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FAILURE OF TOXOGONIN TO REACTIVATE SOMAN-INHIBITED BRAIN ACETYLCHOLINESTERASE IN MONKEYS AND REGENERATION OF THE ENZYME

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SUMMARY

Administration of toxogonin to monkeys intoxicated with pinacolyl methyl phosphonofluoridate (soman) did not result in significant reactivation ($P = 0.05$ of inhibited acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7). "Aging", *i.e.* refractoriness of reactivation due to rapid dealkylation of the soman-inhibited enzyme can be minimized if inhibition of acetylcholinesterase is carried out at pH 8.8 and 0 °C. Nevertheless, addition of $5 \cdot 10^{-5}$ M toxogonin, a concentration which could be expected from physiologically acceptable dosage *in vivo*, did not result in reactivation of the unaged, inhibited enzyme. Therefore, aging and inability of the oxime to cross the blood-brain barrier are of secondary importance in the failure of toxogonin to reactivate brain acetylcholinesterase inhibited by soman. Another part of this study concerned the temporal relationship between the persistent central depression of respiration seen in monkeys after poisoning with soman and the degree of inhibition of brain acetylcholinesterase. The overall acetylcholinesterase activity of the pons and medulla showed significant regeneration within 72 h, although respiratory function did not return during this time. One explanation might be that much of the enzyme activity measured may represent cholinesterase molecules that have not yet arrived at vital sites. Consequently, the functionally active acetylcholinesterase may not have reached a level adequate to maintain respiratory function.

INTRODUCTION

The toxic signs immediately following injection of pinacolyl methylphosphono fluoridate (soman) into experimental animals resemble those obtained with other organophosphate anticholinesterases such as diisopropylphosphorofluoridate (DFP) and isopropyl methylphosphonofluoridate (sarin)[1]. The similarity arises from their common action in phosphorylating acetylcholinesterase (acetylcholine hydrolase EC 3.1.1.7), resulting in inhibition of enzyme activity[2,3] and accumulation of acetylcholine[4]. This interpretation is supported by the close relationship between the

severity of the neurological abnormalities and the degree of depression of brain acetylcholinesterase activity following intoxication with these organophosphates (refs 5, 6 and E. Bay, unpublished observations).

In the monkey, DeCandole *et al.*[1] concluded that respiratory failure in acute anticholinesterase poisoning resulted primarily from paralysis of the central respiratory mechanism. However, respiratory failure in the monkey due to soman intoxication appears to be much more prolonged than that resulting from sarin (E. Bay, unpublished observations). Differences in acetylcholinesterase phosphonylated by these organophosphates have also been reported. For example, the rate of loss of reactivatability of the phosphonylated enzyme, a process designated as "aging", is relatively slow after sarin inhibition ($t_{1/2} = 5.8$ h)[7], and oximes are effective in restoring esterase activity[8,9]. On the other hand, bovine erythrocyte acetylcholinesterase inhibited by soman *in vitro* undergoes a rapid decrease in reactivatability ($t_{1/2} = 2.4$ min)[3]; and oximes were reported to be ineffective in restoring the enzyme activity of rat tissues after inactivation by soman[10]. Our interest in additional characterization of the biochemical lesion resulting from soman-poisoning in the monkey suggested that similar studies be performed. Therefore, the rate of aging of soman-inhibited acetylcholinesterase *in vitro* and the effect of administration of 1-1'-(oxydimethylene)bis(4-formylpyridinium chloride) dioxime (toxogonin) on reactivation of brain tissue acetylcholinesterase in soman-intoxicated monkeys were studied.

Other biochemical observations also appeared to be consistent with the prolonged central depression observed in monkeys after soman poisoning. For example, no decrease in soman-derived radiophosphorus bound to the enzyme was noted for 72 h after incubation with ^{32}P-labeled soman[11], and no recovery of inhibited erythrocyte acetylcholinesterase was noted for the same time interval following injection of soman into rabbits (J. H. Fleisher, G. Bursel and L. W. Harris, unpublished observations). In view of these observations, the possibility that administration of soman might give rise to a stationary, severely depressed level of brain acetylcholinesterase was investigated.

