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In vivo cholinesterase inhibitory specificity of organophosphorus nerve agents[☆]

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Abstract

The purpose of this project was to determine and compare the time-related changes in blood, brain, and tissue acetylcholinesterase (AChE) activity during the first hour after exposure to six organophosphorus nerve agents (GA, GB, GD, GF, VR, and VX) in Hartley guinea pigs. Animals were pretreated with atropine methyl nitrate (1.0 mg/kg, i.m.) to minimize peripheral toxic effects 15 min before they were given a $1.0 \times LD_{50}$ subcutaneous dose of a nerve agent. At 0, 5, 10, 15, 30, and 60 min after nerve agent, animals were humanely euthanized. Blood was collected and brain regions (brainstem, cortex, hippocampus, midbrain, cerebellum, striatum, and spinal cord) and peripheral tissues (diaphragm, skeletal muscle, and heart) were dissected and processed for AChE activity. All six nerve agents produced maximum inhibition of AChE in red blood cells between 5 and 10% of the control within 10 min after exposure. In whole blood, differential effects were observed among the agents: GB, GD, and GF produced more rapid and greater inhibition than did GA, VR, and VX. GF was the most rapid, producing a maximum inhibition to 5% of the control in 5 min, while VR and VX were slower reaching maximum inhibition to 30% of the control at 15 min. The enzyme activity in the majority of the brain regions was more markedly inhibited by the G-agents than by the V-agents. The G-agents caused rapid AChE inhibition, reaching maximum levels (20–30% of control) at 15 min and GA produced the most rapid effects. V-agents produced much slower and less AChE inhibition, reaching maximum (35–60% of control) at 30 min. In the diaphragm, VR, VX, and GD produced more rapid and greater AChE inhibition than other G-agents; GA produced the slowest and least inhibition. In the skeletal muscle, VX induced the most rapid and severe inhibition, while GA the least inhibition. In the heart, all agents produced very rapid inhibition, and GD produced the most severe inhibition of AChE activity. These observations suggest that G-agents and V-agents are tissue compartment specific in their ability to inhibit AChE activity.

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Keywords: Nerve agents; Organophosphorus compounds; Acetylcholinesterase inhibitors; Soman; Sarin; VX; Cholinesterase; Brain; Blood; Tissues

1. Introduction

The potential for exposure to organophosphorus (OP) nerve agents exists on the battlefield for military per-

sonnel and civilian populations (e.g., 1991 Persian Gulf War) and as a terrorist threat to civilian populations (e.g., 1995 Tokyo subway incident). These nerve agents are irreversible inhibitors of the cholinesterase (ChE) enzyme. Their lethal effects are due to hyperactivity of the cholinergic system as a result of inhibition of ChE, in particular, acetylcholinesterase (AChE), and the subsequent increase in the concentration of the cholinergic neurotransmitter acetylcholine (ACh) in the synapse [1].

Tabun (ethyl *N,N*-dimethyl phosphoramidocyanidate; GA), sarin (isopropyl methylphosphonofluoridate; GB),

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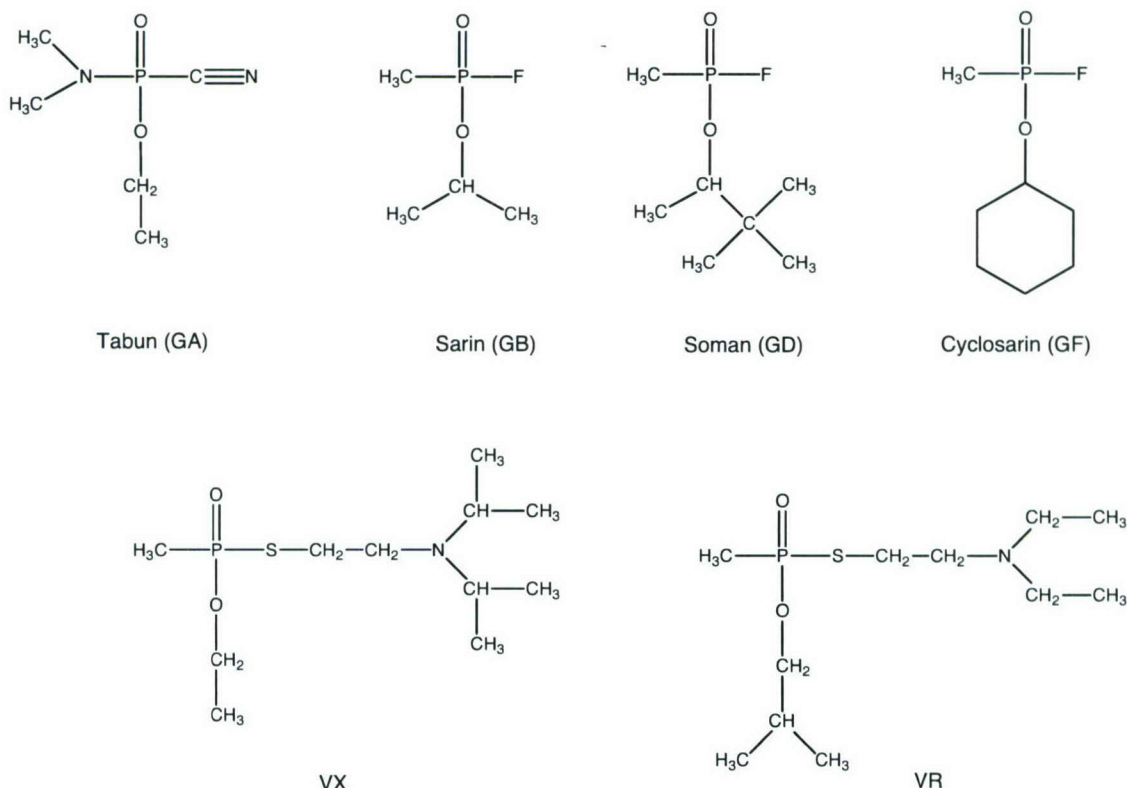


Fig. 1. Chemical structures of Tabun (GA), Sarin (GB), Soman (GD), Cyclosarin (GF), VX, and VR.

soman (pinacolyl methylphosphonofluoridate; GD), cyclosarin (cyclohexyl methyl phosphonofluoridate; GF), VR (*O*-isobutyl *S*-(2(diethylamino)ethyl) methylphosphonothioate) and VX (*O*-ethyl *S*-(2(diisopropyl amino)ethyl) methylphosphonothioate) are OP nerve agents [1]. Their structures are shown in Fig. 1. These military nerve agents are a family of highly toxic phosphoric acid anhydrides, and their structures are very similar to organophosphate pesticides. GA contains a cyanide group; GB, GD, and GF contain a fluoride substituent group. GB, GD, and GF are methylphosphonofluoridate anhydrides. GF contains a cyclohexyl substituent.

