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SOMAN AND SARIN INHIBITION OF MOLECULAR FORMS OF ACETYLCHOLINESTERASE IN MICE

TIME COURSE OF RECOVERY AND REACTIVATION BY THE OXIME HI-6

JOHN G. CLEMENT,* SYLVESTER ROSARIO, ELAINE BESSETTE and NANCY ERHARDT
Biomedical Defence Section, Defence Research Establishment Suffield, Ralston, Alberta, Canada

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Abstract—The *in vivo* sensitivity of the molecular forms of the enzyme acetylcholinesterase to inhibition by either soman or sarin, reactivation by HI-6 and the time course of recovery following inhibition by soman were investigated in mice. Administration of HI-6 (50 mg/kg, i.p.) immediately after soman (100 μg/kg, s.c.) or sarin (150 μg/kg, s.c.) resulted in an apparent selective reactivation of the 10S and 16S molecular forms of acetylcholinesterase and no reactivation of the 4S form of diaphragm acetylcholinesterase. The apparent selectivity of the reactivation of the molecular forms of the acetylcholinesterase was probably due to the fact that the 10S and 16S forms of acetylcholinesterase are located primarily extracellularly and the 4S form intracellularly. The HI-6 was restricted primarily to the extracellular compartment due to its quaternary, hydrophilic nature. If the administration of HI-6 was delayed until 60 min following soman (100 μg/kg, s.c.) injection, no reactivation of any of the molecular forms of acetylcholinesterase could be found in the diaphragm. The soman-inhibited acetylcholinesterase had probably aged and, thus, was not susceptible to reactivation by HI-6. The time course of recovery of the molecular forms in the diaphragm occurred rather quickly with the smaller 4S and 10S forms recovering to control levels faster than the larger 16S form. It took between 8 and 16 days for the 16S form to recover to normal. In the brain, hypothalamic acetylcholinesterase molecular forms such as the 4S recovered faster than the 10S form which had not recovered to control 16 days after soman administration; the 16S form of acetylcholinesterase was not detected in the brain.

The hydrolytic enzyme acetylcholinesterase inactivates acetylcholine at cholinergic synapses. This polymorphic enzyme is found in many neuronal and non-neuronal tissues and is usually composed of several molecular forms [1, 2]. Two general classes of the enzyme are called globular (G) and asymmetric (A). The globular class has three forms called G1, G2 and G4, based on sedimentation analysis, composed of a monomer, dimer and tetramer, respectively, while the asymmetric class has three forms called A4, A8 and A12 which consist of aggregates of 1, 2 and 3 tetramers, respectively, with a collagen tail attached. Three molecular forms of acetylcholinesterase predominate in rodent diaphragm with sedimentation coefficients of 4S, 10S and 16S which correspond to the G1, G4 and A12 forms, respectively, and two molecular forms of acetylcholinesterase predominate in rodent brain with sedimentation coefficients of 4S and 10S which correspond to the G1 and G4 forms, respectively.

Soman (pinacolyl methylphosphonofluoridate) is an extremely toxic organophosphorus compound. The locus of the toxic and lethal effects of this compound is the irreversible inhibition of the enzyme acetylcholinesterase. The recovery of acetylcholinesterase activity following poisoning with a sublethal dose of soman is through resynthesis of the enzyme [3] and not dealkylation [4]. Following soman poisoning, the function of acetylcholinesterase in the periphery can be restored by parenteral administration of the bispyridinium oxime HI-6 [CAS Reg. No. 34433-31-3; 1-(((4-(aminocarbonyl)-pyridinio)methoxy)methyl) - 2 - ((hydroxyimino)-methyl)-pyridinium dichloride] [5]. HI-6 dephosphorylates acetylcholinesterase by nucleophilic attack and the phosphylated HI-6 quickly decomposes [6], thus returning acetylcholinesterase function to normal. However, soman-inhibited acetylcholinesterase gradually undergoes an ageing process where it is converted to a form that cannot be reactivated by oximes [7, 8]. Sarin (isopropyl methylphosphonofluoridate) is another potent organophosphate anticholinesterase which acts in a manner similar to soman. However, the sarin-inhibited acetylcholinesterase is reactivated more easily by HI-6 than soman [5], and its rate of ageing is considerably slower.