MATERIALS AND METHODS

Chemicals

Acetyl[1-^{14}C]choline iodide (2.4 mCi/mmole) and butyryl[1-^{14}C]choline iodide (3.5 mCi/mmole) were purchased from New England Nuclear Corp., Boston, Mass. Amberlite CG 120 resin, sodium form, 200–400 mesh was a product of Mallinckrodt Co., St. Louis, Mo. Naphthalene was obtained from Eastman Organic Chemicals, Rochester, N.Y.; 2,5-diphenyloxazole (PPO) and 1,4-bis(2.5-phenyloxazolyl) benzene (POPOP) were purchased from Packard Instruments Co., Downers Grove, Ill. Lubrol WX was obtained from I.C.I. Organics Incorporated, Stamford, Conn. BW 284-C51 was a gift from Burroughs, Wellcome, Tuckahoe, N.Y.

Measurement of acetylcholinesterase activity

The method used was that described by Siakotos *et al.*[12], in which unreacted substrate is adsorbed on Amberlite CG-120 resin suspended in dioxane. The supernatant solution containing the product of hydrolysis, the free [1-^{14}C]acid, is counted in a liquid scintillation spectrometer. Details concerning the preparation of substrate, the resin, and the scintillation cocktail (fluor) are given by Siakotos *et al.*[12].

Preparation of brain tissues and enzyme assay

Anesthetized monkeys were perfused with heparinized 0.9% NaCl until the brain was virtually free of blood. Unless noted otherwise, 10% (w/v) homogenates were prepared in water from the pons, cerebellum, and medulla. For enzyme assay, further dilution to 1% was made. Homogenates derived from tissues of soman-intoxicated animals were not diluted.

For radiometric assay of acetylcholinesterase, 0.1 ml of brain homogenate and 0.1 ml of 0.1 M sodium phosphate buffer, pH 7.4, containing 0.3 M NaCl and 1% Lubrol WX (PNL), were placed in a centrifuge tube and equilibrated to 37 °C. 100 μl of $3 \cdot 10^{-3}$ M labeled substrate were added and incubated for varying periods of time at 37 °C. Nonenzymatic hydrolysis of the labeled substrate was measured with solutions containing 0.1 ml of water instead of tissue. The reaction was stopped by adding 5 ml of dioxane–resin suspension to each tube. The mixture was made up to 10 ml with dioxane, mixed, and centrifuged at 900 $\times$ *g* for 1 min. 5 ml of the supernatant was transferred to a scintillation vial, 10 ml of a modified Bray's cocktail[12] was added, and the samples were counted in a scintillation spectrometer. The readings (cpm) were referred to a standard curve relating radioactivity to nmoles of substrate. Protein was measured by the biuret procedure[13] or the method of Lowry *et al.*[14] on separate aliquots of the same tissue sample. Enzyme activity per min per mg of protein was calculated from the following:

$$\text{Substrate hydrolyzed} = (\text{nmoles}) \cdot \left(\frac{10\text{ ml}}{5\text{ ml}}\right) \cdot \left(\frac{1}{\text{minutes of incubation}}\right) \cdot \left(\frac{1}{\text{mg of protein}}\right)$$

Subcellular fractionation of brain tissue

Subcellular fractions of monkey pons and medulla were prepared by differential and sucrose-density gradient centrifugation. All procedures were carried out at 0–4 °C or in a refrigerated centrifuge at 2 °C. 10% homogenates (w/v) were prepared in 0.4 M sucrose with a tightly fitting all-glass tissue grinder. The homogenates were separated into crude nuclear (P_1), crude mitochondrial (P_2), microsomal (P_3), and supernatant (S_3) fractions as described by Gray and Whittaker[15]. Fraction P_2 was resuspended in 3 ml of 0.4 M sucrose and layered on a discontinuous gradient of 4 ml of 0.8 M sucrose over 10 ml of 1.2 M sucrose. The preparation was centrifuged in the S.W. 25.3 rotor in the Beckman L-2 ultracentrifuge for 78 700 $\times$ g_{av} for 60 min. The layer forming at the interface between 0.8 M and 1.2 M sucrose corresponding to the nerve ending fraction of Gray and Whittaker[15] was aspirated, diluted with 20 vol. of 0.25 M sucrose and recentrifuged at 105 000 $\times$ *g* for 60 min. The supernatant above fraction P_2 (S_2 in the fractionation scheme shown in Fig. 1) was made to yield a microsomal pellet and a soluble fraction by centrifuging at 78 480 $\times$ g_{av} for 90 min. P_3 and P_4 were also resuspended in 0.25 M sucrose. Aliquots of the original whole homogenate, of the P_3 and P_4 suspensions in 0.25 M sucrose, and of soluble fraction (S_3) were assayed for acetylcholinesterase activity and for protein content.

