
6 New Approaches to Medical Protection against Chemical Warfare Nerve Agents

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I. INTRODUCTION

The acute toxicity of organophosphorus (OP) compounds is usually attributed to their irreversible inhibition of acetylcholinesterase (AChE; EC 3.1.1.7).^{1,2} The resultant increase in the level of acetylcholine at cholinergic synapses, particularly in brain and

diaphragm, produces an acute cholinergic crisis characterized by miosis, increased tracheobronchial and salivary secretions, bronchoconstriction, bradycardia, fasciculation, behavioral incapacitation, muscular weakness, and convulsions culminating in death by respiratory failure.³ Current antidotal regimens for OP poisoning consist of a combination of pretreatment with a spontaneously reactivating AChE inhibitor such as pyridostigmine bromide to protect AChE from irreversible inhibition by OP compounds, postexposure therapy with anticholinergic drugs such as atropine sulfate to counteract the effects of excess acetylcholine, and oximes such as 2-PAM chloride to reactivate OP-inhibited AChE.⁴ Although these antidotal regimens are highly effective in preventing lethality of animals from OP poisoning, they do not prevent the post-exposure incapacitation, convulsions, performance deficits, or in many cases, permanent brain damage.⁵⁻⁷ These symptoms are commonly observed in experimental animals and are likely to occur in humans. An anticonvulsant drug, diazepam, was included as a treatment to minimize convulsions, thereby minimizing the risk of permanent brain damage.⁷ The problems intrinsic to these antidotes stimulated attempts to develop a single protective drug devoid of pharmacological effects, which would provide protection against the lethality of OP and prevent post-exposure incapacitation.⁷

One approach to prevent lethality and minimize side effects or performance decrements is through the use of enzymes such as cholinesterases (ChE) as single pretreatment drugs to sequester highly toxic OP before they reach their physiological targets.⁸⁻¹⁷ This approach turns the irreversible nature of the OP-ChE interaction from disadvantage to advantage; instead of focusing on the OP as an anti-ChE, one can focus on the ChE as an anti-OP. Using this approach, it was shown that administration of fetal bovine serum (FBS) AChE or human serum butyrylcholinesterase (HuBChE), protected animals from a variety of multiple LD₅₀ of highly toxic OP without any toxic effects or performance decrements.⁸⁻¹⁷

The use of enzymes as therapeutic agents is not unique to ChE. In comparison with many drugs, enzymes have many unique advantages; they are specific, catalytically efficient, operate under physiological conditions, and cause essentially no deleterious side effects. Some of the demonstrated uses of enzymes as therapeutic agents include facilitating the digestion of food, wound healing, proteolysis, replacement of defective enzyme in the case of genetic disorders, removal of blood clots, fibrinolysis, and depletion of metabolites in cancer. In almost all instances where enzymes have been employed therapeutically, they have been used for their proteolytic/hydrolytic properties, as replacements for defective or deficient enzymes, or for the improvement or alteration of immune properties. Only recently have enzymes been employed as scavengers or prophylactic drugs for protection from highly toxic substances or as detoxifying or decontamination agents. Both the enzymes for which the toxic agents are substrates that are catalytically hydrolyzed (e.g., organophosphate hydrolases (OPH) or organophosphorous acid anhydride hydrolases (OPAA), and the enzymes which have a very high affinity for these toxic agents and are irreversibly inhibited (e.g., ChE) are potential scavengers for OP compounds. There are requirements for an enzyme to be an effective scavenger for OP toxicity *in vivo*. It should have a relatively high turnover number, a long half-life *in vivo*, be readily

available in sufficient quantities, and not be immunoreactive. In addition, for enzymes such as ChE and CaE, the *in vivo* stoichiometry of sequestration of toxic OP agents should approach 1:1.

The contents of this article describe the progress made in the last decade, by several groups of investigators, in exploring the potential use of enzymes to counteract the toxicity of OP. Among the enzymes which hold promise as scavengers of highly toxic OP nerve agents, significant advances have been made using ChE. Since the biochemical mechanism underlying the prophylaxis by exogenous ChE is established and tested in several species, including non-human primates, this concept should enable a reliable extrapolation of results from animal experiments to human application.

II. STABILITY OF CHOLINESTERASES (ChE) *IN VIVO*

ChE purified from animals such as FBS-AChE, equine serum BChE (EqBChE), and HuBChE were selected as appropriate forms of bioscavengers to be tested as pretreatment drugs for OP toxicity. Their selection was based on the fact that all three enzymes are soluble globular forms,^{18,19} easily purified in large quantities from serum,^{20,21} and have a relatively long half-life *in vivo*.^{9,22–25} Figure 6.1 depicts the time courses of three ChE, administered by three different routes, in mice, rats, guinea pigs, and rhesus monkeys. The determination of half-life of all these ChE in mice,^{9,23–25} rats,^{20,25} guinea pigs,¹⁷ and rhesus monkeys,¹⁶ showed that their mean residence time in circulation was 35–60 h. The route of administration (i.v., i.p., or i.m.) affected the time at which the maximum concentration of enzyme in circulation was reached, but did not affect the mean residence time, and a constant level of enzyme was maintained for a period of approximately 3–10 h. Also, regardless of the route of administration, 60–90 % of administered enzyme was found in the circulation of animals.

All recombinant as well as monomeric forms of native esterases tested so far have a relatively low mean residence time in the circulation of mice.^{23,24} Therefore, in their present form they are not suitable as scavengers of OP. This is discussed in detail later in the chapter. In general, only the tetrameric forms of plasma-derived ChE appear to have relatively long residence times in animals. Enzymes isolated from animal species or from plant or bacterial sources may not be suitable for use in humans, for they will cause adverse immune reaction. At the present time, HuBChE appears to be the most suitable bioscavenger enzyme for human use. Notably, the stability of exogenously administered HuBChE was determined in individuals identified as being homozygous “silent” for serum BChE and half-lives of 8–12 days were reported.^{26–28}

III. SCAVENGER PROTECTION IN RODENTS

The first successful use of AChE or BChE as pretreatment drugs for OP toxicity was demonstrated in rodents.^{8–10} For example, pretreatment of mice with FBS-AChE^{8–10} or HuBChE^{10,22} successfully protected animals against $2\text{--}5 \times \text{LD}_{50}$

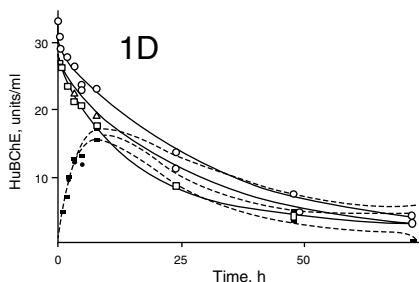
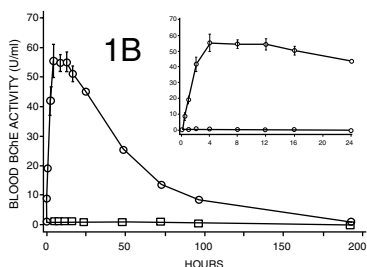
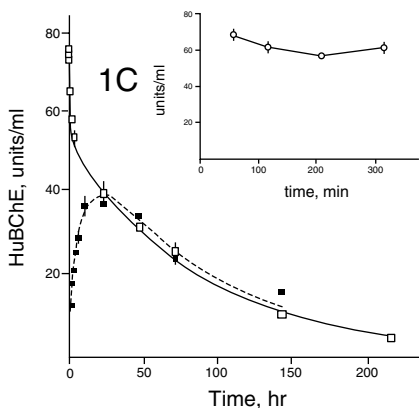
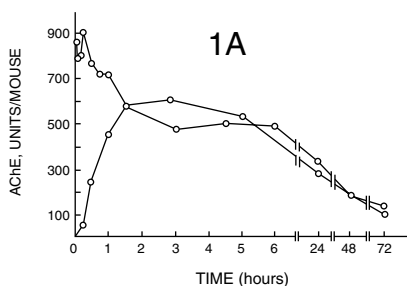


FIGURE 6.1a Average whole blood AChE levels following i.v. or i.p. administration of FBS-AChE: (O) mice administered 920 units/mouse by i.v. route ($n = 2$), and (\square) mice administered 760 units/mouse by i.p. route ($n = 3$). Variations among individual animals administered the same amount of AChE was assumed 15%. Blood volume was to be 7.5% of body weight. From Raveh, L. et al., *Biochem. Pharmacol.*, 41, 37, 1991. With permission.

