30 min in 50 mM phosphate buffer, pH 8.0, with and without the presence of human plasma. Residual AChE activity was plotted against the concentration of OP added to the reaction mixture to indicate the stoichiometry between AChE and OP.

Determination of i.v. LD_{50} of sarin. The i.v. LD_{50} of sarin in mice was determined and calculated by the Spearman–Kerber method as described [21]. The LD_{50} of sarin was also determined in mice pretreated with 50 mg/kg HI-6 (5 min before bolus injection of sarin). The survivability tests were carried out with (a) mice pretreated with 8–9 nmol of FBS AChE (1 hr before bolus injection of sarin), and (b) with 8–9 nmol of FBS AChE and 50 mg/kg HI-6.

Effect of mixing HI-6 with sarin on its ability to inhibit FBS AChE. One millimolar sarin and a 1 mM sarin + 1 mM HI-6 mixture were incubated at 25° for 1 and 2 hr. Appropriate amounts of both of these solutions were diluted to a final concentration of 12 nM each into approximately 13 nM FBS AChE with 0.01% bovine serum albumin. The extent of dilution of both of these solutions was such that the inhibitory ability of sarin would be approximately 75%. Indeed, this theoretical value was found to be in reasonable agreement with the observed values that were determined after a 0.5 hr incubation period. It should be pointed out that the concentration of HI-6 (12 nM) in the diluted solution did not cause any significant reactivation of sarin-inhibited FBS AChE over a 30-min incubation period.

Kinetics of reactivation of OP-inhibited FBS AChE. Inhibition of FBS AChE (0.1 μM) was performed in 50 mM phosphate, pH 8.0, at 25° with aqueous solutions of sarin or soman (1 μM). The AChE activity in the reaction mixture was measured after a 10-min incubation to confirm that greater than 95% inhibition of AChE had occurred. The OP-inhibited AChE was then separated from excess inhibitor by high performance liquid chromatography on a TSK-SW2000 gel filtration column equilibrated with phosphate buffer. Each OP-inhibited AChE sample was compared to a corresponding control

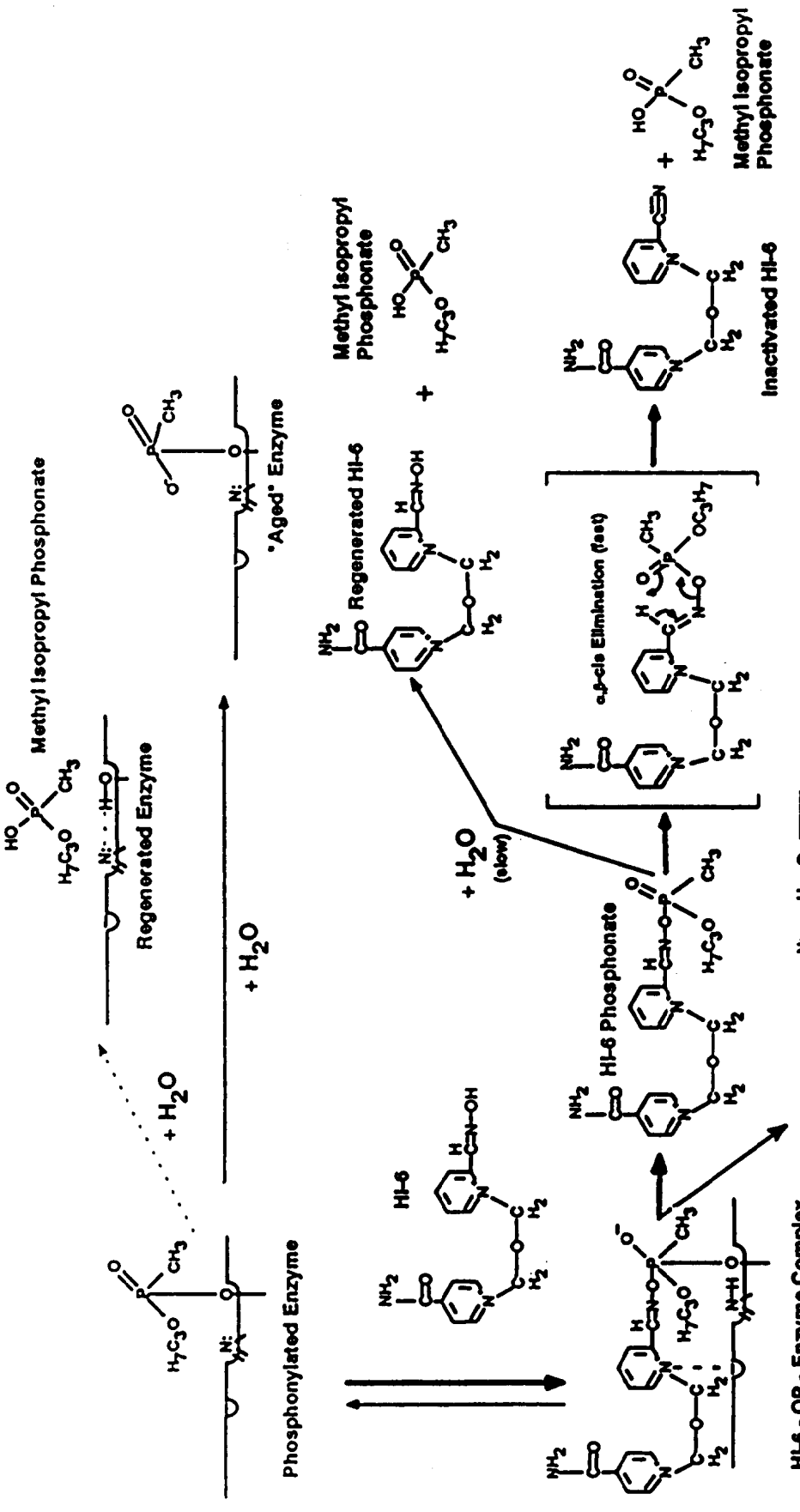

Fig. 1. Reactivation of alkylphosphorylated acetylcholinesterase. AChE after alkylphosphorylation by sarin (upper left) either undergoes slow spontaneous reactivation (dotted arrow) or "aging." The "aged" enzyme is highly resistant to hydrolysis and regeneration by oximes. An oxime such as HI-6 (lower reactions) reacts via its nucleophilic oxime group with an electrophilic P atom, forming an HI-6–OP–enzyme complex. The oxime phosphonate is split off leaving regenerated enzyme. Slow hydrolysis of the oxime–phosphonate complex yields regenerated HI-6 and methylisopropyl phosphonate. Rapid inactivation of HI-6 can also occur simultaneously by α,β,*cis*-elimination.