Experimental procedure

Other procedures used in the separate experiments, including doses of acetylcholinesterase inhibitors and oximes, are described in the appropriate subdivision of the Results section.

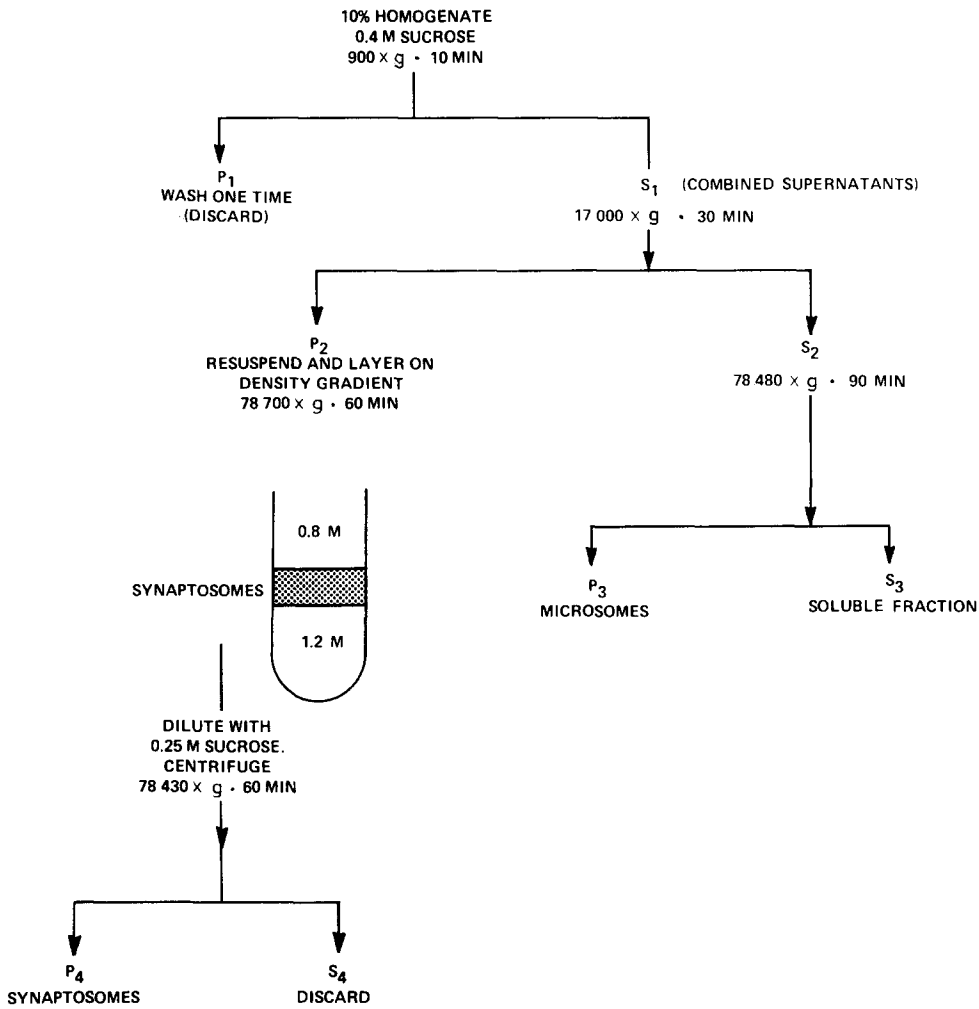

Fig. 1. Fractionation scheme for subcellular distribution of brain acetylcholinesterase.

