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Actions of sarin on fast-twitch and slow-twitch skeletal muscles of the cat and protective action by anticholinergic drugs

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Summary

1. The organophosphate cholinesterase inhibitor sarin has been studied for its effects on the contraction of fast-twitch (flexor hallucis longus, FHL) and slow-twitch (soleus) muscles in the cat.

2. In both muscles lower doses (2·5 to 20 μg intra-arterially) potentiated, and higher doses (20 μg and over) depressed, twitches produced by indirect stimulation. Muscle action potentials became repetitive. There was no simple relationship between degree of inhibition of blood cholinesterase and effect on muscle twitch.

3. The effects of the drug on repetitively stimulated muscles were dependent on both dose and frequency of stimulation. Doses of 2·5 to 10 μg increased the degree of fusion of low frequency tetani (10 Hz for FHL, 5 Hz for soleus) but depressed and caused non-maintenance of tetani at high frequencies (150 Hz and above for FHL, 60 Hz and above for soleus). Doses of 20 μg and above depressed and caused non-maintenance of tetani at all frequencies.

4. The anticholinergic drug N-ethyl-2-pyrrolidylmethylcyclopentylphenyl glycollate (PMCG) injected intra-arterially protected both muscles from the effects of sarin on twitch but less so from effects on tetani. When given after sarin the drug reversed twitch potentiation and repetitive firing. In contrast, atropine had little effect on the responses to sarin.

5. The effects of (+)-tubocurarine were compared with those of PMCG.

6. The possible mode of action of PMCG is discussed.

Introduction

The main effects of the organophosphorus cholinesterase inhibitor sarin at the neuromuscular junction are generally held to be the result of acetylcholine accumulation (see Wills, 1963), although there is evidence of other less significant actions (Cohen & Posthumus, 1955 ; Groblewski, McNamara & Wills, 1956 ; Kunkel, Wills & Monier, 1956). Death in anticholinesterase poisoning results from respiratory failure in which bronchoconstriction and neuromuscular block precede central respiratory failure (De Candole, Douglas, Evans, Holmes, Spencer, Torrance & Wilson, 1953).

Numerous antagonists of the neuromuscular blocking actions of sarin and other anticholinesterases have been studied, with varying success. While atropine and hyoscine undoubtedly relieve central paralysis of respiration after tetraethylpyrophosphate (TEPP) in the cat (Douglas & Matthews, 1952) and the rabbit (Wright, 1954) and after sarin in the rat (Stewart, 1959), these anticholinergic compounds do little to relieve the neuromuscular block at the diaphragm and other sites. However, whereas atropine had no striking effect on the rate of recovery of the twitches of the cat gastrocnemius-soleus muscle group after block by sarin, Kunkel *et al.* (1956) observed that the quaternary derivatives, N-benzyl atropinium chloride and N-phenacyl atropinium bromide, promoted a striking and lasting return of twitch height towards normal.

The search for other anticholinergic drugs which might be better antagonists to anticholinesterase poisoning, primarily as adjuncts to oxime therapy, has revealed that there is no apparent correlation between anticholinergic activity and ability to protect against the lethal effects of sarin (Brimblecombe, Green & Stratton, 1968 ; Brimblecombe, Green, Stratton & Thompson, 1970). However, these workers reported the marked protective activity *in vivo* of N-ethyl-2-pyrrolidylmethyl-cyclopentylphenyl glycollate (PMCG), which is 4 times less active peripherally and 5 times more active centrally than atropine. Brimblecombe & Everett (1969a, 1970) have described other marked pharmacological actions that this compound possesses in cat fast-twitch and slow-twitch skeletal muscle. In an attempt to determine whether such actions contribute to its protective action *in vivo,* the interaction between PMCG and sarin has been studied within these same cat muscles. Preliminary findings have been reported elsewhere (Brimblecombe & Everett, 1969b).

Methods

The muscles studied were the flexor hallucis longus (FHL, fast-twitch muscle) and the soleus (slow-twitch muscle) of the cat hind limb. The methods employed were as described in the preceding paper (Brimblecombe & Everett, 1970). Blood cholinesterase determinations were made using the method of Fleisher, Pope & Spear (1955).

The drugs used were methylisopropyl phosphonofluoridate (sarin, synthesized at C.D.E., Porton), N-ethyl-2-pyrrolidylmethylcyclopentylphenyl glycollate (PMCG, synthesized at C.D.E., Porton), atropine sulphate (B.D.H.) and (+)-tubocurarine (B.D.H.).