FIGURE 6.1b Average BChE levels in rats injected i.p. with 5000 U of EqBChE ($n = 6$, circles) or vehicle ($n = 7$, squares) from blood sampled during 192 h (inset 24 h) following administration. Vertical lines about each point represent \pm SEM. Points above time 0 represent endogenous BChE levels measured 24 h before injection. From Genovase, R.F. and Doctor, B.P., *Pharmacol. Biochem. Behav.*, 51, 647, 1995.

FIGURE 6.1c Time course of HuBChE in blood of guinea pigs after i.v. (\square , 1600 units/animal) and i.m. (2000 units/animal) bolus injections. Each data point is an average from three animals. Endogenous BChE activity (an average 3.4 units/ml of whole blood), was subtracted. (Inset) Expansion of the time between 0 and 5.5 h post-i.v. loading of the enzyme. From Allon et al., *Toxicol. Sci.*, 43, 121, 1998. With permission.

FIGURE 6.1d Individual time course of HuBChE in blood of monkeys following i.v. (open symbols) and i.m. (filled symbols) injections of ~11.5 mg purified enzyme/animal. Each data point is an average of three measurements. Endogenous BChE activity is subtracted. From Raveh, L. et al., *Toxicol. Appl. Pharmacol.*, 145, 43, 1997. With permission.

of VX (ethoxymethyl-S-[2-(diisopropylamino)ethyl]thiophosphonate), or MEPQ (7-(ethoxymethylphosphinyloxy)-1-methylquinolinium iodide) or soman (pinacoloxymethyl-fluorophosphonate) without requiring any other drug treatment. These studies established a quantitative correlation between the degree of protection against OP compounds and the level of inhibition of administered enzyme, although the protected mice were not evaluated for potential behavioral incapacitation or for any detrimental immunologic response from administering an exogenous enzyme. In addition, these results demonstrated that *in vivo* inhibition of exogenously administered AChE or BChE in blood was proportional to the amount of OP administered as challenge, a result consistent with *in vitro* experiments.

Subsequent studies addressed the question whether pretreatment with a ChE can prevent OP-induced cognitive impairments. Behavioral testing was carried out in rats using the Morris Water Maze Task, evaluating learning, memory, and reversal learning processes. Cognitive functioning in rats was significantly impaired following i.v. administration of $0.9\text{--}1.1 \times \text{LD}_{50}$ of soman. HuBChE significantly prevented the development of soman-induced cognitive decrements.²⁹ These results are consistent with previous conclusions that cognitive functions are sensitive to cholinergic manipulations.^{30,31} HuBChE treatment alone was devoid of any impairments in behavioral performance, either motor or cognitive. In that respect, it seems that HuBChE has no undesirable performance decrements. These results further support the concept that pretreatment alone with a scavenger such as HuBChE is sufficient to increase not only survival but also to alleviate deficits in cognitive functioning after exposure to a potent nerve agent such as soman.

IV. PROPHYLAXIS AGAINST SOMAN INHALATION TOXICITY IN GUINEA PIGS WITH HUMAN BUTYRYLCHOLINESTERASE (HuBChE)

The use of a ChE scavenger as a prophylactic treatment against inhalation toxicity, which is a more realistic simulation of exposure to volatile OP, has been described by Allon et al.¹⁷ HuBChE-treated guinea pigs were exposed to a controlled concentration of soman vapors ranging from 417 to 430 $\mu\text{g/l}$ for 45 to 70 s. The correlation between the inhibition of circulating HuBChE and the dose of soman administered by sequential i.v. injections and by respiratory exposure indicated that ~29% of the inhaled dose of soman reached the blood. A HuBChE to soman molar ratio of 0.11 was sufficient to prevent the manifestation of toxic signs following exposure to $2.17 \times \text{LD}_{50}$ of soman (1 LD_{50} inhaled dose = 101 $\mu\text{g/kg}$). It was noted that protection was far superior to the currently used traditional approach (pyridostigmine and post-exposure therapy). The greater-than-calculated values of protection observed were explained by the fact that unlike an i.v. bolus injection, inhalation exposure allows soman to enter the circulation gradually, which increases the efficacy of soman sequestration to below its toxic levels. The following three important observations are advanced regarding the use of scavengers for OP toxicity:¹⁷ (1) the stoichiometry of protection against inhalation exposure agrees reasonably well with that

seen after i.v. challenge, (2) consistent protection is observed across four species of animals, and (3) the pharmacokinetic behavior of HuBChE is similar in mice, rats, guinea pigs, and non-human primates.

V. COMPARISON OF ANTIDOTE PROTECTION AGAINST SOMAN BY PYRIDOSTIGMINE, HI-6, AND ACETYLCHOLINESTERASE (AChE)

Carbamate, oxime, and enzyme scavenger approaches to protection against highly toxic soman were compared by using the prominent example of each type of antidote.³² Pyridostigmine in combination with atropine, HI-6 in combination with atropine, and FBS-AChE alone were used as examples of carbamate, oxime, and enzyme scavenger antidotes, respectively. Each antidotal regimen produced approximately equal maximal protection against the lethal effects of 952 to 1169 nmol/kg ($8-10 \times LD_{50}$) of soman in mice whose carboxylesterase had been inhibited with CBDP (2-(*o*-cresyl)-4H-1:3:2-benzodioxaphosphorin-2-oxide). FBS-AChE was much better than either pyridostigmine/atropine or HI-6/atropine in reducing post-exposure incapacitation from soman as measured by lacrimation, motor dysfunction, activity level, and the inverted screen test. A lower dose of pyridostigmine (566 nmol/kg) or FBS-AChE (1150 nmol/kg) was sufficient to protect against 968 nmol/kg ($8 \times LD_{50}$) of soman than was required for HI-6 (200,000 nmol/kg). The circulatory half-life of FBS-AChE (1550 min) was much greater than that of pyridostigmine (48 min) or HI-6 (11 min). These results suggest that FBS-AChE should be considered a superior alternative to either pyridostigmine/atropine or HI-6/atropine antidotal regimens. The major advantages of bioscavengers for protection against OP toxicity are their rapid removal of OP compounds from circulation and the absence of post-exposure incapacitation and toxic effects that are commonly observed in animals protected by traditional antidotal approaches.³²