The G-type nerve agents are clear, colorless liquids that are volatile at room temperature. GB is the most volatile nerve agent and evaporates at about the same rate as water. In water, GB is very miscible and readily hydrolyzes [2]. VX is a methyl phosphonic acid thio ester. It is an amber-colored, oily liquid with low volatility unless temperatures are high. VX is the least volatile nerve agent. It does not evaporate readily and is nonvolatile when in water. VX is moderately to highly soluble in water. It is fairly resistant to hydrolysis [2]. VR is a Russian V-type agent that is also a phosphonic acid thio ester. It is oily and colorless when pure and has a low volatility. Both VR and VX are charged, whereas

the G-agents do not carry a charge. Both V-agents are more persistent *in vivo* than the G-agents [3].

Four major differences exist in metabolism between the G-agents and V-agents. First, V-agents circulate *in vivo* as a protonated amine. Second, V-agents are hydrolyzed much more slowly than G-agents and some of the hydrolysis products of V-agents are presumably toxic. Also, V-agents may be metabolized by routes that are not available for G-agents such as oxidation reactions at nitrogen and/or sulfur. Last, V-agents react more slowly with carboxylesterases and phosphor-ylphosphatases than do G-agents [3].

Exposure to these OP nerve agents causes a progression of toxic signs, including hypersecretions, fasciculations, tremor, convulsions, respiratory distress and death. A combined regimen of prophylaxis and therapy is the most effective medical countermeasure for dealing with the threat of nerve agent poisoning to military personnel [4,5]. Pretreatment with carbamate ChE inhibitors, such as pyridostigmine bromide, shields a fraction of ChE in the periphery from irreversible inhibition by the nerve agents. In the event of poisoning, immediate therapeutic treatment with an anticholinergic drug, such as atropine sulfate, antagonizes the effects of excess ACh at muscarinic receptor sites, and an oxime, such as

pyridine-2-aldoxime methylchloride (2-PAM), is used to reactivate any unaged, inhibited enzyme [6].

In the past, nerve agent studies have focused primarily on the effects and treatment of GD-induced seizures [7–10]. The ability of different nerve agents to produce seizures has only recently been systematically explored [6,8]. The currently fielded pretreatment (pyridostigmine bromide) and the therapeutic (atropine sulfate and 2-PAM) strategies for OP nerve agents provide reasonable protection and therapy against GD exposure [11,12]. However, more work is needed to support the goal of finding an improved broad-spectrum therapeutic regimen that focuses on efficacy against all nerve agents for our military personnel.

The purpose of this project was to assess the time-course changes in AChE activity in blood, brain, and peripheral tissues after a single toxic exposure to six different nerve agents (GA, GB, GD, GF, VR, and VX) and to compare the AChE inhibitory profiles among these six agents. Understanding the specificity of the various nerve agents' effects on AChE in different tissue compartments may aid in the development of a more effective therapeutic strategy that will work against all of the nerve agents.

2. Materials and methods

2.1. Subjects

Male Hartley guinea pigs (Crl:(HA) BR COBS; Charles River Labs, Kingston, NY, USA) weighing 250–300 g served as subjects. They were housed in individual cages in temperature ($21 \pm 2^\circ\text{C}$) and humidity ($50 \pm 10\%$) controlled quarters that were maintained on a 12-h light/12-h dark cycle (lights on at 06:00 h) and received food and water ad libitum except during experimental periods. In conducting the research described in this report, the investigators adhered to the Guide for the Care and Use of Laboratory Animals by the Institute of Laboratory Animal Resources, National Research Council, in accordance with the stipulation mandated for an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility.

2.2. Materials

Atropine methyl nitrate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Tris(hydroxymethyl) amino methane was purchased from Fischer Scientific (Fair Lawn, NJ, USA). The bicinchronic (BCA) protein reagent kit and DTNB (5,5'-dithiobis(2-nitrobenzoic acid) were purchased from Pierce Biotechnology (Rock-

ford, IL, USA). Heparin sodium was purchased from USP, Inc. (Rockville, MD, USA). GA, GB, GD, GF, VR, and VX were obtained from the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD, USA). DTNB was prepared in Tris buffer (0.05 M, pH 8.2) to a concentration of 0.424 M. Nerve agents were diluted in ice-cold saline prior to injection in a concentration to inject 0.5 mL/kg, s.c. Atropine methyl nitrate was prepared in saline in a concentration to inject 0.5 mL/kg, i.m.

2.3. Experimental procedure

Baseline blood (0.5 mL) was drawn using the toe-nail clip method [13] and was collected into a 1-mL microfuge tube containing 50 μL of heparin sodium (15 units/mL) 1–3 days prior to the experiment. On the day of the study, the animals were pretreated with atropine methyl nitrate (1.0 mg/kg, i.m.) 15 min prior to a nerve agent exposure to minimize peripheral toxic effects. The animals were then injected subcutaneously with a $1.0 \times \text{LD}_{50}$ dose of GA (120 $\mu\text{g/kg}$), GB (42 $\mu\text{g/kg}$), GD (28 $\mu\text{g/kg}$), GF (57 $\mu\text{g/kg}$), VR (11.3 $\mu\text{g/kg}$), or VX (8 $\mu\text{g/kg}$). At 0, 5, 10, 15, 30, or 60 min after nerve agent injection, blood (0.5–1.0 mL) was collected into a 1-mL microfuge tube containing 50 μL of heparin sodium (15 units/mL). Brain regions (brainstem, cortex, hippocampus, midbrain, cerebellum, striatum, and spinal cord) and peripheral tissues (diaphragm, gastrocnemius (skeletal muscle), and heart) were dissected from the animal.

