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PROTECTION OF ANIMALS AGAINST SOMAN (1,2,2-TRIMETHYLPROPYL METHYLPHOSPHONOFLUORIDATE) BY PRETREATMENT WITH SOME OTHER ORGANOPHOSPHORUS COMPOUNDS, FOLLOWED BY OXIME AND ATROPINE

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Abstract—The toxicity of carbamate and some organophosphorus inhibitors of acetylcholinesterase (EC 3.1.1.7) is not reduced by treatment with oximes and atropine. However, the toxicity of such compounds can be greatly reduced by prior administration of some other organophosphorus compounds, followed by oxime and atropine. The latter organophosphorus compounds must be such that their own toxicity is reduced by oxime and atropine. The most effective compound to confer protection when used in conjunction with oxime was ethyl-4-nitrophenyl methylphosphonate, which provided some protection for 48 hr. P2S (2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate) was the only effective oxime of seven examined. A number of cholinolytic drugs were tested in place of atropine, some of which were more effective, both in raising the LD_{50} of the challenging poison, Soman (1,2,2-trimethylpropyl methylphosphonofluoridate) and in mitigating the severity of signs of poisoning. This form of treatment was most effective in the guinea pig, less so in the rabbit, and ineffective in the rat.

POISONING by Soman (1,2,2-trimethylpropyl methylphosphonofluoridate) does not respond to treatment by oximes.[1,2] Pretreatment by carbamates and atropine can be very effective in reducing the lethality of Soman and in hastening the recovery of surviving animals.[1] The protective effect probably depends on temporary inhibition of the excess acetylcholinesterase (EC 3.1.1.7) in vital organs[3,4] during the time that free Soman is present. The protection by carbamates lasts a relatively short time,[1] possibly due to spontaneous decarbamylation of the inhibited acetylcholinesterase. It is apparent that a more prolonged protective effect might be conferred by prolonging the duration of inhibition. One way of doing this would be to administer an organophosphorus compound, followed at a suitable interval by an oxime to reactivate the inhibited enzyme. The organophosphorus compound must be such that inhibition caused by it is readily reversed by oxime. One may thus think of the oxime-responsive organophosphorus compound as conferring a latent protection which is not manifested unless and until the oxime is given: indeed, as will be seen, omission of the oxime actually increases the toxicity of Soman in pretreated animals. For this reason we use the word "protection", in quotation marks, to denote this latent protection without having recourse to circumlocutions.

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Since the effectiveness of carbamates was greatly enhanced by the addition of atropine,[1] the present study has included experiments with it and other cholinolytic drugs.

EXPERIMENTAL

Organophosphorus compounds. The organophosphorus compounds were synthesized in the Chemical Defence Establishment. They were at least 98 per cent pure, by chemical analysis.[5]

Other drugs. The cholinesterase reactivators were also synthesized at C.D.E., except TMB-4, which was obtained from the Aldrich Chemical Co., Inc. The cholinolytics were obtained from various commercial sources, except atropine methanesulphonate, which was prepared in C.D.E. from a commercial sample of atropine base.

The names of the compounds are given in the relevant Tables of results.

Toxicological studies. For most experiments female guinea pigs (Dunkin Hartley pink-eyed whites) (280–360 g) were used. For a few others female rats (180–250 g) (Porton strain albinos) or a mixed strain of rabbits (2–3 kg) were used.

The maximum sign-free doses of various compounds were assessed by observing the effects of graded doses on the gait and muscular coordination of pairs of animals.

In protection studies the animals were given the maximum sign-free dose of organophosphorus compound intramuscularly in a volume of 1 ml/kg of aqueous solution. After a predetermined interval they were poisoned subcutaneously by Soman or other anticholinesterase. Lastly they were given a mixture of cholinolytic and oxime intramuscularly. In earlier experiments the mixture was given at first signs of poisoning, but since these usually developed within 2 min, it was found more convenient to use a fixed interval of 1 min. Details of doses and timing are given in the Tables. The 24-hr mortality was observed and the LD_{50} calculated by the method of moving averages[6] using data from four to six groups of five guinea pigs or rats, or groups of three rabbits.

