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De novo synthesis of acetylcholinesterase in guinea pig retina after inhibition by pinacolyl methylphosphonofluoridate

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EFFECTIVE chemical treatment of cholinergic poisoning by organophosphorus anticholinesterases requires the injection of atropine and an oxime such as pyridinium 2-aldoxime methochloride (2-PAM).[1] The therapeutic contribution of the oxime depends upon its capacity for nucleophilic displacement of the organophosphate moiety attached to inhibited cholinesterase,[2] resulting in restoration of enzyme activity.[3,4] However, acetylcholinesterase (AChE) inactivated by a number of these organophosphates, including the pesticides malathion and diazinon, becomes progressively resistant to reactivation by oximes.[5,6] This effect has been termed "aging".[7,8] Phosphorylated butyrylcholinesterase (BuChE) is also subject to aging.[7] The conversion of BuChE inhibited by diisopropyl phosphorofluoridate (DFP) from a reactivatable to a nonreactivatable condition was shown to depend upon the loss of one isopropyl group from the initially diisopropylphosphorylated enzyme.[9] The loss of the alkyl constituent (dealkylation) promotes a negative charge on the phosphonate bound to the esterase, resulting in resistance to nucleophilic attack by oximes whose active species is oximate anion.[10]

For brain AChE inhibited with ^{32}P-isopropyl methylphosphonofluoridate (sarin), Harris *et al.*[11] showed that the portion of the enzyme which could be reactivated *in vitro* by 2-PAM approximated the percentage of phosphorus released as isopropyl methylphosphonate. The phosphorus retained by the enzyme after incubation with oxime was present entirely as ^{32}P-methylphosphonate and paralleled the proportion of the enzyme not reactivated. The close correlation between the percentage of aged, inhibited enzyme and the per cent of labeled phosphonate retained as methylphosphonate suggested that the latter measurement distinguished phosphorus bound to aged AChE from that bound to other sites.

Pinacolyl methylphosphonofluoridate (soman), like DFP and sarin, phosphonylates both AChE and nonspecific sites.[12] It differs from DFP and sarin in producing an inhibited AChE which is only slightly reactivated by oximes.[13] In explanation, Fleisher and Harris[12] showed that AChE phosphonylated by ^{32}P-soman *in vitro* undergoes rapid decrease in reactivatability in parallel with loss of a pinacolyl group ($t_{\frac{1}{2}}$ = 2·4 min). After aging was completed, the radioactive phosphorus bound to AChE was present exclusively as methylphosphonate.[12] Similar results have been obtained by other laboratories.[14,15] In addition, Coult and Marsh[14] found that the soman-derived methylphosphonate bound to AChE underwent no significant diminution *in vitro* for 72 hr after inhibition.

The rapid aging of inhibited AChE[12] and refractoriness to treatment by oximes[13] characteristic of soman intoxication suggested that this organophosphate could be used to elucidate the mechanism of return of AChE activity which may apply to these conditions.

Since dephosphorylation has been shown to contribute to the recovery of AChE after sarin intoxication,[16] this organophosphate has been included in part of the present study for comparative purposes. The retina, which embryologically as well as functionally is part of the central nervous system,[17] was chosen since one retina could provide a control for subsequent measurements on the contralateral retina of the same animal.

As a preliminary to the studies *in vivo*, the rate of aging of guinea pig retinal AChE after inhibition by soman was estimated by methods previously published.[11,12] The half-time for aging (three preparations) at pH 7·4 and 37° was found to be 6·2 ± 0·7 min.

The parallelism between the rates of aging and dealkylation[11,12,14,15] suggested that soman-derived phosphorus bound to retinal AChE should be almost completely converted from pinacolyl methylphosphonate to methylphosphonate 30 min after giving labeled soman *in vivo*.

This possibility was tested by injecting guinea pigs with 16 mg/kg of atropine intramuscularly followed by 32 μg/kg of ^{32}P-soman subcutaneously (approximately 1·2 LD_{50}). At 0·5 hr after injecting the organophosphate, the guinea pigs were anesthetized and one retina from each of three animals was immediately removed as described[18] and sonicated in 2 ml of ice-cold 0·01 M borate buffer at pH 8·8 to minimize further aging.[12] Sonicate, 0·9 ml, was incubated with 0·1 M 2-PAM for 1 hr at 37°, a concentration of oxime sufficient to release completely inhibitor-derived phosphorus from unaged phosphonylated AChE.[11] An equal volume of sonicate was incubated without oxime as a control. Each preparation was treated with trichloroacetic acid (TCA) to 5 per cent concentration and with one to two drops of concentrated HCl so as to yield a pH approximating 0·5. Carrier bovine albumin, 10 mg, was added and the samples were centrifuged at 2000 rev/min for 10 min; the supernatants were

removed and the ^{32}P was measured. No radioactivity attributable to incubation with 2-PAM was found, indicating that aging was complete. The radiophosphorus remaining in the residues after TCA precipitation and removal of the supernatant was measured as previously described.[12] No difference was detectable in the methylphosphonate content of the residues from oxime-treated sonicates and those incubated in buffer alone. This observation is consistent with no displacement of radiophosphorus into the supernatant after incubation of the retinal sonicates with 2-PAM and with earlier findings that oximes were ineffective in removal of the phosphonyl moiety from the inhibited enzyme after dealkylation.[9,11,12]