VI. EXPERIMENTS WITH NON-HUMAN PRIMATES

The successful demonstration of asymptomatic protection of rodents against a variety of OP by pretreatment with three different ChE prompted the evaluation of sequestration of OP by ChE in non-human primates. The effectiveness of FBS-AChE, EqBChE, and HuBChE as pretreatment drugs was evaluated in rhesus monkeys, which are more sensitive to OP compounds than rodents. Monkeys were exposed to sarin, VX, or soman, the latter OP compound is considered to be the most refractory to current therapy.⁷ Behavioral performance was measured by a highly sensitive test of cognitive function, the serial probe recognition (SPR) task.^{12,14,15,33-38} This behavioral task was chosen because (a) it is a multiple-item memory task that measures short-term memory capacity and decision-making ability,³⁶ (b) it has been used extensively to understand human cognitive processing,³⁷ and (c) it is sensitive to CNS damage in both human and non-human primates.^{37,38} For example, rhesus monkeys with damage to the limbic system and humans suffering from amnesia resulting from

either Parkinson's or Alzheimer's disease show impaired performance on SPR tasks.³⁷ This task was also shown to be sensitive to disruption after exposure to doses of soman as low as 1.5–2.0 $\mu\text{g/kg}$.³⁸ Following i.v. administration of FBS-AChE, the *in vivo* blood AChE activity was elevated more than 100- to 150-fold after 2 h, yet this treatment had very little effect on the SPR performance. The *in vivo* neutralization of soman by FBS-AChE (Figure 6.2) showed a linear relationship between the progressive inhibition of blood AChE activity and the cumulative dose of soman administered.¹⁵ The percent correct and response latencies of monkeys trained on SPR task to a list length of six items showed complete protection against behavioral incapacitation by soman with no apparent sign of OP toxicity. The monkeys failed to respond within the 10-s interval in only 2 of 4200 trials.

This investigation demonstrated that monkeys displayed minimal adverse reactions from FBS-AChE pretreatment. Following OP exposure, even the best pretreatment/therapy regimen, i.e., pyridostigmine pretreatment and atropine/oxime therapy, does not prevent signs of OP intoxication, such as periods of unconsciousness, respiratory distress tremors, and intermittent convulsions.^{6,7} The administration of FBS-AChE prevented the occurrence of all of these signs of OP intoxication. Thus, the ability of FBS-AChE to protect against behavioral incapacitation that results from OP exposure in non-human primates suggests that humans would also be protected.

Concurrently, Broomfield et al. showed in rhesus monkeys that the toxicity of soman ($2 \times \text{LD}_{50}$) can be neutralized by administration of an appropriate amount of EqBChE without any performance decrement as measured by SPR.¹² Also, protection of monkeys against 3 to $4 \times \text{LD}_{50}$ of soman was obtained with EqBChE pretreatment followed by atropine post-exposure treatment. These animals were able to perform the SPR task about 9 h post-exposure, whereas animals treated with conventional atropine/oxime therapy were not able to perform the same task for 14 days. Animals receiving enzyme alone showed only a subtle transient performance decrement on the SPR task.

A second parameter, the Primate Equilibrium Platform (PEP) task^{39–41} was used to demonstrate the protection of rhesus monkeys from the toxicity of as high as $5 \times \text{LD}_{50}$ of soman by pretreatment with FBS-AChE or EqBChE without the occurrence of performance deficits.¹⁴ The PEP is a continuous compensatory tracking device that measures the ability of a monkey to compensate for unpredictable perturbations in the pitch induced by a filtered random noise signal. Subjects performed the PEP task for 2.5 h on each soman-challenge testing day, and results were presented for each 5-min block of testing time. During the 6 weeks of long-term follow-up, PEP tests were conducted for 2 h; Φ was computed for each 5-min block of time; and the mean of the 24 resulting data points was calculated to yield one performance score for the entire 2 h.

The i.v. administration of $\sim 0.5 \mu\text{mol}$ of ChE alone produced a 100-fold increase in blood ChE activity and caused no apparent physiological or neurological effect or deficit, as measured by the PEP task performance. None of the eight monkeys showed any OP toxicity after soman challenges; protection was so complete that there were no fasciculations even at the site of soman injections. Following the first and second soman injections (totaling 25.6 $\mu\text{g/kg}$, $\sim 4 \times \text{LD}_{50}$), the PEP performance of all eight

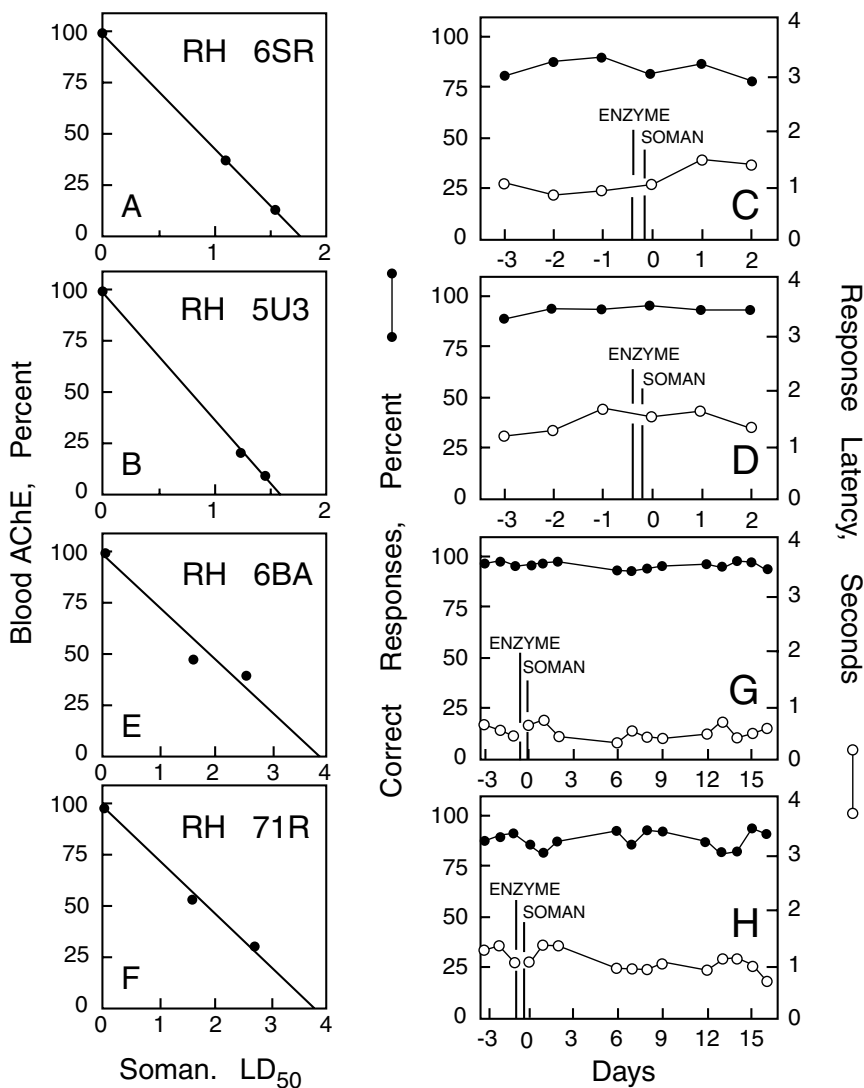


FIGURE 6.2 *In vivo* titration of blood AChE in rhesus monkeys pretreated with 105 nmoles of FBS AChE (ABCD). Soman dose shown is the cumulative LD₅₀. Percent correct responses and response latencies for rhesus monkeys. SPR scores (list length of one item) were obtained at indicated times before administration of 105 nmoles of FBS AChE and after challenge with 1.5 LD₅₀ of soman, i.v., in 2 injections. *In vivo* titration of blood AChE in rhesus monkeys pretreated with 210 nmoles of FBS AChE (EFGH). Monkeys were challenged with 2.5–2.7 LD₅₀ of soman. Percent correct responses and response latencies for SPR scores (list length of 6 items) before injection of 210 nmoles of FBS AChE and after challenge with 2.5–2.7 LD₅₀ of soman, i.v., in two injections. From Maxwell et al., *Toxicol. Appl. Pharmacol.*, 115, 44, 1992. With permission.