RESULTS

(A) Acetylcholinesterase activity of homogenates from brain tissues of unpoisoned monkeys

Varying quantities of 1% homogenates from the pons, medulla and cerebellum were incubated with 10^{-3} M labeled substrate for 10 min at 37 °C. Enzymatic activity was proportional to enzyme concentration for each of the tissues studied.

Utilizing the same homogenates, 100 μl of tissue were incubated with radioactive substrate for varying time intervals up to 32 min. Linearity was obtained well beyond 10 min (Fig. 2). Routinely, homogenates of all tissues were incubated for 5 and 10 min at 37 °C to ensure observance of linearity. Blank values (assay without enzyme) were negligible under these conditions (Fig. 2), but were always run concurrently and deducted from the total radioactivity measured, to yield the net

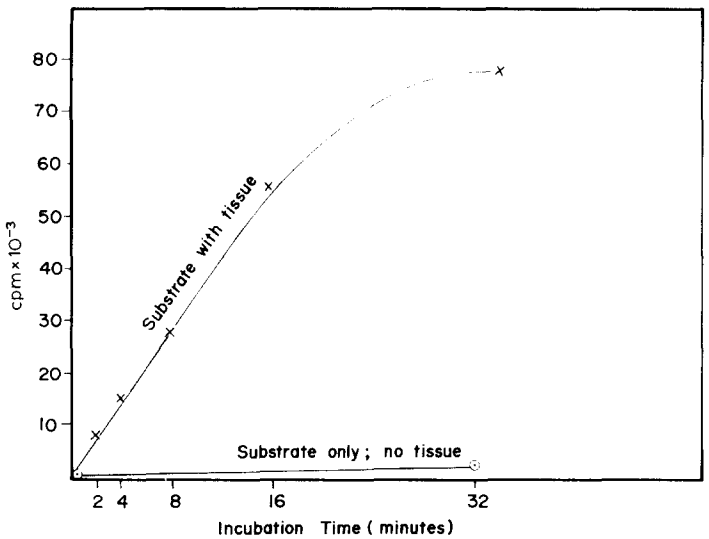

Fig. 2. Relationship between time of incubation and rate of hydrolysis of 100 μl of 1% brain homogenate in 0.3 ml containing 10^{-3} M acetylcholine and pH 7.4 buffer, at 37 °C. 1 cpm = $3.4 \cdot 10^{-12}$ M acetate.

hydrolysis due to enzyme activity. The mean values for the acetylcholinesterase activity of homogenates from eight animals with their 95% confidence limits were found to be: pons, 78.8 (68.0–89.6); medulla, 50.2 (43.8–56.6); cerebellum, 78.6 (71.1–86.1) nmoles/min per mg of protein.

Enzyme measurements were performed with [^{14}C]acetylcholine as substrate. Since butyrylcholinesterase in the central nervous system[16] may contribute to acetylcholine hydrolysis, a preliminary experiment was performed with the selective inhibitor for acetylcholinesterase, 1,5-bis(4-allyldimethylammoniumphenyl) pentane 3-one dibromide (BW 284-C51)[17] to provide information on the specificity of our measurements for this enzyme.

The enzyme activity of 1% homogenates of pons, medulla, and cerebellum from three unpoisoned monkeys was determined with [^{14}C]acetylcholine and [^{14}C]-butyrylcholine iodide in the absence of, and with 10^{-6} M BW 284-C51, a concentration producing virtually complete inhibition of brain acetylcholinesterase without inhibiting butyrylcholinesterase activity[12].

Table I shows that over 93% of the enzyme activity measured with [^{14}C]acetylcholine in the absence of BW284-C51 is due to the presence of acetylcholinesterase. The very small contribution of butyrylcholinesterase to measurement of enzyme activity (less than 7%) with [^{14}C]acetylcholine permitted us to use this substrate routinely without making parallel measurements in the presence of BW 284-C51.