Brain samples (1:20) and peripheral samples (1:5) were diluted in 1% Triton-X 100 solution (in normal saline). The dissected brain and peripheral tissues were homogenized in a 1% Triton-X 100 solution. Homogenates were centrifuged at 4°C for 10 min at $15000 \times g$, and the supernatant was saved for analysis. Two dilutions were made from blood sample. For the whole blood (WB), 20 μL of collected blood was diluted (1:25) in 1% Triton-X 100 solution. For the red blood cells (RBC), the original blood sample was centrifuged for 5 min at 14,000 rpm to separate the RBC and plasma. Ten microliters of the RBC were then diluted (1:50) in 1% Triton-X 100 solution. The brain, tissue, RBC, and WB samples were kept frozen at -80°C until analysis for AChE activity.

2.4. AChE analysis

On the day of AChE analysis, the tissue samples were thawed, and three 7- μL replicates of each were pipetted into a 96-well UV star microplate (Greiner, Longwood,

FL, USA). Three 10 μ L replicates of the WB and RBC samples were pipetted into the UV star microplates. Twenty microliters of deionized water was added to each well containing brain and peripheral tissue samples, and 17 μ L of deionized water was added to each WB and RBC sample. Following the addition of water, 200 μ L of DTNB (0.424 M, pH 8.2) was added to each sample well. Each microplate was then incubated for 10 min at 37 °C before being placed in the Spectramax Plus microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) where it was allowed to shake for 2 min. Immediately after, 30 μ L of the substrate acetylthiocholine iodide (51.4 mM) was added to each well. The samples were read at 412 nm (at 20 s intervals) for 3.5 min, and the activity (μ mol/mL/min) was determined using Softmax plus 4.3 LS software (Molecular Devices Corporation).

2.5. Protein analysis

Protein levels in the tissue samples were determined by a BCA protein assay. The standard curve was created using a bovine serum albumin at the following concentrations: 0.5, 0.75, 1.0, 1.5, and 2.0 mg/mL. Three replicates of 10 μ L for each brain tissue sample were added to individual microplate wells. Two hundred microliters of working reagent (BCA protein reagent kit) was then added to each well of brain tissue samples. Three replicates of 5 μ L for each peripheral tissue sample were added to individual microplate wells. The peripheral tissue samples were further diluted by adding 5 μ L of deionized water before adding 200 μ L of working reagent. The microplates were shaken for 30 s and then incubated at 37 °C for 30 min. The microplates were allowed to cool to room temperature before being read using the Spectramax Plus microplate reader and Softmax Plus 4.3 LS software as described above. Tissue AChE activity was expressed as μ mol/mg protein/min.

2.6. Data analysis

Six nerve agents and six observation times (0, 5, 10, 15, 30, and 60 min) were used in this study to examine the effects of nerve agents at times after agent exposure on AChE levels. One group of animals was used for each agent and observation time combination, yielding 36 groups of animals. The protocol was performed on one agent group at a time during the year. Each agent group had its own control group that was the 0 observation time group.

Agent groups were compared at the 0 observation time using a one-factor analysis of variance (ANOVA)

for each tissue/region to determine whether there were animal differences, since the protocol was run over a 1-year period. A two-factor ANOVA comparing nerve agent groups, observation times, and the interaction of agent groups and observation times was also used. If a significant agent by time interaction was observed, then a one-factor ANOVA was used to compare nerve agents at each observation time and another one-factor ANOVA was used to compare observation times within agents. A post hoc Tukey's test was used for the multiple comparisons of nerve agents and observation time points if the main effect of agent or time was significant.

RBC and WB were collected at the same times as the tissues, but they were also measured on the same animal at a baseline time prior to exposure for each group. RBC and WB were compared with a one-factor ANOVA to determine whether the nerve agent groups were similar with respect to animals in that group. For RBC and WB, the agent groups were significantly different at the baseline measurement, $p < 0.01$. Therefore, RBC and WB were normalized for each animal by taking a percent of each animal's respective baseline measurement and using that for comparison instead of raw data. In all cases, statistical significance was defined as $p < 0.05$.

3. Results

3.1. RBC and WB AChE activity

The range of baseline AChE activity in WB is 2.05–3.98 μ mol/mL/min. The range of baseline AChE activity in RBC is 1.50–3.97 μ mol/mL/min. Fig. 2A shows the time-course changes in RBC AChE activity after the administration of a $1.0 \times \text{LD}_{50}$ dose of the six nerve agents. All six agents produced maximum AChE inhibition in RBCs at 10 min after exposure to 5–10% of the control. At 10 min, the VR group had significantly lower AChE activity than the GB and GD groups. By 30 min after exposure, there is no significant difference among the effects of the six agents.

The time-course changes in WB AChE levels after administration of a $1.0 \times \text{LD}_{50}$ dose of the six nerve agents are shown in Fig. 2B. Unlike in RBC, the WB data suggest that there are significant differences in the results seen in the ability of each of the six agents to inhibit AChE activity. GB, GD, and GF caused a significantly greater inhibition of AChE in WB than did GA, VR, and VX. GF produced the fastest inhibition, causing AChE levels to drop to 5% of the control at 5 min. GB and GD both produced maximum inhibition at 15 min to 10 and 5% of the control, respectively. The other three agents did not have as powerful an inhibitory effect on AChE

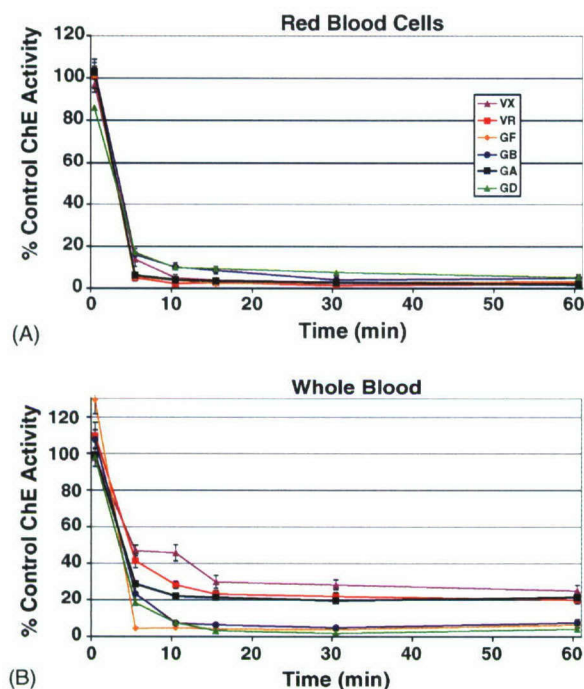


Fig. 2. Effects of nerve agents at $1 \times \text{LD}_{50}$ dose on AChE activity in RBC and WB of guinea pigs.

levels: GA, VR, and VX produced maximum inhibition at 15 min to 22, 25, and 30% of the control, respectively.