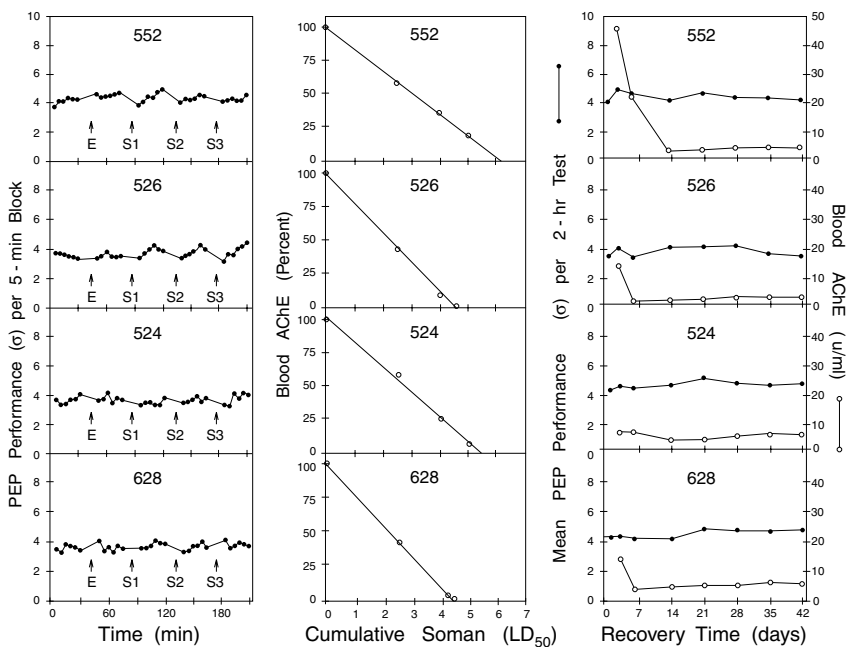


FIGURE 6.3 (Left) Effects of i.v. administered purified FBS AChE on PEP task performance before and after challenge with approximately 2.5, 1.5, and 1.0 LD₅₀ of soman. Four male rhesus monkeys (5–8 kg), trained to perform the primate equilibrium platform (PEP) task, each received approximately 0.4–0.5 μmol of AChE i.v. (greater than 1:1 stoichiometry with soman). The sequence of behavioral testing and soman challenges was (a) 30-min PEP task (baseline); (b) AChE injection, E (15 min); (c) 30-min PEP task to determine the effect of administration of AChE alone, followed by a 15-min pause for obtaining blood samples, AChE assay, and soman injection, S1 (16.0 μg/kg, ≈2.5 LD₅₀, i.m.); (d) 30-min PEP testing, followed by a 15-min pause for obtaining blood samples, AChE assay, and soman injection, S2 (9.6 μg/kg, ≈1.5 LD₅₀, i.m.); (e) 30-min PEP testing, followed by a 15-min pause for obtaining blood samples, AChE assay, and the final i.m. soman injection, S3 (6.4 μg/kg, ≈1.0 LD₅₀, was planned but would be reduced if residual AChE activity was judged insufficient); (f) final 30 min of PEP testing. For each 30 min of PEP testing, the data (filled circles) from 6 sequential, 5-min blocks of time are presented. (Middle) *In vivo* titrations of blood AChE in four rhesus monkeys pretreated by i.v. injection with FBS AChE. Details are as described above. The cumulative dose of soman which reduced ChE activity to the indicated final levels exceeded the amount of AChE administered, suggesting involvement of endogenous esterase. (Right) Long-term effects on PEP task performance of i.v. administered FBS AChE and challenge with a total of approximately 5 LD₅₀ of soman and residual blood AChE levels. PEP performance and blood AChE levels of 4 monkeys were tested weekly for 6 weeks, filled circles, PEP performance; open circles, enzyme level. PEP performance scores are the mean of data from 24 separate, 5-min blocks that compose the 2-h test. From Wolfe, A.D. et al., *Toxicol. Appl. Pharmacol.*, 115, 44, 1992. With permission.

monkeys was completely normal. The four monkeys pretreated with FBS-AChE (Figure 6.3) or EqBChE (Figure 6.4) continued this level of performance even after the third soman challenge. However, the two remaining monkeys that had been

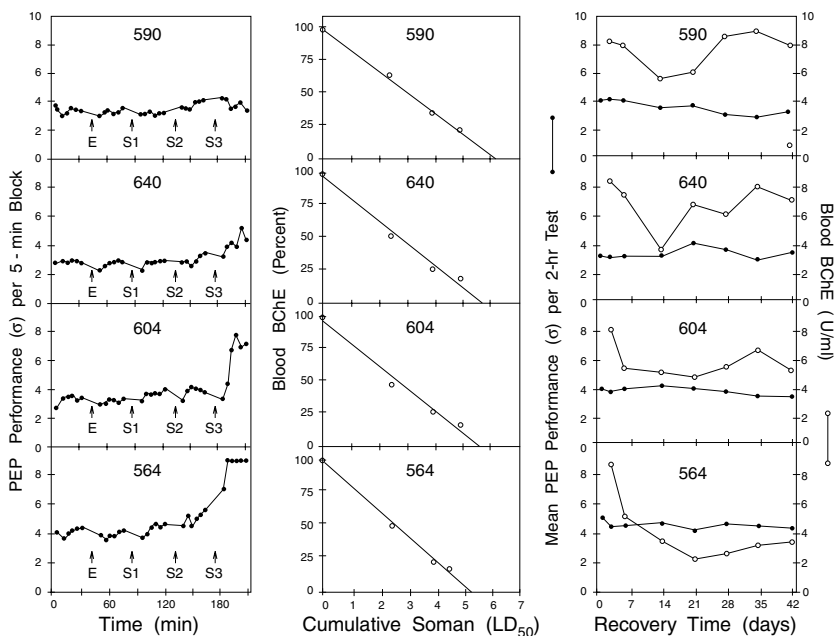


FIGURE 6.4 (Left) Effects of i.v. administered purified horse serum BChE on PEP test performance before and after challenge with approximately 2.5, 1.5, and 1.0 LD_{50} of soman. See legend to Figure 1 for a detailed explanation. (Middle) *In vivo* titrations of blood BChE in four rhesus monkeys pretreated by i.v. injection with horse serum BChE. See legend to Figure 6.1 for details. (Right) Long-term effects on PEP task performance of i.v. administered horse serum BChE and challenge with a total of approximately 5 LD_{50} of soman and residual blood BChE levels. From Wolfe, A.D. et al., *Toxicol. Appl. Pharmacol.*, 115, 44, 1992. With permission.

pretreated with EqBChE exhibited a significant but minor PEP deficit after the third soman injection; this transient PEP performance deficit was similar to that observed after exposure of unprotected monkeys to low doses of soman ($<2.8 \mu\text{g/kg}$).^{40,41} Based upon this comparison, the cumulative protective ratio afforded by ChE pretreatment against soman can be estimated at 10 to 15. During 6 weeks of postsoman testing, none of the monkeys showed any signs of delayed toxicity, convulsions, or other OP symptoms or any abnormality on PEP performance.¹⁴ In non-human primates, the 1:1 stoichiometry between ChE and OP dose, plus the endogenous scavenger (ChE, CaE, and other 3–4 unidentified proteins) present,^{42–45} the LD_{50} of soman may be extrapolated to be approximately 4.3 $\mu\text{g/kg}$.