Sarin was dissolved in iso-propyl alcohol and diluted with saline immediately before use. Doses expressed in the text refer to the bases or cations.

Results

Effect of sarin on twitches

Low doses of sarin (2·5–20 μg) potentiated twitches of FHL by up to 700% and of soleus by up to 230%. Higher doses (20 μg and over) depressed twitches below control levels. Some recovery was observed within 2 to 3 h following dosage with sarin but doses given within 30 min of each other were additive. Muscle action potentials became repetitive within 1 to 2 min of the intra-arterial injection of sarin and became more pronounced with increasing dosage (Fig. 1a and b). The resulting

twitches were considerably distorted and lengthened in time to peak with marked increases in the maximum rate of rise of tension.

Blood cholinesterase

In some experiments blood cholinesterase levels were determined on samples taken from the femoral vein 15 min after injection of sarin. Doses of sarin were usually

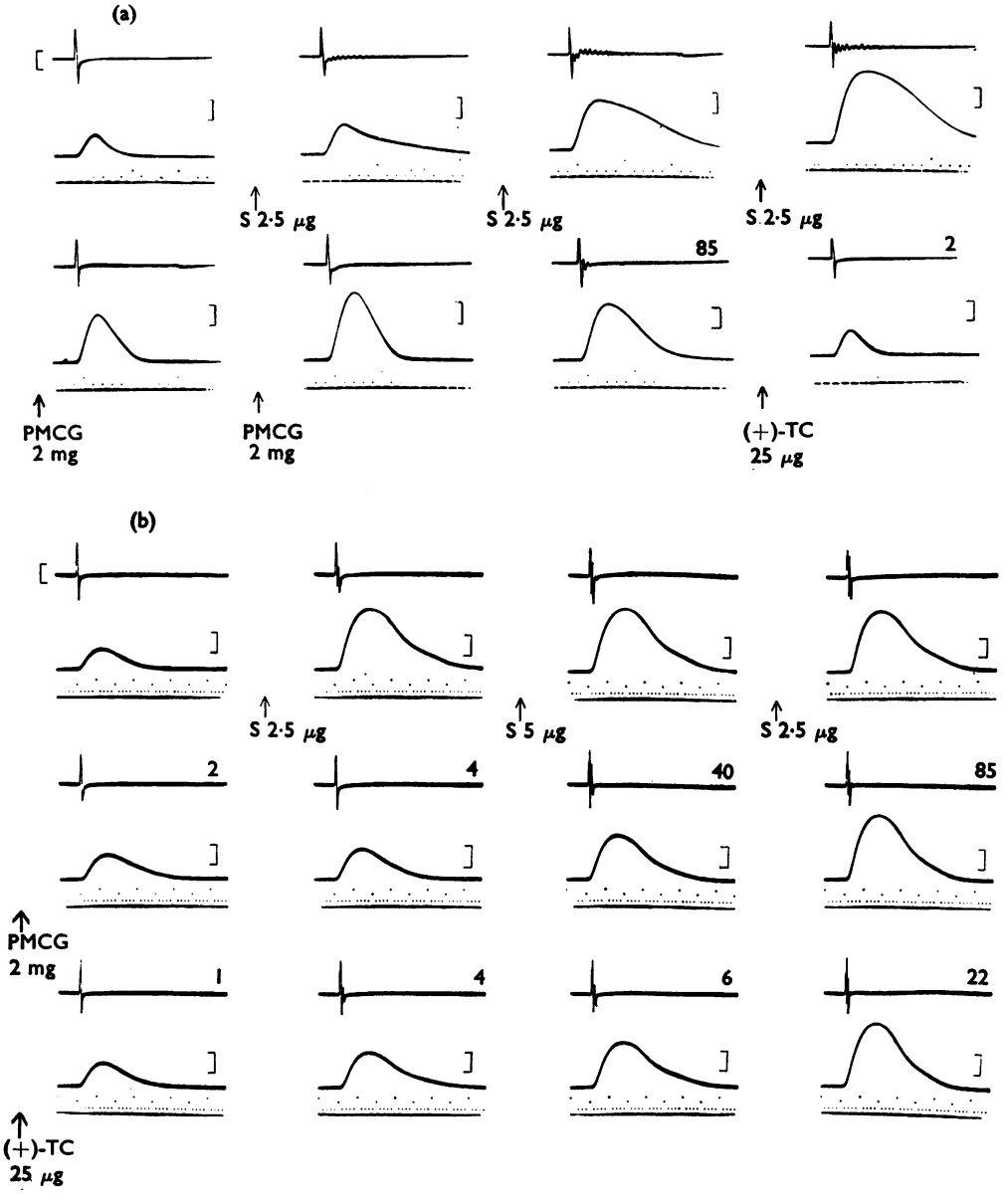

FIG. 1. Twitch potentiation and repetitive firing after sarin: effect of PMCG and (+)-tubocurarine. a, FHL ; b, soleus. At the arrows, sarin (S), PMCG and (+)-tubocurarine ((+)-TC) in the doses indicated. Numerals indicate time in minutes after PMCG or (+)-TC. Calibration: twitch tension 200 g, muscle action potential 5 mV in a, 2·5 mV in b, time base 10 ms.

given at intervals of 30 minutes. Corresponding twitch values (expressed as % control) and blood cholinesterase levels following increasing doses of sarin are shown in Fig. 2. Worthy of note is the fact that in some experiments maximum potentiation of the muscle