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THE USE OF CARBAMATES AND ATROPINE IN THE PROTECTION OF ANIMALS AGAINST POISONING BY 1,2,2-TRIMETHYLPROPYL METHYLPHOSPHONOFLUORIDATE

W. K. BERRY and D. R. DAVIES*

Ministry of Defence, Chemical Defence Establishment, Porton Down, Salisbury, Wiltshire

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Abstract—Doses of physostigmine which produce no clinical signs will, when given to guinea pigs together with atropine before poisoning, raise the LD_{50} of Soman (1,2,2-trimethylpropyl methylphosphonofluoridate) seven to eight times. Prostigmine is equally effective, but other carbamates are less so and simple reversible diquaternary inhibitors are almost ineffective. If physostigmine and atropine are given after Soman, no protection occurs beyond that given by atropine alone; the toxicity of Soman is slightly decreased. Prophylaxis with physostigmine and atropine varies in effectiveness in different species in the order guinea pig > dog > rabbit > mouse = chicken > rat. Physostigmine plus atropine gave rather more protection to guinea pigs against Sarin (isopropyl methylphosphonofluoridate) but not so much as was given by P2S (2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate) and atropine. The protective action of P2S and atropine was not affected by the addition of physostigmine to the mixture.

IN 1946 KOSTER[1] showed that pretreatment of cats with physostigmine protected them against several LD_{50}'s of diisopropyl phosphorofluoridate. Callaway[2] observed a similar phenomenon in rats poisoned with various organophosphorus compounds and Barnes[3] found that prostigmine or 1,5-di(4-ethyldimethylammoniumphenyl)pentan-3-one diiodide were as effective as physostigmine.

The antidotal action of these compounds almost certainly depends upon their common property of being able reversibly to inhibit acetylcholinesterase (EC 3.1.1.7) and upon the fact that vital organs contain much more acetylcholinesterase than is necessary for normal functioning.[4, 5] Inhibition of a proportion of acetylcholinesterase in such organs would prevent complete phosphorylation by an organo-phosphorus compound, and because of the reversible nature of the inhibition, free enzyme would gradually be regenerated. If metabolism of excess organophosphorus compound occurred in parallel with this regeneration, protection could be provided for the whole animal.

If this is the mechanism of the protective action, there is obviously a period during which a great proportion of the enzyme would be inactivated, both reversibly and irreversibly. During this critical period a lethal accumulation of acetylcholine could occur at the nerve endings, needing treatment by atropine. A combination of physostigmine and atropine should therefore be more effective than either alone.

* Present address: Burns Research Unit, Wessex Regional Burns Unit, Odstock Hospital, Salisbury, Wiltshire.

This form of treatment should be effective regardless of the nature of the organophosphorus compound, and should therefore be effective against compounds such as Soman, poisoning by which is resistant to treatment by oximes and atropine.[6, 7]

In the present work this principle has been tested using a number of reversible anticholinesterases, and it has been shown possible to protect guinea pigs, dogs or rabbits against 4–8 LD_{50}'s of Soman. The procedure was less successful in mice or chickens and ineffective in rats.

MATERIALS AND METHODS

Soman was synthesised in the Establishment and was at least 95% pure by chemical analysis.[8]

Physostigmine sulphate, prostigmine methyl sulphate, pentamethonium bromide, procaine hydrochloride and atropine sulphate were obtained from commercial sources and were assumed to be reasonably pure. Other related drugs were synthesised in the Establishment, the carbamates by treating the appropriate phenol or pyridone with methyl- or dimethylcarbamoyl chloride and the dipyridinium alkanes by treating the α,ω-dibromoalkane with pyridine. The compounds were characterised by elemental analysis and melting points.

Soman solutions were prepared by serial dilution in 0·9% (w/v) NaCl and were injected subcutaneously. No solution was kept for more than 1 hr. Reversible anticholinesterases and atropine sulphate were dissolved in distilled water and were given intramuscularly, usually 10 min before Soman, but this was varied in a number of experiments.