The ability of HuBChE to prevent toxicity induced by soman and VX was assessed in rhesus monkeys.¹⁶ A molar ratio of HuBChE:OP ~ 1.2 was sufficient to protect monkeys against an i.v. bolus injection of $2 \times LD_{50}$ of VX, while a ratio of 0.62 was sufficient to protect monkeys against an i.v. dose of $3.3 \times LD_{50}$ of soman, with no additional post-exposure therapy. A remarkable protection was also seen against soman-induced behavioral deficits detected in the performance of a spatial discrimination task (Figure 6.5).

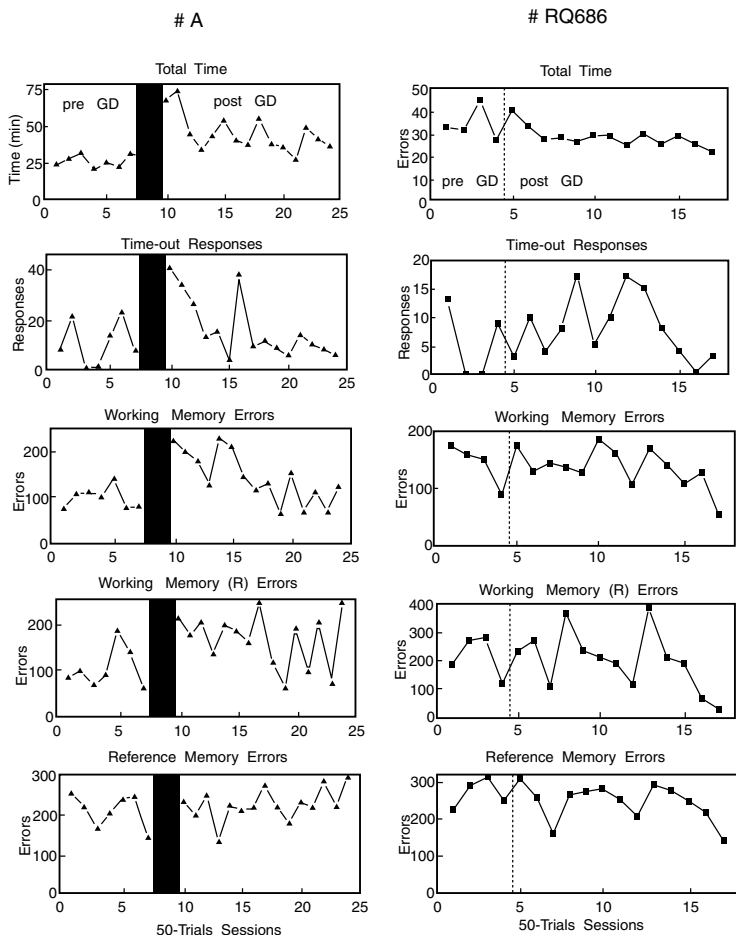


FIGURE 6.5 Effects of soman on performance of the spatial discrimination task in monkeys. (Left) Monkey A, pretreated with 0.1 mg/kg pyridostigmine followed with TAB immediately after exposure to 15 $\mu\text{g/kg}$ soman ($2.7 \times \text{LD}_{50}$). The shaded area represents 2 days in which no performance was obtained during the presentation of the behavioral tests. The five panels represent the behavioral parameters. (Right) Effects of soman (18 $\mu\text{g/kg}$; $3.3 \times \text{LD}_{50}$) on performance of the spatial discrimination task in monkey RQ686, following pretreatment with 26 mg HuBChE. No additional treatment was administered. The animal continued its normal performance with no adverse effects immediately following soman (dotted line). Data points are morning sessions only. Note the different scale on the ordinates of the two panels. From Raveh, L. et al., *Toxicol. Appl. Pharmacol.*, 145, 43, 1997. With permission.

These studies firmly establish that prophylactically administered ChE, with no additional therapy, prevents the toxicity induced by highly toxic OP nerve agents in mice, rats, guinea pigs, and rhesus monkeys. Not only do these bioscavengers prevent lethality, but animals do not show any untoward side effects or performance decrements/deficits determined by the Morris Water Maze task, SPR task, PEP task, or spatial discrimination task.

Of the three ChE investigated so far, only HuBChE appears to be an appropriate candidate for exploration for human use. FBS-AChE and EqBChE are known to induce the production of antibody when administered in heterologous species of animals (unpublished results). The antibody generated by repeated administration of these two enzymes rapidly clears the circulating exogenous ChE from blood, indicating that the use of such enzymes in heterologous species may not be of much value except for a single use. The absence of immunological and physiological side effects following blood and/or plasma transfusions in humans and lack of adverse reaction to partially purified HuBChE administered daily for many weeks,⁴⁷ support the contention that HuBChE is the most promising prophylactic antidote. Also, the stability of the exogenously administered HuBChE in humans (half-life of 8 to 12 days)²⁶⁻²⁸ suggests a long-lasting therapeutic level even after administration of a single dose of enzyme.

The systematic evaluation of the efficacy of HuBChE in protection of four species against nerve agent toxicity offered an extrapolation model from animal to human^{9,10,16,17,29,46} based on the stoichiometry of OP sequestration and pretreatment with HuBChE protection levels in mice, rats, guinea pigs, and monkeys. Further, results show that the stoichiometry of OP sequestration in any given species should depend on the concentration of the circulating enzyme at the time of exposure to challenge. Calculations of protective ratios in humans required quantitative information on the toxicity of OP in humans. These figures were compiled from the literature describing human volunteer studies with non-lethal doses and accidental exposures to nerve agents that enabled an estimate of sign-free doses as well as toxic doses in humans. Predictions were then made by calculating the amount of HuBChE required to reduce toxic levels of OP to below the sign-free doses within one blood circulation time in human (seconds).

It was predicted that 200 mg/70 kg HuBChE would protect against up to $2 \times \text{LD}_{50}$ of VX or soman, without the need for immediate post-exposure treatment.⁴³ Lowering the dose to 50 mg/70 kg is likely to confer protection against long-term exposure to low levels of nerve agents such as soman. It should be noted, however, that the extrapolation from animal-to-human was based on data generated in animals weighing 20 g to 10 kg, and further validation in larger animals may be useful.

VII. IMPROVING THE BIOSCAVENGING CAPABILITY OF ChE

Many approaches have been made to improve the efficacy of stoichiometric bioscavengers. Enzymes that can hydrolyze OP are also being considered as promising bioscavengers. These efforts are summarized below:

1. Amplification of the effectiveness of ChE for detoxification of OP by oximes
2. Site-specific mutagenesis of AChE
3. OP hydrolyzing enzymes, e.g., OPH, OPAA, paraoxonase, parathion hydrolase, etc.

