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DEALKYLATION AS A MECHANISM FOR AGING OF CHOLINESTERASE AFTER POISONING WITH PINACOLYL METHYLPHOSPHONOFLUORIDATE

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Abstract—The decrease in the mole fraction of ^{32}P-pinacolyl methylphosphonic acid (^{32}P-PMPA) derived from acetylcholinesterase inhibited with ^{32}P-labeled pinacolyl methylphosphonofluoridate (Soman) was used to measure the rate of dealkylation of the phosphonylated enzyme *in vitro*. First-order rate constants for dealkylation and for "aging," as measured by decrease in reactivatability by monoisonitrosoacetone were not significantly different. Dealkylation in the brain of the rat poisoned with ^{32}P-Soman *in vivo* reached a maximum within 30 min after poisoning. Eserine, given prior to poisoning with ^{32}P-Soman *in vivo*, raised the LD_{50} of Soman 3·8-fold and appeared to shunt the Soman to nondealkylating sites, as judged by the high mole-fraction of ^{32}P-PMPA and the large retention of cholinesterase activity in the brains of these animals.

THE activity of cholinesterase (ChE) inhibited by organophosphorus compounds can be restored by treatment with nucleophilic agents capable of displacing the phosphorus from the enzyme.[1] However, the fraction of phosphorylated enzyme that can be reactivated decreases exponentially on standing in the absence of prompt addition of effective nucleophiles.[2–4] This effect has been designated "aging." The loss of reactivatability was found to increase with temperature,[5] indicating that a chemical reaction was involved. The nature of the reaction responsible for the conversion of inhibited ChE to a nonreactivatable form was postulated by Berends *et al.*[6] to be hydrolysis of one alkoxy group from the phosphorylated enzyme:

$$(RO)_2\,PO \cdot \text{enzyme} + H_2O \longrightarrow (RO)\,(HO)\,PO \cdot \text{enzyme} + ROH$$

These workers inhibited horse serum butyrocholinesterase with ^{32}P-labeled diisopropylphosphorofluoridate, and the degree of conversion to a nonreactivatable form was found by treatment with pyridine 2-aldoxime methiodide. The ^{32}P liberated during reactivation was found to be diisopropylphosphate; its fractional value of the total protein-bound radioactivity was in agreement with the fraction of the total enzyme activity recovered. The ^{32}P not removed by prolonged incubation with 2-aldoxime methiodide but remaining protein bound was released by alkaline hydrolysis as monoisopropyl phosphate. This indicated that the refractory portion of the phosphorylated enzyme was a hydrogen phosphate known to be resistant to nucleophilic agents.[7]

Data concerning the application of these findings to acetylcholinesterase (AChE) inhibited by other organophosphorus compounds is still scanty. We were therefore

led to study the rate of conversion of AChE phosphonylated with ^{32}P-labeled pinacolyl methylphosphonofluoridate (Soman) to a nonreactivatable form. Concurrent studies on the rate of change of pinacolyl methylphosphonyl enzyme to methylphosphonate enzyme were performed to determine the degree of parallelism between the rates of aging and dealkylation in the case of Soman inhibition *in vitro*. In extension, evidence is presented for phosphonylation and dealkylation after poisoning of rats with ^{32}P-Soman *in vivo*.

MATERIALS AND METHODS

Soman of 96% purity was obtained from the Chemical Process Division of these laboratories. ^{32}P-labeled Soman was supplied by the Defense Research Board of Canada; its specific activity at the time of receipt was 10 mc/mmole. Its purification and the preparation of ^{32}P-pinacolyl methylphosphonic acid (^{32}P-PMPA) and of ^{32}P-methylphosphonic acid (^{32}P-MPA) have been previously described.[8]

Sec-butyl alcohol benzene solvent was prepared by mixing one volume of benzene with one volume of secondary butyl alcohol. The solvent was saturated with the appropriate aqueous medium prior to use.

"Acid Tris" solution: nine volumes of 0·01 M buffer were mixed with one volume of 50% trichloroacetic acid and sufficient 10 N HCl to yield a pH of approximately 0·5.