Since the LD_{50} of Soman to guinea pigs varied from 19·5 to 28 μg/kg during the course of the work, the results are expressed as:

Protective Ratio (PR) = (LD_{50} with treatment):(LD_{50} without treatment). The 95 per cent confidence limits of PR were based on $\pm$ 2 S.D. of the logarithm of the ratio.

RESULTS

In these experiments organophosphorus compounds were used in two ways: first, as potential "protective" drugs, given in sub-toxic doses and used in conjunction with oxime and cholinolytic; and secondly as challenging poisons, with doses in the lethal range. In most experiments TEPP (tetraethyl pyrophosphate) was used as a typical "protective" organophosphorus compound, since it is well known that inhibition of acetylcholinesterase by it is readily reversed by oximes. P2S (2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate) was used as a standard oxime, atropine as the standard cholinolytic and Soman as the toxic agent in most experiments. The effects of varying any one of these compounds at a time have been studied in the experiments reported.

1. *The effectiveness of TEPP as a premedicant*

(a) *Varying the dose of TEPP used with P2S and atropine against Soman poisoning.* An interval of 5 hr was allowed between giving TEPP and poisoning by Soman, in the belief that during this time tissue concentrations of free TEPP would have fallen to negligible levels. Definite signs of poisoning were produced by 0·4 or 0·3 mg/kg of TEPP, but none by 0·2 mg/kg or less. Table 1 shows that the sign-free doses increased

TABLE 1. THE EFFECT OF DOSE OF TEPP ON THE "PROTECTION" OF GUINEA PIGS AGAINST POISONING BY SOMAN (1,2,2-TRIMETHYLPROPYL METHYLPHOSPHONOFLUORIDATE)

Dose of TEPP mg/kg i.m.	Protective ratio and 95 per cent confidence limits	
	Without P2S and atropine	With P2S and atropine
0·4*	—	6·2(4·6–8·3)
0·2	0·53(0·43–0·63)	7·1(4·7–10·3)
0·1	0·62(0·36–0·83)	4·0(2·5–6·3)
0·05	0·79(0·59–1·03)	<2

TEPP was given i.m. 5 hr before Soman, s.c., followed at signs of poisoning by 30 mg/kg of P2S plus 17·4 mg/kg of atropine sulphate, i.m.
* This dose was within the lethal range. To ensure survival the animals were given atropine 10 min before TEPP. Further atropine was given with the P2S after poisoning by Soman.

the toxicity of Soman to guinea pigs, the increase being roughly proportional to the dose of TEPP. When a mixture of 30 mg/kg of P2S and 17·4 mg/kg of atropine sulphate was given as soon as signs of poisoning by Soman were noted, it was found that TEPP had conferred some "protection" at the higher sign-free doses, but the lowest gave no better a PR than P2S and atropine without pretreatment (1·8[1]). In subsequent experiments 0·2 mg/kg of TEPP was used.

(b) *Duration of the effectiveness of TEPP.* Table 2 shows that when TEPP was given only 1 min before Soman, the mixture of P2S and atropine was only slightly more

TABLE 2. VARIATION IN THE "PROTECTIVE" ACTION OF TEPP WITH THE INTERVAL BETWEEN ITS ADMINISTRATION AND THAT OF SOMAN

Interval between TEPP and Soman (hr)	Protective ratio and 95 per cent confidence limits	
	Without P2S and atropine	With P2S and atropine
0·02	—	2·5
0·5	—	7·2(4·7–14·1)
3–4	—	10·5(6·0–18·2)
5	0·53(0·43–0·63)	7·1(4·7–10·3)
16	—	4·9(3·9–6·1)
24	0·60(0·50–0·72)	3·0(1·9–4·8)
72	0·83(0·71–0·98)	2·0(1·6–2·5)

0·2 mg/kg of TEPP was given i.m. At signs of poisoning by Soman 30 mg/kg of P2S plus 17·4 mg/kg of atropine sulphate were given i.m.

effective than if no TEPP had been given. There was marked improvement when TEPP was given 30 min to 5 hr before Soman. Some falling-off was noticeable by 16 hr; and at some time between 24 and 72 hr the protective effect of TEPP was no longer elicited by P2S and atropine. In parallel, during the interval 5–72 hr, the increase in the toxicity of Soman in the absence of treatment with oxime and atropine, declined.