4. Carboxylesterase as a bioscavenger
5. Huperzine A as a pretreatment drug
6. Immobilized ChE for the decontamination of OP

A. AMPLIFICATION OF THE EFFECTIVENESS OF ChE FOR DETOXIFICATION OF ORGANOPHOSPHATES (OP) BY OXIMES

A major limitation for use of ChE as pretreatment drugs for OP toxicity is their 1:1 stoichiometry with OP. An approximately 200-fold difference in molecular weight between OP and ChE necessitates the use of large amounts of enzyme to provide protection. To improve the efficacy of ChE as pretreatment drugs, an approach was developed in which the catalytic activity of OP-inhibited AChE was rapidly and continuously restored by having sufficient amounts of appropriate oxime present.⁴⁸ In general, OP-inhibited ChE can be reactivated rapidly by mono- or bis-quaternary oximes such as 2-PAM and HI-6 so long as it has not undergone aging. The rate of reactivation of OP-inhibited ChE depends on the type and source of ChE, the structure of OP and oxime, as well as the concentration of oxime used. *In vitro* effectiveness of several oximes in reactivating AChE that has been inhibited by a variety of OP showed that oximes, such as TMB₄, 2-PAM, MMB₄ and HI-6, reactivated AChE inhibited by all OP to some extent, but HI-6 was the most effective in reactivating AChE that was inhibited by soman and sarin. The capacity of AChE in combination with 2mM HI-6 to detoxify large amounts of sarin *in vitro* is shown in Figure 6.6a. One mole of enzyme could detoxify a 3200-fold molar excess of sarin or a 64-fold molar excess of soman in the presence of 2 mM HI-6, as compared to a two-fold excess of sarin or soman in the absence of HI-6. Improved detoxification of OP compounds by AChE in combination with oxime has also been demonstrated *in vivo* (Figure 6.6b). Mice receiving 9 nmol of AChE and 1 mg HI-6 could detoxify a cumulative 57-fold excess of sarin when it was administered by repeated injections at 15-min intervals and as long as the HI-6 level was maintained by repeated injections of 1 mg HI-6.⁴⁸ If the level of HI-6 was not maintained, detoxification was less effective as demonstrated by a pronounced decrease in *in vivo* AChE activity.

B. SITE-SPECIFIC MUTAGENESIS OF AChE

Several recent studies have demonstrated that it is indeed possible to improve the bioscavenging performance of cholinesterases by site-directed mutagenesis.⁴⁹ Using this technique, it is possible to obtain mutant enzymes which possess an increased affinity for OP,⁵⁰ or are more easily reactivated by oximes,⁵¹ and/or possess a reduced rate of aging.^{49,52–54} The kinetics of aging were examined in a soman-inhibited mutant enzyme in which the glutamate E202(199), located next to the active-site serine S203(200) of AChE, was converted to glutamine.⁴⁹ For wild-type enzyme, the soman-AChE conjugate aged very rapidly, giving rise to a form of enzyme resistant to reactivation by oximes. In contrast, the E202(199)Q mutant enzyme was largely resistant to aging and could be reactivated by oximes.^{49–52} *In vitro* detoxification of soman and sarin by mouse wild-type and E202Q AChE in the presence of 2 mM HI-6

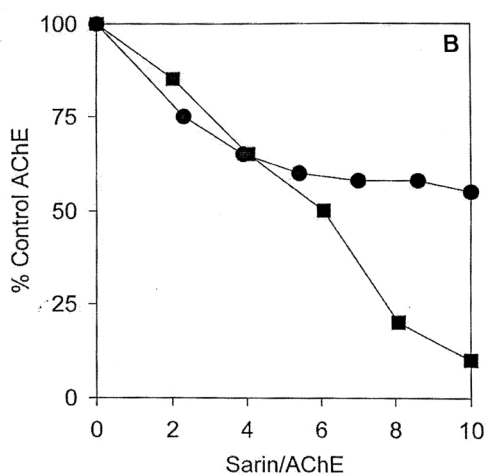
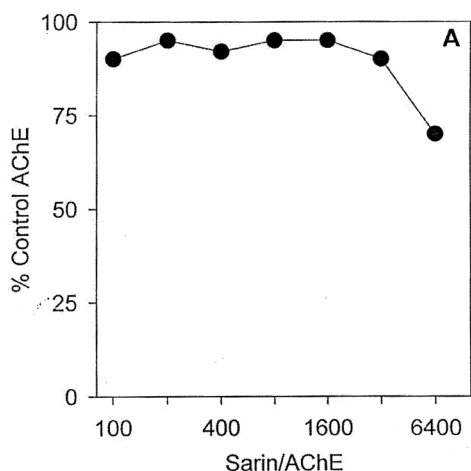


FIGURE 6.6A *In vitro* titration of FBS AChE in the presence of HI-6. Reactivation of FBS AChE (0.125 nmol) in the presence of 2 mM HI-6 at pH 8.0 after repeated additions of sarin at 0.5 h intervals

FIGURE 6.6B *In vivo* detoxification of sarin by FBS AChE in mice. Mice received i.v. FBS AChE (9 nmol) followed by sarin (14 nmol) and 1 mg HI-6. Sarin/HI-6 injections (●) or sarin alone injections (i.v.) were then repeated at 15-min intervals. AChE activity was determined 5 min prior to each sarin injection. All mice survived.

showed that the mutant E202Q AChE was 2–3 times more effective in detoxifying soman and sarin compared to wild-type AChE.⁴⁹ These studies show that these recombinant DNA-derived AChE are a great improvement over wild-type AChE as bioscavengers. They can be used to develop effective methods for the safe disposal of stored OP nerve agents and appropriate formulation for medical surgical and skin decontaminants and also for decontamination of materials, equipment, and the environment.

To evaluate the possible use of recombinant ChE as bioscavengers *in vivo*, the mean residence time of five tissue-derived and two rChE (i.v.) injected in mice were compared with their oligosaccharide profiles.^{23,24} Monosaccharide composition analysis revealed differences in the total carbohydrate, galactose, and sialic acid contents. The molar ratio of sialic acid to galactose residues on tetrameric HuBChE, rMoAChE, and rHuBChE was found to be ~1.0, suggesting that all the terminal galactose residues were capped with sialic acid. However the mean residence time of HuBChE was 9- and 14-fold greater than that of rMoAChE and rHuBChE, suggesting that the capping of galactose with sialic acid by itself is not sufficient to confer circulatory stability to ChE. For *Torpedo* AChE (mean residence time = 44 min) and monomeric FBS-AChE (mean residence time = 304 min), this ratio was ~0.5, suggesting that only half of the terminal galactose residues were capped with sialic acid, yet these enzymes differed greatly in their circulatory stability. In contrast, a molar ratio of 0.5 for sialic acid-to-galactose was observed for the highly stable tetrameric FBS-AChE and EqBChE. These observations suggest that although the presence of sialic acid appears to be essential for maintaining ChE in circulation, the location rather than the number of the non-sialylated galactose residues may be affecting circulatory stability.