Table 1. Effect of mixing HI-6 with sarin on the ability of sarin to inhibit FBS AChE

Addition	AChE (U/mL) Incubation time (hr) 0	1	2
None	3.71 ± 0.21 (100)	3.71 ± 0.21 (100)	3.71 ± 0.21 (100)
Sarin	0.93 ± 0.21 (25.13 ± 4.27)	0.88 ± 0.11 (23.87 ± 4.62)	0.87 ± 0.14 (23.44 ± 4.34)
Sarin + HI-6	0.82 ± 0.12 (22.25 ± 4.43)	0.83 ± 0.13 (23.35 ± 4.01)	0.83 ± 0.14 (22.38 ± 4.20)

The details of the experiment are given under Materials and Methods. Each data point represents the mean ± SD of AChE assays on five samples; numbers in parentheses represent the percent of the control.

AChE sample that had received identical incubation and chromatography treatment except for the absence of OP inhibitor. After chromatographic separation, the sample of OP-inhibited AChE was reactivated at 25° by addition of 2 mM HI-6 in 50 mM phosphate, pH 8.0. Samples (10 μL) of the reaction mixture were assayed (300× dilution in Ellman reagent) sequentially for AChE activity at time intervals up to 1 hr. The observed rate constant (k_{obs}) for oxime reactivation of OP-inhibited AChE was calculated from the equation $v = V_{max}(1 - e^{-k_{obs}t})$ where V_{max} was the maximal recovery of AChE activity and v is the activity at any given time (t). Since the reactivation and aging of OP-inhibited enzymes are parallel first-order reactions, the true rate constants were calculated according to the method of Hovanec and Lieske [22]. Under the experimental conditions described above, the final concentration of HI-6 in the assay mixture did not affect either the rate of the non-specific hydrolysis of AChE or the activity of AChE.

In vitro *detoxification of OPs*. The incubation mixture in 50 mM phosphate buffer, pH 8.0 (total volume 0.5 mL), contained 0.35 mL human plasma, 0.1–0.15 μM FBS AChE, 1–10 mM oxime and variable concentrations of OP compounds. Residual enzyme activity was determined after incubation at room temperature for different intervals of time. Two protocols were used: (A) various concentrations of OP were added at $t = 0$ to the reaction mixture to constitute different OP/AChE molar concentrations. At different time intervals, residual activity of AChE was assayed by diluting the reaction mixture 300-fold into the assay cuvette; (B) to a series of reaction mixtures, equal amounts of OP were added at $t = 0$. After 0.5 hr, the first reaction mixture was discarded after its residual activity was determined. To each of the remaining reaction mixtures the same amount of OP was added and enzyme activity was determined in the second tube after another 0.5 hr. The process of repeated additions of OP to the subsequent mixtures was designed to simulate multiple OP exposure *in vivo*. This procedure was repeated numerous times times until the enzyme activity decreased to 10% of its original value. Residual enzyme activity was plotted against the cumulative concentration of the OP added to each reaction mixture to indicate the ratio of hydrolyzed OP against given enzyme concentration.

In vivo *reactivation of sarin-inhibited FBS AChE by HI-6 in mice*. In one series of experiments, mice (N = 5) were injected i.v. with approximately 4000 U (10 nmol active site) of FBS AChE. One hour later, blood was withdrawn for AChE determination. Each mouse was then injected i.v. with a freshly prepared mixture of 2 μg (14.3 nmol) of sarin and 1 mg HI-6 (2.6 μmol) followed 15 min later by blood withdrawal and AChE determination. Five additional sarin/HI-6 mixtures were injected into each mouse, and blood samples were withdrawn 15 min after each injection for AChE determination. In a second series of experiments, mice were pretreated with FBS AChE (3500 U/mouse) 1 hr before the initiation of the titration experiment. HI-6 (50 mg/kg; 3.2 μmol) was injected i.v. 5 min before the first i.v. administration of 2.6 μg of sarin/mouse. The subsequent four sarin injections did not contain HI-6.

RESULTS

Effect of HI-6 on sarin potency. It has been reported that some OPs react to form phosphorylated oximes, which are potent inhibitors of AChE [23]. To test this possibility, sarin and HI-6 were preincubated for 1 and 2 hr at 25°, and diluted to greater than 10,000-fold. This mixture was used to test the ability of sarin to inhibit FBS AChE. As shown in Table 1, the ability of sarin to inhibit FBS AChE was not changed by incubating the OP with HI-6 at room temperature for up to 2 hr.

In vitro *reactivation of OP-inhibited FBS AChE by oximes*. Figure 2A shows the results of the *in vitro* titration of FBS AChE by MEPQ in the presence and absence of HI-6. The titration was carried out in the presence of 70% human plasma in 50 mM phosphate buffer, pH 8.0, in order to mimic the physiological conditions. As expected, 1:1 stoichiometry between FBS AChE and MEPQ was observed (Fig. 2A, dotted line). Figure 2B shows the reactivation of MEPQ-inhibited FBS AChE in the presence of 1, 2, 5 and 10 mM HI-6. The rate of reactivation of MEPQ-inhibited FBS AChE under these experimental conditions depended on the