Toxicity

Female rats weighing 120 to 160 g were used throughout. All estimates of the LD_{50} of ^{32}P-Soman and of unlabeled Soman were based upon 24-hr mortalities in groups of six rats given four dose levels having a constant logarithmic increment between successive doses. The values for the LD_{50} were determined by the method of moving averages developed by Thompson[9] and the mortality tables constructed by Weil.[10] The Soman dissolved in 0·85% NaCl solution was injected by the subcutaneous or by the intravenous route in a volume of 2·5 ml/kg.

Cerebral ChE activity

The animals were sacrificed and their brains rapidly removed, washed, drained, and homogenized in sufficient 0·3 M KCl to yield a 2% (fresh weight) homogenate. The ChE activity of these homogenates was determined by colorimetric estimation of acetylcholine remaining after 10-min incubation at 25°. (Conditions: 1 ml homogenate and 1 ml 0·004 M acetylcholine chloride in 0·134 M phosphate buffer at pH 7·4). Details of the colorimetric procedure have been published earlier.[11]

Preparation of tissues from rats injected with ^{32}P-Soman

Immediately prior to sacrifice the rats were injected i.v. with 0·2 ml heparin. Immediately after sacrificing, lungs, liver, and kidneys were promptly perfused *in situ* with sufficient 0·85% NaCl to remove virtually all remaining blood. Brain was excised and blotted to remove all superficial blood vessels and blood. Approximately 1-g portions of each organ were washed three times in 0·85% NaCl, blotted, weighed, and homogenized in H_2O. Trichloroacetic acid was added to a final concentration of 5%, and the sample was allowed to stand in the cold for 20 min. The preparation was centrifuged for 10 min at 3,000 rpm and the supernatant discarded. Two additional

cycles of mixing with 5% TCA, centrifugation, and decantation followed. The TCA residue was then digested on the steam bath with 2 ml of 1 M NaOH; 1-ml aliquots were transferred to planchets and counted in the liquid state with an end-window counter. Known amounts of ^{32}P-Soman were added to planchets containing digests comparable to the experimental samples and counted as standards. From the count corrected for background and the known weight of the tissue, the count per gram of tissue and the corresponding amount of Soman bound to protein per gram of tissue could be calculated.

Calculation of mole fractions of ^{32}P-PMPA and ^{32}P-MPA in phosphonate mixtures derived from protein phosphonylated by Soman

Analytical procedures previously described for characterization and estimation of pinacolyl methylphosphonic acid and methylphosphonic acid after enzymatic hydrolysis of Soman *in vitro*[8] were utilized to obtain the data required for the theoretical treatment, which follows.

The mean partition coefficients (eight trials) for ^{32}P-PMPA and ^{32}P-MPA in sec-butyl alcohol : benzene, 1 : 1, and an equal volume of aqueous medium were found to be $36{\cdot}7 \pm 5{\cdot}5$ and $0{\cdot}053 \pm 0{\cdot}012$ ($P = 0{\cdot}05$) respectively. These values were not significantly changed when determined in the presence of the various tissues used in the studies undertaken in this paper.

Let $A = {}^{32}$P-PMPA and $B = {}^{32}$P-MPA derived from ^{32}P-Soman; let $R = T_o/T_w$ where R is the observed value of the ratio of total counts/min in equal volumes of organic phase, T_o, and aqueous phase, T_w, respectively.

$$T_w = C_A \text{ and } C_B \tag{1}$$

where C_A and C_B represent the concentrations of A and B, respectively, in the aqueous phase.

$$T_o = D_A C_A + D_B C_B \tag{2}$$

where D_A = concentration of A in organic phase/C_A and D_B = concentration of B in organic phase/C_B.