Female guinea pigs, 300–400 g, were used for the main experiments, but for special problems both males and females of other species were used.

LD_{50} values were calculated by the method of Irwin and Cheeseman[9] on the basis of deaths occurring up to 24 hr after poisoning.

RESULTS

The maximum effective dose of reversible anticholinesterase

The maximum sign-free doses of the reversible anticholinesterases were determined by observing the effects of graded doses on the animals' gait and balance. Table 1 shows the results obtained in six species with physostigmine. In the absence of atropine the doses were very similar in all six species, but in the presence of atropine there was greater variation for the four species tested.

TABLE 1. THE MAXIMUM SIGN-FREE DOSE OF PHYSOSTIGMINE IN THE PRESENCE AND ABSENCE OF ATROPINE IN VARIOUS SPECIES

Species	Maximum sign-free dose, mg/kg	
	Without atropine	With atropine, 17·4 mg/kg
Chicken	0·16	—
Dog	< 0·10	—
Guinea pig	0·16	0·63
Mouse	0·10	< 0·10
Rabbit	0·10	0·25
Rat	0·10	0·3

TABLE 2. THE EFFECT OF DOSE OF PHYSOSTIGMINE ON THE MORTALITY OF SOMAN-POISONED GUINEA PIGS GIVEN 17·4 mg/kg OF ATROPINE SULPHATE

Dose of Soman μg/kg	126	200	316
		Deaths/total	
Physostigmine, 0·16 mg/kg	0/5	2/5	4/5
Physostigmine, 0·63 mg/kg	1/5	1/5	3/5

The antidotes were given i.m. 10 min before Soman, s.c.
The LD_{50} of Soman was 28 μg/kg.

The results of pretreatment of atropinised guinea pigs with 0·16 or 0·63 mg/kg of physostigmine, the maximum sign-free doses in the absence or presence, respectively, of atropine, are shown in Table 2. Over this 4-fold range the dose of physostigmine was not critical, consequently in all subsequent experiments the smaller dose was used.

In a further experiment to assess the variation of protective effect with dose of physostigmine, eighty animals were given atropine plus doses of physostigmine varying between 0·02 and 0·16 mg/kg 10 min before Soman. The results may be expressed as

$$\text{Probit mortality} = -4{\cdot}89 - (2{\cdot}63 \pm 0{\cdot}66)p + (6{\cdot}97 \pm 1{\cdot}17)s$$

where p and s are the logarithms of the doses, in μg/kg, of physostigmine and Soman respectively. Since the partial regression coefficient of p is four times its standard error, the experiment demonstrates that the protective effect of physostigmine diminishes significantly as the dose is lowered.

The effects of physostigmine, atropine and P2S in Soman-poisoned guinea pigs

The LD_{50} of Soman in guinea pigs pretreated with physostigmine, atropine and P2S†, singly or in combination, is shown in Table 3.

P2S alone had no effect, nor did it significantly alter the effect of atropine. Atropine

TABLE 3. THE SUBCUTANEOUS LD_{50} OF SOMAN IN GUINEA PIGS GIVEN PHYSOSTIGMINE ATROPINE AND P2S INTRAMUSCULARLY EITHER ALONE OR IN COMBINATION 10 min BEFORE SOMAN

Treatment	Dose (mg/kg)	LD_{50} (μg/kg) (95% limits)	Factor by which LD_{50} is increased
None		28(25–32)	
Atropine	17·4	43(36–50)	1·5
P2S	30·0	28 approx.	1·0
Physostigmine	0·16	50 approx.	1·8
Atropine + P2S	17·4 + 30·0	51(45–59)	1·8
Atropine + Physostigmine	17·4 + 0·16	230(148–355)*	8·2
Atropine + Physostigmine + P2S	17·4 + 0·16 + 30·0	300(174–416)*	10·7

* P that these are identical, 0·30.

