
7 Nerve Agent Bioscavengers: Protection against High- and Low- Dose Organophosphorus Exposure*

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I. INTRODUCTION

Organophosphorous anticholinesterases (OPs), usually acid anhydride derivatives of phosphoric acid, are among the most toxic substances identified.¹ Originally, OP were developed for use as insecticides,² but their extreme toxicity toward higher vertebrates has led to their adoption as weapons of warfare.³ The OPs most com-

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monly utilized as chemical weapons (referred to as nerve agents) are anhydrides of hydrocyanic acid, hydrofluoric acid, or of a derivative of thiocholine: tabun (GA), sarin (GB), soman (GD), cyclohexylmethyl phosphonofluoridate (GF), and ethyl-S-diisopropylaminoethyl methylphosphonothioate (VX). Their molecular weights range from 140 to 267 Daltons (Da) and, under standard conditions, they are all liquids that differ in their degrees of volatility.⁴ They have median lethal dose (LD₅₀) values in mammals, including estimates for humans, in the $\mu\text{g/kg}$ dose range for all routes of exposure except dermal, where LD₅₀ doses are in the mg/kg range.³

OPs produce their acute toxic effects by irreversibly inhibiting the enzyme acetylcholinesterase (AChE, E.C. 3.1.1.7).^{5,6} This inhibition leads to an increase in the concentration of acetylcholine in the cholinergic synapses of both the peripheral and central nervous systems. The physiological consequences of elevated acetylcholine include alterations in the function of the respiratory center and over-stimulation at neuromuscular junctions.⁷⁻¹³ A sufficiently high level of acetylcholine or a sufficiently rapid increase in acetylcholine concentration precipitates a cholinergic crisis, resulting in dimming of vision, headache, shortness of breath, muscle weakness, and seizure. In the extreme, organophosphorus intoxication can be a life-threatening event, with death usually resulting from respiratory failure. This is often accompanied by secondary cardiovascular components including hypotension, cardiac slowing, and arrhythmias.⁶ The toxic effects ensuing from low-level exposure have not been well defined and are still the subject of some debate. To provide some context for the ensuing discussion of biological scavengers, the following definitions, suggested by the Gulf War Research Coordination Board, will be adopted to define low-level exposure:

- Level 1: An exposure that results in no clinical signs (and for humans no subjective symptoms) and minimal AChE inhibition (0–20% reduction in red blood cell [RBC] AChE).
- Level 2: An exposure that results in no clinical signs (and for humans no subjective symptoms) and moderate AChE inhibition (>20% reduction in RBC AChE).
- Level 3: An exposure that results in mild clinical signs, such as salivation, miosis, and tachycardia. In humans such an exposure would also be expected to cause symptoms such as shortness of breath.

These low-level exposure definitions refer to the effects observed in a single exposure of less than a 24-h duration.¹⁴ While a level of AChE inhibition is not mentioned for Level 3 exposures, the symptoms described can be considered cholinergic in nature, probably resulting from inhibition of synaptic AChE. This suggests that a prophylactic approach based on the reduction of the concentration of OP toxicant in the blood before it can reach its site of action (synaptic endplates) should be particularly effective; potentially incapacitating or even toxic exposures could be mitigated to Level 3-type outcomes, and lower level exposures could be rendered inconsequential.

II. CURRENT THERAPY FOR NERVE AGENT EXPOSURE

The conventional approach to treatment of OP intoxication involves efforts to counteract the effects of AChE inhibition. Cholinolytic drugs such as atropine are administered at the onset of signs of OP intoxication to antagonize the effects of the elevated acetylcholine levels that result from the inhibition of AChE.¹⁵ Additionally, an oxime nucleophile is given, which reacts with the inhibited (phosphonylated) enzyme to displace the phosphonyl group and restore normal activity.¹⁶ In the United States, the oxime of choice for treatment of nerve agent poisoning is the chloride salt of 2-PAM, usually referred to as 2-PAM Cl, although bis-pyridinium oximes may be more effective depending on the particular OP agent.¹⁷ Anticonvulsant drugs such as diazepam are also administered to control OP-induced tremors and convulsions. In conjunction with therapy, individuals at high risk for exposure to nerve agents are pretreated with a spontaneously reactivating AChE inhibitor such as pyridostigmine, which temporarily masks the active site of a fraction of AChE molecules and thus protects the enzyme from irreversible inhibition by the OP agent.¹⁸

While these treatment regimens have been the standard for many years, they are not ideal and suffer from a number of disadvantages. The major drawback of current approaches is that, while they can be effective in preventing lethality, they do not prevent performance deficits, behavioral incapacitation, loss of consciousness, or permanent brain damage, all of which can result from acute OP toxicity.¹⁹

Several nerve agents, including GF, sarin and, in particular, soman, present an additional therapeutic challenge in that after they inhibit AChE, they undergo a second reaction in which the phosphonyl group attached to the inhibited enzyme is dealkylated. This process, known as aging, results in a phosphonylated AChE that is refractory to either spontaneous or oxime-mediated reactivation.²⁰ The ineffectiveness of therapeutically administered oxime as a treatment for some nerve agents explains the continued research efforts aimed at alternative approaches to protection.²¹ In particular, efforts have focused on approaches that prevent the critical enzyme AChE from becoming inhibited in the first place. Although the currently used pretreatment/therapy regimen is able to protect soldiers against the otherwise lethal effects of nerve agents, it does not adequately protect against the incapacitation that results from high levels of nerve agent exposure. Furthermore, it appears that greater than marginal improvement of these pharmacological approaches will not be possible, because stronger drugs or higher doses are likely to produce unacceptable performance decrements by themselves.^{21,22}

With respect to low-dose exposures, there are no standardized treatment regimens. Indeed, due to the subtlety of the symptoms and the difficulty in detecting decrements in cholinesterase activity associated with Level 1, 2, or 3 exposures, many such exposures may go unnoticed and unreported. Individuals at risk for low-dose exposures (such as laboratory researchers working with OPs, chemical plant staff, and farmers using pesticides) can be routinely monitored for red blood cell (RBC) AChE activity; should lowered AChE activity be detected, responses include removal of the individual from the work environment, closer monitoring of RBC AChE levels, and

reassessment of procedures and practices to reduce the chance of subsequent exposures. The long-term consequences of low-dose exposure to OP, if any exist, remain unknown.