Multiply (1) by D_B

$$D_B T_w = D_B C_A + D_B C_B \tag{3}$$

Subtract (2) from (3)

$$D_B T_w - T_o = D_B C_A - D_A C_A$$

$$D_B T_w - T_o = C_A (D_B - D_A) \tag{4}$$

$$C_A = \frac{D_B T_w - T_o}{D_B - D_A} \tag{5}$$

$$F_A, \text{ the mole fraction of } A, = \frac{C_A + D_A C_A{}^*}{T_w + T_o} \tag{6}$$

* The mole fraction of A is taken as the ratio of the concentrations of solute A in both the aqueous and organic solvent phases (designated as $C_A + D_A C_A$ respectively) to the total solute concentration in both phases, designated by $T_w + T_o$.

and

$$F_A = \frac{C_A\,(1 + D_A)}{T_w + T_o}$$

Substituting for the value of C_A from (5)

$$F_A = \frac{D_B\,T_w - T_o}{D_B - D_A} \times \frac{1 + D_A}{T_w + T_o} \tag{7}$$

Since

$$R = \frac{T_o}{T_w},\ T_o = RT_w \tag{8}$$

Substituting for T_o in (7)

$$F_A = \frac{D_B\,T_w - RT_w}{D_B - D_A} \times \frac{1 + D_A}{T_w + RT_w} \tag{9}$$

Factoring:

$$F_A = \left[\frac{D_B - R}{D_B - D_A}\right] \times \frac{1 + D_A}{(1 + R)}$$

Multiplying numerator and denominator of bracketed portion by -1 and rearranging

$$F_A = \frac{D_A + 1}{D_A - D_B} \times \frac{R - D_B}{R + 1} \tag{10}$$

Substituting the experimentally derived values for D_A and D_B

$$F_A = 1{\cdot}03 \times \frac{R - 0{\cdot}053}{R + 1} \tag{11}$$

The mole fraction of ^{32}P-PMPA in Soman-phosphonylated protein can be derived by determining R at any time after inhibition with this agent, uncombined Soman having first been removed by standing with TCA followed by the centrifugation procedures described in this report.

EXPERIMENTAL AND RESULTS

Aging and dealkylation in vitro

Bovine erythrocyte AChE (Winthrop Laboratories, Inc.), containing 10 mg of enzyme preparation/ml, was incubated with 2×10^{-7} M ^{32}P-Soman in 0·01 M borate buffer at pH 8·8 and 0° for 16 min. Preliminary experiments showed that virtually complete inhibition with minimal aging occurred under these conditions. The preparation was immediately transferred to a thermostated bath at 37° and allowed to equilibrate to this temperature. Samples were taken before, and at suitable time intervals after, rapidly adjusting pH to 7·35 with phosphate buffer (final concentration 0·2 M) to initiate aging. For reactivation, 0·1-ml samples were transferred into 1·9 ml of 9×10^{-2} M monoisonitrosoacetone in 0·05 M phosphate –0·3 M KCl at pH 7·65. Thirty min at 25° was allowed for reactivation. At the end of this time, the samples were diluted tenfold with 0·3 M KCl–0·05 M phosphate buffer, pH 7·65. One ml of this diluted sample was added to 1 ml of 0·004 M ACh in the same KCl–phosphate

buffer solution. The AChE activity was estimated colorimetrically in terms of ACh hydrolyzed in 16 min at 25° as described elsewhere.[11]

Appropriate controls of uninhibited enzyme with the same concentrations of oxime used in the experimental samples were run concurrently. The final concentration of $4{\cdot}5 \times 10^{-3}$ M monoisonitrosacetone present in the ChE activity determination was found to produce little or no inhibition of AChE.

Concurrently, 2-ml samples were taken for study of the rate of dealkylation from the same preparations used above. These were delivered into cold 7·5% TCA within 10 sec of the time at which the samples were added to the monoisonitrosacetone solution above. After 20 min in the TCA solution, 0·1 ml bovine albumin containing 20 mg protein was added as a carrier. Controls containing albumin alone in the absence of ChE enzyme were run concurrently. All samples were centrifuged at 3,000 rpm and the supernatants discarded. The residues were washed twice with 5% TCA, centrifuged, and the supernatants discarded. Three cycles of washing with ether, centrifugation, and decantation followed. The samples were dried and digested with 0·2 N NaOH for 1–2 hr on a steam bath to cleave the phosphorus-containing moiety bound to protein. The pH was then adjusted to approximately 1·0, TCA was again added to 5% concentration, the mixture was centrifuged, and the supernatant collected. The residue was washed twice with "acid Tris" solution, centrifuging after each wash and adding these supernatants to the first. This solution was then extracted with an equal volume of 1 : 1 sec-butyl alcohol : benzene. The phases were separated and clarified by centrifugation and appropriate aliquots were transferred to planchets, dried and counted in an end-window counter.

