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The protective actions of some anticholinergic drugs in sarin poisoning

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Summary

1. The central and peripheral anticholinergic activities of a series of drugs comprising atropine, hyoscine, caramiphen and one of its analogues, and three glycollic acid esters, have been measured.

2. The ability of the same drugs used alone, and in conjunction with N-methyl pyridinium-2-aldoxime methanesulphonate (P2S), to protect mice, rats and guinea-pigs from the lethal effects of sarin has been assessed.

3. No correlation was found to exist between central or peripheral anticholinergic activity and ability to protect from sarin.

4. On the indirectly stimulated isolated rat phrenic nerve-diaphragm preparation all drugs with the exception of hyoscine caused potentiation of responses to low frequency stimulation but partial block of responses to high frequency stimulation. The drugs did not reverse the effects of sarin on the phrenic nerve-diaphragm preparation.

6. It is concluded that a pharmacological action other than an anticholinergic one is involved, in part, in the protective actions against sarin of some of the drugs studied. Whether their effects on skeletal muscle are of any relevance in this respect is unresolved.

Introduction

The treatment of poisoning with organophosphorus anticholinesterase agents involves the use of an anticholinergic drug and an oxime. The role of the latter, in reactivating inhibited cholinesterase, seems to be of prime importance in the periphery although statements (Brown, 1960; Hobbiger, 1963) that quaternary aldoximes do not penetrate into the central nervous system may have to be modified in view of more recent findings, for example those of Firemark, Barlow & Roth (1964), who showed that after large doses of labelled N-methylpyridinium-2-aldoxime it is possible to detect small amounts of the compound in the brain.

On the other hand, the actions of the anticholinergic drugs seem to be of greatest importance centrally, mainly in protecting the medullary respiratory centre from the depressant effect of anticholinesterases (Douglas & Matthews, 1952). Again, however, there must be some peripheral component in their therapeutic action, due presumably to their antimuscarinic activity, because the quaternary atropine methyl nitrate displays some protective action against organophosphate poisoning when used alone or in conjunction with an oxime (Coleman, Little & Bannard, 1963).

It might be expected that anticholinergic drugs which are more active centrally than the standard therapeutic drug atropine would be more effective in the treatment of anticholinesterase poisoning since Coleman, Little & Bannard (1962) failed to show any correlation between the therapeutic effectiveness of a series of drugs used in conjunction with an aldoxime in sarin-poisoned mice and their peripheral anticholinergic activity. The experiments reported here were designed to make a comparison between therapeutic effectiveness and both peripheral and central anticholinergic potency. To obtain information on the latter oxotremorine was used. This drug has marked parasympathomimetic actions, blockade of which can be used to measure the peripheral anticholinergic potency of atropine-like drugs. In addition it produces muscular tremors in a variety of species of animals, and although there has been much discussion as to its mode of action in producing these tremors there is now evidence (Bebbington & Brimblecombe, 1965 ; Cox & Potkonjak, 1969a, b) to suggest that it is due to an interaction with central acetylcholine receptors analogous to peripheral muscarinic receptors. Thus, blockade of oxotremorine-induced tremors can be used to give a measure of central anticholinergic potency.

Methods

Protection experiments

Male mice (18–25 g), rats (190–210 g) and guinea-pigs (250–300 g) were used. Sarin (*iso*propyl methylphosphonofluoridate) solutions were administered subcutaneously and the drugs being studied for protective actions were given intramuscularly into the thigh 15 min before the sarin. When the aldoxime, N-methylpyridinium-2-aldoxime methanesulphonate (P2S) was also used, it was injected intramuscularly into the opposite thigh also 15 min before the sarin. The anticholinergic drugs were always given in doses of 50 μmol/kg and P2S in a dose of 140 μmol/kg (30 mg/kg). These are the same doses as those used previously by other workers (Davies, Green & Willey, 1959).

Groups of six animals per dose of sarin were used ; the ratio between doses of sarin varied between 1·3 to 2·0. LD50s were computed by probit analysis (Finney, 1947). The degree of protection afforded by any procedure was expressed as a protection ratio: that is, LD50 of sarin in treated animals÷LD50 of sarin in untreated animals. Final observations of numbers of animals dead or surviving were made 24 h after injection of sarin.