Differences in oligosaccharides of ChE from various sources and the micro-heterogeneity in glycans on each ChE were elucidated by charge- and size-based separation analyses. However, neither the carbohydrate composition nor the oligosaccharide profile could be completely correlated with the pharmacokinetic parameters of these enzymes. The glycans of recombinant ChE and monomeric FBS-AChE displayed a remarkable heterogeneity in size and consist of hybrid and complex bi-, tri-, and tetra-antennary structures. *Torpedo* AChE also contains high-mannose structures. The three plasma ChE, on the other hand, contain mature glycans which are predominantly of the complex biantennary type, suggesting that these structures are responsible for the extended mean residence times of the enzymes. *Torpedo* AChE, rChE, and monomeric FBS-AChE showed a distinctive shorter mean residence time (44–304 min) compared with tetrameric forms of plasma ChE (1902–3206 min). Differences in the pharmacokinetic parameters of ChE appear to be due to the combined effect of the molecular weight and charge- and size-based heterogeneity in glycans. Site-specific analysis of glycan structures may elucidate the structures responsible for the rapid clearance of non-plasma ChE and suggest suitable manipulations for improving the circulatory stability of rChE.

C. OP HYDROLYZING ENZYMES, E.G., OPH, OPAA, PARAOXONASE, PARATHION HYDROLASE, ETC.

Lenz et al.'s chapter provides a comprehensive discussion of this topic.

D. CARBOXYLESTERASE AS A BIOSCAVENGER

Although the development of bioscavenger protection against toxic OP has focused primarily on the use of AChE and BChE, studies of carboxylesterase (CaE) have demonstrated that this esterase has some advantages as an OP bioscavenger. A recent comparison of AChE, BChE, and CaE as bioscavengers has described some of these advantages.⁵⁵ AChE, BChE, and CaE are all members of the α/β hydrolase family and have a high degree of overall homology in their amino acid sequences, but they differ in several critical regions that produce distinct differences in their biochemical properties.⁵⁶ The most significant biochemical differences in these esterases are related to the extent of aging of the OP-inhibited esterase, the size of the active site, and the ability of the OP-inhibited esterase to undergo spontaneous or oxime-induced reactivation. The ideal OP scavenger would have a fast rate of reactivity for a broad spectrum of OP compounds, a slow rate of aging, and the ability to reactivate to increase its stoichiometry as a scavenger. Evaluation of CaE on these criteria suggests that it is a major candidate as an OP bioscavenger.

One of the primary concerns in the use of esterases as bioscavengers for OP compounds is the 1:1 stoichiometry of their detoxication of OP compounds. The major limitation on the stoichiometry of esterases as OP scavengers is the aging of OP-inhibited esterases that prevents their reactivation. One of the most important advantages of CaE is that OP-inhibited CaE does not undergo the rapid aging that prevents oxime reactivation of OP-inhibited cholinesterases.⁵⁷ This means that OP-inhibited CaE can be reactivated to an active enzyme for further sequestration of OP molecules. The effectiveness of this process *in vivo* has been demonstrated by the protection that is produced by diacetylmonoxime, an oxime that reactivates OP-inhibited CaE but does not reactivate OP-inhibited AChE.⁵⁸ Oxime reactivation of soman-inhibited CaE by diacetylmonoxime in rats increased soman detoxication enough to produce a two-fold increase in the LD₅₀ of soman.⁵⁹

Another advantage of CaE is the size of its active site. Saxena et al. have developed a method to estimate the size of the active site of esterases in which the volume of the active site corresponds to the area defined by the van der Waals surface.⁶⁰ The active site volumes of AChE, BChE, and CaE were calculated from the X-ray crystallographic structure of *Torpedo californica* AChE and models of BChE and CaE that were created from the homology of these enzymes with *Torpedo californica* AChE and *Geotrichum candidum* lipase, respectively. The active site volume of CaE was 10 \times larger than that of AChE and 6 \times larger than that of BChE.⁶⁰ The larger size of the active site of CaE is important inasmuch as Taylor et al. have demonstrated that substitution of smaller aliphatic amino acid residues for bulky aromatic residues in the active site of AChE increases the volume of the active site and the ease with which oximes can reactivate OP-inhibited AChE.⁶¹ Their site-directed mutagenesis studies showed that changing phenylalanines in the active site of AChE to smaller groups enhanced oxime reactivation 10- to 20-fold. The reasons for this beneficial effect are complex, but a primary factor is that a more spacious active site allows more avenues of nucleophilic attack by oximes on the phosphorylated serine of AChE and increases the probability of a successful reactivation reaction. Jarv discussed the importance of the direction of nucleophilic attack for oxime reactivation of OP-inhibited ChE.⁶² His analysis concluded that

oxime reactivation is an S_N2 reaction in which the reaction intermediate undergoes inversion of configuration that can be prevented by steric hindrance in a small constrained active site. The large active site volume of CaE, therefore, minimizes steric hindrance in the active site and maximizes the potential for reactivation.

The importance of active site volume is also evident in the decreasing stereospecificity of esterases as the volumes of their active sites increase. In site-specific mutagenesis studies of mammalian AChE, Taylor et al. observed that the stereoselectivity of AChE was reduced 3-fold and 230-fold by substitution of small aliphatic groups for phenylalanine at positions 295 and 297, respectively.⁶¹ Furthermore, in a comparison of the stereoselectivity of AChE, BChE, and CaE, whose relative active site volumes are 3:5:30, the reported ratio of reaction rates of C(+)P(−) and C(+)P(+) stereoisomers of soman for AChE, BChE, and CaE are 17500, 290, and 135, respectively.^{63,64} Even though the stereospecificity of CaE is reduced by its larger active site volume in comparison to ChE, it still maintains a 135-fold greater reactivity with the most toxic stereoisomers [i.e., C(+)P(−) soman].

Another advantage of a large active site is that it confers an enzyme specificity for a wider range of OP inhibitors. By measuring the rate constants for esterase inhibition by a spectrum of OP inhibitors, Maxwell et al. compared the structural specificity of AChE, BChE, and CaE.⁵⁵ This specificity study found that AChE could accommodate OP inhibitors containing only one bulky group (e.g., isopropyl, pinacolyl, or phenyl); BChE could accommodate OP inhibitors containing two of the smaller bulky groups (i.e., isopropyl); and CaE could accommodate OP inhibitors containing up to two of the largest bulky groups (e.g., phenyl groups). Therefore, CaE had the ability to detoxify the broadest spectrum of OP inhibitors. The only exception to this observation is that the fewer aromatic residues in the active site of CaE in comparison to ChE reduces the affinity of CaE for positively charged OP inhibitors.⁵² However, this is not a major deficiency inasmuch as few nerve agents or pesticides are positively charged.

The final advantage of CaE as a bioscavenger is shown by the recent observations that OP-inhibited CaE undergoes spontaneous reactivation. Jokanovic et al. observed that OP-inhibited CaE in rats exhibited spontaneous reactivation after inhibition with dichlorvos, sarin, or soman.⁶⁵ In mechanistic studies of this process, Maxwell et al. found that spontaneous reactivation of sarin-inhibited CaE had a pH profile that suggested the involvement of an amino acid residue with a pK_a of 6.1.⁵⁵ Subsequent examination of the amino acid sequences of CaE from six mammals and two insects revealed a highly conserved histidine that met this pK_a requirement and was not part of the catalytic triad of CaE. This conserved histidine was not found in any wild-type ChE and was located immediately adjacent to the glycines that comprise the oxyanion hole of AChE, BChE, and CaE. This oxyanion hole region appears to be particularly important for spontaneous reactivation of OP-inhibited esterase and OP hydrolysis since mutagenesis of this region has produced profound changes in these activities. For example, Lockridge et al. produced OP hydrolase activity in rHuBChE by site-directed mutagenesis in which a glycine in this region was changed to a histidine.⁶⁶ In addition, Newcomb et al. found a mutant blowfly CaE in which a glycine in the oxyanion region was changed to an aspartate, which converted this CaE to an OP hydrolase.⁶⁷ This conversion was so effective that the mutant blowfly was found to be OP resistant, requiring four to five times more diazinon than was necessary to produce lethality in wild-type blowflies.

