Biochemical Pharmacology, Vol. 33, No. 4, pp. 683–685, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00
Pergamon Press Ltd.

SHORT COMMUNICATIONS

Sodium pentobarbital alteration of the toxicity and distribution of soman (pinacolyl methylphosphonofluoridate) in mice*

(*Received* 14 *February* 1983; *accepted* 4 *August* 1983)

Soman (pinacolyl methylphosphonofluoridate) is an extremely toxic organophosphorus anticholinesterase. In mice, lethal poisoning is characterized by salivation, diarrhea, intense tremors and convulsions and finally death due to anoxia, the result of respiratory arrest. Soman poisoning is treated most effectively using a combination of atropine and either bispyridinium oximes such as HI-6 [1, 2] or pretreatment with a carbamate anticholinesterase such as pyridostigmine [3, 4].

Previous investigators reported that anticholinesterase poisoning enhances the action of barbiturates *in vivo* [5–7]. Recently, Clement [8] showed that the toxicity of soman could be reduced by sub-chronic treatment with a barbiturate, sodium phenobarbital. The purpose of this investigation was to determine the effect of anesthesia with another barbiturate, sodium pentobarbital, on the toxicity of soman in mice.

Materials and Methods

Toxicology. Male CD-1 mice (25–30 g) were obtained from Canadian Breeding Farms and Laboratories Ltd., St. Constant, Quebec, Canada. The animals were acclimatized for at least 1 week following their arrival at Defence Research Establishment Suffield prior to use. Animals had food and water *ad lib.*

All drugs were dissolved with 0.9% saline and injected immediately after preparation. Sodium pentobarbital was injected intraperitoneally (i.p.), and soman was injected subcutaneously (s.c.). The injection volume in all cases was 1% of body weight. Ten animals per dose and at least five different doses were used in constructing the LD_{50} curves. Twenty-four hour LD_{50} determinations and 95% confidence limits were calculated by the method of Litchfield and Wilcoxon [9].

Acetylcholinesterase determination. Acetylcholinesterase activity was determined by the method of Siakotos *et al.* [10], as modified by Clement [8]. All incubations were carried out at 37°.

Aliesterase determination. Plasma aliesterase activity was determined according to the procedure outlined by Clement [8].

Materials. Soman was obtained from the Organic Chemistry Group, Defence Research Establishment Suffield. The soman was 97–99% pure. Fresh soman solutions were prepared immediately before use by diluting the neat material in 0.9% saline to the required concentration. Sources of other drugs used were as follows: sodium pentobarbital (Nembutal; Abbott Laboratories); tributyrin (Fisher Scientific); [^{14}C]acetylcholine iodide (4.0 mCi/mmole; New England Nuclear); and atropine sulfate (British Drug House).

Statistics. Significance of difference between means was examined by Student's *t*-test. Significance of difference in toxicology data was examined using the potency ratio.

Results and Discussion

Anesthesia with sodium pentobarbital increased the toxicity of soman in mice. Following an i.p. injection of sodium pentobarbital (70 mg/kg), mice lost their righting reflex within 146 ± 10 sec ($\bar{x}$ ± S.D.; N = 15). Within 15 min of receiving sodium pentobarbital, the mice were injected with various doses of soman. Sodium pentobarbital pretreatment increased the toxicity of soman significantly ($P < 0.05$) as evidenced by the decrease in the s.c. soman LD_{50} value from 136 (129–142; 95% limits) μg/kg in control unanesthetized mice to 78 (67–90) μg/kg in sodium pentobarbital-pretreated mice.

The results presented in Table 1 demonstrate that sodium pentobarbital inhibited plasma and erythrocyte acetylcholinesterase as well as inhibiting plasma aliesterase. The nature of the sodium pentobarbital-induced increase in soman toxicity is perhaps the result of sodium pentobarbital occupying sites to which soman would normally be attached. If binding sites for soman *in vivo* are already occupied by sodium pentobarbital, then the plasma concentration of free soman may be increased and the distribution of soman may be altered. This was evident in Table 2 where in sodium pentobarbital-pretreated mice there was a significant increase in soman-induced inhibition of brain acetylcholinesterase. A similar effect of sodium pentobarbital pretreatment on inhibition of diaphragm acetylcholinesterase by soman was not evident. Due to the high lipid content of the brain and the lipophilic nature of soman combined with the large blood flow to the brain, it is not unreasonable to expect an increase in the amount of soman in the brain which is reflected in an increase in the inhibition of acetylcholinesterase in the brain over the diaphragm. Anesthetics such as ketamine and pentobarbital increase the permeability of the blood–brain barrier [12]. Since soman is lipophilic and is not excluded from the brain, an increase in permeability of the blood–brain barrier would not account for the significant increase in soman inhibition of brain acetylcholinesterase following pentobarbital pretreatment.