Measurement of anticholinergic potency

(*a*) *Antagonism of oxotremorine effects.* The anticholinergic drugs were injected intraperitoneally into mice 15 min before the intravenous injection of 0·49 μmol/kg (100 μg/kg) oxotremorine. (ED90 for salivation = 0·16 μmol/kg ; for tremors = 0·34 μmol/kg.) Animals were examined at 5, 10 and 15 min after the oxotremorine injection for the presence of salivation and the occurrence of tremors. The mice were grasped by the skin at the nape of the neck and turned on their backs. Any dampness of the lips was taken to indicate presence of salivation ; the hind limbs were observed for tremors which occurred in these extremities before whole body tremors became apparent. Four groups, each containing five mice, were used and ED50s for prevention of salivation and of tremors were calculated by probit analysis.

TABLE 1. *Anticholinergic activities in mice*

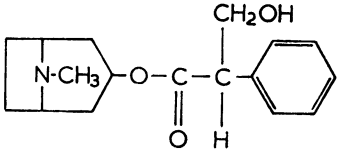
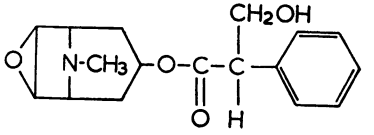
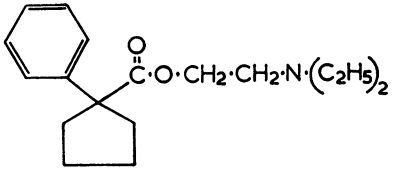
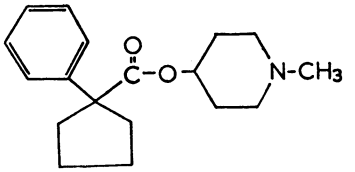
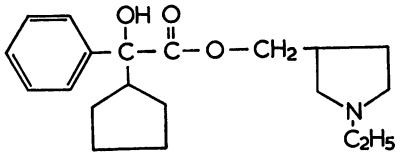
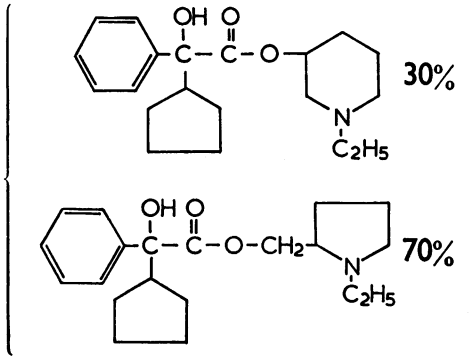
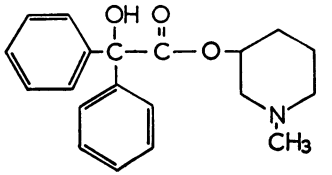
Drug	Structural formula	ED50 (μmol/kg with 95% confidence limits) for antagonism of oxotremorine-induced		Production of mydriasis in mice. Dose (μmol/kg) to produce a mean pupil diameter of 20 arbitrary units
		Salivation	Tremor	
Atropine (sulphate)		0·44(0·33–0·66)	16·2(10·0–26·0)	0·13
Hyoscine (hydrobromide)		0·05(0·02–0·08)	1·1(0·6–2·5)	0·026
Caramiphen (HCl)		23·5(13·2–41·2)	40·3(24·0–67·5)	6·5
G3063 (HCl)		5·45(3·4–8·6)	4·7(1·9–11·3)	0·39

TABLE 1— *continued*

Drug	Structural formula	ED50 (μmol/kg with 95% confidence limits) for antagonism of oxotremorine-induced		Production of mydriasis in mice. Dose (μmol/kg) to produce a mean pupil diameter of 20 arbitrary units
		Salivation	Tremor	
PMCG (HCl)		1·7(0·4–7·1)	3·5(2·2–5·7)	0·13
Ditran (HCl)		0·77(0·15–1·2)	0·88(0·50–1·4)	0·21
N-methyl-3-piperidyl-benzilate (HCl)		1·05(0·5–1·9)	1·8(1·0–3·2)	0·65