The oxime HI-6 is a bisquaternary, hydrophilic compound which restricts its distribution *in vivo*, primarily if not totally, to the extracellular compartment [9, 10]. The purpose of the present study was 2-fold; (1) to determine if the oxime reactivator HI-6 selectively reactivated a particular molecular form of acetylcholinesterase in the mouse diaphragm following administration of a sublethal dose of an organophosphate anticholinesterase such as soman or sarin; and (2) to determine the time course of the recovery of the various molecular forms of acetylcholinesterase in mouse diaphragm and brain following inhibition by a sublethal dose of soman.

* Correspondence: Dr. J. G. Clement, Defence Research Establishment Suffield, Box 4000, Medicine Hat, Alberta, Canada, T1A 8K6.

MATERIALS AND METHODS

Materials

Soman, sarin and HI-6 were prepared at Defence Research Establishment Suffield and were in excess of 99% pure. Other substances used were obtained from various commercial sources: 4-methylumbelliferyl phosphate, 4-methylumbelliferyl galactoside, acetylthiocholine and dithiobis nitrobenzoic acid (Sigma); ISO-OMPA (ICN Biochemicals); [^{3}H]acetylcholine chloride (NEN); Triton X-100 (BDH); alkaline phosphatase (EC 3.1.3.1), molecular weight of 80,000, from *Escherichia coli* (Sigma); catalase (EC 1.11.1.6), molecular weight of 244,000, from bovine liver (Sigma); and β-galactosidase (EC 3.2.1.23), molecular weight of 540,000, from *Escherichia coli* (Sigma).

Animals

CD-1 male mice (20–25 g) were obtained from Charles River Canada Ltd., St. Constant, Quebec. The mice were housed in the vivarium at Defence Research Establishment Suffield for at least 1 week prior to use in the experiments. The light schedule was 12 hr light and 12 hr dark with the lights on at 7:00 a.m. The mice had access to food and water *ad lib*. The temperature was in the range of 22–24°.

HI-6 reactivation of diaphragm acetylcholinesterase

Mice were injected with either soman (70 or 100 μg/kg, s.c.) or sarin (150 μg/kg, s.c.) and then either immediately or 60 min later were injected with HI-6 (50 mg/kg, i.p.). Appropriate controls received saline in place of HI-6. The mice were decapitated and exsanguinated 30 min following the administration of either HI-6 or saline. The diaphragm was extirpated and the homogenate prepared.

Time course of recovery of brain and diaphragm acetylcholinesterase

Mice were injected with soman (100 μg/kg, s.c.) and then at various time periods (1, 2, 4, 8 and 16 days) after poisoning were decapitated and exsanguinated. The tissues (diaphragm and hypothalamus) were isolated and homogenates prepared.

Tissue preparation

Mice were killed and the tissues removed and rinsed in 0.9% saline, blotted dry on filter paper, and weighed. The hypothalamus homogenate was prepared by 10–20 strokes in a glass–teflon homogenizer in an extraction buffer (4°) containing 1 M NaCl, 0.05 M $MgCl_2$, 0.01 M Tris–HCl and 1% Triton X-100, pH 7.4 [1]. The diaphragm, from individual mice, was homogenized in the same extraction buffer (4°) using a Brinkmann Polytron with a micro probe for a period of 20–30 sec. The tissue concentration of the final homogenate was 10 and 100 mg wet weight of tissue/mL of extraction buffer for the hypothalamus and diaphragm, respectively. The homogenates were then centrifuged at 20,000 *g* for 20 min at 4° in a Beckman model J2-21 M centrifuge and a JA 21 rotor. Fresh, unfrozen tissue was used in all experiments since the use of frozen tissue produced changes in the relative proportions of the various molecular forms of acetylcholinesterase detected ([1, 11]; Clement JG and Bessette E, unpublished observations).