(B) Effects of oxime administration in vivo on soman-inhibited brain acetylcholinesterase activity

Soman (5 μg/kg) was injected intravenously over a 2-min period into eight monkeys. At the first sign of intoxication, 0.1 mg/kg of atropine sulfate was injected intramuscularly. A second group of eight animals was given the same dose of soman

TABLE I

ACETYLCHOLINESTERASE AND BUTYRYLCHOLINESTERASE IN SOME BRAIN TISSUES OF THE MONKEY

Enzyme source	*Acetylcholinesterase activity (cpm/100 µl of 1% homogenate per 10 min incubation)*				*Relative acetylcholinesterase activity (%) Column 1–3*
	Without BW 284C-51		*With BW 284C-51*		
	Acetylcholine (1)	*Butyrylcholine (2)*	*Acetylcholine (3)*	*Butyrylcholine (4)*	*Column 1*
Pons	21 461	1361	831	378	95.8
	30 420	3451	2439	2113	92.0
	30 962	3515	1652	2226	94.8
					Mean 94.2
Medulla	11 937	996	498	669	95.8
	18 760	2429	1506	2139	92.0
	18 901	3985	1347	3184	92.8
					Mean 93.5
Cerebellum	23 900	1162	1568	227	93.3
	18 518	516	1258	741	93.2
	21 243	1553	1031	660	94.8
					Mean 93.1

followed by intramuscular injection of 7.5 mg/kg of toxogonin and 0.1 mg/kg of atropine at the first signs of poisoning. After 72 h the monkeys were sacrified, the brain perfused, and the acetylcholinesterase activity of homogenates from the pons and medulla was measured. The percent inhibition of acetylcholinesterase activity in the pons and medulla of the treated animals was 58.6 ± 3.0% and 59.3 ± 7.4% ($P = 0.05$), respectively, when compared to unpoisoned control values (Results, Section A). The acetylcholinesterase activity in the pons and medulla of soman-poisoned monkeys not receiving toxogonin showed 62.7 ± 6.3% and 60.4 ± 6.8% ($P = 0.05$) inhibition, respectively. The values for the percentage inhibition in treated and untreated animals overlap and are, therefore, not considered significantly different. This observation suggests that significant reactivation of soman-inhibited acetylcholinesterase activity did not occur following administration of toxogonin.

(C) Rate of loss reactivatability ("aging") in vitro of brain acetylcholinesterase inhibited by soman

Homogenates of pons, medulla and cerebellum (10%, w/v) from unpoisoned monkeys were prepared in 0.02 M borate buffer at pH 8.8 and 0 °C. The homogenates were incubated with $2 \cdot 10^{-7}$ M soman (final concentration) for 12 min at 0 °C. About 90% inhibition with minimal aging occurred under these conditions. Without delay, the preparations were transferred to a thermostated bath at 37 °C and allowed to equilibrate to this temperature. Samples were taken before, and at suitable time intervals after, the pH was rapidly lowered to 7.4 with a small volume of 2 M phosphate buffer (pH 6.0). Change in reactivatability was measured by taking 0.1 ml samples into 0.1 ml of 10^{-1} M monoisonitrosoacetone at pH 7.8 in 0.1 M phosphate buffer. 60 min at 37 °C was allowed for reactivation. At the end of this time, the samples were diluted to 1 ml with buffer–salt–detergent mixture at pH 7.4 and the

enzyme activity estimated as described in the Methods section. Appropriate controls of uninhibited enzyme with the same concentration of oxime were run concurrently. The final concentration of $3.3 \cdot 10^{-3}$ M monoisonitrosoacetone present in the enzyme determination produced little or no inhibition of acetylcholinesterase. The logarithm of the percent of reactivated enzyme was plotted as a function of time of aging before transfer into oxime solution. The graph was consistent with first-order kinetics. By interpolation the half time for loss of reactivatability was 1.0 ± 0.1 min ($P = 0.05$) for six preparations (two for each brain tissue).