(b) Production of mydriasis in mice. Male mice (18–25 g) were used. Drugs were injected into a tail vein. Preliminary experiments were carried out on a limited number of animals to obtain an indication of suitable dose levels. Then, using groups of ten mice at each of three doses, the pupil diameter was measured at various times after the injection to cover, as far as possible, the total period of action of the drug. The eyes were held 20 cm from a Watson microscope lamp and the measurement was made in arbitrary units using an eyepiece graticule in a $\times 20$ microscope. The mean pupil diameter from both eyes was used and the mice were kept in the dark before and between readings. The duration of effect varied with dose, so in calculations of potency relative to atropine the maximum mean pupil diameter reached at each dose was used irrespective of time and the results calculated on the basis of a six-point assay. Doses of atropine used were always 0·2, 0·1 and 0·05 μmol/kg and in a standard assay these doses gave pupil diameters of 25·5, 17·0 and 10·0 units respectively. The mean pupil diameter in untreated eyes was 6 units and in maximally dilated pupils it was 34 units. From these results it is also possible to calculate the potency of the anticholinergic drug as the dose required to produce a given effect, for example to give a mean pupil diameter of 20 units.

Effects on isolated rat phrenic nerve-diaphragm preparation

The phrenic nerve-diaphragm preparation was prepared as described by Bülbring (1946). The preparation was suspended in a 50 ml organ bath containing Krebs solution at 32° C through which was passed a mixture of 95% oxygen and 5% carbon dioxide. The nerve was stimulated using rectangular pulses of 0·5 ms duration and of twice the voltage strength required to evoke a maximal twitch. Contractions were recorded on a smoked drum using a spring-loaded lever giving a 10-fold magnification.

Drugs

The anticholinergic drugs used, with their structural formulae, are listed in Table 1. Atropine was purchased from B.D.H. Ltd. and hyoscine from McFarlan-Smith Ltd. G3063 was obtained from Dr. I. W. Coleman of the Defence Chemical, Biological and Radiation Laboratories, Ottawa, Canada. The remaining anticholinergic compounds, oxotremorine, sarin and P2S were synthesized in these laboratories. All doses are expressed in terms of bases (for atropine sulphate the molecular weight was halved since two molecules of base are present in this salt).

Results

Anticholinergic activity

In Table 1 are listed the anticholinergic activities of the drugs as measured by their abilities to antagonize oxotremorine-induced salivation and tremor and to produce mydriasis in mice. With the exception of the values for caramiphen and G3063, these results were published previously by Brimblecombe & Green (1968).

Protection experiments

The results of the protection experiments using mice, rats and guinea-pigs are given in Tables 2, 3 and 4. None of the anticholinergic drugs used alone was

effective in protecting mice from more than 1·5 LD50s of sarin; when P2S was used in addition the ratio of protection increased, the highest observed value being 6·3 with the combination G3063 and P2S. In rats, anticholinergic drugs used alone gave protection ratios ranging between 1·2 (atropine and hyoscine) and 9·3 (G3063). With P2S also present the range of protection ratios was 9·3 (hyoscine and P2S) to 79·5 (G3063 and P2S). In guinea-pigs no anticholinergic drug alone was effective in protecting from more than 1·5 LD50s of sarin; P2S increased the protection ratios to between 18·6 (caramiphen and P2S) and 84·2 (PMCG and P2S). High protection ratios were also given by the combination of P2S with Ditran (70·2) and G3063 (58·3). P2S alone gave protection ratios of only 1·1 for rats and guinea-pigs and 1·2 for mice.

TABLE 2. *Protection of mice against sarin*

Drug (Dose 50 μmol/kg)	Drug alone: LD50 μmol/kg with 95% confidence limits	Drug alone: Protection ratio	Drug +140 μmol/kg P2S: LD50 μmol/kg with 95% confidence limits	Drug +140 μmol/kg P2S: Protection ratio
Atropine	1·6(1·3–1·9)	1·1	3·9(3·2–5·1)	2·6
Hyoscine	2·0(1·7–2·3)	1·3	2·9(2·5–3·4)	2·0
Caramiphen	1·7(1·4–2·0)	1·1	3·7(2·5–5·2)	2·5
G3063	2·0(1·7–2·5)	1·4	9·3(6·3–13·5)	6·3
PMCG	2·2(1·9–2·5)	1·5	4·4(3·4–6·4)	3·0
Ditran	2·0(1·7–2·5)	1·4	3·6(2·8–4·7)	2·4
N-methyl-3-piperidyl benzilate	1·9(1·7–2·1)	1·3	2·7(2·0–3·7)	1·8
P2S	1·8(1·6–2·1)	1·2		

Drugs were given intramuscularly 15 min before sarin subcutaneously.
LD50 for sarin alone = 1·48 μmol/kg (207·2 μg/kg).