Sucrose density gradient centrifugation

Sucrose density gradients (5–30%) were formed by manual layering and were allowed to diffuse overnight in the cold room (4°) to form a continuous gradient. The linearity of the gradient was confirmed in preliminary experiments by determining the sucrose concentrations of the various fractions by refractometry. Tissue homogenate supernatant (200 μL) was layered on top of the sucrose gradient, and the tubes (Polyallomer Bell-top Quick Seal tubes, Beckman Instruments) were sealed using the Beckman tube sealer. The tubes were centrifuged at 65,000 rpm for 2 hr at 4° in a Beckman L8-55M ultracentrifuge and a VTi 65.1 rotor. The tubes were pierced at the bottom of the tube and fractionated (0.2-mL fractions collected from the top of the tube) using an ISCO (model 640) fraction collector. For the time course of recovery of the various molecular forms following soman poisoning, a 5–20% sucrose gradient was used.

To determine the sedimentation coefficients of the various molecular forms of acetylcholinesterase, alkaline phosphatase (6.1S), catalase (11.4S) and β-galactosidase (16S) were used as markers. The marker enzymes were layered on gradients and centrifuged using the same conditions as those used in the separation of the various molecular forms of acetylcholinesterase. The tubes were fractionated and the enzyme activities of the various fractions were determined. The marker enzymes sediment in the same way whether they are added to the homogenate and layered on the gradient or layered directly on the gradient [12]. Therefore, the positions of the marker enzymes were determined in separate experiments, and this positioning was used as the indicator in the remaining experiments examining the molecular forms of acetylcholinesterase.

Data analysis

The acetylcholinesterase activity for the various fractions was determined and plotted, and the area under the curve (AUC) of the various molecular forms was determined using a LOTUS 123 template. All fractions were corrected for nonenzymatic hydrolysis using fractions from a run where extraction buffer was used as the blank. The 4S form included fractions 1–14, the 10S form fractions 15–23, and the 16S form fractions 24–32. The data were analyzed by Student's *t*-test. A $P < 0.05$ was considered statistically significant.

Enzyme assays

Acetylcholinesterase. The difference in the procedures for the measurement of acetylcholinesterase activity over the course of these studies was due to the evolution of the analysis techniques used in our laboratory and the introduction of laboratory robot technology.

(1) *Radiometric assay.* In the study of the time course of recovery of the various molecular forms of acetylcholinesterase in the diaphragm and hypothalamus, each fraction was analyzed for

acetylcholinesterase activity by the method of Johnson and Russell [13] using [^{3}H]acetylcholine chloride as the substrate. In each sample, ISO-OMPA (10 μM) was present to selectively inhibit pseudocholinesterase activity [14]. The radioactivity was quantitated by liquid scintillation counting in a Beckman liquid scintillation counter model LS9800.

(2) *Spectrophotometric assay*. In the reactivation experiments, each fraction was analyzed for acetylcholinesterase activity using the method of Ellman *et al.* [15] with acetylthiocholine as the substrate. Each fraction contained ISO-OMPA (10 μM) to selectively inhibit pseudocholinesterase activity [14].

Catalase. The activity of catalase was determined by following the disappearance of H_2O_2 spectrophotometrically [16].

Alkaline phosphatase. A 10-μL sample was added to a well in a 96-well immunoassay plate. The reaction was started by the addition of 100 μL of enzyme substrate 4-methylumbelliferyl phosphate (100 μM) in 0.1 M diethylamino buffer, pH 9.8, containing 20 mM $MgCl_2$ and incubated at room temperature in the dark. The relative fluorescence was measured in a MicroFluor microplate reader (Dynatech Laboratories) at an excitation wavelength of 365 nm and an emission wavelength of 450 nm at 5 min intervals.

β-Galactosidase. A 10-μL sample was added to a well of a 96-well immunoassay plate. The reaction was started by the addition of 100 μL of the enzyme substrate 4-methylumbelliferyl galactoside (100 μM) prepared in 10 mM sodium phosphate buffer, pH 7.0, which contained 0.1 M NaCl, 0.1 mM $MgCl_2$ and 0.1% (w/v) bovine serum albumin (BSA), and incubated at room temperature in the dark. The relative fluorescence was measured in a MicroFluor microplate reader at an excitation wavelength of 365 nm and an emission wavelength of 450 nm at 5 min intervals.