III. NERVE AGENT BIOSCAVENGERS: AN ALTERNATIVE TO CONVENTIONAL APPROACHES

While successful, current treatments for acute nerve agent poisoning always result in the victim suffering a toxic insult that subsequently must be therapeutically managed. In contrast, recent efforts have focused on identifying proteins that can act as biological scavengers of organophosphorus compounds and can remain stable in circulation for long periods of time. The concept of using a protein that can react with a nerve agent, either stoichiometrically or catalytically, to protect against the toxic effects of those compounds, either acute or low level, is not new. As early as 1956 it was shown that injection of exogenous paraoxonase could protect rats against several times the LD_{50} of paraoxon.²³ This approach avoids the side effects associated with current antidotes and the requirement for their rapid administration, by prophylactically inactivating (through sequestration or hydrolysis) anticholinesterase agents before they can react with the target AChE.^{21,24-31} The time frame for this inactivation to occur before endogenous AChE is affected is quite narrow (estimated to be approximately 2 min in humans), so especially for situations involving acute exposure, the scavenger function must be very rapid, irreversible, and specific.³² Ideally, the scavenger would enjoy a long residence time in the bloodstream, would be biologically innocuous in the absence of nerve agent, and would not present an antigenic challenge to the immune system. For these reasons, prime efforts to identify candidate bioscavengers have focused on enzymes of mammalian (usually human) origin.

Candidate bioscavenger proteins, in general, function either by stoichiometrically binding and sequestering the anticholinesterase or by catalytically cleaving the OP substrate into biologically inert products. In the former category are naturally occurring human proteins that bind nerve agents, including enzymes such as cholinesterases (ChE) and carboxylesterases (CaE), as well as antibodies specific for nerve agent haptens. Each of these stoichiometric scavengers has the capacity to bind one or two molecules of nerve agent per molecule of protein scavenger. While this approach has been proven to be effective in laboratory animals, it has the disadvantage that the extent of protection is directly proportional to the concentration of unexposed, active scavenger in the bloodstream at the time of nerve agent exposure. Since the molecular weight of a protein scavenger is in the range of 80,000 Da and the molecular weight of the nerve agents is about 160 Da, the concentration mass ratio of scavenger to nerve agent is 500:1. Thus, a high concentration of scavenger protein in circulation is necessary to protect against exposure to multiples of an LD_{50} dose of nerve agent, although lower concentrations would be sufficient to prevent inactivation of synaptic AChE after a low-dose exposure. It might be possible to mitigate the need for large amounts of scavenger by also administering, either prophylactically or immediately post-exposure, a currently fielded oxime. Oxime treatment would allow

for the continual reactivation of the bioscavenger *in vivo*, in effect converting the stoichiometric scavenger into a pseudo-catalytic one.

Candidate enzymes with bona fide catalytic activity against nerve agents include the human organophosphorus acid anhydride hydrolases (OPAH), such as paraoxonase (hu-Pon). Additionally, the ability to generate catalytic antibodies in response to appropriate transition state analogs suggests that nerve agent-specific antibodies that catalyze hydrolysis of their ligands could be effective bioscavengers.^{33,34} Finally, the ability to engineer site-specific amino acid mutations into naturally occurring scavenger enzymes can allow investigators to alter the binding and/or catalytic activities of these enzymes. In general, the use of scavengers with catalytic activity would be advantageous because small amounts of enzyme, meaning lower concentrations in circulation, would be sufficient to detoxify both large amounts of nerve agent (as in an acute exposure) or lower amounts of agent associated with low-dose exposure Levels 1, 2, and 3.

By nearly all criteria, the use of biological scavengers, either stoichiometric or catalytic, as a prophylactic approach to providing protection against an exposure to either a low-level or a lethal dose of a nerve agent offers numerous advantages over conventional treatments. In fact, the half-time for reaction of a nerve agent with a biological scavenger can be calculated using some very conservative assumptions. Based on toxicity estimates in humans, the expected concentration of a nerve agent in the blood at an LD₅₀ dose would be about 8×10^{-7} M.³⁵ The bimolecular rate constant for reaction of soman with AChE is $\sim 9 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$.^{36,37} If a scavenger were present in blood at a concentration of 1 mg/mL (1×10^{-5} M), then the rate constant for reaction of scavenger with toxicant would be pseudo first order and the $t_{1/2}$ for the reduction of toxicant would be $\sim 3 \times 10^{-4}$ min. Under those conditions, which assume perfect mixing and that all of the scavenger and all of the toxicant remain in the bloodstream, the concentration of toxicant would be reduced to 1/1000 of its initial concentration within 10 half-times (2×10^{-3} min). In practical terms, the inhibition of AChE by nerve agent essentially would be 0 under Levels 1, 2, and 3 exposure definitions given above. Where actual measurements have been made of the rate of reduction of concentration of soman in animals (guinea pigs), it was found that, in the absence of an exogenous scavenger, the concentration of a $2 \times \text{LD}_{50}$ dose of soman in circulation was reduced by 1000-fold in about 1.5 min.³⁸ These results support our contention that, if a bioscavenger were present in circulation at the time of exposure, the reduction in toxicant concentration to a physiologically insignificant level (with no measurable inhibition of AChE) would be very rapid, and would certainly occur in less than one circulation time at any concentration of OP that could produce a low-level effect in an untreated animal. The need to administer, repetitively, a host of pharmacologically active drugs with a short duration of action at a precise time following exposure is all but eliminated if a scavenger is used. The potential for having to use mission-oriented protective posture (MOPP) gear is greatly reduced. Finally, with the appropriate scavenger(s), such an approach could afford protection against all of the current threat agents, including those that induce rapid aging of AChE and are refractory to treatment by the current atropine and oxime treatment regime.

IV. STOICHIOMETRIC SCAVENGERS AND THE PROTECTION THEY OFFER

A. ANTIBODIES

More than 25 years ago efforts were undertaken to protect animals by actively immunizing them with analogs of paraoxon or soman attached to appropriate protein carrier molecules, to elicit an antibody response against these two highly toxic organophosphorus compounds.^{39,40} As summarized in Table 7.1, rabbits that developed antibodies against paraoxon were protected against 2 to 3 times the LD₅₀ of paraoxon.⁴¹ The extent of protection was found to be directly related to the concentration of the paraoxon-specific antibodies in circulation. Significantly, the protected animals were essentially asymptomatic and did not require the administration of any additional therapeutic drugs. Rabbits immunized with an analog of soman were not protected against the administration of a lethal dose of that compound. Subsequently it was determined that the polyclonal antibodies induced in these animals were not of sufficiently high affinity to successfully compete with AChE for the binding of soman.⁴⁰

Based on these limited but promising results, efforts were made to generate high-affinity monoclonal antibodies that could be used to afford passive protection from nerve agents. Hunter et al.⁴² reported the production of the first anti-soman monoclonal antibodies, which were subsequently shown to be of sufficiently high affinity to compete with AChE for soman binding *in vitro*.⁴⁰ When mice were passively immunized with these antibodies they failed to show any protection against the *in vivo* toxicity of soman, although the time to death was almost doubled in the animals pretreated with antibody.⁴⁰ Further *in vitro* characterization of the monoclonal antibodies showed that their anti-soman binding constants were only in the micro-molar range, but that they were highly soman-specific, in that they did not bind the structurally related nerve agent sarin.⁴³ Subsequent calculations suggest that to afford protection on a stoichiometric level against soman or sarin, a monoclonal antibody must have a binding constant in the 50 nano-molar range.³⁵