(D) Effects of toxogonin in vitro on "unaged" soman-inhibited brain acetylcholinesterase

Homogenates of the pons and cerebellum (10%, w/v) from unpoisoned monkeys were prepared in 0.9% saline buffered to pH 8.8 with 0.02 M borate buffer. The preparations were incubated with $2 \cdot 10^{-7}$ M soman at 0 °C as in Results, Section C. Varying concentrations of toxogonin in the same borate-buffered medium were added to aliquots of the soman-inhibited preparation and the samples incubated at 37 °C for 60 min. Control tissue with and without toxogonin, and a soman-inhibited sample without oxime were run concurrently. All samples were centrifuged at 105 000 × *g*, the supernatant removed, and the pellet washed with 0.9% saline. Two additional cycles of washing with 0.9% NaCl, centrifugation and removal of the supernatant followed. The samples were diluted to 1.0 ml with buffer–salt–detergent mixture at pH 7.4 and the enzyme activity estimated as described in the Methods section. The enzyme activity of the control sample treated with toxogonin was 99% that of the control not treated with toxogonin indicating removal of the oxime. The percent reactivation corresponding to each concentration of toxogonin used for the pons and cerebellum is given in Table II. Negligible reactivation was obtained by the oxime at concentrations of $2.5 \cdot 10^{-5}$ and $5.0 \cdot 10^{-5}$ M. At very high concentrations reactivation was obtained, indicating that a substantial portion of the inhibited enzyme was capable of being reactivated, *i.e.* remained unaged under the conditions used.

(E) Acetylcholinesterase activity in brain tissues of monkeys following soman intoxication

Monkeys were given 1 mg/kg of atropine intramuscularly and 30 min later received 9.5 μg/kg of soman by the same route. Additional atropine and artificial respiration were administered as needed to insure survival. At 1 h and at 72 h after giving soman, the animals were anesthetized, sacrificed, and the brains perfused and excised. Brain homogenates from soman-intoxicated animals were prepared as described, using a lesser dilution to provide sufficient residual enzyme activity for accurate measurement. The results, given in Table III, show greater than 90% reduction of acetylcholinesterase activity 1 h after poisoning when referred to the unpoisoned control values. The corresponding brain tissues in animals surviving for 72 h show a significant increase ($P = \pm 0.05$) in enzyme activity over the values found 1 h after poisoning with soman.

(F) Acetylcholinesterase activity in subcellular fractions from the pons and medulla of control and soman-intoxicated monkeys

Subcellular fractions of the pons and medulla were prepared from soman-intoxicated monkeys and control animals as described under the Methods section.

TABLE II

EFFECTS OF TOXOGONIN ON "UNAGED" SOMAN-INHIBITED ACETYLCHOLINESTERASE *in vitro*

*Toxogonin** (molarity)	*Reactivation (%)*	
	Pons	*Cerebellum*
$1.0 \cdot 10^{-2}$	49.0	53.0
$2.5 \cdot 10^{-3}$	49.0	45.0
$5.0 \cdot 10^{-4}$	20.0	21.5
$1.0 \cdot 10^{-4}$	7.2	6.0
$5.0 \cdot 10^{-5}$	3.0	4.1
$2.5 \cdot 10^{-5}$	1.2	4.3
None**	0.0	0.0

* Toxogonin in 0.02 M borate buffer at pH 8.8 was added to the cold preparations which were then incubated for 60 min at 37 °C. The oxime was removed by centrifuging at 105 000 × g and washing with 0.9% saline as described in Results, Section D.

** $2 \cdot 10^{-7}$ M soman produced 96% inhibition of the acetylcholinesterase in the pons and 94% inhibition in the cerebellum.

TABLE III

RECOVERY OF ACETYLCHOLINESTERASE *in vivo* IN SOME BRAIN TISSUES OF THE MONKEY AFTER SOMAN INTOXICATION

*Tissue**	*Acetylcholinesterase activity of tissue homogenates** (% of normal; 95% confidence limits)*	
	1 h	*72 h*
Pons	5.3 (1.4–9.3)	32.2 (27.9–36.3)
Medulla	3.5 (1.60–6.38)	32.3 (25.5–39.1)
Cerebellum	2.2 (0.1–4.2)	24.2 (20.1–28.3)

* Tissues from six or more animals were examined for each time interval.