RESULTS

The doses of soman and sarin administered to mice were sublethal (LD_{50} values of soman and sarin used in this study were 130–140 and 170 μg/kg, respectively) and produced typical signs of anticholinesterase poisoning such as salivation, tremors, lacrimation, and diarrhea. The administration of HI-6 typically reduced the degree of the signs of poisoning and also increased the speed of recovery. No quantitation of the degree of poisoning was performed on these animals.

Diaphragm acetylcholinesterase activity was decreased to approximately 34–40% of control activity at 30 and 90 min after soman administration (Fig. 1; Table 1), respectively. There did not appear to be any selectivity in the inhibition of the various molecular forms of acetylcholinesterase. Administration of HI-6 immediately following soman resulted in reactivation of soman-inhibited acetylcholinesterase as indicated by an increase in total acetylcholinesterase activity to 47% of control at 30 min following soman and HI-6 administration (Table 1). The reactivation of acetylcholinesterase was isolated to the 10S and 16S forms of the enzyme

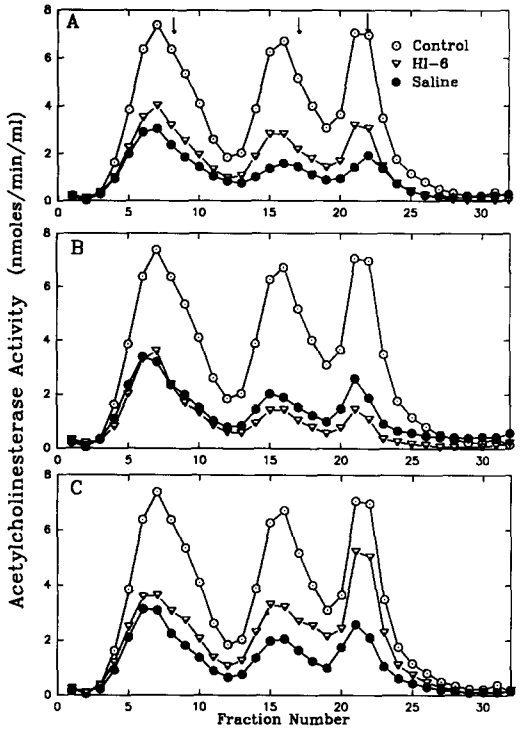

Fig. 1. Reactivation of the various molecular forms of acetylcholinesterases by HI-6 following inhibition by soman. (A) Mice were administered soman (100 μg/kg, s.c.) and were injected immediately with HI-6 (50 mg/kg, i.p.). The mice were killed 30 min following the administration of HI-6. In the saline group saline was injected in place of HI-6. The symbol key in this panel applies to all panels of the figure. (B) Soman (100 μg/kg, s.c.) was administered and 60 min later HI-6 (50 mg/kg, i.p.) was injected. The mice were killed 30 min following administration of the oxime. In the saline group, saline was administered in place of HI-6. (C) Soman (70 μg/kg, s.c.) was administered and HI-6 (50 mg/kg, i.p.) was injected immediately thereafter. The mice were killed 30 min following oxime administration. In the saline group, saline was administered in place of HI-6. The position of the marker proteins is as follows: left arrow, alkaline phosphatase (6.1S); middle arrow, catalase (11.4S); and right arrow, β-galactosidase (16S). The number of observations is indicated in Table 1. All samples were analyzed in duplicate.

with significant reactivation of the 4S form not occurring (Fig. 1A; Table 1).

If the administration of HI-6 was delayed until 60 min after soman poisoning, there was no significant reactivation of total diaphragm acetylcholinesterase or the various molecular forms of the enzyme (Fig. 1B; Table 1), indicating that the soman–acetylcholinesterase complex had aged by this time.

When the dose of soman was decreased to 70 μg/kg, the reactivation produced by HI-6 was significantly greater (Fig. 1C; Table 1) than that following the higher dose of soman. There was significant reactivation of all of the molecular forms of acetylcholinesterase with the largest increases in the 10S and 16S forms.