3.2. Brain regional AChE activity

The baseline AChE activities in each of the seven brain regions are shown in Table 1.

The levels of AChE activity in the spinal cord, brainstem, cerebellum, striatum, and midbrain following exposure to a $1.0 \times \text{LD}_{50}$ dose of six nerve agents are shown in Fig. 3. In these brain regions, the G-agents caused a greater inhibition of AChE activity over time in comparison to the V-agents. The G-agents also exerted their effects much more quickly than the V-agents, as can be seen by the differences in their slopes. The lines representing the G-agents quickly decline from 0 to 15 min

to 20–30% of control and then level off for the remainder of the time. Among the G-agents, GA induced the most rapid and potent inhibitory effects on AChE. In contrast, the activities of VR and VX decline much more slowly to reach their nadir at 30 min, indicating a much slower inhibition of AChE activity over time. Additionally, the inhibition (to 35–60% of control) was less severe compared with G-agents.

Fig. 3A illustrates how apparent the split in inhibition levels between the G-agents and V-agents is in the spinal cord. GA produced maximum inhibition at 5 min (to 43% of control). It was the fastest-acting nerve agent in the spinal cord, followed by GF. GB and GD caused maximum inhibition at 15 min, reducing enzyme activity to 38 and 42% of the control, respectively. VR and VX reached maximum inhibition at 30 min, but only reduced AChE activity to 55 and 65% of the control, respectively.

The effects of nerve agent exposure on AChE activity in the brainstem over time are shown in Fig. 3B. GA and GF produced the fastest AChE inhibition, lowering enzyme levels to ~30% of the control within the first 5 min. This was followed by GB, which produced maximum AChE inhibition at 10 min to 30% of the control. GD produced maximum AChE inhibition at 15 min to 35% of the control. Again, VR and VX inhibited more slowly and caused less inhibition than all the other agents. They produced maximum AChE inhibition in the brainstem at 30 min to 37 and 45% of the control, respectively.

The difference in inhibition levels of the G-agents and V-agents is also apparent in the results obtained for the cerebellum as shown in Fig. 3C. Similar to what was observed in spinal cord and brainstem, both GA and GF produced the fastest slope of decline in AChE activity. They reached maximum inhibition at 10 and 15 min to ~25% of the control. GB and GD followed with a maximum AChE inhibition at 15 min to ~30% of the control. The V-agents did not reach maximum inhibition until 30 min, with VR reducing AChE activity to 30% of the control and VX reducing activity to 45% of the control.

Table 1
Mean baseline AChE activity ($\mu\text{mol}/\text{mg}$ protein/min) for each brain region studied

	Brainstem	Cerebellum	Cortex	Hippocampus	Midbrain	Spinal cord	Striatum
GA	208.25	212.24	58.76	98.77	147.92	190.61	351.09
GB	232.75	244.94	73.82	109.38	185.05	220.64	435.33
GD	196.79	241.08	57.81	98.92	150.97	195.80	383.66
GF	231.12	252.29	69.77	107.12	175.22	221.14	389.36
VR	240.97	260.59	73.32	111.56	186.19	219.50	413.70
VX	208.13	233.21	63.46	108.81	155.37	196.38	388.08