** When referred to control values for [^{14}C]acetylcholine hydrolysis, *i.e.* 78.8 nmoles per mg protein per min for the pons; 50.2 nmoles for the medulla; and 78.6 nmoles for the cerebellum.

Enzyme activity and protein content were assayed. The results, given in Table IV, show regeneration of acetylcholinesterase activity in the microsomal and soluble fractions of pons and medulla 72 h after poisoning. Recovery of acetylcholinesterase in the synaptosomal membrane (NEP) fraction appears substantially less at 72 h than that in the microsomal and soluble fractions.

DISCUSSION

The inability of toxogonin, a quaternary oxime, to reactivate monkey brain acetylcholinesterase inhibited by soman may be due in part to inability to cross the hematoencephalic barrier. This possibility is supported by our recent observation that toxogonin given at 34 mg/kg intramuscularly (4.5 times the dose used in the present study) showed little capacity to penetrate the retinal portion of the blood-

TABLE IV

ACETYLCHOLINESTERASE ACTIVITY IN SUBCELLULAR FRACTIONS FROM THE PONS AND MEDULLA OF CONTROL AND SOMAN-INTOXICATED MONKEYS

Tissue	*Control cholinesterase activity (nmoles [^{14}C]acetylcholine/mg protein per min)*	*After soman (% of control)*	
		1 h	*72 h*
Pons			
Whole homogenate	73.3 (64.6– 82.1)*	3.3**	32.8**
Microsomal fraction	215.7 (153.0–278.4)	3.1	32.2
Soluble fraction	50.2 (32.2– 68.2)	3.7	35.4
Synaptosomal membrane fraction (NEP)	114.2 (88.6–139.8)	3.0	18.5
Medulla			
Whole homogenate	48.3 (43.3– 53.4)	2.8	29.1
Microsomal fraction	121.3 (89.7–153.0)	2.2	30.5
Soluble fraction	34.7 (23.6– 46.0)	2.8	34.7
Synaptosomal membrane fraction (NEP)	68.6 (45.0– 92.2)	1.9	21.7

* Mean values and 95% confidence limits for the acetylcholinesterase activity from 6 monkeys.

** Mean of the values obtained from two monkeys at each time interval.

brain barrier based on less than a 2-fold increase in retinal acetylcholinesterase compared to more than a 20-fold increase in the red blood cell acetylcholinesterase activity of sarin-poisoned guinea pigs[18].

Intoxication with soman produces an inhibited acetylcholinesterase in peripheral tissues which is resistant to reactivation by oximes[10,19]. Such resistance has been attributed to the rapid aging of the phosphonylated enzyme. However, our laboratory showed that the enzyme inhibited by soman at pH 8.8 and 0 °C could be restored to 80% of the initial control activity by prompt incubation with $9 \cdot 10^{-2}$ M monoisonitrosoacetone[3]. This finding suggested that the active center of the enzyme inhibited by soman under these conditions remains susceptible to reactivation. This interpretation was supported by our later later observation that high concentration of pyridinium 2-aldoxime methochloride (2-PAMCl) could restore the enzyme activity of red blood cell acetylcholinesterase inhibited by soman at 0 °C and pH 8.8[20]. The current finding of substantial reactivation of inhibited enzyme activity following addition of $2.5 \cdot 10^{-3}$ M and 10^{-2} M toxogonin at pH 8.8 (Table II) agrees with earlier observations[3,20] and further supports the interpretation that the enzyme inhibited by soman under these conditions remains, at least in part, reactivatable.

Hence, the ineffectiveness of $5 \cdot 10^{-5}$ M toxogonin to reactivate the unaged inhibited enzyme (Table II) suggests that an additional mechanism may be involved. Perhaps the bulky pinacolyl methylphosphonate grouping characteristic of the initial phosphonylated enzyme[3] may sterically hinder the approach of the reactivator molecules. This possibility is supported by the contrasting effectiveness of similar low concentrations of toxogonin to reactivate acetylcholinesterase inhibited by sarin[19,21], the latter yielding the less bulky isopropyl methylphosphonylated enzyme.