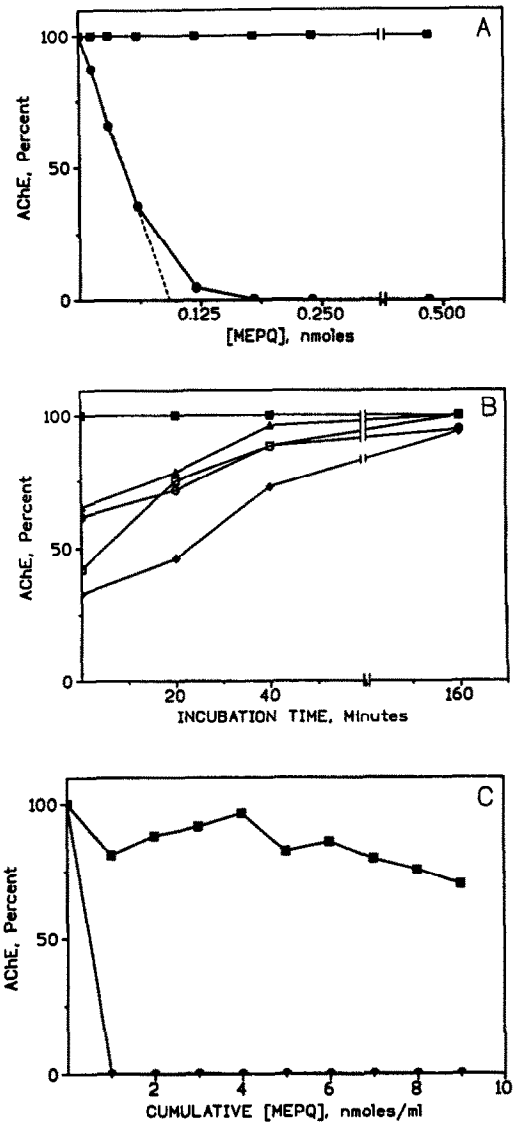

Fig. 2. (A) *In vitro* titration of FBS AChE by MEPQ in the presence or the absence of HI-6. The incubation mixture contained 0.7 mL human plasma, FBS AChE (37 U; 0.095 nmol), and increasing amounts of MEPQ in a final volume of 1 mL (50 mM phosphate buffer, pH 8.0). The mixture was incubated at room temperature for 1 hr and the residual AChE activity was measured by the method of Ellman *et al.* [20]. Key: (●—●) without HI-6; (■—■) with 2 mM HI-6. The dashed line shows the extrapolation of the linear portion of the curve to indicate the amount of MEPQ required for 100% inhibition of enzyme. (B) Time course of reactivation of MEPQ-inhibited FBS AChE in the presence of various concentrations of HI-6. The incubation mixture contained 0.35 mL human plasma, 15 U (30 U/mL; 0.075 nmol/mL) FBS AChE, 1 nmol (2 nmol/mL; 27-fold molar excess) MEPQ, and 1 (◆—◆), 2 (□—□), 5 (○—○) and 10 (△–△) mM HI-6 in final volume of 0.5 mL (50 mM phosphate buffer, pH 8.0). The control sample (■—■) contained no MEPQ. The samples were incubated at room temperature, and aliquots were assayed for residual AChE activity. (C) Reactivation of FBS AChE in the presence of HI-6 after repeated additions of MEPQ. To 0.145 nmol/mL of FBS AChE with 2 mM HI-6 and 0.7 mL of human plasma (50 mM phosphate buffer, pH 8.0) 1-nmol aliquots of MEPQ were added at 30-min intervals. The residual activity of AChE in the incubation mixture was measured before each addition of MEPQ. The reaction mixture after the addition of 9 nmol MEPQ contained 62-fold molar excess of MEPQ over the enzyme. Key: (■—■) with 2 mM HI-6; and (●—●) without HI-6.

molar concentration of oxime. In all cases more than 90% of the enzyme activity was restored after 160 min. This amount corresponds to the hydrolysis of approximately 1 nmol of MEPQ by a mixture of 0.037 nmol of FBS AChE and 2 mM HI-6. Figure 2C shows the results of reactivation of FBS AChE in the presence of 2 mM HI-6 after repeated additions of MEPQ. Repeated additions of MEPQ to FBS AChE in the presence of HI-6 at 0.5-hr intervals (see Materials and Methods, Protocol B) appeared to continuously reactivate the enzyme. All the added MEPQ was destroyed. This conclusion was based on the observation that the addition of an aliquot of the last reaction mixture to fresh FBS AChE solution (containing no HI-6) did not show any enzyme inhibition.

The results of the *in vitro* titration of FBS AChE by sarin, in the presence and absence of 2 mM HI-6 are shown in Fig. 3A. Since only the (*P*-) of the two enantiomers of sarin has been shown to inhibit AChE [24], twice the molar concentration of a racemic mixture of sarin was required to completely inhibit a molar amount of the enzyme. Greater than stoichiometric amounts of sarin did not inhibit FBS AChE in the presence of 2 mM HI-6. Figure 3B shows that repeated addition (see Materials and Methods, Protocol B) of 10 times the molar concentration of sarin to FBS AChE in the presence of 2 mM HI-6 every 0.5 hr (total of 10 times) did not result in any inhibition of the enzyme. A total of 28.8 nmol of sarin was neutralized by 0.144 nmol of FBS AChE in the presence of 2 mM HI-6 (approximately 100 times more than the expected amount) without any loss of enzyme activity. This continuous neutralization of sarin was further evident, as shown in Fig. 3C, when progressively larger molar excesses of sarin were added to FBS AChE in the presence of HI-6 (see Materials and Methods, Protocol A). Inhibition of enzyme activity was observed only when the total amount of sarin exceeded the molar concentration of enzyme by greater than 3200. All of the cumulative sarin added to the reaction mixture was neutralized completely, since an aliquot of this reaction mixture taken after the last sarin addition did not inhibit fresh enzyme solution without HI-6.

To evaluate the kinetics of sarin neutralization by a single cycle of HI-6 reactivation, FBS AChE was inhibited with an excess of sarin, separated from free sarin on HPLC, and reactivated with HI-6. The kinetics of HI-6 reactivation of sarin-inhibited FBS AChE are shown in Fig. 3D. Sarin-inhibited AChE was reactivated almost completely by 2 mM HI-6 in 10 min with a first-order rate constant of 0.35 min^{-1}. This suggests that the half-life for AChE reactivation is approximately 2 min and that the HI-6 reactivation enables FBS AChE to detoxify one-half its molar equivalent of sarin every 2 min under these experimental conditions.