B. ENZYMES

A number of different enzymes that react with OPs but do not catalyze their hydrolysis have been tested for their ability to provide protection against nerve agent poisoning. Wolfe et al.⁴⁴ first reported the use of exogenously administered AChE as a bioscavenger (Table 7.2). In that study, fetal bovine serum acetylcholinesterase (FBS-AChE) was administered to mice 20 h before a multiple LD₅₀ challenge of VX was administered. Complete protection was afforded against a $2 \times \text{LD}_{50}$ dose of VX (100% survival of exposed animals), while moderate protection (80% survival rate) was observed after a challenge of $3 \times \text{LD}_{50}$. No protection was observed against higher multiple LD₅₀ challenges of VX. When animals pretreated with FBS-AChE were exposed to soman, little protection was afforded. However, FBS-AChE pretreatment in conjunction with post-exposure atropine and 2-PAM treatment protected mice from $2 \times \text{LD}_{50}$ of soman. The authors reported that animals displayed no detectable side effects in response to administration of FBS-AChE.

TABLE 7.1
Protection from Organophosphorus Intoxication by Antibody Bioscavengers

Bioscavenger	Test Species	Nerve Agent	Protection (LD ₅₀) ^a	Serum T _{1/2} ^b	Ref.
Polyclonal Antibodies ^c	Rabbit	Paraoxon	2–3	Days to Weeks	41
Polyclonal Antibodies	Rabbit	GD	-	Days to Weeks	41
Monoclonal Antibody ^c	Mouse	GD	—/extended mean survival time	(6–8 days)	40, 77

^aValues represent multiples of median lethal doses (LD₅₀) of nerve agent survived after antibody administration.

^bHalf-life of antibodies in blood circulation.

^cPolyclonal antibodies: the endogenous serum titer after priming with nerve agent analogs. Monoclonal antibody: produced *in vitro* by a hybridoma, then passively administered to naïve mice.

TABLE 7.2
Protection from Organophosphorus Intoxication by the Bioscavenger FBS-AChE

Bioscavenger	Test Species	Nerve Agent	Protection (LD ₅₀) ^a	Serum T _{1/2} ^b	Ref.
FBS-AChE	Rhesus Monkey	GD	2–5	30–40 h	45, 48
FBS-AChE	Mouse	GD	2 (w/ Atropine + 2-PAM)	40–50 h	44
FBS-AChE	Mouse	GD	2 (after CBDP treatment)	~24 h	61
FBS-AChE	Mouse	GD	2–8	24–26 h	46, 50, 62
FBS-AChE	Mouse	MEPQ	4	~24 h	50, 61
FBS-AChE	Mouse	VX	2–3.6	~24–50 h	44, 50, 61

^aValues represent multiples of median lethal doses (LD₅₀) of nerve agent survived after FBS-AChE administration.

^bHalf-life of administered FBS-AChE in blood circulation.

Maxwell and co-workers carried out a similar set of experiments using rhesus monkeys pretreated with the scavenger FBS-AChE.⁴⁵ When monkeys pretreated with FBS-AChE were challenged with either 1.5 or $2.5 \times \text{LD}_{50}$ of soman, there was protection (Table 7.2) with no decrements in performance on the serial probe recognition (SPR, discussed in Behavioral Effects, below) task as compared with animals treated with FBS-AChE alone. The animals were also monitored for the generation of an antibody response against the administered FBS-AChE, but none was detected. The authors caution, however, that whenever a foreign protein is administered to an animal, the potential for an antibody-mediated immune response must be assessed on a case-by-case basis. Maxwell and co-workers also compared the relative protection against soman afforded to mice by three different treatments: pyridostigmine pretreatment with atropine therapy post-exposure, post-exposure oxime (HI-6) and atropine therapy, or FBS-AChE pretreatment alone.⁴⁶ The authors concluded that the FBS-AChE pretreatment offered superior protection against both soman toxicity (survival after 8 to $10 \times \text{LD}_{50}$ doses) and behavioral incapacitation. The results of these and other studies using FBS-AChE are summarized in Table 7.2.

Broomfield and co-workers reported that equine butyrylcholinesterase (EqBChE) afforded complete protection against a $2 \times \text{LD}_{50}$ challenge dose of soman in rhesus monkeys (Table 7.3) with no supporting therapy and against 3 to $4 \times \text{LD}_{50}$ doses when atropine was also administered (post-exposure).⁴⁷ Protection against a single LD_{50} dose of sarin was also demonstrated. In all cases (Table 7.3) there were no fatalities. Furthermore, when animals were assessed for behavioral deficits again using an SPR task, they all returned to baseline performance within 9 h after soman exposure (*vide infra*).²²

In a related study, Wolfe et al. assessed the ability of pretreatment with either FBS-AChE or EqBChE to protect rhesus monkeys against multiple LD_{50} doses of soman (Tables 7.2 and 7.3).⁴⁸ Survival and the ability to perform a different behavioral test, the Primate Equilibrium Platform (PEP) task, were the variables assessed. Those animals that received FBS-AChE as a pretreatment were protected against a cumulative exposure of $5 \times \text{LD}_{50}$ of soman and showed no decrement in the PEP task. Two of the four monkeys that received purified EqBChE did show some transient decrement in PEP task performance when the cumulative dose of soman exceeded $4 \times \text{LD}_{50}$. All of the experimental animals were observed for an additional 6 weeks, and none displayed any residual or delayed performance decrements suggesting no residual adverse effects. These results were reviewed and expanded upon by Doctor et al., wherein mice pretreated with FBS-AChE were also administered the oxime HI-6 immediately post-exposure to sarin.⁴⁹ In theory, the oxime will continuously regenerate the inhibited scavenger enzyme *in vivo*; this approach is predicted to increase the amount of sarin that could be scavenged by a given amount of AChE, making this stoichiometric scavenger pseudo-catalytic. The therapeutic addition of HI-6 after pretreatment with FBS-AChE was found to enhance the efficacy of the scavenger enzyme against sarin *in vivo*, increasing the ratio of neutralized OP compound per FBS-AChE molecule from $1:1$ (in the presence of AChE alone) to roughly $65:1$.

Maxwell et al. identified carboxylesterase as another enzyme with the potential to be a good anti-organophosphorous scavenger molecule (summarized in Table 7.4).⁵⁰

TABLE 7.3
Protection from Organophosphorus Intoxication by the Bioscavenger EqBuChE

Bioscavenger	Test Species	Nerve Agent	Protection (LD ₅₀) ^a	Serum T _{1/2} ^b	Ref.
EqBChE	Rhesus Monkey	GB	1	620 h	47
EqBChE	Rhesus Monkey	GD	2 (4 w/ atropine)	620 h	47
EqBChE	Rhesus Monkey	GD	5	30–40 h	48

^aValues represent multiples of median lethal doses (LD₅₀) of nerve agent survived after EqBChE administration.