The AChE activity of the original sonicate was obtained by diluting 0·1 ml of the sonicate to 2·5 ml with 0·1 M sodium phosphate buffered at pH 7·8 and containing 1% Lubrol WX* and 0·3 M NaCl. One-tenth ml of 3×10^{-2} M acetyl β-methyl-l-^{14}C choline iodide (^{14}C–MCh) was added to 0·9 ml of the diluted sonicate and incubated at 37° for 20 min (enzyme activity was linear with time under these conditions). The unreacted substrate was removed by Amberlite CG-120 resin suspended in dioxane. The supernatant solution containing the product of hydrolysis, the free acid-l-^{14}C, was counted in a liquid scintillation spectrometer. Details concerning the preparation of the resin and the scintillation mixture (fluor) are given by Siakotos *et al.*[19]

The results in Table 1 show that no dephosphorylation in the soman-derived methylphosphonate bound to retinal AChE occurred between 0·5 and 24 hr in surviving animals after intoxication with ^{32}P-soman. Measurements of the AChE activity in aliquots of the retinal tissue from the intoxicated animals were compared with those from unpoisoned controls to give the per cent residual AChE

TABLE 1. BINDING OF RADIOPHOSPHORUS AND INHIBITION OF RETINAL ACETYLCHOLINESTERASE ACTIVITY IN GUINEA PIGS INTOXICATED WITH ^{32}P-SOMAN

Time after soman (hr)	No. of preparations	^{32}P bound* (μg/retina $\times 10^4$)	Inhibition† (%)
0·5	9	0·95 (0·82–1·08)‡	97·2 (95·8–98·6)‡
24	9	0·98 (0·88–1·08)	82·5 (78·5–86·5)

* Measured as ^{32}P-methylphosphonate.[12]

† Based upon a value of 3·13 μmoles acetyl-l-^{14}C-beta-methylcholine hydrolyzed per sonicated retina per hour from normal guinea pigs.[18]

‡ The 95% confidence limits are in parentheses.

activity. The per cent inhibition, taken as equal to 100 per cent minus the per cent residual enzyme activity, is shown in Table 1. A significant decrease in the inhibition of retinal AChE was obtained between 0·5 and 24 hr after the injection of the labeled soman. The regeneration of AChE activity in the retinas of the experimental animals in the absence of dephosphorylation suggests that synthesis of new enzyme protein may have occurred.

The possible contribution of *de novo* synthesis of AChE to the return of enzyme activity and survival was evaluated by giving cycloheximide, an inhibitor of protein synthesis,[20] to guinea pigs poisoned with soman or sarin. The cycloheximide was injected intraperitoneally as follows: 5 mg/kg 1 hr before injecting the organophosphate; 5 mg/kg at 0·5 hr after giving soman or sarin; and 2·5 mg/kg at 2, 3, 4 and 5 hr after organophosphate intoxication. The animals were injected with 16 mg/kg of atropine intramuscularly followed by a subcutaneous injection of 32 μg/kg of soman or 50 μg/kg of sarin (approximately 1·2 LD_{50}). Atropinized controls given cycloheximide alone (LD_{50} = 237 ± 69 mg/kg; P = 0·05) without organophosphorus treatment, and organophosphate without cycloheximide, were run concurrently.

The values for the AChE activity of the intact retinas removed at 0·5 hr after intoxication with soman or sarin were compared with those of the contralateral retinas obtained 6 hr after giving the organophosphates. These values and per cent survival over 24 hr are given in Table 2. Our results indicate that the AChE activity of intact retinas recovered significantly (P = 0·05) in surviving animals between 0·5 and 6 hr after poisoning with either organophosphorus compound. However, AChE

* Obtained from I.C.I. Organics Inc., Stanford, Conn.

recovery was significantly greater ($P = 0.05$) in retinas from sarin-poisoned (24·6 per cent) than from soman-poisoned (16·7 per cent) guinea pigs when the enzyme activities obtained at 6 hr were referred to the corresponding control value of 303 mμmoles of ^{14}C-acetyl-β-methylcholine hydrolyzed per retina per hr (Table 2).