TABLE 1. AGING AND DEALKYLATION OF PINACOLYL METHYLPHOSPHONYLATED ACHE AT PH 7·35 AND 37°

	Aging*			Dealkylation†		
	Reactivation obtained at zero time (%)	$t_{1/2}$ (min)	Rate constant (min^{-1})	Alkylated enzyme at zero time (%)	$t_{1/2}$ (min)	Rate constant (min^{-1})
	82	2·7	0·26	96	2·7	0·26
	82	2·4	0·29	100	2·4	0·29
	72	1·9	0·37	92	2·3	0·30
	78	2·0	0·35	94	2·6	0·27
	80	2·0	0·35	98	2·1	0·33
mean ± S.D.	78·8 ± 4	2·2 ± 0·3	0·32 ± 0·04	96·0 ± 3	2·4 ± 0·3	0·29 ± 0·03

* Obtained by 30-min incubation in 9×10^{-2} monoisonitroacetone at pH 7·65 and 25°.
† Corrected for 0·02 mole fraction of PMPA not undergoing dealkylation after 24 hr at pH 7·35 and 37° in the bovine AChE preparation used in this study.

From the data obtained, the logarithm of the per cent of reactivated AChE and of the fraction of pinacolyl methylphosphonyl enzyme remaining were plotted as a function of time of aging before sampling (Fig. 1). It may be seen that the data are consistent with first-order kinetics. Four additional trials yielded similar results. The half-times for both aging and the decrease in ^{32}P-PMP–AChE were interpolated from the curves and used to calculate the rate constants shown in Table 1.

Phosphonylation of rat tissues after poisoning with Soman in vivo

The binding of ^{32}P from labeled Soman to rat tissue proteins *in vivo* was studied by injecting 75 μg ^{32}P-Soman/kg i.v. into rats pretreated with 10 mg atropine/kg i.p. After 1 hr, the survivors were sacrificed; the brain, diaphragm, kidney, liver, and lungs were removed and treated as described under Methods. Results for ten rats are shown