TABLE 3. *Protection of rats against sarin*

Drug (Dose 50 μmol/kg)	Drug alone: LD50 μmol/kg with 95% confidence limits	Drug alone: Protection ratio	Drug+140 μmol/kg P2S: LD50 μmol/kg with 95% confidence limits	Drug+140 μmol/kg P2S: Protection ratio
Atropine	0·89(0·56–1·24)	1·2	20·1(14·3–42·1)	27·5
Hyoscine	0·91(0·83–1·0)	1·2	6·8(5·4–9·2)	9·3
Caramiphen	3·00(1·49–10·2)	4·1	8·6(3·5–16·8)	11·8
G3063	6·81(3·9–16·9)	9·3	58·0(35·1–110·7)	79·5
PMCG	1·51(1·22–1·87)	2·0	45·5(26·5–134·8)	62·3
Ditran	1·46(1·17–1·84)	2·0	24·6(11·1–72·7)	33·6
P2S	0·81(0·71–0·94)	1·1		

Drugs were given intramuscularly 15 min before sarin subcutaneously.
LD50 for sarin aline = 0·73 μmol/kg (103 μg/kg).

TABLE 4. *Protection of guinea-pigs against sarin*

Drug (Dose 50 μmol/kg)	Drug alone: LD50 μmol/kg with 95% confidence limits	Drug alone: Protection ratio	Drug+140 μmol/kg P2S: LD50 μmol/kg with 95% confidence limits	Drug+140 μmol/kg P2S: Protection ratio
Atropine	0·36(0·33–0·40)	1·3	10·2(6·9–16·3)	35·8
Hyoscine	0·40(0·33–0·48)	1·4	6·1(1·8–10·4)	21·4
Caramiphen	0·31(0·26–0·36)	1·1	5·3(1·7–17·2)	18·6
G3063	0·43(0·36–0·51)	1·5	16·6(8·9–30·0)	58·3
PMCG	0·43(0·33–0·57)	1·5	24·0(15·4–38·8)	84·2
Ditran	0·40(0·33–0·48)	1·4	20·0(13·3–30·1)	70·2
P2S	0·31(0·24–0·40)	1·1		

Drugs were given intramuscularly 15 min before sarin subcutaneously.
LD50 for sarin alone = 0·28 μmol/kg (40 μg/kg).

Correlation coefficients were calculated between the various sets of values for anticholinergic activity of the drugs and the protection they afforded either alone or in combination with P2S, but in no case was a significant correlation demonstrated.

Rat phrenic nerve-diaphragm preparation

Some of the anticholinergic drugs were tested for their ability to modify single twitch and tetanic contractions elicited by stimulation of the phrenic nerve in isolated rat diaphragm preparations. A bath concentration of 27 μmol/l. of PMCG resulted in a gradual increase in the muscle response to single stimuli. Responses to 5 and 30 Hz stimulation were also increased but with a frequency of stimulation of 50 Hz a partial block of the Wedensky type was seen and there was a decrease in the initial contraction height. Similar results were obtained with most of the other drugs studied. Potentiation of both single twitches and tetani occurred with a concentration of 14 μmol/l. of PMCG, caramiphen, G3063, Ditran and N-methyl-3-piperidyl benzilate. With atropine a concentration of 140 μmol/l. was required to produce similar degrees of potentiation. Hyoscine was without effect at 220 μmol/litre.

The effects of PMCG and G3063 alone, or in combination with P2S, were studied on tetanic contractions of rat diaphragm preparations poisoned with sarin. At a bath concentration of 12 nmol/l. sarin the amplitude of responses to single stimuli applied every 10 s rapidly increased. Fifteen minutes after addition of sarin responses to 5, 30 and 50 Hz stimulation were recorded. The response to 5 Hz stimulation was partially maintained but responses to 30 and 50 Hz stimulation were not maintained. The drugs alone, or in combination with P2S, were added to the bath 20 min after sarin and tetanic responses were recorded at 5 min intervals. The sarin was left in the bath throughout the experiment.