† 2-hydroxyiminomethyl-*N*-methylpyridinium methane sulphonate.

alone or physostigmine alone each had a slight protective effect, but a mixture raised the LD_{50} of Soman eight times. This figure was not significantly increased when P2S was added to the mixture.

Physostigmine and atropine proved more effective in reducing the toxicity of Soman, both by decreasing its lethality and in hastening recovery from poisoning. Surviving guinea pigs treated with P2S and atropine remained distressed and even moribund for periods of up to 72 hr after poisoning, but those treated with physostigmine and atropine showed definite signs of recovery in 2–4 hr and appeared normal after 24 hr.

The influence of time and order of administration of physostigmine and atropine upon their effectiveness in Soman poisoning

(i) *Duration of action.* The duration of the protective action of physostigmine was assessed by injecting it i.m. at various times before Soman, with the atropine always given 10 min before Soman. The dose of Soman was the maximum that had failed to kill any of five animals pretreated 10 min beforehand with both drugs in the experiments of Table 3. Table 4 shows that the protective effect against this dose was gone by 120 min.

TABLE 4. THE DURATION OF THE PROTECTIVE ACTION OF PHYSOSTIGMINE IN GUINEA PIGS

Interval between physostigmine and Soman (min)	Deaths/total
10	0/5
30	0/5
60	1/5
120	5/5

The dose of Soman, 126 μg/kg, was the largest found to give 0/5 deaths when physostigmine and atropine were given 10 min beforehand (Table 3). Here, physostigmine, 0·16 mg/kg given at various times before Soman, but atropine sulphate, 17·4 mg/kg, given 10 min before Soman.

(ii) *The effects of time and order of administration.* The alteration in LD_{50} of Soman in guinea pigs produced by varying the timing of the doses of physostigmine and atropine is shown in Table 5.

Provided physostigmine was given before poisoning, a highly significant protective action was seen. If it was not given until after poisoning the protective effects disappeared, i.e. the mixture was no more effective than atropine alone, giving a slight but significant increase in LD_{50}. The timing of the atropine injection did not seem to be critical, provided it was given not later than 1 min after poisoning.

Physostigmine did not hasten the onset of toxic signs when given with atropine after poisoning. Thus 20 μg/kg (0·7 $\times$ LD_{50}) of Soman produced no signs of poisoning in 25 min. The administration of physostigmine and atropine at this stage did not precipitate any toxic signs, and the animals were all alive 24 hr later. After 32 μg/kg (1·15 $\times$ LD_{50}) toxic signs developed in 5–6 min in all animals. They were then treated

TABLE 5. THE INFLUENCE OF TIME OF ADMINISTRATION OF PHYSOSTIGMINE AND ATROPINE RELATIVE TO SOMAN ON THE LD_{50} IN THE GUINEA PIG

Time of administration (min)		LD_{50} of Soman, μg/kg (95% limits)
Physostigmine	Atropine	
No treatment		28(25–32)
−10	−10	230(148–355)
−10	+ 1	290
− 1	− 1	170(107–269)
+ 1	+ 1	54 approx.
None	At signs*	32(29–36)
At signs*	At signs*	37(33–42)

Atropine sulphate, 17·4 mg/kg and physostigmine sulphate, 0·16 mg/kg given i.m. at the times shown before (−) or after (+) s.c. injection of Soman.
* As soon as signs of poisoning by Soman were observed.

with atropine or atropine plus physostigmine. The subsequent severity of toxic signs was similar in both groups. In 24 hr 2/5 treated with atropine alone died, and 0/5 of those given both drugs. This difference is not significant.