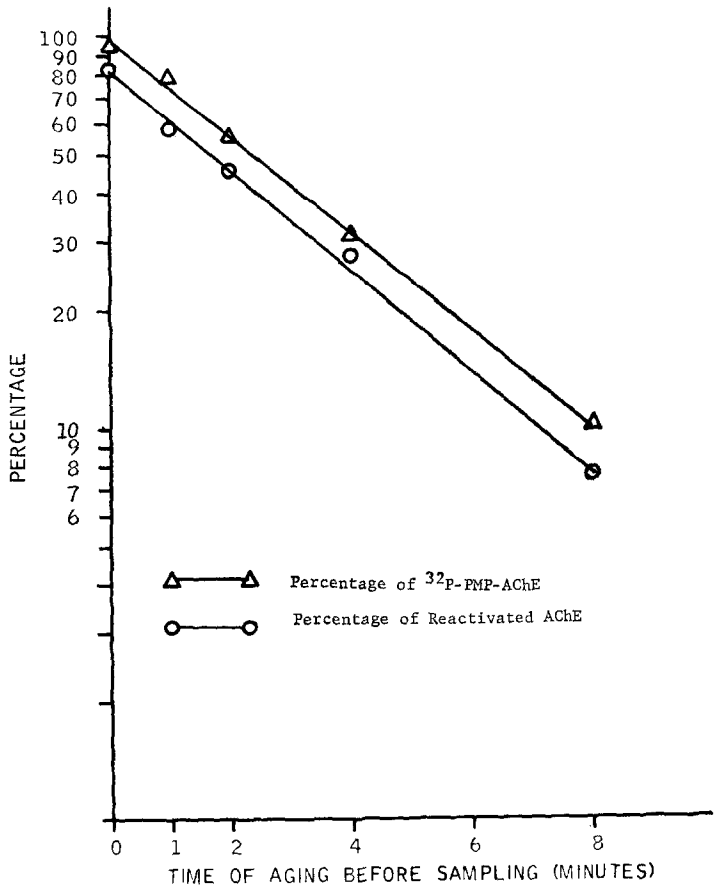

FIG. 1. Decrease in ^{32}P-PMPA derived from ^{32}P-Soman-inhibited AChE and the aging process

in Table 2. The tissues examined may be arranged in the order of increasing amounts of ^{32}P bound, with diaphragm least, followed by brain, liver, kidney and lungs, respectively.

Dealkylation in rat tissue after poisoning with ^{32}P-Soman in vivo

As a preliminary, it was necessary to establish that no further dealkylation of the alkylated phosphonic acid (PMPA) occurred after NaOH cleavage from the enzyme. ^{32}P-PMPA was therefore added to the preparation after NaOH treatment in the presence of each kind of tissue studied, followed by the extraction with sec-butyl

alcohol : benzene; 95 to 100% recovery of ^{32}P-PMPA was obtained indicating that no dealkylation had occurred.

After administration of the compounds used in this study, all tissue samples were processed as described in the Methods section through the washes with TCA. The residues were then washed twice with ethanol, once with ethanol : ether, 3 : 7, and twice with ether, centrifuging after each wash. The dried residue was then digested with 0·1 N NaOH for 3 hr to cleave the phosphonate moiety bound to tissue protein. Subsequent procedure was the same as that used in the *in-vitro* study above.

TABLE 2. BINDING OF ^{32}P FROM SOMAN IN CERTAIN RAT TISSUES

Tissue	Protein-bound, Soman derived ^{32}P (μg/g tissue wet wt $\pm$ P = 0·05 $\times$ 10^3)*
Brain	15·3 $\pm$ 3·4
Diaphragm	13·2 $\pm$ 4·8
Kidney	57·5 $\pm$ 22·4
Liver	27·2 $\pm$ 8·4
Lung	297·8 $\pm$ 52·0

* Mean value for 10 animals.

For investigating dealkylation *in vivo*, 10 mg atropine/kg was injected into rats i.v., followed 20 min later by 75 μg ^{32}P-Soman/kg i.v., a dose equivalent to 1·5 LD_{50} in nonatropinized animals. Surviving animals were sacrificed in groups of eight for each time interval studied. Since Koelle[12] had reported that eserine protected ChE against inhibition by diisopropylfluorophosphate (DFP) *in vitro*, we were led to try the effect of eserine on the PMPA/MPA ratio after poisoning with ^{32}P-Soman. For this the rats were injected with atropine as above: 5 min later they received 1 mg eserine/kg i.v. and 15 min thereafter the same dose of ^{32}P-Soman used in the absence of eserine. The results are shown in Table 3.

TABLE 3. MOLE FRACTION OF PINACOLYL METHYLPHOSPHONIC ACID (PMPA) DERIVED FROM RAT BRAIN AFTER POISONING WITH ^{32}P-SOMAN *IN VIVO**

Time of Sacrifice	Mole fraction PMPA (mean $\pm$ P = 0·05)	
	Without eserine pretreatment	Pretreatment with eserine
1 min	0·70 $\pm$ 0·05	0·92 $\pm$ 0·04
30 min	0·51 $\pm$ 0·05	0·96 $\pm$ 0·03
2 hr	0·54 $\pm$ 0·08	0·96 $\pm$ 0·12
24 hr	0·40 $\pm$ 0·06	0·93 $\pm$ 0·03
72 hr	0·50 $\pm$ 0·10	

* The mole fraction of PMPA is taken as the ratio of the concentrations of solute A in both the aqueous and organic solvent phases (designated as $C_A + D_A C_A$ respectively) to the total solute concentration in both phases, designated by $T_w + T_o$. Eight animals were used for each time interval studied.