E. HUPERZINE A AS A PRETREATMENT DRUG

Huperzine A (HUP), an alkaloid isolated from moss *Lycopodium Huperzia Serrata* is a selective, slow reversible inhibitor of mammalian AChE, with K_i of 20–40 nM.^{68–70} HUP has been demonstrated in mice,^{71,73} guinea pigs,⁷² and monkeys⁷³ to protect against nerve agent toxicity by treatment of animals prior to challenge with soman. Pretreatment of four monkeys with a sign-free dose of (–)-HUP protected them even 14 h post-loading of HUP. The monkeys displayed only minor toxic signs and survived without the need for post-exposure therapy. Similarly, a protective ratio of 2.0 was obtained 6 h after pretreatment of mice with HUP, with no post-challenge supporting therapy. In both monkeys and mice, the long-lasting antidotal efficacy conferred by HUP correlated well with the time course of blood-AChE inhibition. In guinea pigs, pretreatment with HUP was shown to prevent seizures and neuropathological damage to the hippocampus following exposure to soman. These studies highlighted the superiority of HUP as an antidote against nerve agent toxicity compared to pyridostigmine and physostigmine inasmuch as the duration of protection conferred following administration of a single dose of the prophylactic drug.

HUP is more than 1000-fold less potent inhibitor of BChE than mammalian AChE, a finding that is attributed to the reduced aromaticity of the active site gorge of BChE compared with AChE. It was thought that the combined administration of HUP and BChE would have a synergistic effect in terms of protection and will allow decreasing the amount of protein required for adequate protection. Preliminary observations indeed show that pretreatment with HUP and HuBChE protected mice against soman with the sum of the individual contribution of each drug alone.⁷³ Thus, a protective ratio of 2.6 was observed while the predicted value from the separate experiments was at 2.5. These studies with HUP as a potential antidote suggest that this slow inhibitor is a promising pretreatment drug that confers protection by a relatively long-lasting reversible inhibition of AChE at physiologically important sites.

F. IMMOBILIZED ChE FOR THE DECONTAMINATION OF OP

It has been demonstrated that a variety of enzymes exhibited enhanced mechanical and chemical stability when immobilized on a solid support, producing a biocatalyst. Munneke first immobilized a pesticide detoxification extract from bacteria by absorption on glass beads.⁷⁴ The absorbed extract retained activity for what was then a remarkable full day. Wood and co-workers, using isocyanate-based polyurethane foams (Hypol®), found that a number of enzymes unrelated to OP hydrolysis could be covalently bound to this polymer.⁷⁵ Later, Havens and Rase immobilized a parathion hydrolase.⁷⁶ Furthermore, Turner observed that polyurethane foams are excellent adsorption materials for OP such as pesticide vapors.⁷⁷

As described above (see Section VII.A), soluble ChE and oxime together detoxify OP compounds. These features were combined to develop a sponge product composed of ChE (FBS-AChE and EqBChE), organophosphate hydrolase (rabbit or bacterial OPH), oxime (2-PAM or HI-6), and polyurethane foam combinations for the removal and decontamination of OP compounds from medically important biological surfaces such as skin.⁷⁸ This is an important extension of the bioscavenger approach to external decontamination and protection against organophosphate toxicity, since currently

accepted methods for decontamination of personnel and materials use bleach, which is caustic and harmful and also poses a significant environmental burden. Additionally, the ChE-sponge has unique attributes, making it a biosensor for OP for use in any environmental condition, such as vapor, water, soil, and long-term remote sensing.

ChE, OPH, and other enzymes may be immobilized with a Hypol[®] toluene diisocyanate polyurethane prepolymer creating the enzyme sponges in less than 20 min at ambient temperature in any desired size or shape. Since the enzymes predominantly attach (covalently) at surface lysines to the inert foam at multiple points during the polymerization process, they become an integral part and acquire the structural integrity of the resultant polymerized matrix. This is clearly evident in the enhanced mechanical and chemical stability of immobilized AChE and BChE; they were stable at room temperature for more than two years without any special treatment. The enzymes remained covalently attached to the polymer even after 20 washes over many days; did not wash off; and were very resistant to environmental assaults such as salt water, proteolytic degradation, or saturated organic fumes. Due to the large capacity of the prepolymer for protein, high activity sponges can be synthesized from purified ChE, substantially increasing their efficacy. Multiple OP-hydrolyzing enzymes can be co-immobilized on one sponge, including phosphotriesterases (paraoxonase or OP hydrolases) and/or cholinesterases. The advantage of including OP hydrolases in the multi-enzyme component is that they detoxify all phosphorylated oxime intermediates with little substrate specificity.

Since the enzyme is likely attached to the polymer at multiple points and therefore becomes partially distorted, it is not unexpected that the K_m values for the immobilized ChE and OPH were about 10-fold greater than for the corresponding soluble enzyme, but the combined effects on affinity for substrate and k_{cat} resulted in approximately a 20- to 50-fold decrease in acylation (k_{cat}/K_m). Yet there was no observed shift in the pH profile of the enzymes, and, more important, the bimolecular rate constants for the inhibition of AChE-sponge and BChE-sponge and the soluble enzymes by MEPQ showed no significant difference between soluble and covalently bound enzymes. Therefore, the OP interacts similarly with soluble and immobilized ChE.

OP such as diisopropylfluorophosphate or MEPQ inhibited the activity of ChE-sponges, and the oxime HI-6 restored activity of the AChE-sponge until the molar concentration of MEPQ reached approximately 1000 times that of the cholinesterase active site, demonstrating that the bioscavenger approach works externally as well as *in vivo*. In addition, the AChE-sponge could be recycled many times by rinsing the sponge with HI-6 in the absence of OP. In this case, most of the original cholinesterase activity could then be restored to the sponge for another cycle of detoxification of OP. The ability of the immobilized enzyme-sponge and HI-6 to detoxify the MEPQ was dependent upon the efficiency of the sponge to decontaminate particular surfaces. The sponge alone could decontaminate MEPQ from non-porous plastic and steel surfaces (>97%), and an AChE-sponge with HI-6 detoxified the removed MEPQ. However, the sponge alone (without enzyme) was not more effective than the M291 decontamination kit for removing neat soman applied to a guinea pig (shaved skin). To improve the removal/extraction of OP from skin surfaces, additives were incorporated into the polyurethane matrix both during synthesis and postsynthesis. Liquid additives to the sponges possessing partial organic

solubilizing characteristics such as tetraglyme and also oximes such as 2-PAM and HI-6 were particularly effective in protecting guinea pigs from soman exposure, yielding protective ratios about 4-fold (LD_{50} 80 mg/kg) better when compared to the M291 kit (LD_{50} 20mg/kg). Sponges synthesized with activated carbon incorporated into the polymer matrix, a process that did not interfere with the immobilization of ChE, were also useful at removing soman from skin, and might be effective in removing other toxic agents such as vesicants. The sponges should be suitable for a variety of biological surface detoxification and decontamination schemes for both chemical weapons, and for civilians and first-responders exposed to pesticides or highly toxic OP such as sarin or soman.

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