^bHalf-life of administered EqBChE in blood circulation.

TABLE 7.4
Protection from Organophosphorus Intoxication by Endogenous Plasma CaE

Bioscavenger	Test Species	Nerve Agent	Protection (LD ₅₀) ^a	Ref.
CaE ^b	Mouse	GD	16	56
CaE	Guinea Pig	GD	3.5	56
CaE	Rabbit	GD	3	56
CaE	Rat	GD	8–9	56, 82
CaE	Rat	GB	8	84
CaE	Rat	GA	4–5	84
CaE	Rat	VX	1	84
CaE	Rat	Paraoxon	2	84

^aValues represent multiples of median lethal doses (LD₅₀) of nerve agent survived due to the presence of CaE. Because CaE is an endogenous plasma protein in these species, the protection it offers was measured by comparing LD₅₀ values in untreated and CBDP-treated animals; 2 mg/kg CBDP completely abolishes endogenous plasma CaE activity.⁸⁴

^bFor each species, the activity of the host's endogenous CaE was tested.

While AChE and BChE were found to be more efficient scavengers for soman in mice than CaE (i.e., they have higher bimolecular rate constants), the latter enzyme was capable of affording equal protection on a molar basis. Carboxylesterases (CaE; EC 3.1.1.1) catalyze the hydrolysis of a wide variety of aliphatic and aromatic esters and amides.⁵¹ As with AChE, catalysis occurs by a two-step process in which the substrate acylates the active site serine of CaE, which subsequently deacylates by the addition of water.⁵² CaE can be distinguished from AChE and BChE by the fact that AChE and BChE react with positively charged carboxylesters, such as acetylcholine and butyrylcholine, and are readily inhibited by carbamates, while CaE does not react with positively charged substrates and is inhibited by carbamates only at high concentrations.⁵² These differences in substrate specificity also extend to the reaction of CaE with OP compounds. Positively charged OP compounds, such as VX, react poorly with CaE while neutral OP compounds, such as soman, sarin, and paraoxon, react rapidly. Dephosphorylation of the active-site phosphorylated serine of CaE is a slow process compared to deacylation,⁵³ and therefore CaE has usually been considered to be a stoichiometric detoxification mechanism for OP compounds.

CaE is 60-kDa enzyme that is found in many mammalian tissues—lung, liver, kidney, brain, intestine, muscle, and gonads—usually as a microsomal enzyme. In some species CaE is also found in high concentration in plasma; plasma CaE is probably synthesized in the liver and secreted into the circulation via the Golgi apparatus.⁵⁴ Secretion of CaE appears to be controlled by the presence or absence of a retention signal at the carboxy terminal of the enzyme (Figure 7.1). CaE that is retained in the liver has a highly conserved carboxy terminal tetrapeptide sequence (HXEL in single-letter amino acid code, where X represents any amino acid), while the secretory form of CaE has a disrupted version of this retention signal in which the terminal leucine residue is replaced by either histidine-lysine or histidine-threonine.⁵⁴ Mammalian species that have high levels of secretory CaE in their plasma require much larger doses of OP compounds to produce toxicity than species with low levels of plasma CaE.⁵⁰ For example, the LD₅₀ dose for soman in rats is 10-fold larger than the LD₅₀ in non-human primates, which correlates with the differences in the plasma concentrations of CaE found in these species (Figure 7.2). Although human CaE has been cloned and expressed,⁵⁵ there is no commercial source of highly purified CaE for use in *in vivo* testing of protective efficacy. Therefore, the primary evidence demonstrating the effectiveness of CaE as a stoichiometric scavenger against OP, especially sarin and soman, has been by comparison of OP LD₅₀ in animals with high endogenous plasma levels of CaE to OP LD₅₀ levels in animals of the same species whose plasma CaE has been chemically inhibited.⁵⁶ For example, inhibition of plasma CaE prior to the LD₅₀ determination of soman in rats reduces its LD₅₀ by approximately 8-fold (Table 7.4), strongly suggesting that circulating CaE is an effective bioscavenger against OP compounds.

Recent investigations of the reactivation of OP-inhibited CaE have suggested that it may be possible to increase its potential as an OP scavenger by exploiting its turnover of OP compounds. Maxwell et al. observed that OP-inhibited CaE does not undergo the aging process that prevents oxime reactivation of OP-inhibited cholinesterases,⁵⁷ while Jokanovic et al. found that OP-inhibited CaE from plasma

Biochemical Basis for CaE Cellular Trafficking

Enzyme	COO-HTerminal Residues	Reference
<i>Intracellular CaEs</i>		
Rabbit Es-1	..TE H IEL	[88]
Rabbit Es-2	..QK H TEL	[89]
Hamster AT51p	..GK H SEL	[90]
Human CaE-1	..TE H SEL	[91]
Human CaE-2	..ER H TEL	[92]
Pig CaE	..IK H AEL	[93]
Rat Es-10(pI6.1)	..WK H VEL	[94]
Rat Es-B	..PH H NEL	[95]
Mouse Es-X	..RE H VEL	[96]
Mouse Es-22	..TE H TEL	[97]
Consensus	.. H XL	
<i>Secreted CaEs</i>		
Mouse Es-1	..TE H TE H K	[98]
Rat Es-1	..TE H TE H T	[99-101]

FIGURE 7.1 The carboxy-terminal amino acid residues of carboxylesterase enzymes from disparate species are aligned to show the conserved “HXEL” motif found among intracellular enzymes (shown in bold letters), and the disrupted versions of this retention motif found in the mouse and rat secreted carboxylesterase isoenzymes (alterations to the motif shown in italics). The capacity of the carboxy-terminal “HXEL” motif to act as an endoplasmic reticulum retention signal has been directly demonstrated.¹⁰²