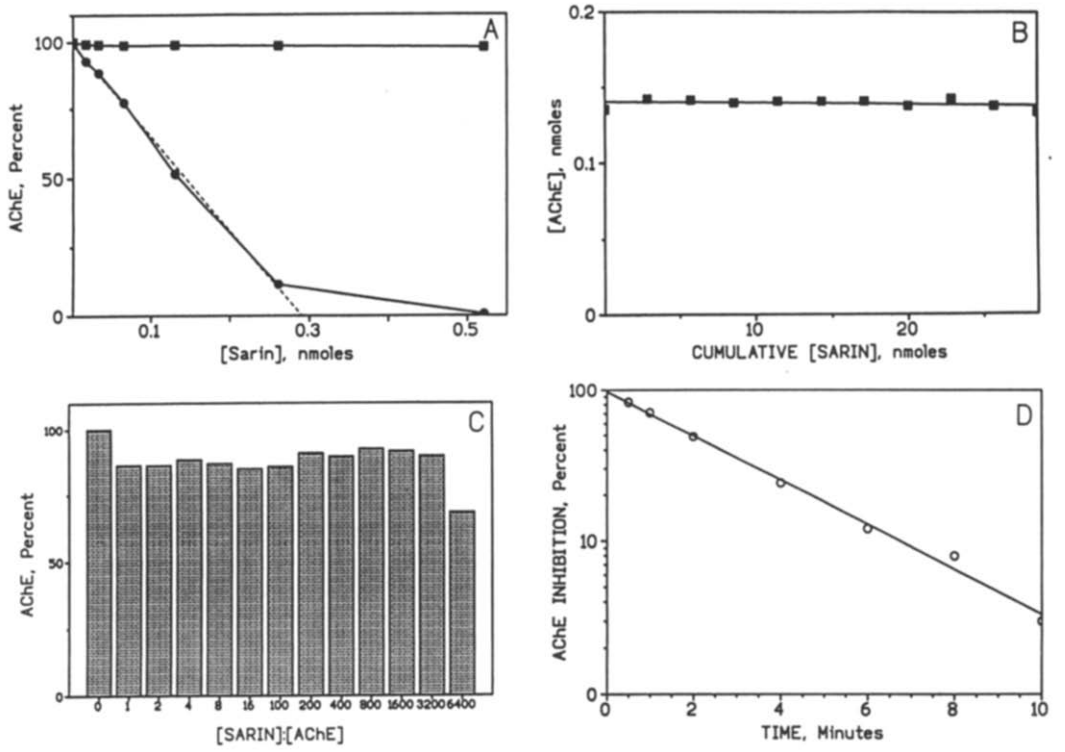

Fig. 3. (A) *In vitro* titration of FBS AChE by sarin in the presence and absence of HI-6. The procedure described under Fig. 2A was used. Fifty-five units (0.14 nmol) of FBS AChE was used. Only (*P*-) of the two enantiomers of sarin inhibits FBS AChE; thus, twice the molar amount of sarin was required to inhibit the enzyme. Key: (●—●) without HI-6; and (■—■) with 2 mM HI-6. (B) Reactivation of FBS AChE in the presence of HI-6 after repeated additions of sarin. The reactivation of FBS AChE (0.144 nmol) by HI-6 (2 mM) was carried out in the presence of 70% human plasma after repeated additions of sarin (3 nmol; approximately 20-fold excess) at 0.5-hr intervals. The residual AChE activity was measured, 30 min after each addition of sarin. (C) *In vitro* reactivation of sarin-inhibited FBS AChE by HI-6. The incubation mixture contained 0.7 mL human plasma, 50 mM phosphate buffer, pH 8.0, 2 mM HI-6, 0.125 nmol FBS AChE and the indicated molar excess (concentration) of sarin in a final volume of 1 mL. The residual AChE activity was measured after incubation for 0.5 hr at room temperature. (D) Rate of reactivation of sarin-inhibited FBS AChE by HI-6. Details of the experiments are given under Materials and Methods.

Figure 4A shows the results of *in vitro* titration of FBS AChE by soman in the absence and presence of 1 mM HI-6. The addition of 0.33 nmol of soman to the reaction mixture containing 0.14 nmol of FBS AChE completely inhibited enzyme activity in the absence of HI-6 (the extrapolated amount of soman needed to completely inhibit this amount of enzyme would be 0.276 nmol), whereas only 17% (0.024 nmol) of the AChE activity was inhibited in the presence of 1 mM HI-6. Under these experimental conditions, approximately 2 nmol of soman is needed to inhibit enzyme activity 100% in the presence of HI-6. Thus, the same amount of FBS AChE is able to neutralize 7.2 times more soman in the presence of 1 mM HI-6 (2.0/0.275 = 7.2) than in the absence of HI-6.

The concentration of HI-6 was increased to 2 mM and the amount of soman added to reaction mixture was lowered to less than the amount required to inhibit all of the FBS AChE. The results shown in Fig. 4B demonstrate that FBS AChE (0.144 nmol) in the presence of 2 mM HI-6 continued to neutralize repetitive additions of soman (0.22 nmol) as long as the soman concentration did not exceed the concentration of FBS AChE during the incubation period (see Materials and Methods, Protocol B). Enzyme activity was determined before each addition of soman (30 min). The addition of a higher concentration of soman to a reaction mixture containing a constant amount of FBS AChE in the presence of 2 mM HI-6 and the determination of residual enzyme activity after 30 min showed that an increasing amount of enzyme was inhibited but not in proportion to the amount of added soman (Fig. 4C; see Materials and Methods, Protocol A).

To evaluate the kinetics of soman neutralization by a single cycle of HI-6 reactivation, FBS AChE was inhibited by an excess of soman, separated from soman on HPLC, and reactivated with HI-6 as previously described for sarin. The kinetics of HI-6 reactivation of soman-inhibited AChE (Fig. 4D) differed from sarin-inhibited AChE both in the extent of reactivation and the rate constant for reactivation. Only 48% of the soman-inhibited