twitches was obtained before measurable inhibition of blood cholinesterase occurred. In different experiments maximum potentiation occurred with blood cholinesterase levels of 40–100% control values. It appeared that maximum potentiation occurred at lower cholinesterase inhibitions when the initial dose of sarin was relatively large. At higher doses of sarin (20 μg and over) twitch tensions fell, and those below control values were accompanied by cholinesterase inhibitions of 80–90%.

Effect of sarin on tetani

The responses to tetanic stimulation after sarin were dependent on the frequency of stimulation and the dose of sarin. Low doses (2·5–10 μg) increased the degree of fusion of low frequency tetani (10 Hz for FHL, 5 Hz for soleus), but depressed tetani at high frequencies (150 Hz and above for FHL, 60 Hz and above for soleus). In addition these latter tetani waned during the stimulation period (0·5 to 3 s). Usually there was a small range of intermediate frequencies at which tetani showed no apparent change. Higher doses (20 μg and above) depressed tetani at all frequencies, tension of those at the higher frequencies waning rapidly to zero. The effects of sarin (5 μg) on FHL tetani at 10, 20, and 160 Hz for 3 s and the extent of recovery within 140 min is shown in Fig. 3.

Blood cholinesterase

Waning of tetanic tension, which occurred in the presence of low doses of sarin (5–10 μg), occurred when blood cholinesterase levels were still at least 40% control levels.

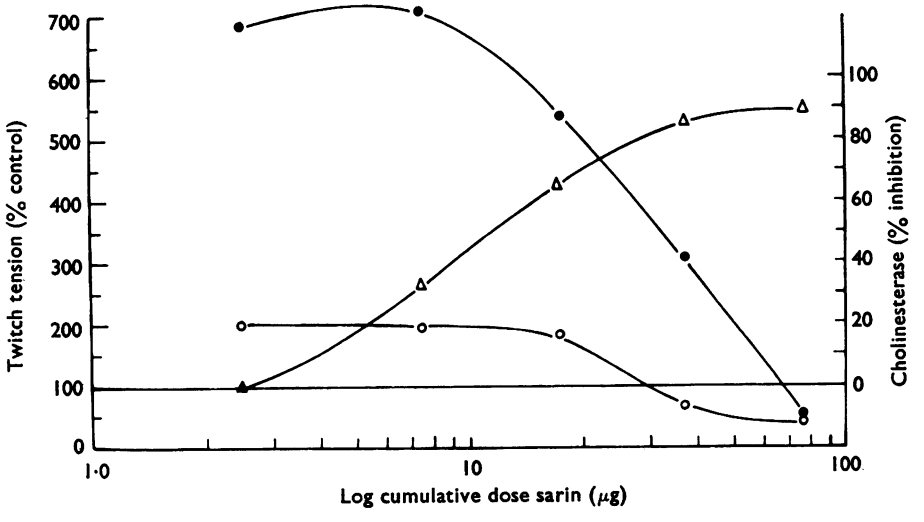

FIG. 2. Cumulative effects of sarin on twitch tensions of FHL (●) and soleus (○) and inhibition of cholinesterase (△).