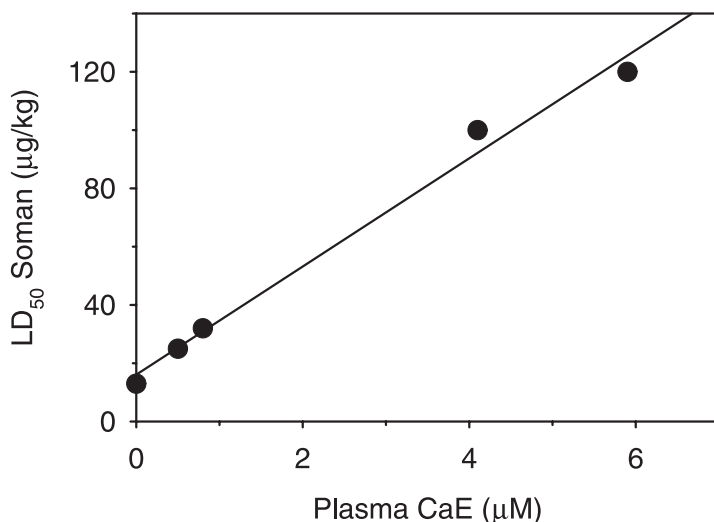


FIGURE 7.2 Effect of plasma CaE concentration on soman LD₅₀ (administered s.c.) in different species. Data points (from lower left to upper right of graph) for species were monkey, rabbit, guinea pig, rat, and mouse. Data taken from Maxwell et al.⁵⁰

underwent spontaneous reactivation with a half-time of 1 to 2 h.⁵⁸ Comparisons of the amino-acid sequences of CaE, AChE, and BChE are informative with regard to the critical amino acid residues required for occurrence of aging vs. spontaneous reactivation. Of the seven conserved amino acid residues that have been identified by theoretical studies and confirmed by site-directed mutagenesis to be important for aging of OP-inhibited AChE and BChE, only two are conserved in CaE.⁵⁹ Conversely, a highly conserved histidine found in CaE from six mammalian species and two insect species, but not in mammalian cholinesterases, correlates with the higher level of spontaneous reactivation of OP-inhibited CaE in comparison to OP-inhibited cholinesterase.⁵⁹ Interestingly, introduction of a histidine into BChE at a position nearly identical to the position of the conserved histidine of CaE produces spontaneous reactivation of OP-inhibited BChE.⁶⁰

A more detailed discussion of the relative merits of FBS-AChE, EqBChE, and plasma CaE as scavengers, which describes the extent of protection they offer against a variety of nerve agents, both *in vitro* and *in vivo* in mice, was presented by Doctor et al.⁶¹ The authors note that some of the *in vivo* differences in sensitivity and protection seen may be due to variations in the circulatory pharmacodynamics of the different OP compounds, such that those inhibitors that distribute more slowly from circulation are more readily scavenged. This concept supports the feasibility of using scavengers to protect against low-level exposures of nerve agent. Raveh et al. have provided additional examples that agree with those conclusions.^{63,64} The extent of protection afforded by FBS-AChE against soman in marmosets and rhesus monkeys with respect to survival was determined and found to be the same in both species. Significantly, the stoichiometry of the protective dose of FBS-AChE scavenger to OP compound was experimentally determined to be one-to-one on a molar basis in both species of monkey, suggesting that a similar ratio will be maintained in other species, including man. Finally, none of the animals pretreated with scavenger displayed any adverse symptoms following a LD₁₀₀ challenge dose of soman.

Ultimately, the goal of research on scavenger molecules is to generate a means to protect humans from the toxic effects of nerve agents. In an effort to minimize any physiological, immunological, or psychological side effects of scavenger use in humans, research efforts have begun to focus on the use of human BChE (HuBChE), human CaE, and/or FBS-AChE (which does not induce an immune response in rhesus monkeys).⁴⁵ In a series of studies, Ashani and his co-workers examined the scavenger properties of FBS-AChE and particularly HuBChE in mice, rats, and rhesus monkeys with respect to several different nerve agents as well as other OP compounds (Table 7.5).⁶²⁻⁶⁴ They found that following administration of exogenous cholinesterase, there was a linear correlation between the concentration of cholinesterase in the blood and the level of protection against OP poisoning. Furthermore, the extent of protection granted to mice was sufficient to counteract multiple LD₅₀ doses of soman. When the protective effect of pretreatment with HuBChE was compared in mice and rats, it was found that in both species the same linear correlation existed between blood concentration of HuBChE and protection against soman, sarin, or VX (Table 7.5). They further noted that to be effective, a scavenger had to be present before exposure to the OP compound, because (as discussed above) the nerve agent had to be scavenged within

TABLE 5
Protection from Organophosphorus Intoxication by HuBChE Bioscavengers

Bioscavenger	Test Species	Nerve Agent	Protection (LD ₅₀) ^a	Serum T _{1/2} ^b	Ref.
HuBChE	Rhesus Monkey	GD	2	~30 h	64
HuBChE	Rhesus Monkey	VX	1.5	~30 h	64
HuBChE	Rat	GD	2–3	46 h	63
HuBChE	Rat	VX	2	46 h	63
HuBChE	Mouse	GD	2.1	21 h	64
HuBChE	Mouse	GB	1.6	21 h	64
HuBChE	Mouse	GA	1.8	21 h	64
HuBChE	Mouse	VX	4.9	21 h	64

^aValues represent multiples of median lethal doses (LD₅₀) of nerve agent survived after HuBChE administration.

^bHalf-life of administered HuBChE in blood circulation.

one blood circulation time period.⁶³ In the final paper in this series, the authors report similar protection results against a 3.3 LD₅₀ dose of soman or a 2.1 LD₅₀ dose of VX in rhesus monkeys.⁶⁴ They also report considerable protection against soman-induced behavioral deficits in a spatial discrimination task.

V. CATALYTIC BIOSCAVENGERS

While stoichiometric scavengers are able to afford good protection as long as they reside at high levels in the blood stream, they suffer the disadvantage that they are all molecules of high molecular weight (*vide supra*); a comparatively large quantity is required to neutralize a small amount of nerve agent. A catalytic scavenger, even having the same high molecular weight, could be administered in smaller quantities and could produce the same or greater degree of protection. It would also have the advantage of not being consumed in the process of detoxifying the nerve agent, so it would be available to protect against multiple exposures of either high or low dose. Some of these potential bioscavenger proteins along with parameters of their catalytic activities are summarized in Tables 7.6–7.8. As discussed above, in conjunction with an oxime such as HI-6, cholinesterases that have not undergone aging can be continually reactivated to function pseudo-catalytically, eliminating substantially more moles of OP compounds than would be predicted based on binding alone. Furthermore, some enzymes, such as the OPAH from *Pseudomonas diminuta*⁶⁵ or the hu-Pon,⁶⁶ have intrinsic catalytic anti-organophosphorus activity. The former enzyme has been shown to afford protection against soman lethality in mice and to protect against behavioral side effects (Table 7.6).⁶⁷ However, since this bacterially derived enzyme has no known mammalian homologues, it will likely be a potent initiator of immune responses and is therefore unlikely to be appropriate for use as a prophylactic scavenger in humans. Nonetheless, the *Pseudomonas diminuta* OPAH could be used as a one-time pretreatment either in addition to or in place of conventional therapy, since in the short term this enzyme is highly effective against GD, GB, and VX, and alone induces no known behavioral effects. The hu-Pon enzyme has been identified as having a similar potential for affording protection (Table 7.6), but without the complication of inducing an immune response (being an endogenous self-antigen in humans); this enzyme has not yet been tested for efficacy in a mammalian model system.⁶⁸