Concentrations of 27 μmol/l. of PMCG or G3063 had no effect on the Wedensky inhibition produced by sarin. P2S at a concentration of 0·5 mmol/l. completely reversed the effects of sarin, with tetanic contractions being restored to their normal levels. When P2S was used together with either PMCG or G3063 there was only partial restoration of the tetanic contractions especially at low rates of stimulation.

Discussion

These results show clearly, even without statistical analysis which is scarcely justified with so few compounds, that no correlation exists between the peripheral anticholinergic potencies of these drugs (as measured either by production of mydriasis or by blockade of oxotremorine-induced salivation) and their capacity, when used alone or in conjunction with P2S, to protect from the lethal effects of sarin. This finding is in agreement with that of Coleman *et al.* (1962). Additionally, the results indicate no correlation between protection effectiveness and central anticholinergic potency, as measured by blockade of oxotremorine-induced tremors.

There seem to be two possible explanations for this lack of correlation. In the first place there is the possibility that in the protection experiments the concentration of drugs at those sites which are important for survival may not be the same as the concentrations reached in the mydriasis and oxotremorine experiments. This is very difficult to investigate experimentally. The second possible explanation is that in view of the large doses used a pharmacological action other than the ability

to compete with acetylcholine at muscarinic receptors might have some significance in protection.

Since neuromuscular blockade is one of the main consequences of anticholinesterase poisoning, and one of the main causes of death, it seemed relevant to investigate whether the anticholinergic drugs used here showed any effect at the neuromuscular junction. It had been reported by Abood & Biel (1962) that PMCG potentiated isometric contractions of the isolated frog sartorius muscle produced by submaximal stimulation of the attached sciatic nerve. The experiments reported here using the rat phrenic nerve-diaphragm preparation confirmed this effect with PMCG and other glycollic acid esters and with caramiphen and its analogue G3063. Atropine and hyoscine were only weakly active and inactive, respectively.

The significance, if any, of this effect in the sarin-poisoned animal is open to question. In the experiments reported here using the rat phrenic nerve-diaphragm preparation there was no evidence that either PMCG or G3063 reversed the action of sarin; indeed these drugs seemed to antagonize to some extent the beneficial effects of P2S. However, the actions of PMCG have been studied in more detail *in vivo* in the cat by Brimblecombe & Everett (1969a). They showed that this drug potentiated contraction of skeletal muscle by an action mainly on the muscle membrane. Higher doses depress twitches and tetani by a similar action and probably partly by a curare-like action. In a further study (Brimblecombe & Everett, 1969b) the effects of PMCG in sarin-poisoned cats were investigated. It was shown that pretreatment with the drug protected both fast and slow muscles (flexor hallucis longus and soleus) from the effects of sarin in potentiating twitches and, to a lesser extent, in causing a decrease in tetanic tension. Administration of PMCG subsequent to sarin reduced the established effects of the latter. However, the dose of PMCG used (about 2 mg intra-arterially) was relatively high and further studies are required before the possible significance of these findings in relation to the protective action of the drug can be established.

In all three species P2S given alone afforded very little protection, with protection ratios of only 1·1 or 1·2. With the exception of caramiphen and G3063 in rats, none of the anticholinergic drugs used alone showed protection ratios in excess of 2. Combinations of anticholinergic drugs with P2S gave greater protection than would be expected for an additive action of the two individual components. This probably lends weight to the point made in the **Introduction** that the main sites of action of the two components are central and peripheral respectively; for maximum protection both are necessary.

There were marked differences in the degree of potentiation found when drugs were used in combination with P2S. No fully acceptable explanation can be offered for this at present. Differences in the rate of onset and duration of action of the anticholinergic drugs may be contributory factors, but preliminary studies do not reveal any simple relationship. Neither can any satisfactory explanation be given for the marked species differences observed in these experiments. There were differences in the toxicity of sarin, which may be explicable on the basis of species variation in its rates of hydrolysis. In addition there were differences in the ease with which sarin-poisoning could be treated; mice in which sarin had the highest LD50 were more difficult to treat than guinea-pigs in which sarin had the lowest LD50.

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