(c) *Varying the interval between Soman and the mixture of P2S and atropine.* When designing experiments to assess the effect of giving the P2S and atropine at varying times relative to Soman, it had to be borne in mind that P2S given between TEPP and Soman would probably reactivate at least some of the TEPP-inhibited acetylcholinesterase, thus lessening the "protective" effect. Since it was already known that physostigmine, given before Soman, could protect against it, and that it seemed to

TABLE 3. THE EFFECT OF P2S AND ATROPINE, WITH AND WITHOUT PHYSOSTIGMINE, WHEN GIVEN AT VARIOUS TIMES RELATIVE TO SOMAN TO GUINEA PIGS PRETREATED WITH TEPP

Time of giving therapy relative to Soman	Protective ratio, and 95 per cent confidence limits, conferred by P2S and atropine	
	No physostigmine	With physostigmine
30 min before	3·8(2·5–5·9)	8·1(4·5–14·8)
10 min before*	6·0(4·1–8·9)*	13·3(8·9–20·9)*
2 min before	10·0(6·6–15·1)	9·6(6·5–14·1)
1 min after	7·1(4·7–10·8)	7·2(4·9–10·7)
* C. effects in animals not pretreated with TEPP, ref. 1:—		
10 min before	1·8(1·1–2·4)	10·8(7·6–18·2)

0·2 mg/kg of TEPP given i.m. 5 hr before Soman. At time stated 30 mg/kg of P2S, 17·4 mg/kg of atropine sulphate given i.m. with or without 0·16 mg/kg of physostigmine sulphate.

act independently of P2S,[1] the present experiments included, for comparison, treatment by a mixture of P2S, physostigmine and atropine. Table 3 suggests that the mixture of P2S and atropine was best given 2 min before Soman, although there are no significant differences among the group between 10 min beforehand and 1 min after. The addition of physostigmine significantly increased the "protective" effect at 30 and 10 min before Soman [t = (difference between logs PR) / (S.D. of difference); $P \sim 0{\cdot}05$] but did not produce any change at 2 min before or 1 min after. The timing of the injection of the supporting drugs was therefore less critical when physostigmine was included.

2. *TEPP, P2S and atropine used against anticholinesterases other than Soman*

As the result of the previous experiments the interval between the dose of TEPP or other organophosphorus compound tried as a "protective" agent and the dose of anticholinesterase poison was standardized at 30 min in this and most subsequent experiments. Oxime and atropine were given 1 min after Soman.

Table 4 shows that TEPP, supported by P2S and atropine, was effective against a

TABLE 4. PREMEDICATION WITH TEPP IN GUINEA PIGS POISONED BY DIFFERENT ANTICHOLINESTERASES

Anticholinesterase *Organophosphorus compounds*	Protective ratio and 95 per cent confidence limits
oxime-responsive	
diethyl 4-nitrophenyl phosphate (paraoxon)	>53
isopropyl methylphosphonofluoridate (Sarin)	24·5(22·5–26·5)
oxime-resistant	
1,2,2-trimethylpropyl methylphosphonofluoridate (Soman)	7·1(4·7–10·8)
Ethyl-*N*,*N*-dimethylphosphoramidocyanidate (Tabun)	14·0(10·1–19·5)
Carbamates	
1,3-di(3-*N*-dimethylcarbamoyl-5-trimethylaminophenoxy)propane diiodide (CT 3113)	7·5(6·3–9·1)
Physostigmine	7·2(5·9–9·4)
3-methylcarbamoyloxy trimethylaminophenyl bromide hydrobromide	7·2(5·9–9·4)

0·2 mg/kg of TEPP given 30 min before poisoning; P2S and atropine 1 min after.