Table 1. Reactivation of soman-inhibited molecular forms of acetylcholinesterase by HI-6*

Treatment	Acetylcholinesterase activity (nmol/min/mL) Total	4S	10S	16S	N
Untreated control	97.91 ± 10.07† (100)	41.03 ± 3.00 (100)	31.98 ± 5.50 (100)	24.90 ± 5.40 (100)	9
Soman (100 μg/kg) + HI-6 at "0" time					
Saline	33.31 ± 6.09 (34)	17.25 ± 5.43 (42)	8.27 ± 2.25 (26)	7.80 ± 1.97 (31)	8
HI-6	46.47 ± 5.01‡ (47)	21.88 ± 3.17 (53)	14.29 ± 3.86§ (45)	10.29 ± 2.34‖ (41)	7
Soman (100 μg/kg) + HI-60 min later					
Saline	39.38 ± 14.53 (40)	18.65 ± 7.09 (45)	10.24 ± 4.46 (32)	10.48 ± 3.97 (42)	6
HI-6	28.49 ± 2.60 (29)	17.41 ± 3.33 (42)	6.78 ± 1.26 (21)	4.31 ± 1.14§ (17)	5
Soman (70 μg/kg) + HI-6 at "0" time					
Saline	36.16 ± 6.48 (37)	16.90 ± 2.97 (41)	10.53 ± 2.09 (33)	8.74 ± 2.24 (35)	8
HI-6	57.47 ± 2.76‡ (59)	22.44 ± 1.68§ (55)	17.99 ± 2.03‡ (56)	17.04 ± 1.66‡ (68)	4

* Mice were treated with soman (100 or 70 μg/kg, s.c.) and either at the same time or 60 min later received HI-6 (50 mg/kg, i.p.). The mice were killed 30 min after administration of the oxime and the homogenate was prepared.
† Values are means ± SD with percent control given in parentheses.
‡–‖ Significantly different from the appropriate saline control group: ‡ $P < 0.001$, § $P < 0.01$, and ‖ $P < 0.05$.

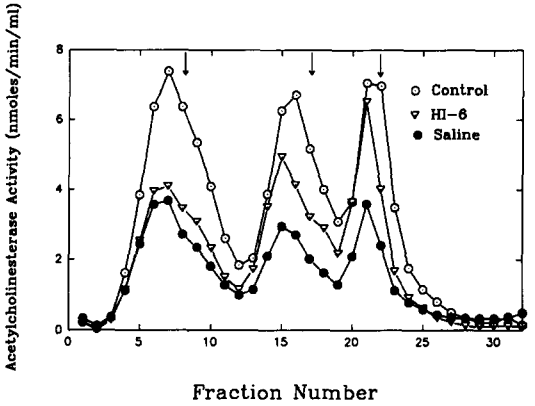

Fig. 2. Reactivation of the various molecular forms of acetylcholinesterase by HI-6 following inhibition by sarin. Mice were administered sarin (150 μg/kg, s.c.) and were injected immediately with HI-6 (50 mg/kg, i.p.). The mice were killed 30 min following administration of the oxime. In the saline group, saline was injected in place of HI-6. The position of the marker enzymes is as in Fig. 1. The number of observations is indicated in Table 2. All samples were analyzed in duplicate.

The effect of sarin and HI-6 administration on the activity of the various molecular forms of acetylcholinesterase in the mouse diaphragm was also investigated. Sarin produced a profound inhibition of all of the various molecular forms with no apparent selectivity of inhibition (Fig. 2 and Table 2). Administration of HI-6 immediately after sarin resulted in significant reactivation of the 10S and 16S but not the 4S form of acetylcholinesterase.

The time course of recovery of the various molecular forms of acetylcholinesterase in the diaphragm and hypothalamus is presented in Fig. 3 and 4. In the mouse diaphragm, three molecular forms, the 4S, 10S and 16S, were detected. The various forms appeared to be affected to the same degree following soman poisoning (Fig. 3). One day after poisoning all of the molecular forms of acetylcholinesterase had started to recover activity with the smaller forms, 4S and 10S, recovering to control levels faster than the 16S form. By day 8 after soman, the 4S and 10S forms had recovered to control levels, whereas the 16S form took between 8 and 16 days to recover to control level.