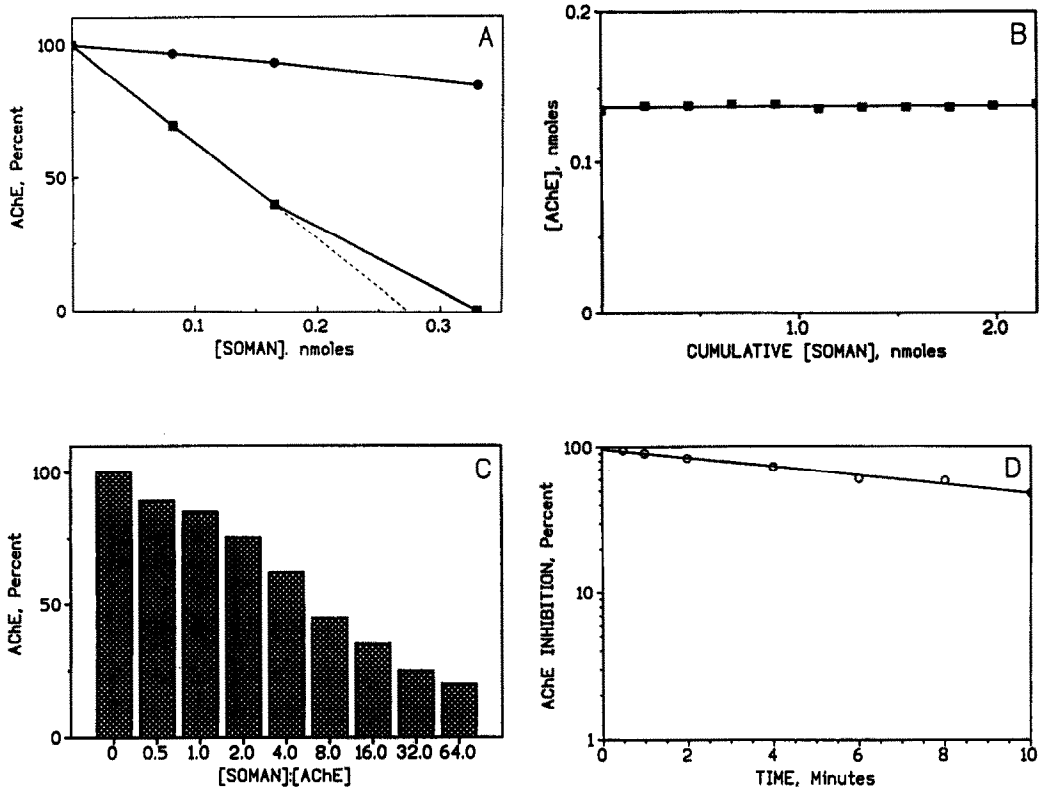

Fig. 4. (A) *In vitro* titration of FBS AChE by soman in the presence and the absence of HI-6. The procedure utilized was the same as described under Fig. 2A. Only two of the four stereoisomers of soman inhibit FBS AChE; thus, twice the molar amount of soman was required to inhibit the enzyme. Key: (●—●) without HI-6; and (■—■) with 1 mM HI-6. (B) Reactivation of FBS AChE in the presence of HI-6 after repeated additions of soman. The reactivation of FBS AChE (0.144 nmol) by HI-6 (2 mM) was carried out in the presence of 70% human plasma after repeated additions of soman (0.2 nmol; 1.5-fold excess). (C) *In vitro* reactivation of soman-inhibited FBS AChE by HI-6. The incubation mixture contained 0.7 mL human plasma, 50 mM phosphate buffer, pH 8.0, 2 mM HI-6, 0.125 nmol FBS AChE and the indicated molar concentration of soman in a final volume of 1 mL. (D) Rate of reactivation of soman-inhibited FBS AChE by HI-6. Details of the experiments are given under Materials and Methods.

AChE was reactivated, presumably because of aging that occurred during the approximately 10 min required to inhibit and separate free inhibitor from inhibited enzyme by HPLC. The apparent rate constant for reactivation by 2 mM HI-6 was 0.075 min^{-1}, which was 4.7 times less than that for HI-6 reactivation of sarin-inhibited FBS AChE. This rate constant suggests that, under these conditions, the half-life for AChE reactivation is 9.2 min and that HI-6 reactivation enables FBS AChE to detoxify one-half its molar equivalent of soman every 9.2 min. Recently HLo-7, another bis-quaternary oxime, was shown to be equally as effective an antidote against soman toxicity as HI-6 [25]. Therefore, 1, 2, and 5 mM HLo-7 (see Materials and Methods, Protocol A) were tested and compared with the same concentrations of HI-6 to determine their abilities to reactivate soman-inhibited FBS AChE. The results are shown in Fig. 5. The oxime, HLo-7, was a better reactivator of soman-inhibited FBS AChE than HI-6 at the 1 or 2 mM concentration. Both oximes were equally effective as reactivators at the 5 mM concentration.

The reactivation of soman-, sarin-, tabun-, and VX-inhibited FBS AChE by HI-6 is shown in Fig. 6 (see Materials and Methods, Protocol A). Other oximes, such as pralidoxime chloride, TMB_4, and MMB_4 were also effective in reactivating OP-inhibited FBS AChE. The results of the effectiveness of these oximes for reactivation of the four OP-inhibited FBS AChE are described in Table 2. With a 2 mM concentration of HI-6, the activities of all four OP-inhibited FBS AChE were restored almost completely. The rank order of the effectiveness of these four oximes against soman toxicity was HI-6 > MMB_4 > pralidoxime chloride > TMB_4, against tabun was HI-6 > TMB_4 > pralidoxime chloride > MMB_4, and against VX was HI-6 = MMB_4 > TMB_4 > pralidoxime chloride. All the oximes were equally effective against sarin at a 2 mM concentration.

In vivo *reactivation of sarin-inhibited FBS AChE by HI-6*. To determine the effectiveness of HI-6 for