It may be noted that approximately 50% decrease in PMPA content of rat brain occurs within 30 min after poisoning with ^{32}P-Soman. No further significant lowering occurred in the time intervals investigated through 72 hr after poisoning. Dealkylation occurred to only a small extent (7%) in the animals receiving eserine prior to ^{32}P-Soman at all time intervals tested (Table 3).

To determine whether the PMPA content would vary with the tissue studied, the mole fraction of PMPA in rat brain was compared with that present in liver and lung in animals sacrificed 30 min after the injection of ^{32}P-Soman. Table 4 shows the values for PMPA content in liver and lung to be much higher than the corresponding value for rat brain from the eight animals sacrificed 30 min after injection of ^{32}P-Soman.

TABLE 4. MOLE FRACTION OF PINACOLYL METHYLPHOSPHONIC ACID (PMPA) IN RAT BRAIN, LIVER, AND LUNG 30 MIN AFTER POISONING WITH ^{32}P-SOMAN *IN VIVO**

Tissue	Mole fraction PMPA (mean ± P = 0·05)
Brain	0·51 ± 0·05
Liver	0·93 ± 0·02
Lung	0·95 ± 0·02

* See footnote to Table 3.

Effects of eserine pretreatment on toxicity and on cerebral ChE activity after Soman poisoning

Pretreatment with eserine resulted in a marked decrease in dealkylation after Soman poisoning in rats. The altered ratio of PMPA could have resulted from temporary binding of ChE sites by eserine, owing to its reversible anticholinesterase action,[12] while Soman is bound to other tissue components which do not dealkylate. To test this, a study of the effect of eserine pretreatment on toxicity and on cerebral ChE activity in rats poisoned with Soman was performed. For this the LD_{50} of Soman was determined by the subcutaneous route, with and without eserine pretreatment, in atropinized rats. Results, given in Table 5, show that i.v. injection of

TABLE 5. PROTECTIVE EFFECTS OF ATROPINE ALONE OR WITH ESERINE ON THE TOXICITY AND CHE ACTIVITY AFTER SOMAN POISONING IN RATS*

Prophylaxis prior to Soman	Subcutaneous LD_{50} (P = 0·05) (mg/kg)	Cerebral ChE activity†	
		AChE hydrolyzed (P = 0·05) (μmoles)	Relative activity (%)
Atropine	0·083 (0·079–0·087)	0·29 (0·26–0·32)	15·4
Atropine followed by eserine	0·32 (0·30 –0·34)	1·10 (1·06–1·14)	58·5

* Atropine injected i.v. at 10 mg/kg; eserine injected i.v. at 1·0 mg/kg.

† ChE activity measured in 10 surviving animals 24 hr after injection of 1·5 LD_{50} (124 μg/kg) of Soman s.c. for each treatment studied.

† Based upon a control value of 1·88 μmoles of ACh hydrolyzed by rat brain homogenates from normal animals.

1 mg eserine/kg 15 min prior to poisoning elevated the LD_{50} of Soman 3·8 times. In addition, the protective effect of eserine was demonstrated by the finding that animals receiving atropine and eserine followed by s.c. injection of 124 μg Soman/kg (1·5 LD_{50}) were asymptomatic 24 hr later and retained 58·5% of normal ChE activity. Rats receiving atropine alone prior to Soman suffered 50% mortality. The survivors retained only 15·4% of normal ChE activity (Table 5).

DISCUSSION

The hypothesis of Berends *et al.*[6] that aging closely parallels the decrease in the alkylated fraction of DFP-inhibited butyrocholinesterase appears basically applicable to conditions prevailing with Soman-inhibited AChE *in vitro*. This conclusion is supported by the virtually parallel slopes of the first-order curves for aging and for the decrease in pinacolyl methylphosphonyl enzyme as a function of time (Fig. 1). An analysis of variance for the difference of the mean rate constants for both processes shown in Table 1 yields a Students t of 0·009. The tabulated t value required for a significant difference to exist between the slopes of the paired curves for each of the five trials is approximately 2·02, based upon 95% confidence limits.[13] It appears reasonable to conclude that we are dealing with essentially parallel phenomena.