Sodium pentobarbital inhibited plasma and erythrocyte acetylcholinesterase and plasma aliesterase (Table 1). Sodium pentobarbital was reported previously to affect acetylcholinesterase activity in the rat [13]. The obvious question is which enzyme is the major one responsible for altering the distribution and toxicity of soman. Inhibition of plasma cholinesterase only by iso-OMPA (tetraisopropyl pyrophosphoramide) has no effect on the toxicity of soman in mice, and inhibition of acetylcholinesterase by pyridostigmine greater than that observed here did not affect soman toxicity.† Evidence from this laboratory and others [14–17] has shown that plasma aliesterase is a soman depot [18] or a site of attachment (and thus detoxification) for a very large amount of soman which is administered to the animal [19]. Myers [11] concluded that only 5% of the

* Suffield Report No. 336.

† Iso-OMPA (0.25 mg/kg, i.v.) inhibited plasma cholinesterase approximately 85% while having no significant effect on either plasma or liver aliesterase or erythrocyte acetylcholinesterase. Following pretreatment with iso-OMPA (0.25 mg/kg, i.v.) the soman LD_{50} was 138 mg/kg, s.c. Pyridostigmine (1.25 mg/kg, i.v.), a carbamate anticholinesterase which does not inhibit aliesterase, inhibited diaphragm acetylcholinesterase 57% yet soman toxicity was not altered (J. G. Clement, unpublished observations).

Table 1. Effect of anesthesia induced by sodium pentobarbital on plasma and erythrocyte acetylcholinesterase and plasma aliesterase activity*

	Acetylcholinesterase†				Aliesterase†	
Group	Plasma	P	Erythrocyte	P	Plasma	P
Control	1131 ± 61‡	<0.05	261 ± 15	<0.02	2019 ± 101	<0.05
Pentobarbital	961 ± 39		199 ± 11		1617 ± 111	

* Mice were injected i.p. with sodium pentobarbital (70 mg/kg). Fifteen minutes later, when the mice were deeply anesthetized, they were decapitated, and blood was collected.

† Units of activity: plasma acetylcholinesterase, nmoles acetylcholine hydrolysed · (ml plasma)$^{-1}$ · min^{-1}; erythrocyte acetylcholinestase, nmoles acetylcholine hydrolysed · (ml erythrocytes)$^{-1}$ · min^{-1}; plasma aliesterase, nmoles tributyrin hydrolysed · (ml plasma)$^{-1}$ · min^{-1}.

‡ Mean ± S.E.M.; N = 5–10 observations.

Table 2. Effect of sodium pentobarbital pretreatment on soman inhibition of brain and diaphragm acetylcholinesterase*

	Acetylcholinesterase (nmoles · mg^{-1} · min^{-1})	
Treatment	Brain	Diaphragm
Control	11.60 ± 0.25†	1.45 ± 0.11
Atropine + pentobarbital	11.06 ± 0.28	1.34 ± 0.12
Atropine + soman	3.97 ± 0.34	0.62 ± 0.11
Atropine + pentobarbital + soman	2.85 ± 0.70‡	0.72 ± 0.06

* Atropine (17.4 mg/kg) or atropine (17.4 mg/kg) + sodium pentobarbital (70 mg/kg) were administered i.p. 15 min before soman (100 μg/kg; s.c.). One hour after soman administration the mice were decapitated and exsanguinated, and brain and diaphragm homogenates were prepared. Atropine pretreatment protected the mice against the lethal effects of this dose of soman. Atropine (17.4 mg/kg; i.p.) pretreatment only increases soman LD_{50} by a factor of 1.42 [19].

† Mean ± S.D.; N = 4–6.

‡ Significantly different ($P < 0.02$) from atropine + soman group.

lethal dose of sarin (isopropyl methylphosphonofluoridate) administered to an animal is actually used to inhibit acetylcholinesterase. The majority of the administered dose is bound to aliesterase.

Since the early 1950's, it has been assumed that the cause of respiratory arrest produced by soman and other extremely toxic organophosphorus poisons was primarily due to central respiratory depression (for references see Ref. 20). However, De Candole *et al.* [21] stated that "the detailed picture varies with the species studied, the drug used and the dosage administered". All of these early experiments were performed in anesthetized animals. Based on the results of this study, it is possible that the results and the subsequent conclusions of the authors were correct for anesthetized animals, but would not be truly representative of what was actually occurring in the acutely poisoned unanesthetized animal. It appears that the altered distribution of soman produced by pretreatment with a barbiturate (this study) which enhanced the inhibition of brain acetylcholinesterase would favor an effect of soman on the central nervous system. This combined with the depressant effect of barbiturates on the respiratory centre [22] would favor a central effect which may not be representative of the situation in the unanesthetized animal.