In the hypothalamus (Fig. 4), only two molecular forms of acetylcholinesterase were detected, the 4S and 10S forms. The 4S form did not appear to be inhibited to as great a degree as the 10S form, and it had recovered by 24 hr. The time course of recovery of the 10S form was slower than that found in the diaphragm, and it was not totally recovered even at 16 days after soman poisoning.

DISCUSSION

The results of this study demonstrate that the various molecular forms of acetylcholinesterase can be separated by the use of a vertical tube rotor. The time for the completion of the centrifugation run was shortened from the 16–18 hr it would normally take using a swinging bucket rotor to 2 hr using the vertical tube rotor, thus reducing the time that the tissue had to be handled prior to the enzyme being separated into its various molecular forms and fractionated, which, in turn, reduced the possibility of proteolytic-induced changes in the relative proportions of the various molecular forms of acetylcholinesterase [1, 11].

Table 2. Reactivation of sarin-inhibited molecular forms of acetylcholinesterase by HI-6*

Treatment	Acetylcholinesterase activity (nmol/min/mL) Total	4S	10S	16S	N
Untreated control	97.91 ± 10.07† (100)	41.03 ± 3.00 (100)	31.98 ± 5.50 (100)	24.90 ± 5.40 (100)	9
Saline	47.74 ± 9.53 (49)	21.22 ± 4.26 (52)	14.32 ± 4.46 (45)	12.20 ± 1.78 (49)	5
HI-6	64.80 ± 12.52‡ (66)	24.49 ± 5.81 (60)	23.57 ± 5.31‡ (74)	16.74 ± 2.35§ (67)	5

* Mice were treated with sarin (150 μg/kg, s.c.) and immediately thereafter (i.e. within 10 sec) received HI-6 (50 mg/kg, i.p.). The mice were killed 30 min after oxime administration.

† Values are means ± SD with percent control given in parentheses.

‡, § Significantly different from saline control group: ‡ $P < 0.05$, and § $P < 0.01$.

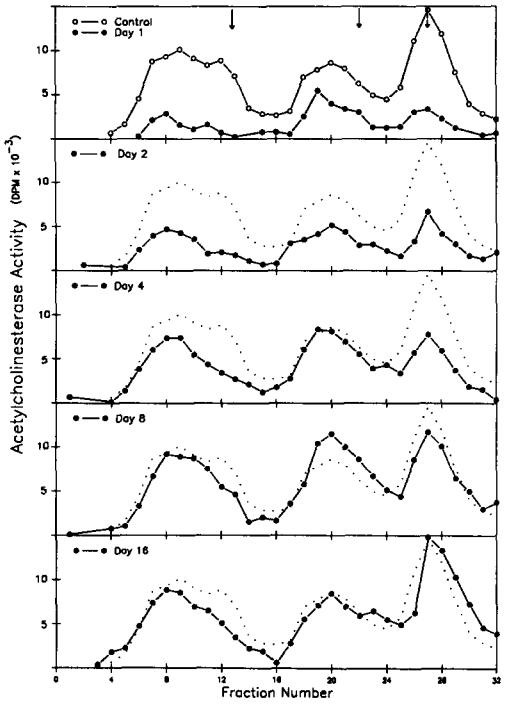

Fig. 3. Time course of recovery of the various molecular forms of mouse diaphragm acetylcholinesterase following poisoning with soman. Mice were killed at various times after the administration of soman (100 μg/kg, s.c.) and the molecular forms of acetylcholinesterase separated. Each point is the mean of at least three separate observations. The control acetylcholinesterase activity is included in each panel of the figure as a dotted line.