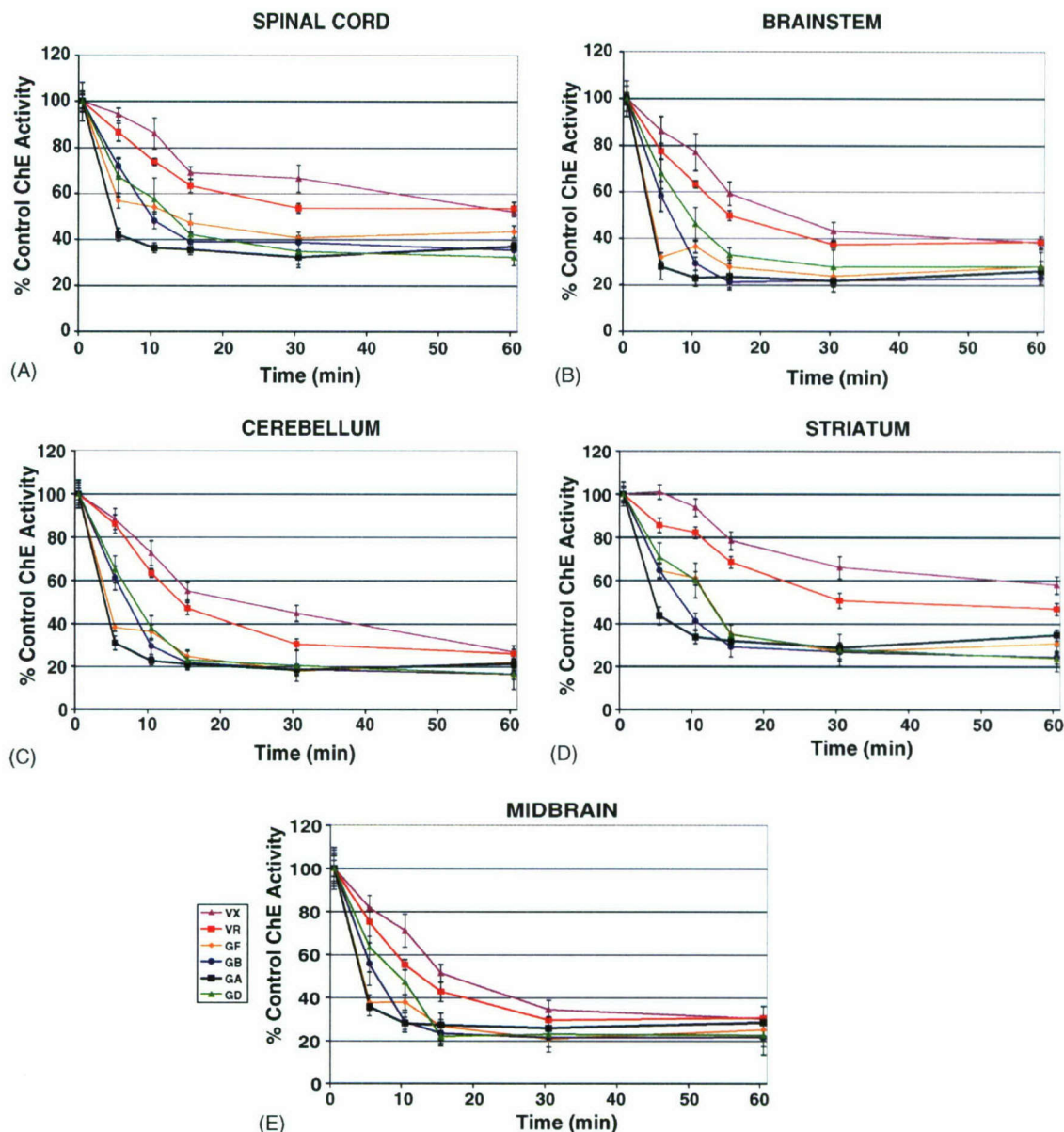


Fig. 3. Effects of nerve agents at $1 \times \text{LD}_{50}$ dose on AChE activity in spinal cord (A), brainstem (B), cerebellum (C), striatum (D), and midbrain (E) of guinea pigs.

Fig. 3D illustrates the time-course of changes in AChE activity in the striatum after administration of the six nerve agents. The graph indicates that the striatum is the area of the brain where the differences between the actions of the G-agents and V-agents are most apparent. Among G-agents, GA had the sharpest decline in the slope of AChE reduction, reaching nadir at 10 min. GB, GD, and GF produced maximum inhibition at 15 min to 30–35% of the control. VR and VX had much lower slopes and produced maximum inhibition at 30 min, but not to levels as low as the G-agents: VR inhibited AChE

activity to 50% and VX inhibited only to 65% of the control.

Fig. 3E shows the time-course changes in AChE activity in the midbrain after exposure to the nerve agents. The split between the G-agents and V-agents is still apparent; however, it is not as pronounced as in the results obtained for the spinal cord, brainstem, cerebellum, and striatum. The differences between the G-agents and V-agents are only significant until the 30-min time point. GA and GF were the most rapid and reached maximum inhibition at 10 and 15 min (to 30% of the control), respectively. This

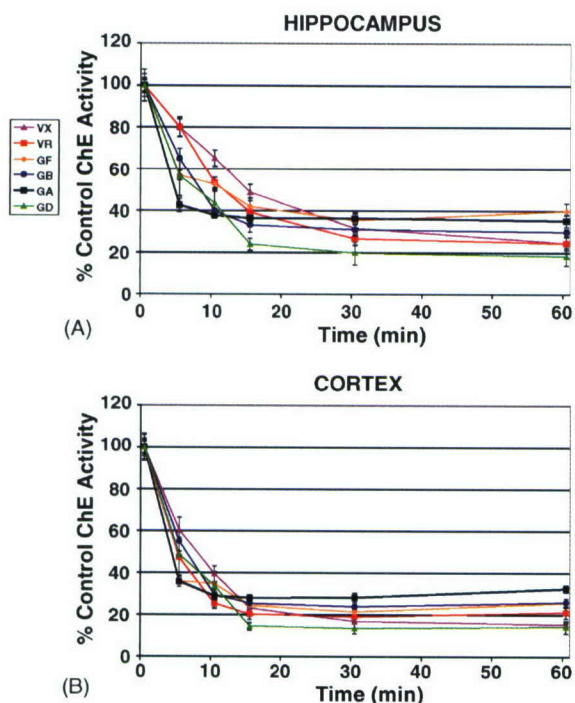


Fig. 4. Effects of nerve agents at $1 \times LD_{50}$ dose on AChE activity in hippocampus (A) and cortex (B) of guinea pigs.

was followed by GB and GD, which reached maximum inhibition at 15 min to 25% of the control. VR and VX acted much more slowly, producing maximum inhibition at 30 min to 30 and 35% of the control, respectively.

As shown in Fig. 4, in the hippocampus and cortex, the time-course patterns of AChE inhibition were different compared with the other brain regions described above. In both of these regions, there was no clear difference between the effects of the G-agents and V-agents, although some separations were still visible during the first 15 min in the hippocampal region.

Fig. 4A shows the time-course of AChE inhibition in the hippocampus. The difference in the effects of the G-agents and V-agents became less apparent. GA was still the most rapid acting. It produced maximum inhibition at 5 min to maintain 43% of the control through the observation period. This was followed by GD, GB, VR, GF, and VX during the first 15 min after nerve agent exposure. GD is the most potent inhibitor of AChE, producing maximum inhibition to 25% of the control in 15 min. Both GA and GF were the least potent inhibitors after 30 min, producing maximum inhibition to ~40% of the control. VR and VX produced maximum inhibition at 30 min to ~30% of the control.

Fig. 4B illustrates the time-related changes of AChE in the cortex after the six nerve agents. There is no

Table 2

Mean baseline AChE activity ($\mu\text{mol}/\text{mg}$ protein/min) for each peripheral tissue studied

	Diaphragm	Heart	Skeletal muscle
GA	14.49	21.15	13.46
GB	18.98	22.82	15.67
GD	12.26	16.39	9.95
GF	17.00	21.17	13.92
VR	15.39	18.22	13.63
VX	12.29	17.11	10.71

apparent difference in the actions of the G-agents and V-agents. Here again GA and GF still induced the most rapid inhibition of AChE, while VX was the slowest inhibitor. All six nerve agents produced inhibition of AChE activity to reach maximum at 10–15 min and then leveled off beyond this point. All nerve agents inhibited AChE activity within a narrow range of each other to 20–30% of the control. Similar to the finding in the hippocampus, GD was the most potent (to 17% of the control) and GA was the least potent (to 30% of the control) inhibitor of AChE in the cortex.