While the enzymes discussed above possess the desired catalytic activity, none of them is fast enough for use as a nerve agent pretreatment. Since the OP anticholinesterases have been in the environment for only a little over 50 years, it is not likely that any of the enzymes we identify as OPAH have as their primary function the destruction of OP. In fact, an OPAH from an *alteromonas* species has been identified as a prolidase, a dipeptidase that cleaves at a penultimate proline from the carboxyl end of a peptide.⁶⁹ Recently, hu-Pon was shown to be a homocysteine thiolactone hydrolase that can protect against protein N-Homocysteinylolation.⁷⁰ A functional catalytic scavenger must have a lower K_m (a measure of the strength of binding of a substrate to the enzyme) and a higher turnover number than has been found to date among these naturally occurring catalytic enzymes, since agent must be

TABLE 6
Kinetic Properties of Naturally Occurring Catalytic Bioscavengers

Bioscavenger	Source Species	Substrate Specificity	km (μM)	Vmax ($\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	Ref.
Phosphotriesterase	<i>P. Diminuta</i>	GD	36/500	15/7.3	67/85
Phosphotriesterase	<i>P. Diminuta</i>	GB	700	N.D. ^a	85
Phosphotriesterase	<i>P. Diminuta</i>	Paraoxon	50	3200	85
Phosphotriesterase	<i>P. Diminuta</i>	DFP	100	64	85
Bimolecular Rate Constant ($k_{\text{cat}}/\text{km} [\text{M}^{-1} \cdot (\text{min}^{-1})]$)					
Q191/R191 ^b					
hu-Pon	Human	GD	$2.8 \times 10^6/2.1 \times 10^6$		68
hu-Pon	Human	GB	$9.1 \times 10^5/6.8 \times 10^4$		68
hu-Pon	Human	DFP	$3.7 \times 10^4/\text{N.D.}$		68
hu-Pon	Human	Paraoxon	$6.8 \times 10^5/2.4 \times 10^6$		68

^aNot determined.

^bTwo naturally occurring allelic variants of hu-Pon (Q191 and R191) have been identified. The activity of each form is shown.

TABLE 7
Kinetic Properties of Catalytic Mutated HuBChE Bioscavengers

Bioscavenger	Substrate Specificity	Spontaneous Reactivation Rate Constant ($\times 10^3 \text{ min}^{-1}$)	Ref.
Wild type HuBChE	GB	<0.05	74
Wild type HuBChE	VX	<0.05	74
Wild type HuBChE	GD	<0.05	74
G117H HuBChE ^a	GB	5	74
G117H HuBChE	VX	7	74
G117H HuBChE	GD	<0.05	74
G117H E197Q HuBChE ^b	GB	62	74
G117H E197Q HuBChE	VX	78	74
G117H E197Q HuBChE	GD (P _S C _R) ^c	6	74
G117H E197Q HuBChE	GD (P _R C _R) ^c	6	74
G117H E197Q HuBChE	GD (P _S C _S) ^c	77	74
G117H E197Q HuBChE	GD (P _R C _S) ^c	128	74

Note: The rate-limiting step in the hydrolysis of organophosphate nerve agents by mutated HuBChEs is the enzyme reactivation step.⁷⁴

^aA version of HuBChE in which the glycine at amino acid residue 117 has been replaced by histidine.

^bA double mutant of HuBChE containing both histidine (rather than glycine) at amino acid residue 117 and glutamine in place of glutamic acid at residue 197.

^cThe reactivity with each of the four stereoisomers of GD was determined independently.

TABLE 8
Kinetic Properties of Mouse-Derived Catalytic Antibody Bioscavengers

Bioscavenger	Substrate Specificity	km (μM)	Vmax ($\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	Ref.
Antibody IIA12-ID10	GD, others?	330	25	33
Antibody DB-108Q	GD, others?	110	16	75
Antibody DB-108P	GD, others?	100	53	75

cleared from the bloodstream within the 1 to 2 min before it reaches critical targets.³² Therefore, it was decided to attempt to create such an enzyme by specific mutation of existing human enzymes. Obvious candidates for such attempts include members of the cholinesterase family (including carboxylesterase) and the paraoxonases, which already possess the desired activity but at insufficient levels. The rationale for the design of mutations in the cholinesterase family was based on the fact that for these enzymes, the OP inhibitors are in reality hemisubstrates; their initial reaction with enzyme is similar to that of normal substrates. However, the subsequent reaction, equivalent to deacylation of the active site serine, is blocked because of the geometry of the active site. The amino acid group responsible for deacylation is not in an appropriate position to effect dephosphorylation.⁷¹

The perceived solution to this problem was to insert a second catalytic center into the active site specifically to carry out the dephosphorylation step of the reaction.⁷² Applying this rationale, the human form of BChE has been mutated (Figure 7.3) to

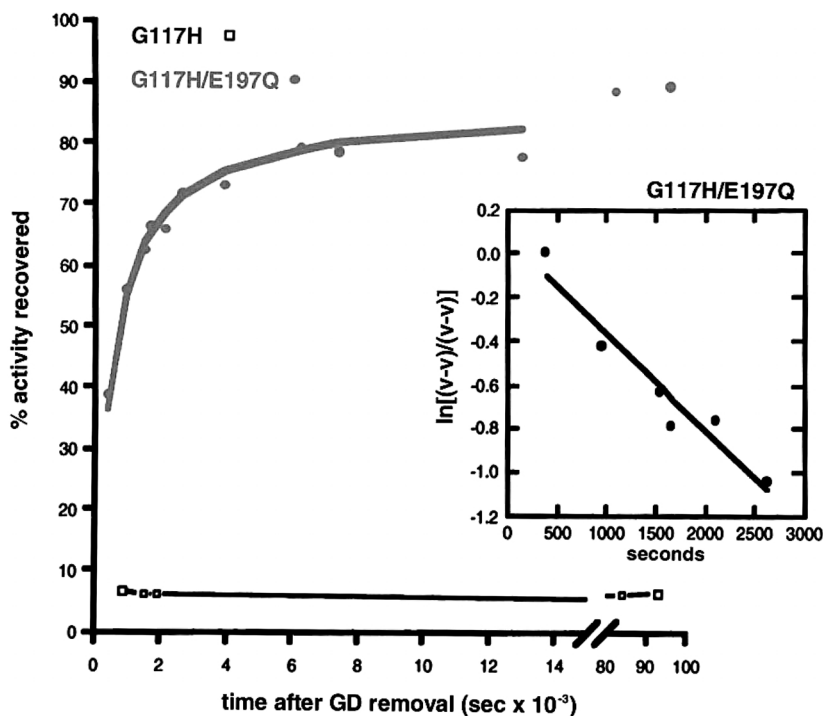


FIGURE 7.3 Comparative reactivation kinetics of soman-inhibited human butyrylcholinesterase single mutant G117H (\square) and double mutant G117H/E197Q (\bullet). Note that the recovery rate of the double mutant is very fast (with reaction rates of 77,000 and 128,000 per minute for the $P_S C_R$ and $P_S C_R$ isomers of soman, respectively), while the single mutant does not recover measurably. The insert shows that reactivation of the double mutant with soman can be treated as a first-order reaction for at least 2.5×10^3 s.