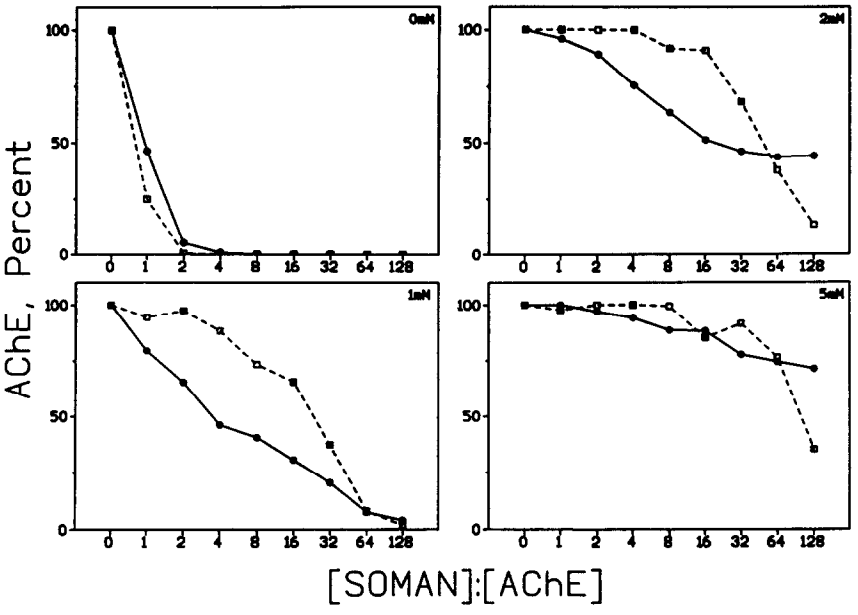

Fig. 5. *In vitro* reactivation of soman-inhibited FBS AChE by various concentrations of HI-6 and HLo-7. The incubation mixture contained 50 mM phosphate buffer, pH 8.0, 0.7 mL human plasma, 0, 1, 2, and 5 mM HI-6 and 0, 1, 2, and 5 mM HLo-7, 0.125 nmol FBS AChE and the indicated molar excess of soman in a final volume of 1 mL. The residual AChE activity was measured after incubation for 0.5 hr at room temperature. Key: (●—●) HI-6; and (□—□) HLo-7.

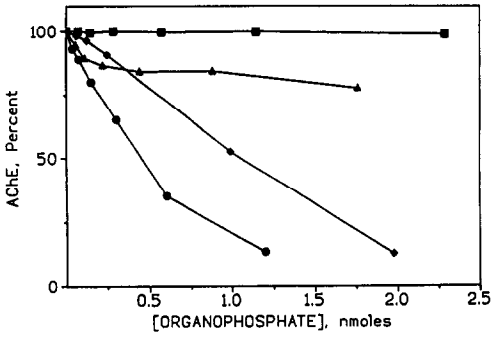

Fig. 6. Reactivation of various OP-inhibited FBS AChE by HI-6. The incubation mixture, 1 mL in 50 mM phosphate buffer, pH 8.0, contained 0.125 nmol FBS AChE, 0.7 mL human plasma, 2 mM HI-6 and various concentrations of soman, sarin, tabun and VX. The residual AChE activity of the samples was determined after 0.5 hr of incubation at room temperature. Key: (■—■) sarin; (▲—▲) soman; (◆—◆) tabun; and (●—●) VX.

in vivo reactivation of sarin-inhibited exogenously administered FBS AChE in mice, a mixture of sarin and HI-6 was injected i.v., repeatedly (5 or 6 times) every 15 min, into a group (N = 5) of mice 1 hr after i.v. administration of FBS AChE. The blood AChE levels were determined before each sarin + HI-6 injection. The results described in Fig. 7A show the *in vivo* reactivation of sarin-inhibited FBS AChE by HI-6 in mice. Approximately 7–8 nmol of enzyme was present in the circulation of each mouse before the start of the sarin + HI-6 injections. One nanomole of FBS AChE (400 U = 1 nmol) is inhibited by 280 ng (2 nmol of racemic mixture) of sarin in the absence of HI-6. Based upon *in vitro* titrations, to inhibit the 7–8 nmol of FBS AChE, 14–16 nmol (1.96–2.24 μg) of sarin would be required. The average decrease in blood AChE level per each μg of sarin injected with HI-6 was approximately 25 U as compared with 1450 U per μg of sarin without HI-6 (see *in vitro* data). Thus, the stoichiometry of AChE:sarin *in vivo* was enhanced 57-fold in the presence of HI-6 in the i.v. injected challenge. Since the half-life of HI-6 in rodents is relatively shorter than FBS AChE (approximately a factor of 1:60), the repeated sarin doses also contained HI-6 so that a constant level of HI-6 was maintained for reactivation of sarin-inhibited FBS AChE in the blood of the mice.

The effect of a single dose of HI-6 (50 mg/kg) on the ability of FBS AChE to protect mice is shown in Fig. 7B. The average decrease in blood AChE level per each μg of sarin injected after a single HI-6 pretreatment was approximately 115 U as compared with 25 U/μg with multiple HI-6 administration and 1450 U/μg of sarin without HI-6. The stoichiometry of AChE:sarin in these *in vivo* experiments was 1:12 with a single administration of HI-6. These results show that an adequate amount of oxime reactivator is required to continuously reactivate the *in vivo* sarin-inhibited FBS AChE.

DISCUSSION

Three approaches may be considered for improving the efficacy of ChEs as pretreatment drugs. First,

Table 2. *In vitro* reactivation of OP-inhibited FBS AChE by oximes

Oxime (mM)	AChE Activity (%)			
	Soman (150 ng/mL)	Sarin (150 ng/mL)	Tabun (150 ng/mL)	VX (150 ng/mL)
TMB_4				
0	0.0	1.1 ± 0.2	52.0 ± 3.5	0.0
2	20.7 ± 1.0	93.4 ± 3.7	87.0 ± 6.7	86.9 ± 5.9
Pralidoxime chloride				
0	0.0	1.1 ± 0.2	52.0 ± 3.5	0.0
2	38.1 ± 2.6	97.4 ± 5.1	84.9 ± 5.8	81.7 ± 4.9
MMB_4				
0	0.0	1.1 ± 0.2	52.0 ± 3.5	0.0
2	54.3 ± 7.5	100	82.0 ± 3.2	95.0 ± 3.3
HI-6				
0	0.0	1.1 ± 0.2	52.0 ± 3.5	0.0
2	94.5 ± 2.1	98.8 ± 2.0	97.1 ± 1.5	97.4 ± 2.8

FBS AChE (46 U/mL), 0.35 mL plasma in 50 mM phosphate buffer, pH 8.0 (final volume 0.5 mL), were incubated with and without 2 mM oximes and OPs for 4 hr at room temperature. The residual AChE activity was determined by the assay of Ellman *et al.* [20]. Each data point represents the mean ± SD of duplicate AChE assays on four samples.

production of catalytic antibodies that hydrolyze OPs. This approach has been attempted [26]. The second approach is the use of hydrolytic enzymes such as OP hydrolases [27–30]. Parathion hydrolase was shown to hydrolyze tabun with a sufficient rate to be useful as a pretreatment drug [30] but it has a very short half-life in mice [27]. A third approach is to rapidly restore the catalytic activity of OP-inhibited ChEs and to minimize the aging of the enzyme. This can be achieved by having sufficient amounts of the appropriate oxime present. Indeed, the results presented here show that the addition of HI-6 or other oximes amplifies the effectiveness of exogenous AChE by continuously restoring its catalytic activity and thus detoxifying the OP.