3.3. Peripheral tissue AChE activity

The baseline AChE activities in the three peripheral tissues are shown in Table 2.

Patterns of enzyme inhibition were different in the three peripheral tissues (diaphragm, skeletal muscle, and heart) studied. Unlike the brain regions, there was no apparent split between the effects of the G-agents and V-agents. There were, however, three other noteworthy patterns of AChE inhibition in the peripheral tissue. First, in the diaphragm and skeletal muscle, all of the agents produced a very gradual decline in the inhibition of AChE activity over time. In the heart, however, there was a very fast decline in AChE activity during the first 5 min, after which inhibition levels began to level off. Second, in these tissues, GA produced significantly less inhibition and with less speed than all of the other agents. This was especially obvious in the diaphragm. In the heart, GA produced significantly less inhibition than most of the other agents. This is a much different pattern of inhibition produced by GA than that seen in the brain regions. Third, unlike in the brain regions, VR and VX produced greater inhibition in the diaphragm and especially in the skeletal muscle. In the diaphragm and skeletal muscle, both VR and VX produced high levels of inhibition that were comparable to levels attained by GD. Again, this was a pattern not found in the brain regions.

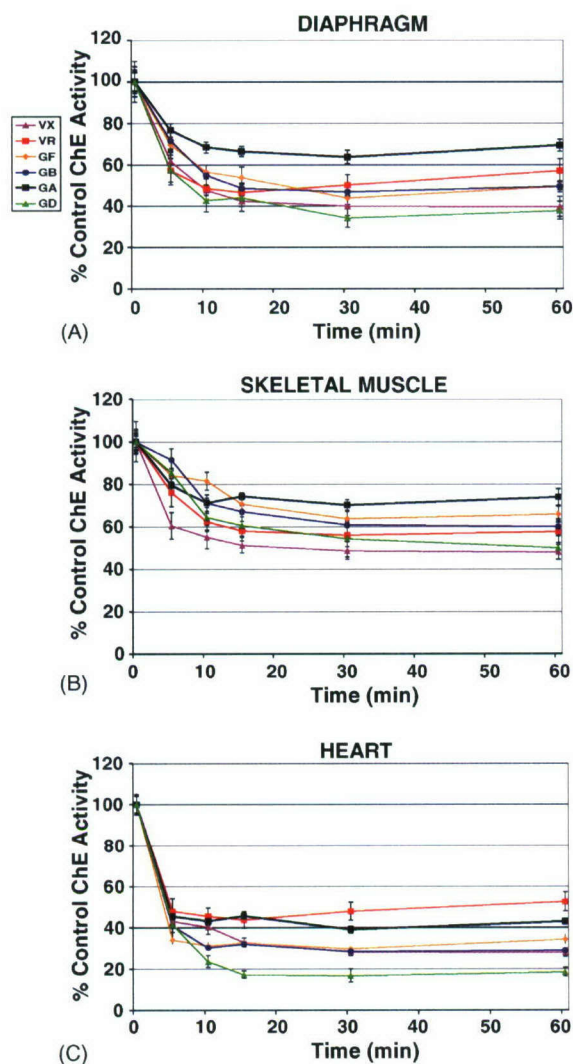


Fig. 5. Effects of nerve agents at $1 \times \text{LD}_{50}$ dose on AChE activity in diaphragm (A), skeletal muscle (B), and heart (C) of guinea pigs.

Fig. 5A shows the time-course of changes in AChE activity in the diaphragm. GD and VR produced the fastest effect, reaching maximum inhibition at 10 min and reducing AChE activity to 43 and 50% of the control, respectively. This was followed by VX, GF, GB, and GA. VX reached maximum inhibition at 15 min and reduced AChE activity to 43% of the control. GB and GF reached maximum inhibition at 15 min, producing a change that was 50–55% of the control. GA reached maximum inhibition at 10 min and reduced AChE activity to 65% of the control, at a level of remaining AChE that was significantly higher than that of all of the other agents.

Fig. 5B shows the time-course changes in AChE activity in the skeletal muscle. In this tissue, VX produced

the rapid inhibition of AChE, reducing maximum AChE inhibition to 60% of the control. This was followed by VR, GA, GD, GF, and GB. VR and GD produced maximum inhibition at 10 min and reduced AChE activity to ~65% of the control. GB produced maximum inhibition at 15 min and GF at 30 min, reducing enzyme activity to 70 and 65% of the control, respectively. GA produced maximum inhibition at 5 min, but it reached only 30% total inhibition of AChE activity, making it the least potent inhibitor in this tissue.

Fig. 5C shows the time-course changes in AChE activity in the heart. There was a very fast decline in AChE activity during the first 5 min after all nerve agents. GD had the greatest effect in the heart, producing maximum inhibition at 10 min and lowering AChE activity to 25% of the control. GF produced maximum inhibition at 5 min to 35% of the control. GB and VX produced maximum inhibition at 10 min to 30% of the control. GA and VR can be seen as outliers in the heart data over the 60-min time-course. GA and VR produced levels of maximum inhibition that were less than the other nerve agents at 45 and 50% of the control (at 5 min), respectively.

4. Discussion

The six nerve agents (GA, GB, GD, GF, VR, and VX) studied exerted differential effects on the AChE enzyme in guinea pigs. All of the nerve agents at a $1.0 \times \text{LD}_{50}$ dose inhibited RBC similarly and to a point where only 5–10% of the control levels were still active at 10 min. In the whole blood, however, there was a split seen between the agents' ability to inhibit AChE. GB, GD, and GF caused a much greater inhibition over time than did GA, VR, and VX. In all of the brain regions studied, the G-agents caused more rapid and greater inhibition of AChE than did the V-agents. With G-agents, maximum inhibition was reached in 15–30 min, while with V-agents, maximum inhibition was reached in 30–60 min. In the cortex and hippocampus, the differential effects of the G-agents and V-agents became less apparent. However, among G-agents, GA induced the most rapid AChE inhibition in all brain regions. In the peripheral tissue, the inhibitory patterns changed. In the diaphragm, and especially in the skeletal muscle, GA had a much slower and less of an inhibitory effect than the other agents. In these tissues, V-agents induced much more rapid and greater inhibition. In the heart, both GA and VR had less of an effect than the other agents, while GD produced the most severe inhibition of AChE activity. These observations suggest that G-agents and V-agents are tissue compartment specific in their ability to inhibit ChE activity.