TABLE 5. EFFECT OF VARIOUS CHOLINESTERASE REACTIVATORS USED IN CONJUNCTION WITH ATROPINE SULPHATE IN GUINEA PIGS PRETREATED WITH TEPP AND POISONED BY SOMAN

Reactivator and dose (mg/kg)	Protective ratio and 95 per cent confidence limits
P2S, 30	7·4(4·7–14·1)
4-PAM (4-hydroxyiminomethyl-*N*-methylpyridinium iodide), 30	<2·4*
TMB-4 (1,3-di(4-hydroxyiminomethylpyridinium) propane dichloride), 15	<2·4*
Toxogonin (1,3-di(4-hydroxyiminomethylpyridinium)-2-oxapropane dibromide), 46	<2·4*
MINA, isonitrosoacetone, 35	<2·4*
3-methyl-4-hydroxyiminomethylthiazole methanesulphonate, 100	<2·4*
3-methyl-5-hydroxyiminomethylthiazole methanesulphonate, 100	3·2(2·7–3·8)
Salicylhydroxamic acid, 150	<2·4*

0·2 mg/kg of TEPP given 30 min before poisoning. Oxime and atropine given 1 min after.

* No survivors from 50 μg/kg of Soman, the smallest dose routinely used in this experiment.

variety of anticholinesterase poisons, including organophosphorus compounds whose effects were responsive to oximes (paraoxon, Sarin) or resistant (Soman, Tabun[2]), and carbamates, against which oximes are ineffective.[7]

3. *The effectiveness of various reactivators used in conjunction with TEPP and atropine against Soman poisoning*

Seven oximes and one hydroxamic acid were examined as substitutes for P2S. The largest safe dose of MINA (monoisonitrosoacetone) was not determined by its immediate action on motor coordination. It is metabolized to cyanide, and the dose given was that which was found earlier[8] not to kill by cyanide poisoning. Most of the reactivators were therapeutically ineffective, except 3-methyl-5-hydroxyiminomethyl-thiazole methanesulphonate, which showed a small effect, less than half that of P2S (Table 5).

4. *The effectiveness of various cholinolytic drugs used in conjunction with TEPP and P2S against Soman poisoning*

The dose of atropine used was 50 μequiv./kg, and this dose was used in comparable experiments with other cholinolytic drugs (see also ref. 9). Table 6 shows that three were decidedly better than atropine sulphate, the best being hyoscine hydrobromide, which gave a PR 2·5 times that of atropine sulphate. A striking effect with these three compounds was the relatively mild nature of the convulsions and the speed of recovery.

TABLE 6. EFFECTIVENESS OF VARIOUS CHOLINOLYTICS IN CONJUNCTION WITH P2S ON THE TOXICITY OF SOMAN TO GUINEA PIGS PRETREATED WITH TEPP

Cholinolytic	Protective ratio	Remarks
Atropine sulphate	7·3 (4·7–14·0)	Survivors not completely recovered by 24 hr
Atropine methanesulphonate	10·9(6·7–17·6)	Convulsions for short periods only. Survivors normal in 3 hr
Atropine methylnitrate	No survivors at 2·4 LD_{50}'s	
Hyoscine hydrobromide	18·4(16·8–19·2)	Immediate sedation; slight convulsions after Soman, very rapid recovery
Hyoscine methanesulphonate	15·1(12·0–20·2)	
Ditran*	14·1(6·9–28·9)	Sedation; slight convulsions after Soman.
N-methyl-3-periperidyl benzilate hydrochloride	10·0(8·1–12·3)	Animals comatose; mild convulsions after Soman; survivors normal in 30 min
Amitryptyline	5·6(4·0–7·0)	Heavy sedation
Parpanit	5·0(3·4–7·4)	
Diethazine	4·1(2·6–5·3)	
Orphenadrine citrate	3·3(2·7–4·0)	

0·2 mg/kg of TEPP given 30 min before Soman. 50 μequiv./kg of cholinolytic plus 30 mg/kg of P2S given 1 min after.