express an enzyme with the ability to catalyze the hydrolysis of sarin, DFP, paraoxon, VX, and other non-aging nerve agents.^{60,73} Aging and reactivation are parallel first-order reactions in phosphorylated enzymes. In the reactivation reaction the phosphoryl group is removed from the active site serine residue, restoring activity, whereas in the aging reaction one of the alkyl groups is removed from the phosphoryl group, rendering the group non-reactivable. To effect the hydrolysis of rapidly aging nerve agents such as soman, it is necessary to inhibit the aging reaction so that reactivation is faster. This was accomplished by replacing the carboxyl group (glutamic acid) adjacent to the active site serine with an amide (glutamine) (Figure 7.4).⁷⁴ Unfortunately, these mutants have catalytic activities that are too slow for practical use (Table 7.7), and thus the search for a faster enzyme continues. For example, human CaE and hu-Pon are currently being subjected to mutation in efforts to generate additional, faster catalytic anti-nerve agent enzymes. It is important to note that

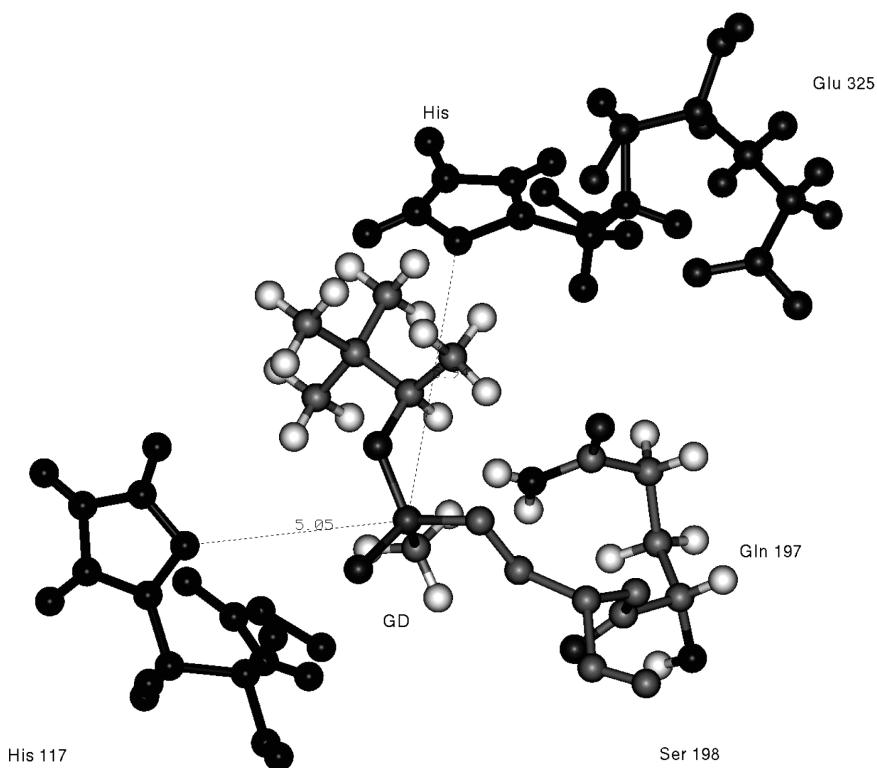


FIGURE 7.4 A ball-and-stick computer model of the active site of the double mutant of butyrylcholinesterase G117H/E197Q. In addition to the His 117 and Gln 197, the active site triad amino acid residues of His 438, Ser 198, and Glu 325 are also depicted with soman at the active site. The distances between the phosphorus atom of soman and His 117 is 5.05 Å and distance between the phosphorus atom of soman and the active site His 438 is 5.94 Å.

in the case of hu-Pon, the desired catalytic activity is present at low levels in the native enzyme; since OP are “accidental” substrates for hu-Pon (see above),⁷⁰ it is likely that improvement in activity can be realized through protein engineering.

Finally, through the careful design and synthesis of transition state analogs of the hydrolysis of soman, it has been possible to immunize mice and recover hybridomas whose antibodies display slow catalytic activity (Table 7.8) towards soman.^{33,75} Such catalytic antibodies could be “humanized” to reduce their immunologic antigenicity,⁷⁶ thereby prolonging their serum half-life into the range of days to weeks, as reported for other mammalian species.⁷⁷ While most of these catalytic enzymes and antibodies have not yet been tested in mammalian systems, they are indicative of the types of drugs that may soon be available for use in animals, including humans. Since mutated BChE, CaE, and hu-Pon are based on human proteins, and catalytic antibodies can be rendered predominantly human in structure, the expectation is that these proteins would have no immunological or behavioral side effects.

VI. BEHAVIORAL EFFECTS

Since overt signs, symptoms, or physiological responses may not accompany many low-level exposures, behavioral toxicological measures may be chosen to detect any toxic changes wrought. Under such conditions, it is important to ensure that biological scavengers, either elevated levels of naturally occurring proteins or mutagenized forms thereof, do not elicit behavioral effects of their own after administration. Other considerations are potential behavioral effects that might result after pretreatment with a biological scavenger followed by exposure to a nerve agent, as well as a comparison of the extent of behavioral side effects that ensue from pretreatment with scavenger followed by nerve agent exposure vs. exposure to nerve agent followed by conventional therapy. The discussion here will be limited only to the side effects, if any, resulting from administration of scavengers alone. The other topics, including the ability of scavengers to ameliorate behavioral side effects following nerve agent exposure and the advantages of scavengers vs. conventional therapy, are discussed in detail elsewhere.⁷⁸

VII. BEHAVIORAL EFFECTS OF SCAVENGERS ALONE

Most studies that have examined the behavioral effects of biological scavengers have done so by comparing a behavior before scavenger administration, after scavenger administration, and then after exposure to nerve agents.⁷⁸ There are, however, several studies that have examined the behavioral effects of the biological scavengers themselves in the absence of cholinesterase inhibitors. In a study by Genovese and Doctor, rats were trained to perform three behavioral paradigms: a passive avoidance task, a motor activity, and a scheduled-controlled behavior (Table 7.9).⁷⁹ The performance of animals before and after administration of purified EqBChE at a dose that would be expected to provide protection against an exposure of several LD₅₀ of an OP compound was assessed. They determined the pharmacokinetic profile of EqBChE in rats

TABLE 7.9
Extent of Behavioral Deficits following Bioscavenger Administration or Conventional Therapy