TABLE 2. EFFECTS OF CYCLOHEXIMIDE TREATMENT ON RETINAL ACETYLCHOLINESTERASE AND SURVIVAL IN GUINEA PIGS INJECTED WITH SOMAN OR SARIN

Compounds injected*	Retinal AChE activity (μmoles ^{14}C-MCh × 10^3/retina/hr) Left retina (0·5 hr after poisoning)	Right retina (6 hr after poisoning)	Survival† (%)
Atropine and soman	9·5 (6·0–13·0)‡	50·7 (44·8–56·6)‡	90
Atropine and sarin	5·6 (0·0–11·2)	74·3 (58·6)–90·0)	100
Atropine, soman and cycloheximide	5·1 (1·9–8·3)	24·1 (18·6–29·6)	30
Atropine, sarin and cycloheximide	3·1 (0·0–6·2)	44·7 (35·0–54·4)	80
Atropine and cycloheximide	372·0 (317·1–429·9)	328·0 (283·6–372·4)	100
Atropine		303·0 (272·8–323·2)	100

* Atropine was injected i.m. at 16 mg/kg; 32 μg/kg of soman or 50 μg/kg of sarin was injected subcutaneously; and 20 mg/kg of cycloheximide was injected i.p.

† Based upon 24-hr survival of 10 animals.

‡ The 95% confidence limits are in parentheses.

Without cycloheximide, the controls showed 90 and 100 per cent survival 24 hr after injecting soman and sarin respectively. Cycloheximide alone caused no deaths; however, when given to sarin-poisoned guinea pigs, the recovery of AChE was reduced to 13·7 per cent of the cycloheximide control, and survival to 80 per cent (Table 2). With soman, cycloheximide reduced AChE recovery to 7·3 per cent and survival to 30 per cent. The greater recovery of retinal AChE and lesser susceptibility of sarin-poisoned guinea pigs to cycloheximide are consistent with an additional mechanism for recovery, i.e. dephosphorylation, which appears to be independent of *de novo* synthesis. However, in soman-poisoned guinea pigs, the return of AChE activity and survival appears strongly related to synthesis of new enzyme protein.

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Species and strain differences in the epimerization of 3β, 17β-dihydroxy-17α-ethynyl-$\Delta^{5(10)}$-estrene to the 3α-hydroxy epimer*

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THE CONVERSION of 3β,17β-dihydroxy-17α-ethynyl-$\Delta^{5(10)}$-estrene to 3α,17β-dihydroxy-17α-ethynyl-$\Delta^{5(10)}$-estrene, both intermediary metabolites of norethynodrel (17α-ethynyl-17β-hydroxy-$\Delta^{5(10)}$-estrene-3-one), is catalyzed by a 3β-hydroxy-$\Delta^{5(10)}$-steroid epimerase found in the soluble fraction of rat liver.[1] Since much new information has been published on the effects of drugs on different strains of the same species and on different species of animals,[2,3] it is of great importance that all the biochemical pathways of each species be determined, especially if it is unique to one species. The importance of species differences in drug metabolism is shown by the number of recent reviews[4–6] and symposia[7,8] correlating species differences in drug metabolism with the mechanism of action of many drugs.

A study was undertaken to determine whether the 3β-hydroxy-$\Delta^{5(10)}$-steroid epimerase was unique to the Charles River rat, CD strain, in which it was originally found,[1] or whether it exists in other rat strains, in other rodent species commonly used in laboratory experimentation, and in man. In addition, the Michaelis constant (K_m) for the epimerization reaction and the inhibitor constant (K_i) for the 3α-hydroxy epimer were determined.

NADP was purchased from Sigma Chemical Co. The animals and their source follow: Golden Syrian Hamster, Petterson Hamstery; New Zealand White Rabbit, Franklin's Rabbitry; Mongolian Gerbil, Chick Line Co., Guinea Pig, Hartley strain, Flow Laboratories; CF-1 mouse, Carworth Farms; Charles River CD strain rat, Charles River Breeding Laboratory; Blue Spruce Farms hood rat (LE); Blue Spruce Farms Sprague–Dawley rat (SD); Holtzman rat; Carworth CFN rat; Carworth CFE rat. The 3β,17β-dihydroxy-17α-ethynyl-$\Delta^{5(10)}$-estrene was synthesized by Dr. Ivy Carroll of this laboratory's organic synthesis group using the method of Palmer *et al.*[9]

Two young adult male and two young adult female animals from each rodent species or strain were used in this study. The animals were killed by decapitation, the livers quickly removed and a 1-g section from each liver was homogenized in 10 ml of 0·1 M potassium phosphate buffer, pH 7·4, using a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 10,000 *g* for 20 min. The supernatant obtained by centrifuging the 10,000 *g* supernatant in a Beckman L2-65B at 105,000 *g* for 60 min was used as the enzyme source.

All incubations were performed in duplicate. A ratio of 1 mg of the 3β-hydroxy steroid substrate per gram animal liver was consistently used in all experiments except in those incubations used to determine the kinetic constants. Thus, 1 mg of substrate dissolved in 0·5 ml ethanol and 1·0 mM NADP were incubated with the 105,000 *g* supernatant from 1 g rat liver for 20 min in a 50-ml Erlenmeyer flask (total fluid vol. 10 ml) using a Dubnoff metabolic shaker (37°). The reaction was

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