It is important to consider factors like regional blood flow, ionization pK_a , and the chemical structures and physical properties of these OP nerve agents when trying to explain how they affect brain tissue and peripheral tissue differently. All of the nerve agents produced maximum AChE inhibition in RBC at 10 min after exposure. Enzyme activity was reduced between 5 and 10% of the control in that time. After 10 min, there were no significant differences found among these agents. RBC contain mostly AChE enzyme. All of the nerve agents seemed to have very similar effects on inhibiting AChE activity in RBC. The reason for the fast decrease in AChE activity exhibited by all of the nerve agents lies in the fact that the blood is the first thing the agents encounter after being injected subcutaneously. The concentration of agent in the blood is greater than in any other body tissue. There is a plentiful supply of each agent available to bind to enzyme in the blood. In an autoradiography study, radiolabeling of GD showed that during a 32-min period, blood continues to maintain the highest level of labeling by a factor of 10 times that of surrounding tissues [14].

However, in whole blood (WB) there was a significant separation of the agents' ability to inhibit AChE activity. GB, GD, and GF caused a significantly greater inhibition of AChE in WB than did GA, VR, and VX. One explanation of this phenomenon is that WB contains both RBC and plasma. Plasma contains, in addition to AChE, butyrylcholinesterase (BuChE) and carboxylesterase (CaE), although to a lesser extent in the guinea pigs [15]. It is possible that GA is less readily bound to BuChE and CaE in the plasma than are GB, GD, and GF. It is also known that VR and VX react slowly with CaE [3]. All of the blood enzymes appear to act as buffers for the enzymes in body tissue. There is little inhibition of tissue enzyme until much of the blood enzyme is inhibited [5].

In most of the brain regions there is an apparent difference in the level of AChE inhibition between the G-agents and V-agents, although it became less apparent in the hippocampal and cortical regions. This phenomenon is mostly seen in the caudal brain regions from spinal cord, brainstem, cerebellum, striatum to midbrain region. In a study conducted by Traub [14], it was found that at 2 min, radiolabeled GD is already concentrated in a detectable differential amount in the area postrema, nucleus of the XIIth cranial nerve and medial vestibular, prepositus hypoglossal and paragigantocellular reticular nuclei.

Overall, the G-agents caused more rapid and greater inhibition of AChE than did the V-agents. A plausible explanation for these findings is the fact that the V-agents are charged and have a more difficult time

crossing the blood–brain barrier than do the uncharged G-agents. The toxicities of VX and VR are very similar to each other. The diethylaminoethanthiol group of VR has been reported to have a pK_a of 8.3 [16], while VX has a pK_a of 8.5 [17]. This means that a portion of VR and VX would be positively charged at physiological pH (7.4). Uncharged species are able to distribute into the central nervous system, while charged species become confined to the peripheral nervous system [18]. The G-agents, then, cross readily into the brain circulation and are able to produce their effects much more quickly than the V-agents.

Even though they are charged species, a small number of molecules of V-agents are still able to cross the blood–brain barrier. In comparison to the $1 \times LD_{50}$ dose of each of the G-agents, the amount of molecules present in the $1 \times LD_{50}$ dose of both VR and VX is very small. The quick rates at which G-agents cross the blood–brain barrier, and thus, their large concentrations in various regions of the brain, could also play a role in the split seen between the G-agents and V-agents. The LD_{50} dose for GA is greater than that of any other nerve agent ($120 \mu\text{g/kg}$). Thus, there are more molecules of GA by mass action crossing into the brain than of any other nerve agent. There is a vast amount of GA present in the brain, then, to bind to AChE and cause more inhibition of the enzyme than do any of the other agents. The other G-agents have the following LD_{50} 's: GB ($42 \mu\text{g/kg}$), GD ($28 \mu\text{g/kg}$), and GF ($57 \mu\text{g/kg}$). Next to GA, these three agents produce the mid-level amounts of inhibition seen in the brain regions. VR and VX are the most toxic and have LD_{50} 's of 11.3 and $8 \mu\text{g/kg}$, respectively. Compared with the G-agents, these dosages are very small, and thus, there are only a small number of molecules of VR and VX present in the brain to bind to and inhibit AChE.

From the hippocampus to cortex, however, the split between the G-agents and V-agents became less and less apparent. In the hippocampus, VR and VX still displayed slower and less action during the first 15 min after injection. Even in the cortex, the inhibition by VX remained slow, but all other nerve agents appeared to produce similar effects. All six nerve agents produced an inhibition that was 20% of the control, the lowest level of activity observed among all the brain regions. One factor to consider in explaining this is the cerebrovasculature of the brain. The cortex receives a rich blood supply from the anterior, middle, and posterior arteries that surround its surface [19]. This would mean that all of the nerve agents get spread over the entire cortex very rapidly and become available in mass quantities to cause inhibition in this area.

Another possibility is that a nerve agent-induced opening of the blood–brain barrier occurs in the cortex [20]. Vascular occurrences like acidosis, increased blood flow, and hypertension, which are all known to accompany nerve agent-induced seizures, could play a role in the opening of the blood–brain barrier around the cortex [21]. This would allow for an influx of every nerve agent into the cortex. The cortex and hippocampus are connected by a dense network of cholinergic fibers, so damage to the blood–brain barrier in the cortex and the influx of agent into that area could lead to similar inhibition of AChE activity in the hippocampus.