Previously, it was shown that MEPQ-inhibited FBS AChE was reactivated in mice by post-exposure administration of TMB_4 [10] and the reactivated FBS AChE was effective in the protection of the mice against exposure to additional MEPQ. The complete and rapid *in vitro* reactivation of MEPQ-inhibited FBS AChE (Fig. 2) further emphasizes the advantage of being able to continuously reactivate the inhibited enzyme and consequently hydrolyze the OP.

Both MEPQ and sarin are reactivatable inhibitors of ChEs [11]; thus, it is not surprising that the inhibited FBS AChE was reactivated by HI-6 under the experimental conditions described. *In vitro* reactivation of soman-inhibited FBS AChE by HI-6 (Figs. 4, A–C and 5) showed that AChE is reactivated in the presence of the oxime. The concentration of HI-6 required for such a reactivation is dependent on the rate of "aging" of the OP-inhibited FBS AChE. Since sarin-inhibited AChE "ages" relatively slowly, a large amount of sarin is neutralized continuously as long as the oxime concentration is maintained. This is not quite the same with soman-inhibited AChE since it "ages" much more rapidly. However, with a higher concentration of HI-6 and a relatively low concentration of HLo-7, the continuous neutralization of soman is accomplished. The 4.7-fold slower rate of HI-6 reactivation of soman-inhibited AChE versus sarin-inhibited AChE is probably a steric effect resulting from the larger size of the pinacolyl group of soman compared with the isopropyl group of sarin which may have reduced the accessibility of the soman-inhibited active site to oxime. Nevertheless, any OP can be neutralized by incubation with AChE in the presence of an appropriate concentration of oxime (Table 2). The hydrolysis of OPs by FBS AChE in the presence of HI-6 was substantiated by demonstrating at the end of the incubation period that the addition of an aliquot of the mixture to a fresh AChE solution without HI-6 produced no inhibition of the AChE activity.

In vivo experiments in mice (Fig. 7) demonstrated that pretreatment of animals with FBS AChE and subsequent repeated challenges with sarin and administration of HI-6 continuously reactivated exogenously administered AChE and protected mice. When FBS AChE-pretreated mice were challenged with sarin without HI-6 treatment, the equivalent of 1 mol of exogenously administered AChE was inhibited by 2 mol of sarin. In the presence of HI-6, however, the equivalent of 57 mol of sarin was neutralized by 1 mol of AChE. Each challenge with sarin was approximately 1.5 to 2.0 times the molar concentration of circulating enzyme. However, when the sarin was administered in a single bolus dose that exceeded the molar equivalent (2× enzyme amount), OP toxicity symptoms appeared (not shown). This is probably due to the rapid transfer of the OP from the blood into other physiological targets which are not accessible to the oxime. A single administration of HI-6, 5 min before the repeated sarin challenges (Fig. 7B), resulted in a less effective reactivation of exogenously administered FBS AChE. Thus, it is important to

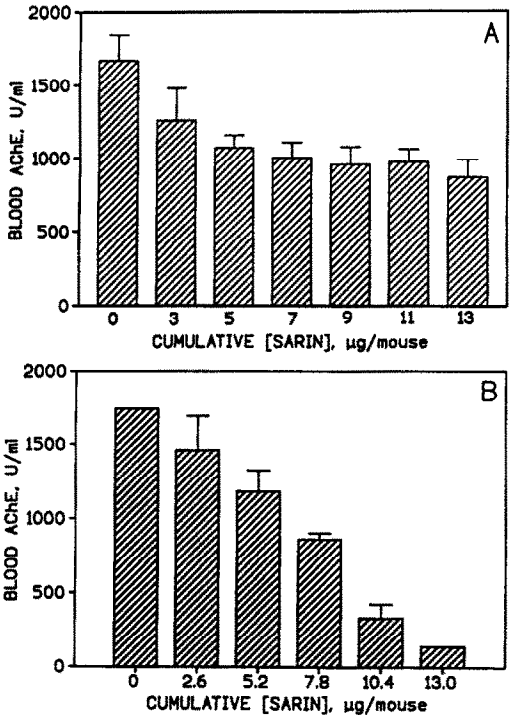

Fig. 7. *In vivo* reactivation of sarin-inhibited FBS AChE by HI-6 in mice. (A) Each mouse (N = 5) was injected i.v. with 3600 U (9 nmol) of FBS AChE. One hour later they were challenged with approximately 2 µg of sarin (14 nmol) and 1 mg HI-6. Sarin/HI-6 injections were repeated six times at 15-min intervals. Blood AChE levels were determined 5 min prior to each sarin challenge. The LD_{50} for sarin is 20 nmol/mouse (2.8 µg); all mice survived. Values are means ± SD. (B) Each mouse (N = 3) was injected i.v. with 3400 U of FBS AChE. After 30 min, 1.25 mg of HI-6 (50 mg/kg) was administered by i.v. injection. Five minutes later, the mice were challenged with 2.6 µg of sarin only. Sarin injections were repeated five times at 15-min intervals. Blood AChE levels were determined 5 min prior to each sarin challenge. All mice survived. Values are means ± SD.

maintain an optimal blood level of HI-6 for long periods of time to enable the rapid reactivation of the circulating inhibited enzyme. Lundy and Hand [31] have shown that repeated administration of HI-6 is more effective against soman toxicity in rodents than a single dose.

The greater protection of FBS AChE *in vitro* and *in vivo* in the presence of HI-6 arises as a consequence of continuous bimolecular reactivation of the organophosphates and not from the direct reaction between HI-6 and OP. No reaction appears to take place between HI-6 and sarin when placed together under the experimental conditions used (Table 1).

The *in vitro* reactivation of OP-inhibited FBS AChE by oximes has a very useful application in many areas. An appropriate formulation can be developed for medical, surgical and skin decontamination. It can also be used for decontamination of materials, equipments, and the environment. Such a formulation can be employed as a protective shield (skin) against exposure to OP pesticides by crop dusters. This approach can be used to develop effective methods for the safe disposal of stored organophosphate nerve agents. The advantages of this approach will be its relative ease of handling, cost effectiveness, and relative safe disposal of detoxification products. In addition, the presence of residual AChE in the detoxifying medium itself will serve as an end point for the completeness of the process. The *in vivo* reactivation of sarin-inhibited FBS AChE in mice by HI-6 certainly provides much needed credence to the practicality of cholinesterase use as a pretreatment against the toxicity of this OP nerve agent.