Protection	Species	Behavioral Test(s)	Impairment	Recovery Time	Ref.
Atropine	Rat	Passive Avoidance, VI56 s Schedule	Total	>1 Week	79
EqBChE	Rat	Passive Avoidance, Motor Activity, VI56 s Schedule	None	Immediate	79
HuBChE	Rat	Morris Water Maze	None	Immediate	81
Pyridostigmine	Rhesus Monkey	Primate Equilibrium Platform (PEP)	Substantial	N.D.	86
EqBChE	Rhesus Monkey	Serial Probe Recognition (SPR)	None	Immediate	80
EqBChE	Rhesus Monkey	Observation, SPR	Subtle SPR defect	~6 Days	87
EqBChE	Rhesus Monkey	Observation, SPR	None	Immediate	80
HuBChE	Rhesus Monkey	Spatial Discrimination	Minor (1/4 had errors)	>1 day	64

Note: Impairment, behavioral impairment relative to untreated animals; Recovery Time, time elapsed before performance returns to pretreatment levels; N.D., not determined.

and then examined the behavior of the animals in the passive avoidance task when the levels of administered EqBChE were maximal. Subsequently, the animals were tested after enzyme levels had started to diminish, to enhance the opportunity of detecting any behavioral effects. During the activity tests, individually housed animals were allowed to habituate. Enzyme was given such that maximum levels would be present in circulation about 1 h before the beginning of dark cycle. Motor activity was then monitored for 10 days. As a final test, the effects of excess enzyme were examined in rats trained to perform a VI56 s schedule of food reinforcement. Previously, cholinergic compounds had been shown to disrupt performance of this task. Animals were observed for 10 days to ensure that any prolonged or delayed effects would be noted. In all cases for all test paradigms, the authors report that EqBChE did not disrupt performance of any of the learned tasks, did not upset the circadian cycle of light/dark activity, and had no effect on motor activity. They noted that these outcomes were in contrast to those observed when the standard cholinolytic, atropine, was administered. Finally, they evaluated the protective effects of the levels of enzyme given to the rats in the behavioral studies against MEPQ, a peripherally active OP compound. While the level of protection observed was lower than the theoretical prediction, the authors suggested that the simultaneous administration of scavenger and MEPQ might have reduced the efficacy of the administered EqBChE.

In a separate study also using EqBChE, rhesus monkeys were trained to perform a SPR task.⁸⁰ Using a six-object list, the monkeys were tested for same-different discrimination and delayed same-different discrimination. Once the animals became proficient at the task (80% correct for three successive sessions on 3 consecutive days), they received EqBChE in a dose similar to that reported by Broomfield et al. as sufficient to afford protection against 2 or 3 multiples of an LD₅₀ soman challenge (*vide supra*).⁴⁷ The authors reported that in their study, repeated administration of commercially prepared EqBChE had no effect on the behavior of the monkeys as measured by the SPR studies (Table 7.9). Given the lack of behavioral effects and the relatively long *in vivo* half-life of the EqBChE, they concluded that this biological scavenger was potentially more effective than current chemotherapeutic treatments for OP intoxication. Other studies in rats or monkeys using human BChE also showed virtually no behavioral effects following administration of this enzyme.^{64,81}

VIII. SUMMARY

Organophosphorous nerve agents represent a very real threat not only to warfighters in the field but also to the public at large.⁸² Nerve agents have already been used by terrorist groups against a civilian population and, due to their low cost and relative ease of synthesis, are likely to be used again in the future.⁸³ In addition, many commonly used pesticides and chemical manufacturing by-products can act as anticholinesterases, and may be a low-dose exposure threat to workers in a variety of professions. Current therapeutic regimes for acute nerve agent exposure are generally effective at preventing fatalities if administered in an appropriate time frame. While the current therapeutic drugs, atropine and 2-PAM, have not been tested against a

low-level exposure, their requirement for timely administration following symptoms makes it unclear whether under low-level exposure conditions these therapeutic interventions could be effectively implemented on a large scale. For acute multi-LD₅₀ levels of exposure, pyridostigmine pretreatment coupled with post-exposure administration of an oxime, atropine, and an anti-convulsant does not prevent the substantial behavioral incapacitation or, in some cases, permanent brain damage that can result from OP poisoning. For low-level exposures that result in the Level 1 or Level 2 effects described above, the current therapy will probably not be administered at all since it is to be given at the onset of overt physiological signs. It is therefore important from both military and domestic security perspectives to develop novel defenses against nerve agents, including the use of bioscavenger molecules that avoid many of the difficulties associated with current treatments. While the use of nerve agents on the battlefield may be somewhat predictable, their use in a terrorist situation will be, in all probability, an unanticipatable event. The ability to afford long-term protection for first-responders exposed to toxic, incapacitating or Level 3 doses of OP, thereby reducing the severity of outcomes to Level 2 or Level 1 symptoms (and eliminating the impact of Level 1 and 2 exposures completely), is a notable potential advantage of biological scavengers.

The use of bioscavengers as a defense against OP intoxication has many advantages and few apparent disadvantages. As discussed in detail above, bioscavengers can afford protection against not only mortality, but also most or all of the adverse physiological and behavioral effects of nerve agent exposure. They can be administered prophylactically, precluding the need for immediate post-exposure treatment. In addition, the use of bioscavengers has several psychological benefits that are likely to result in a higher degree of user acceptability than exists for conventional therapy. No post-exposure auto-injectors are necessary, and protection is afforded with little chance of short- or long-term side effects. Of particular significance is the fact that current candidate bioscavenger proteins are, for the most part, enzymes of human origin. From a scientific standpoint, these proteins are good candidates because they are less likely to be recognized by cells of the immune system, and will enjoy prolonged residence times in circulation. From a user point of view, individuals are, in essence, being protected against nerve agents using a substance that their bodies already produce, rather than being injected with drugs and enzyme inhibitors that alone can produce potent side effects; such a distinction may enhance the comfort and compliance of end users.

There are several challenges that must be met in the future before bioscavengers can augment or replace the current therapeutic regimes for nerve agent intoxication. First, scavenger proteins, either alone or in combination, with a range of specificities that encompasses all known nerve agents, must be defined. The immunogenicity and serum half-life of the scavenger(s) must be determined in humans, and efforts may be required to minimize the former and maximize the latter. Finally, appropriate dosages of scavenger(s) must be determined that will, based on animal models, protect against concentrations of nerve agents likely to be encountered under a wide range of scenarios. While the majority of the research to date has focused on stoichiometric scavengers, the use of either naturally occurring or genetically engineered enzymes with

catalytic activity holds the greatest theoretical promise for the development of a broad specificity prophylactic scavenger. Future efforts are likely to focus on generating, characterizing, and utilizing such enzymes in rodent and non-human primate models.

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