The six nerve agents followed a much different pattern of AChE inhibition in the peripheral tissue compared with that seen in the brain regions. The split between the G-agents and V-agents was observed in the diaphragm and skeletal muscle, but it was in the opposite direction, compared with brain tissues. Both VR and VX had a more rapid and greater effect on AChE inhibition in the diaphragm and especially in the skeletal muscle than they did in whole blood or brain regions. Blood must circulate numerous times throughout the body before enough of the contents it carries reach peripheral tissues like the diaphragm and skeletal muscle. It is possible that since the V-agents are more viscous and less water-soluble than the G-agents, they are able to persist longer in the bloodstream and have a greater effect on these peripheral tissues. Also, since most of the molecules of the uncharged G-agents readily enter the brain and bind to AChE there, probably not a plentiful supply of the G-agents is left in the bloodstream to bind to AChE in areas like the diaphragm and skeletal muscle. In addition, as mentioned earlier, the V-agents circulate in vivo as a charged species. Tissues like the diaphragm and skeletal muscle do not restrict the entry of charged particles. VR and VX would have no trouble getting into the diaphragm and skeletal muscle and binding to AChE there. In the diaphragm, skeletal muscle, and heart, GA caused significantly less inhibition of AChE than with the other nerve agents over time, while in the brain GA caused significantly more rapid inhibition of AChE. The reason for GA's weak inhibitory action on peripheral tissue AChE is not clear.

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References

- [1] P. Taylor, Anticholinesterase agents, in: J.G. Hardman, L.E. Limbird, A.G. Gilman (Eds.), *Goodman and Gillman's The Pharmacological Basis of Therapeutics*, 10th ed., McGraw-Hill, NY, 2001, pp. 175–191.
- [2] N.B. Munro, S.S. Talmage, G.D. Griffin, L.C. Waters, A.P. Watson, J.F. King, V. Hauschild, The sources, fate, and toxicity of chemical warfare agent degradation products, *Environ. Health Perspect.* 107 (12) (1999) 933–974.
- [3] M.J. van der Schans, B.J. Lander, H. van der Wiel, J.P. Langenber, H.P. Benschop, Toxicokinetics of the nerve agent ($\pm$)-VX in anesthetized and atropinized hairless guinea pigs and marmosets after intravenous and percutaneous administration, *Toxicol. Appl. Pharm.* 191 (2003) 48–62.
- [4] M.A. Dunn, F.R. Sidell, Progress in medical defense against nerve agents, *J. Am. Med. Assoc.* 262 (1989) 649–652.
- [5] F.R. Sidell, Nerve agents, in: F.R. Sidell, E.T. Takafuji, D.R. Franz (Eds.), *Textbook of Military Medicine, Part I: Medical Aspects of Chemical and Biological Warfare*, Office of the Surgeon General, Department of the Army, Washington, DC, 1997, pp. 129–179.
- [6] T.-M. Shih, S.M. Duniho, J.H. McDonough, Control of nerve agent-induced seizures is critical for neuroprotection and survival, *Toxicol. Appl. Pharm.* 188 (2003) 69–80.
- [7] T.-M. Shih, T.A. Koviak, B.R. Capacio, Anticonvulsants for poisoning by the organophosphorus compound soman: pharmacological mechanisms, *Neurosci. Biobehav. Rev.* 15 (1991) 349–362.
- [8] T.-M. Shih, I. Koplovitz, J.H. McDonough, Evaluation of anti-convulsant drugs for soman-induced seizure activity, *J. Am. Coll. Toxicol.* 15 (Suppl. 2) (1996) S43–S60.
- [9] J.H. McDonough, T.-M. Shih, Pharmacological modulation of soman-induced seizures, *Neurosci. Biobehav. Rev.* 17 (1993) 203–215.
- [10] J.H. McDonough, T.-M. Shih, Neuropharmacological mechanisms of nerve agent-induced seizures and neuropathology, *Neurosci. Biobehav. Rev.* 21 (1997) 559–579.
- [11] J.R. Keeler, C.G. Hurst, M.A. Dunn, Pyridostigmine used as a nerve agent pretreatment under wartime conditions, *J. Am. Med. Assoc.* 266 (1991) 693–695.
- [12] D.H. Moore, C.B. Clifford, I.T. Crawford, G.M. Cole, J.M. Baggett, Review of nerve agent inhibitors and reactivators of acetylcholinesterase, in: D.M. Quinn, A.S. Balasubramanian, B.P. Doctor, P. Taylor (Eds.), *Enzymes of the Cholinesterase Family*, Plenum Press, NY, 1995, pp. 297–304.
- [13] A. Vallejo-Freire, A simple technique for repeated collection of blood samples from guinea pigs, *Science* 114 (1951) 524–525.
- [14] K. Traub, In vivo distribution of carbon-14 radiolabeled soman ((3,3-dimethyl-2-butoxy)-methylphosphoryl fluoride) in the central nervous system of the rat, *Neurosci. Lett.* 60 (2) (1985) 219–225.
- [15] J. Bajgar, The influence of inhibitors and other factors on cholinesterases, *Sb Ved Pr Lek Fak Univerzity Karlovy Hradec Kralove* 34 (1991) 3–75.
- [16] A.J.J. Ooms, The reactivity of organic phosphor combinations in regards to a number of esterases, Dissertation, University of Leiden, Leiden, The Netherlands, 1961.
- [17] J. Epstein, J.J. Callahan, V.E. Bauer, Kinetics and mechanisms of hydrolysis of phosphonothiolates in dilute solution, *Phosphorus* 4 (1974) 157–163.

- [18] D.M. Maxwell, K.M. Brecht, I. Koplovitz, Characterization and treatment of the toxicity of *O*-isobutyl *S*-(2-(diethylamino)ethyl)methylphosphonothioate, a structural isomer of VX, in guinea pigs, *J. Am. Coll. Toxicol.* 15 (Suppl. 2) (1997) S78–S88.
- [19] N.R. Carlson, *The Physiology of Behavior*, fourth ed., Allyn and Bacon, Boston, MA, 2000, pp. 159–175.
- [20] P. Carpentier, I.S. Delamanche, M. Le Bert, G. Blanchet, C. Bouchaud, Seizure-related opening of the blood–brain barrier induced by soman: possible correlation with the acute neuropathology observed in poisoned rats, *Neurotoxicology* 11 (1990) 493–508.
- [21] S.I. Rapoport, Opening of the blood–brain barrier by acute hypertension, *Exp. Neurol.* 52 (3) (1976) 467–479.