* 70% *N*-ethyl-2-pyrrolodylmethyl phenylcyclopentyl glycollate.
30% *N*-ethyl-3-piperidyl phenylcyclopentyl glycollate.

TABLE 7. ORGANOPHOSPHATES AS PREMEDICANTS IN GUINEA PIGS POISONED BY SOMAN

Organophosphorus compound	Maximum sign-free dose (1)*	LD_{50} (2)*	Ratio (1):(2)	Protective ratio
TEPP	200	ca. 400	0·5	7·2(4·7–14·1)
Paraoxon	350	1070	0·33	7·0(5·3–9·3)
Ethyl methylphosphonofluoridate	55	112	0·60	3·2(2·4–3·9)
diethyl dimethylpyrophosphonate	300	620	0·48	4·9(4·2–5·8)
ethyl 4-nitrophenyl methylphosphonate	60	160	0·38	8·5(5·8–10·7)
2-chloroethyl-2,2-dichlorovinyl ethylphosphonate	150	—	—	3·3(2·5–4·0)
2-chlorocyclopentyl methylphosphonofluoridate	25	42·5	0·59	<2·5
Sarin	15	33	0·44	4·9(2·7–6·0)
diisopropyl phosphorofluoridate (DFP)	1500	—	—	<2·5

* μg/kg i.m.
Stated dose of organophosphate given 30 min before Soman; P2S and atropine 1 min after.

TABLE 8. DURATION OF THE "PROTECTIVE" ACTION OF ORGANOPHOSPHORUS COMPOUNDS USED IN GUINEA PIGS AS PREMEDICANTS AGAINST POISONING BY SOMAN

Organophosphorus compound	Protective ratio with this interval, hr, between organophosphorus compound and Soman				
	0·5	5	24	48	72
Diethylphosphates					
TEPP	7·3(4·7–14·1)	7·1(4·7–10·8)	3·0(1·9–4·8)	—	2·0(1·6–2·5)
Paraoxon	7·0(5·3–9·3)	—	<2·5	—	—
Ethyl methylphosphonates					
diethyl dimethylpyrophosponate	4·9(4·2–5·8)	—	5·6(3·8–8·5)	2–3*	—
ethyl 4-nitrophenyl methylphosphonate	8·5(6·8–10·7)	—	6·3(4·0–10·0)	2·7(2·1–3·4)	—
Isopropyl methylphosphonate					
Sarin	4·0(2·7–6·0)	<1·7	—	—	—

* Shallow dose–response curve, very wide limits of estimate of LD_{50}.
Maximum sign-free dose of organophosphorus compound given at time stated before Soman. P2S and atropine given 1 min after.

5. *The "protective" effects of organophosphorus compounds other than TEPP, used with* P2S *and atropine against Soman poisoning*

Tables 7 and 8 show the results obtained by using a variety of anticholinesterase organophosphates as premedicants against Soman poisoning. The most effective, when given 30 min before Soman, were TEPP, paraoxon (diethyl *p*-nitrophenyl phosphate) and diethyl dimethylpyrophosphonate. The others were not very effective.

There were marked differences in the duration of the "protective" action of some of these compounds. That of diethyl dimethylpyrophosphonate, which was initially as good as that of TEPP, persisted longer, a significant effect remaining after 48 hr. Likewise the action of ethyl 4-nitrophenyl methylphosphonate was moderately good for 24 hr but fell off sharply thereafter. Although Sarin (isopropyl methylphosphonofluoridate) was reasonably effective at 30 min its "protective" action had disappeared by 5 hr.