The percentage of reactivatable enzyme was always found to be consistently less than the proportion of alkylated enzyme for every time interval studied, including the zero-time sample (Fig. 1, Table 1). This discrepancy is probably accounted for by our observation that several minutes are required for the 9×10^{-2} M monoisonitrosacetone to reactivate the AChE phosphonylated by Soman. During this time, the unreactivated portion is aging. (It is estimated that at the temperature of 25° and a pH of 7·65 used for incubation with monoisonitrosacetone, that the half-time for aging would be 15 min in the absence of oxime.*) In contrast, the AChE in the corresponding sample taken for study of the rate of dealkylation is very rapidly denatured upon mixing with TCA, so that further change in the relative proportion of alkylated phosphonylated enzyme is prevented. The content of PMPA based upon this technique would always be greater than the amount of reactivatable enzyme (Table 1) because of the time lag inherent in the reactivation process as described above.

It is likely that similar studies with anticholinesterases having slower rates of aging[6, 14–16] would yield smaller differences between the relative amounts of reactivatable enzyme and of nondealkylated phosphorylated ChE under the conditions used in this report. On the other hand, the use of lower concentrations of oximes in *in vitro* studies probably would increase the difference in time between cessation of dealkylation by TCA denaturation and maximal reactivation of a rapidly aging phosphorylated enzyme.

It is of interest that dealkylation, as measured by the decrease of alkylated phosphonate bound to brain tissue, progressed to approximately 50% in animals poisoned with ^{32}P-Soman (Table 3). Since no additional significant dealkylation of the phosphonylated protein in brains from Soman-poisoned rats occurred between 30 min and 72 hr after injection of Soman, 50% is thought to represent the maximal dealkylation that occurs in rat brain under the conditions used. The existence of stereoisomers of Soman raises the possibility that differences in dealkylation for the various isomers might account for the observation that only 50% dealkylation was obtained in the rat brain preparations after Soman poisoning. However, the finding that the same preparation of ^{32}P-Soman, when used to phosphonylate the purified AChE preparation, underwent 98% dealkylation (see second footnote to Table 1) seems to exclude this possibility. It appears more likely that the remaining alkylated fraction is bound to brain aliesterase[17] or to nonspecific sites other than brain ChE.

* J. H. Fleisher and L. W. Harris, unpublished observation.

A high mole fraction of alkylated phosphonic acid was obtained without eserine pretreatment from liver and lung tissue derived from rats poisoned with ^{32}P-Soman (Table 4). In the case of liver, this probably is due to the high liver aliesterate content,[17] and in the case of lung to a relatively high nonspecific binding capacity[18] (Table 2). The high proportion of ^{32}P-PMPA of rat liver tissue after poisoning with ^{32}P-Soman is also consistent with the observation of Oosterbaan *et al.*[19] that the phosphoryl residue of liver aliesterase inhibited by DFP does not undergo dealkylation.

The contribution of ^{32}P binding to sites other than AChE to the level of non-dealkylating phosphonylated protein noted with rat brain after Soman poisoning is also supported by the results in Table 5. Here the addition of eserine to prophylaxis with atropine is seen to protect cholinesterase enzyme in the brain of the rat from irreversible inhibition by Soman. This finding, considered along with those in Table 3 wherein pretreatment with eserine is shown to prevent dealkylation of phosphonylated protein in brain, suggests that blockage of reaction between ChE and Soman by prior occupation of the active site of the enzyme by eserine directs the phosphonylating agent to other non-ChE proteins. Furthermore, the findings that only about 6% of the phosphonylating moiety is dealkylated (Table 3) in brains in which 41·5% of the ChE is inactivated in the presence of eserine (Table 5), whereas about 50% of the phosphonylating moiety is dealkylated in brains in which 84·6% of the ChE is inactivated in the absence of eserine, indicate the importance of active ChE in the dealkylation of the phosphonyl residue attached to proteins including ChE.

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