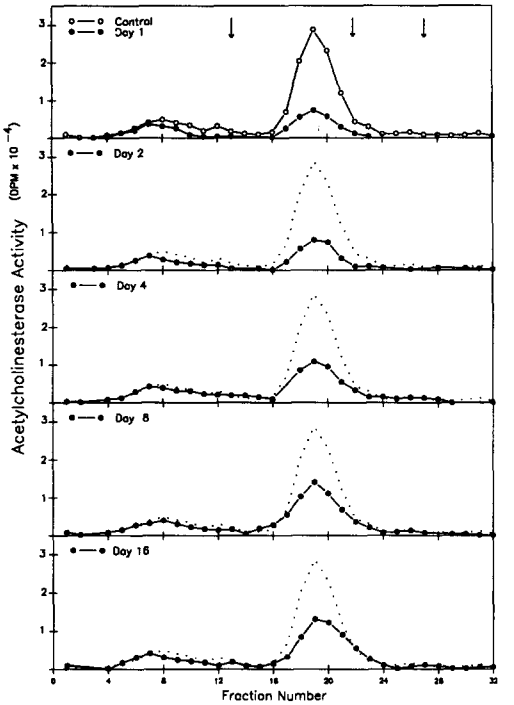

Fig. 4. Time course of recovery of the various molecular forms of mouse hypothalamic acetylcholinesterase following poisoning with soman. Mice were killed at various times after the administration of soman (100 μg/kg, s.c.) and the molecular forms of hypothalamic acetylcholinesterase separated. Each point is the mean of at least three separate observations. The control acetylcholinesterase activity is included in each panel of the figure as a dotted line.

The effects of soman and sarin on the molecular forms of acetylcholinesterase in the mouse diaphragm were investigated. The three major peaks found, the 4S, 10S and 16S, correspond to the G1, G4 and A12 forms, respectively. The A12 form is primarily located extracellularly, bound to the membrane [17, 18] and concentrated at the endplate region in the mouse diaphragm [19], the G1 form is primarily located intracellularly [17, 20] and the G4 form is distributed approximately equally between the intracellular and extracellular compartments [18]. Both soman and sarin produced a profound inhibition of all molecular forms of acetylcholinesterase in the mouse diaphragm without any apparent selectivity. This is not surprising since soman and sarin, both being very lipid soluble compounds, would interact with target proteins in the intracellular and extracellular compartments.

HI-6 is a powerful reactivator of soman-inhibited acetylcholinesterase [21, 22]. When the oxime was administered immediately following soman or sarin, there appeared to be a selectivity with regards to the reactivation of the individual molecular forms of acetylcholinesterase, with the larger forms, the 10S and 16S, being more susceptible to reactivation by the oxime. The reason for the apparent selectivity may be due to a definite selectivity of the particular molecular form of acetylcholinesterase to reactivation by HI-6 or simply due to the accessibility of HI-6 to the phosphorylated acetylcholinesterase. The latter seems the more plausible explanation. The various molecular forms of acetylcholinesterase react in a similar manner to various inhibitors [20, 23–26]; thus, it would be unlikely that the various phosphorylated acetylcholinesterase molecular forms would interact with the oxime in a selective fashion. Since HI-6 is a hydrophilic, bispyridinium, quaternary ammonium compound with limited ability to pass plasma membranes, HI-6 could only reactivate the "unaged" molecular forms of acetylcholinesterase that it came in contact with, primarily, in the extracellular compartment. Based on the distribution of the various molecular forms of acetylcholinesterase [17–20], the results of this study suggest that in the mouse diaphragm the 4S form is located primarily intracellularly and the 10S and 16S forms are located primarily extracellularly. The lack of reactivation of the soman-inhibited acetylcholinesterase when the HI-6 was administered 60 min after soman was probably due to the fact that the inhibited enzyme had aged and thus was not susceptible to reactivation by the oxime HI-6. It was reported that the half-time for ageing of soman-inhibited acetylcholinesterase in rodents is in the range of 20–30 min [22]. The reactivation of the 4S form of acetylcholinesterase at the lower soman dose (70 μg/kg) suggests that there is some movement of HI-6 across cell membranes but at the higher dose of soman the degree of inhibition is too great for the small concentration of HI-6 to reactivate the 4S form of acetylcholinesterase.

In the mouse brain region examined, only the 4S and 10S forms of acetylcholinesterase were found with the 10S form predominating and the 16S form absent in agreement with the results of others [1, 2, 27]. The recovery of the molecular forms in the brain was much slower than that of the diaphragm and is characteristic of brain tissue where the recovery of acetylcholinesterase activity to control levels following organophosphate exposure takes from 16 to 32 days depending upon the region examined [27–30]. The differences in the time course of recovery of the molecular forms of acetylcholinesterase following soman poisoning may be an expression of the rate of synthesis and assembly of the molecular forms in the various regions examined.

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