REFERENCES

1. Holmsted B, Third symposium on prophylaxis and treatment of chemical poisoning. *Fundam Appl Toxicol* **5**: 51–59, 1985.
2. Taylor P, Anticholinesterase agents. In: *The Pharmacological Basis of Therapeutics* (Eds. Gilman AD, Goodman LS, Hall TW and Murad F), pp. 110–129. Macmillan, New York, 1985.
3. Somani S (Ed.), *Chemical Warfare Agents*. Academic Press, New York, 1992.
4. Gray AP, Design and structure–activity relationships of antidotes to organophosphorus anticholinesterase agents. *Drug Metab Rev* **15**: 557–589, 1984.
5. McLeod CG Jr, Pathology of nerve agents: Perspectives on medical management. *Fundam Appl Toxicol* **5**: S10–S16, 1985.
6. Dunn MA and Sidell FB, Progress in medical defense against nerve agents. *JAMA* **262**: 649–652, 1989.
7. Wolthuis OL and Benschop HP, Problems in the therapy of soman poisoning. *Fundam Appl Toxicol* **1**: 188–193, 1981.
8. Castro CA, Larsen T, Finger AV, Solana BP and McMaster SB, Behavioral efficacy of diazepan against nerve agent exposure in rhesus monkeys. *Pharmacol Biochem Behav* **41**: 159–164, 1991.
9. Wolfe AD, Rush RS, Doctor BP, Koplovitz I and Jones D, Acetylcholinesterase prophylaxis against organophosphate toxicity. *Fundam Appl Toxicol* **9**: 266–270, 1987.
10. Raveh L, Ashani Y, Levy D, De La Hoz D, Wolfe AD and Doctor BP, Acetylcholinesterase prophylaxis against organophosphate poisoning. Quantitative correlation between protection and blood-enzyme level in mice. *Biochem Pharmacol* **38**: 529–534, 1989.
11. 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.
12. Doctor BP, Raveh L, Wolfe AD, Maxwell DM and Ashani Y, Enzymes as pretreatment drugs for organophosphate toxicity. *Neurosci Biobehav Rev* **15**: 123–128, 1991.
13. Maxwell DM, Wolfe AD, Ashani Y and Doctor BP, Cholinesterase and carboxylesterases as scavengers for organophosphorus agents. In: *Cholinesterases: Structure, Function, Mechanism, Genetics, and Cell Biology* (Eds. Massoulie J, Bacou F, Barnard E, Chatonnet A, Doctor BP and Quinn DM), pp. 206–209. American Chemical Society, Washington DC, 1991.
14. Wolfe AD, Maxwell DM, Raveh L, Ashani Y and Doctor BP, *In vivo* detoxification of organophosphate in marmosets by acetylcholinesterase. In: *Proceedings*

of the 1991 Medical Defense Bioscience Review, pp. 547–550. U.S. Army Medical Research Institute of Chemical Defense, Edgewood, MD, 1991.
15. Maxwell DM, Castro CA, De La Hoz DM, Gentry MK, Gold MB, Solana BP, 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.
16. Wolfe AD, Blick DW, Murphy MB, Miller SA, Gentry MK, Hartgraves SL and Doctor BP, Use of cholinesterases as pretreatment drugs for the protection of non-human primates against soman toxicity. *Toxicol App Pharmacol* **117**: 189–193, 1992.
17. 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.
18. Wilson IB, Molecular complementarity and antidotes for alkyl phosphate poisoning. *Fedn Proc* **18**: 752–758, 1959.
19. De La Hoz D, Doctor BP, Ralston JS, Rush RS and Wolfe AD, A simplified procedure for the purification of large quantities of fetal bovine serum acetylcholinesterase. *Life Sci* **39**: 195–199, 1986.
20. Ellman GL, Courtney KD, Andres V Jr and Featherstone RM, A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **7**: 88–95, 1961.
21. Finney DJ, The Spearman–Kerber method. In: *Statistical Methods in Biological Assay* (Ed. Finney DJ), pp. 524–530. Charles Griffin, London, 1964.
22. Hovanec JW and Lieske CN, Spontaneous reactivation of acetylcholinesterase inhibited with para-substituted phenyl methylphosphonochloridates. *Biochemistry* **11**: 1051–1056, 1972.
23. Schoene K, Phosphonyloxime aus soman; bildung und reaktion mit acetylcholinesterase *in vitro*. *Biochem Pharmacol* **22**: 2997–3003, 1973.
24. Boter HL and Van Duk C, Stereospecificity of hydrolytic enzymes on reaction with butyrylcholinesterase and acetylcholinesterase prophylaxis against soman poisoning in mice. *Biochem Pharmacol* **18**: 2403–2407, 1969.
25. Clement JG, Hansen AS and Boulet CA, Efficacy of HLo-7 and pyrimidoxime as antidotes of nerve agent poisoning in mice. *Arch Toxicol* **66**: 216–219, 1992.
26. Brimfield AA, Lenz DE, Maxwell DM and Broomfield CA, Catalytic antibodies as biologic scavengers for organophosphorus poisons. In: *Army Science: The New Frontiers* (Eds. Kamely D and Sasmor R), pp. 19–27. Borg Biomedical Services, The Woodlands, 1993.
27. Ashani Y, Rothschild N, Segall Y, Levanon D and Raveh L, Prophylaxis against organophosphate poisoning by an enzyme hydrolysing organophosphorus compounds in mice. *Life Sci* **49**: 367–374, 1991.
28. Cohen JA and Waring MGPJ, Purification and properties of dialkylfluorophosphatase. *Biochim Biophys Acta* **26**: 29–39, 1957.
29. Broomfield CA, A purified recombinant organophosphorus acid anhydrase protects mice against soman. *Pharmacol Toxicol* **70**: 65–66, 1992.
30. 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 hydrolysing organophosphate esters. *Biochem Pharmacol* **44**: 397–400, 1992.
31. Lundy PM and Hand B, Studies of mechanisms involved in the enhanced protection afforded by multiple doses of HI-6 against soman poisoning. In: *Proceedings of the 1985 Chemical Defense Bioscience Review*, p. 6. U.S. Army Medical Research Institute of Chemical Defense, Edgewood, MD, 1985.