6. *The effectiveness of TEPP, used with* P2S *and atropine, against Soman poisoning in other species*

The object of this experiment was to find whether, as with physostigmine,[1] there were species differences in response to this form of pretreatment. Only two other species were examined, the rat, which responded badly to physostigmine, and the rabbit, which responded better, though not as well as the guinea pig. Table 9 shows

TABLE 9. EFFECTIVENESS OF TEPP AGAINST POISONING BY SOMAN IN THREE SPECIES

Species	Max. sign-free dose of TEPP(mg/kg)	Protective ratio
Guinea pig	0·20	7·1(4·7–10·8)
Rabbit	0·15	3·7(2·3–6·0)
Rat	0·15	All died from 2 LD_{50}'s

TEPP given 30 min before Soman; P2S and atropine 1 min after. The LD_{50}'s of Soman were given in ref. 1.

that the pattern of response to treatment with TEPP in the three species paralleled that to physostigmine and establishes the existence of species differences in the "protection" against Soman poisoning.

DISCUSSION

The hypothesis that animals could be protected against the lethal effects of Soman by pretreatment with an oxime-responsive organophosphorus anticholinesterase, supported by oxime and cholinolytic after poisoning, has been verified. Three compounds have been shown to raise the LD_{50} of Soman to the guinea pig by factors of 7–9 when used with P2S and atropine. One of them, TEPP, was shown to confer at least this degree of "protection" against several anticholinesterases, carbamates as well as oxime-responsive and oxime-resistant organophosphorus compounds.

The three compounds, TEPP, paraoxon and *O,O*-diethyl dimethylpyrophosphonate, form phosphylated* acetylcholinesterases which can easily be reactivated by oximes. Sarin was less effective, and DFP was ineffective. This ranking corresponds to ease of reactivation, as far as can be assessed from observations by different laboratories.[11,12]

The two compounds which form ethyl methylphosphonoacetylcholinesterase, viz. ethyl 4-nitrophenyl methylphosphonate and diethyl dimethylpyrophosphonate, have

* A term suggested by Hudson and Keay,[10] to denote any group covalently bound through phosphorus.

a longer-lasting "protection" than the two which form diethylphosphorylacetylcholinesterase (TEPP and paraoxon). That of Sarin (isopropyl methylphosphonoacetylcholinesterase) was much shorter still. The duration of the "protective" action is thus governed by the nature of the phosphylating group.

Loss of "protective" action could occur through breakdown of the phosphylated enzyme. Spontaneous reactivation (dephosphylation) would decrease the proportion of active enzyme "protected" against Soman, while aging would decrease the proportion of phosphylated enzyme reactivated by oxime. Examination of published data[11–13] shows that rates of aging increase in the order ethyl methylphosphonyl, diethylphosphoryl and isopropyl methylphosphonyl enzyme. Rates of spontaneous reactivation decrease in the same order. We have attempted to assess the importance of these factors by measuring the activity of red cell and diaphragm acetylcholinesterases after administration of organophosphate and P2S *in vivo*, but the results were inconclusive. It was impossible to use each animal as its own control, therefore individual variation in normal acetylcholinesterase activity could not be separated statistically from individual variation in response to treatment. In consequence the fiducial limits of estimates of percent reactivation, whether spontaneous or oxime-induced, were too large to establish any differences.

The choice of an effective oxime for reactivation of the TEPP-inhibited acetylcholinesterase *in vivo* proved surprisingly limited. Of the eight compounds tested, only P2S was outstandingly good, despite the fact that Toxogonin and TMB-4 are better reactivators *in vitro*.[2,14–17] Work to be reported elsewhere[18] has shown that the reactivation of TEPP-inhibited acetylcholinesterase from the particulate fraction of guinea pig diaphragm by TMB-4 is much more rapid and extensive than that by P2S. If this occurs *in vivo* it may be postulated that reactivation would lag behind the detoxification and elimination of Soman, allowing recovery of enzyme activity, whereas reactivation by TMB-4 would occur while there was still free Soman in the organ to re-inhibit the reactivated enzyme.

Although the success of this form of pretreatment probably depends on reactivation of the fraction of acetylcholinesterase inhibited by the premedicant, e.g. TEPP, the very large protective ratio against paraoxon or Sarin is probably related to the ease with which the fraction of enzyme inhibited by the poison can be reactivated. The high protective ratio observed in Tabun poisoning cannot be so explained, since Tabun is a P2S-resistant poison.[2] Possibly the rate of elimination of Tabin is unusually high.

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