The protection of guinea pigs against poisoning by Sarin (isopropyl methylphosphonofluoridate)

It has already been shown in Table 3 that the addition of P2S to the combination of physostigmine and atropine did not alter the level of the protective effect against Soman. This was not unexpected, since poisoning by Soman is oxime-resistant. However, Sarin poisoning responds very well to P2S and atropine[10] and the combination of P2S, physostigmine and atropine was expected to be more effective than either P2S plus atropine or physostigmine plus atropine. It was found that P2S and atropine increased the LD_{50} of Sarin to guinea pigs by a factor of about 40, whether or not physostigmine was included, but that physostigmine plus atropine increased it by a factor of only 18.

Physostigmine and atropine against Soman in various species

The maximum sign-free dose of physostigmine (0·16 mg/kg) was given together with an appropriate dose of atropine 10 min before Soman. The doses are shown in Table 6. Guinea pigs, rabbits and dogs responded well, with increases of 4·5–8 times the apparent LD_{50}. Chickens, mice and rats did not respond to any great extent.

TABLE 6. PHYSOSTIGMINE AND ATROPINE AGAINST SOMAN POISONING IN VARIOUS SPECIES

Species	Dose (mg/kg)		LD_{50} of Soman (μg/kg)	
	Physostigmine	Atropine	Untreated	Treated
Rat	0·10	17·4	160	320
Chicken	0·16	1·0	ca. 50	100
Mouse	0·10	17·4	160	440
Rabbit	0·10	17·4	ca. 20	90
Dog	0·10	5·0	12	ca. 75
Guinea pig	0·16	17·4	28	230

Treatment was given i.m. 10 min before Soman s.c.
In all cases the effect of treatment was significant, $P < 0·05$.

An unexpected finding in dogs was the slowness with which they developed symptoms from subcutaneous injection of Soman. It has been reported above that symptoms from quite small doses (1·15 × LD_{50}) appeared in guinea pigs in about 5 min. This also occurs in other species tested. In dogs, however, two given 0·6–0·8 of the LD_{50} did not develop signs at all; two others given 1·2 and 1·7 times the LD_{50} developed signs only after 1·5 hr. Both of these were unconscious after 2·75 hr and one of them died without convulsions 0·75 hr later. The remaining dog died overnight.

When dogs were pretreated with physostigmine and atropine the development of poisoning was, by contrast with the guinea pig, quite irregular, the animals passing through a rhythm of acute and quiet phases. Repeated treatment with atropine seemed to be effective.

The effectiveness of other reversible anticholinesterases

In these experiments other reversible anticholinesterases, in maximum sign-free doses, were given with atropine to guinea pigs 10 min before Soman. Table 7 shows

TABLE 7. THE EFFECTIVENESS OF SOME REVERSIBLE ANTICHOLINESTERASES AGAINST SOMAN POISONING IN GUINEA PIGS

Compound	Dose/mg/kg	*Factor by which LD_{50} of Soman was increased
Dipyridinium propane	50	All dead at 2·2 LD_{50}'s
Dipyridinium pentane	15	All dead at 2·2 LD_{50}'s
Pentamethonium	30	About 2·5
Procaine hydrochloride	20	All dead at 2·2 LD_{50}'s
Carbamyl choline	0·025	All dead at 2·2 LD_{50}'s
Physostigmine sulphate	0·16	8·1(5·6–11·5)
3-(2-dimethylaminoethyl) phenyl-*N*-methylcarbamate	0·08	7 approx.
3-isopropylphenyl-*N*-methyl carbamate	5	3 approx.
3-trimethylaminophenyl-*N*-methylcarbamate	0·025	5·5(4·5–6·8)
Prostigmine methyl sulphate	0·10	7 approx.
N-methylpyridinium-3-dimethyl carbamate (Pyridostigmine)	0·10	4 approx.
2-piperidinomethylpyridine-3-dimethylcarbamate dihydrochloride	0·25	3 approx.
2-dimethylaminomethylpyridine-3-dimethylcarbamate dihydrochloride	0·05	6 approx.

The drugs, bromides unless otherwise stated, were given in maximum sign-free dose with 17·4 mg/kg of atropine sulphate 10 min before Soman.