In light of the results of this study, the question of the contribution of central and peripheral mechanisms involved in respiratory depression produced by soman and other organophosphates needs to be re-examined.

Acknowledgements—Ms. J. D. Shiloff provided technical assistance. Mrs. L. Wall decyphered the first draft and typed the final manuscript.

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Biochemical Pharmacology, Vol. 33, No. 4, pp. 685–687, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

The interaction of rat liver mitochondrial monoamine oxidase with clorgyline plus *d*-amphetamine

(*Received* 13 *June* 1983; *accepted* 15 *September* 1983)

Copeland *et al.* [1] have reported clorgyline to promote the binding of a spin-labelled amphetamine derivative to the soluble fraction of monoamine oxidase (MAO) activity from rat liver. This enhancing effect was found to occur rapidly in a time-scale corresponding to the initial non-covalent interaction between clorgyline and the enzyme. This initial interaction has been shown to be competitive with respect to the substrate and selective towards the A-form of MAO [2, 3]. *d*-Amphetamine has also been shown to be a competitive inhibitor with selectivity towards MAO-A [4], but the apparent synergism reported by Copeland *et al.* [1] would imply that both these compounds should be able to bind to the enzyme simultaneously. Since the ability of one inhibitor to enhance the binding of another could have important consequences for the therapeutic use of mixtures of inhibitors we have investigated the phenomenon using *d*-amphetamine and clorgyline as reversible inhibitors of rat liver mitochondrial MAO.

Materials and methods

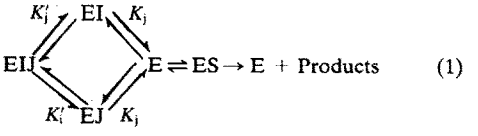
Rat liver mitochondria, prepared as previously described [5] and stored frozen, were used as the source of the enzyme. For studying the activity towards MAO-A the B-form was inhibited by preincubating samples of the mitochondria for 60 min at 37° with 3×10^{-7} M *l*-deprenyl [5]. The mitochondria were then extensively washed by sedimentation and resuspension to remove any excess inhibitor. For studies on the activity towards MAO-B, the activity of the A-form was inhibited by treatment in a similar manner with 10^{-7} M clorgyline [5]. Assay of the preparations treated in these ways indicated that no detectable activity of the susceptible form remained whereas the activity of the remaining form had not been significantly affected.

Monoamine oxidase activity was determined at 37° by a modification [6] of the method of Otsuka and Kobayashi [7] using 100 μM 5-hydroxytryptamine-(side-chain-2-^{14}C) creatinine sulphate as a substrate for MAO-A and 10 μM 2-phenethylamine-[ethyl-1-^{14}C] hydrochloride as a substrate for MAO-B. In all cases the assay was initiated by adding the enzyme sample to the buffer, substrate, inhibitor mixture so that only the initial phase of inhibition was determined. Care was taken to restrict the time of the assay to the period where product formation was a linear function of time so that no significant time-dependent, irreversible, inhibition by cloryline was occurring.

The radioactive substrates were obtained from Amersham International p.l.c. Amersham, U.K. Clorgyline hydrochloride and *l*-deprenyl hydrochloride were kind gifts from May & Baker, Dagenham, Essex, U.K. and from Professor J. Knoll, Department of Pharmacology, Semmelweis University of Medicine, Budapest, Hungary, respectively. *d*-Amphetamine was a kind gift from Smith, Kline & French Ltd., Welwyn Garden City, Herts, U.K.

Analysis

Since both clorgyline and *d*-amphetamine can bind to the free enzyme as competitive inhibitors but the results suggesting synergism between their binding [1] indicate that they must both be able to bind to the enzyme at the same time, the simplest model that could account for this behaviour would be:



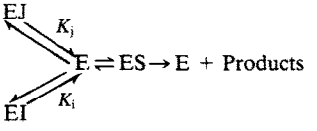
(1)

where I and J represent clorgyline and *d*-amphetamine respectively and K_i, K_j etc. are dissociation constants. This mechanism gives rise to an initial rate equation of the form:

$$v = \frac{V}{1 + \frac{K_m}{[S]}\left(1 + \frac{[I]}{K_i} + \frac{[J]}{K_j} + \frac{[I][J]}{K_iK_j'}\right)} \quad (2)$$

Where v and V are the initial and maximum velocities, respectively, and the square brackets denote concentrations.

Alternatively if the two inhibitors bound to the enzyme at the same site, a mechanism that would not allow for synergism between them, the system could be represented by:



(3)

the initial-rate equation would be

$$v = \frac{V}{1 + \frac{K_m}{[S]}\left(1 + \frac{[I]}{K_i} + \frac{[J]}{K_j}\right)} \quad (4)$$