$5 \cdot 10^{-5}$ M toxogonin corresponds to a plasma oxime concentration of 18 μg/ml. Since this level approximates or exceeds that expected from intramuscular injection, and greatly exceeds oxime levels from oral administration[22,23], prophylaxis by

plasma concentrations of toxogonin resulting from conventional dosage is unlikely.

Oxime concentrations far greater than those obtainable in plasma by therapeutic dosage will reactivate unaged soman-phosphorylated acetylcholinesterase *in vitro*[3,20] (and Table II above). However, the enzyme in monkey brain inactivated by soman *in vitro* undergoes very rapid aging ($t_{1/2} = 1.0 \pm 0.1$ min; Results, Section C). The half-time for aging *in vitro* has been shown to approximate that *in vivo* after soman[20], and other organophosphates[24,25]. Once the enzyme has aged, it cannot be reactivated by any known means, since aging is due to dealkylation of the pinacolyl methylphosphonylated enzyme[3,11]. The loss of the alkyl constituent promotes a negative charge on the phosphonate bound to the esterase. This cannot react with the anion which is the active form of the oxime[26].

Our finding that 7.5 mg/kg of toxogonin administered at the first signs of poisoning after giving soman did not restore any significant proportion of brain tissue acetylcholinesterase (Results, Section B) is consistent with the ineffectiveness of therapeutically acceptable concentrations of the oxime *in vitro* (Table II) as well as with the rapidity of aging of soman-inhibited enzyme.

The enzymically-inactive product of soman inhibition shows no evidence of spontaneous dephosphorylation *in vitro*[11] or *in vivo*[27]. Therefore, the reappearance of enzyme *in vivo* (Table III) is assumed to represent newly synthesized acetylcholinesterase. This interpretation is supported by our recent observation that recovery of retinal acetylcholinesterase in soman-poisoned guinea pigs is markedly inhibited by cycloheximide[27], an inhibitor of protein synthesis[28].

Consistent with earlier findings[29,30], acetylcholinesterase activity in subcellular fraction of brain tissues of the monkey is found mainly in microsomes (Table IV). Further fractionation of the crude mitochondrial pellet, P_2 showed the next highest level of enzyme activity to be in the synaptosomal membrane fraction (Table IV). This observation parallels the findings of DeRobertis *et al.*[31].

72 h after soman poisoning, the enzyme recovery in the microsomal fraction of pons and medulla (Table IV) agrees closely with the recovery found in whole brain homogenates of six monkeys sacrificed at the same time interval after soman intoxication (Table IV). Substantially less recovery was found in the synaptosomal membrane fraction than in the microsomal fraction (Table IV). The acetylcholinesterase activity of the microsomal and synaptosomal membrane fractions reflect primarily in the enzyme levels within the cell bodies[29,30] and nerve endings, respectively[31]. Therefore, the greater recovery in the microsomal component at 72 hours after anticholinesterase inhibition is consistent with synthesis of enzyme protein within the cell body of the neuron and subsequent transportation to the nerve endings by axonal flow[32]. Although the possibility of some synthesis in the nerve endings cannot be excluded, the lesser recovery in the nerve ending fraction makes this doubtful. Our interpretation, therefore, parallels the view recently offered by Austin and James[33] based on regeneration of acetylcholinesterase in subcellular fractions of rat brain following DFP inhibition.

A capacity for sustained respiration did not return in most of the monkeys within 72 h after giving soman (E. Bay, unpublished observations). During this time, the acetylcholinesterase activity of the medulla and pons, tissues associated with the central control of respiration[34], showed a recovery (Table III). Much of this enzyme activity may be ascribed to cholinesterase molecules which are not yet functionally

active, *i.e.* they are still on their way to their final locations in the synapses. Consequently, the measured recovery of overall acetylcholinesterase activity in the pons and medulla may coincide with an inadequate level of functionally active cholinesterase; and this may be responsible for the central depression of respiration.

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