* The LD_{50} of Soman varied from 19·5 to 28 μg/kg during the course of these experiments, therefore the results have been expressed in this manner.

that dipyridinium propane and dipyridinium pentane, compounds related to oximes previously found to be very effective therapeutically against poisoning by tetraethyl pyrophosphate,[11, 12] were inactive, as was carbamyl choline. Several mono- and dimethylcarbamates exhibited varying degrees of effectiveness. There are no obvious structural features related to effectiveness. No compound was significantly better than physostigmine.

DISCUSSION

Protection by oximes and atropine in Soman poisoning is only marginal, and in looking for a new approach to the problem of providing effective protection the

original work of Koster[1] and the later work of Callaway[2] and Barnes[3] has been extended. It has been shown that the prophylactic administration of reversible anticholinesterases, in doses which do not produce signs of poisoning and in conjunction with atropine, can protect guinea pigs against several lethal doses of Soman. The best inhibitor was physostigmine, although two others, prostigmine and 3-(2-dimethylaminoethyl) phenyl-*N*-methylcarbamate, were nearly as good. Each of these drugs was much more effective than P2S similarly used. The treatment is essentially prophylactic, for the effect disappears when the antidote is given after poisoning. The evidence shows that with therapeutic treatment neither the lethality of Soman, nor the severity of toxic signs of Soman poisoning are increased.

The antidotal effectiveness of physostigmine and atropine varies in different species, and follows the decreasing order guinea pig, dog, rabbit, mouse and chicken, rat. This may explain why other workers who have studied the effects of a mixture of atropine and reversible anticholinesterase on organophosphorus poisoning did not find good protection. Parkes and Sacra[13] and Lewis, McKeon and Lands[14] used mice, and the latter also used birds (pigeons), species which, according to the present work, might be very resistant to treatment.

Further evidence of marked species differences in response to treatment of organophosphorus poisoning was provided by DeCandole and McPhail[15] who found large species differences in response to atropine given as an antidote to poisoning by Sarin or diethyl-*p*-nitrophenyl phosphate. Monkeys were the most responsive, mice the least responsive. Davies, Green and Willey[10] found that P2S plus atropine given prophylactically or therapeutically against Sarin was much less effective in mice or rats than in guinea pigs or rabbits. The reasons for such species differences may be associated with differences in the metabolism of the drugs concerned, or even in the properties of cholinesterases and drug receptors. In order to assess the probable application of this work to man, it is necessary to discover the reasons and provide a rational explanation for the difference.

Reasons for using atropine in conjunction with physostigmine were given in the Introduction, and since this combination of drugs has resulted in an enhancement of the antidotal effect of physostigmine, there is support for the existence of the mechanism of action proposed. On this hypothesis it is possible to explain the varying effectiveness of different reversible anticholinesterases. One of us (W.K.B., unpublished results) has found that the dipyridinium alkanes and pentamethonium are competitive reversible inhibitors of acetylcholinesterase. The existence of the enzyme–inhibitor complex therefore depends on the continuing presence of free inhibitor. The carbamates, on the other hand, form moderately stable carbamylated acetylcholinesterases[16, 17] which would persist after the removal of free inhibitor. Since the former compounds were less effective therapeutically than the latter, the rate of breakdown of the enzyme–inhibitor complex appears to be an important factor: excessively rapid breakdown, as would occur on elimination of a simple reversible inhibitor, might leave the enzyme exposed to Soman which had not yet been detoxified or excreted. The least effective carbamate, carbamylcholine, is known to yield a carbamylated acetylcholinesterase which breaks down more rapidly than the mono- or dimethylcarbamates produced by the remaining compounds[17] and this could account for its ineffectiveness. But the rate of breakdown of the enzyme–inhibitor complex cannot furnish the entire explanation for varying effectiveness. Although the mono-

and dimethyl carbamylated enzymes break down at much the same rate, there is evidence to show that the rate of carbamylation varies considerably with the structure of the remaining part of the inhibitor.[17]

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