Interactions between sarin and PMCG

Since the effects of sarin persist over many hours it is virtually impossible to use any muscle as its own control. Therefore, in another series of experiments, similar muscles were prepared in both hind legs and stimulated alternately every 10 s. Control twitches and tetani were recorded for each muscle and generally compared closely. PMCG (2 mg) was then used to pretreat one muscle in one leg only. The dose chosen had little or no potentiating action of its own—particularly in the case of soleus. Five minutes later, sarin (5 μg) was injected intra-arterially into both

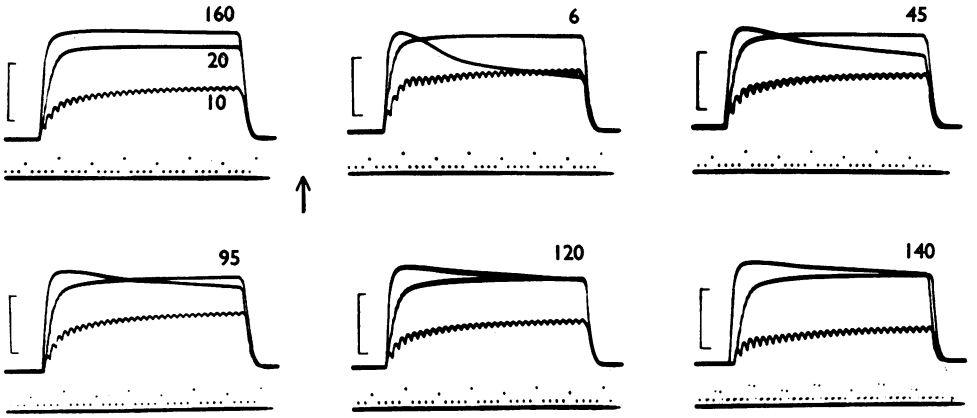

FIG. 3. FHL. Effect of sarin on tetanic stimulation of 3 s duration at frequencies of 10, 20 and 160 Hz as indicated. At the arrow sarin 5 μg. Numerals indicate time in min after sarin. Calibration: tetanic tension 1 kg, time base 100 ms.

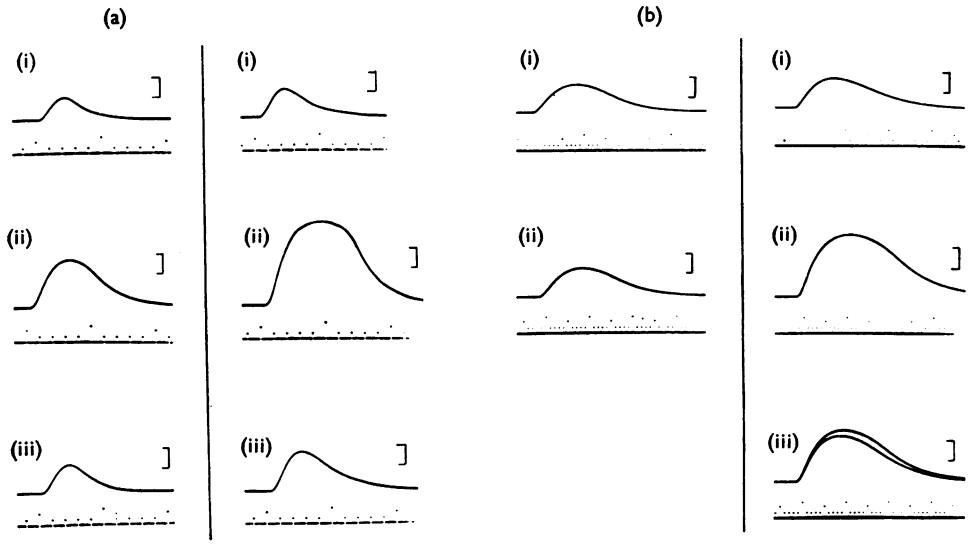

FIG. 4. Both legs. a, FHL ; b, soleus (different cats). All muscles, sarin 5 μg given between (i) and (ii). Left hand records in a and b: muscles pretreated with PMCG 2 mg. Right hand records in a and b: no pretreatment but PMCG (2 mg in a and 4 mg in b) given between (ii) and (iii). In b twitches before and after (smaller twitch) PMCG have been superimposed. Calibration: twitch tension 200 g, time base 10 ms.

legs. Typical results for both FHL and soleus are shown in Fig. 4. The protective action of PMCG against the effect of sarin is clearly demonstrated in the pretreated muscles while the twitches of the unprotected leg were grossly abnormal after injection of sarin. The protective action of PMCG lasts for 15 to 30 min and sometimes up to an hour, after which it slowly declines. It can be renewed by further dosage of the same order. Similarly, as shown in Fig. 4, PMCG (2–10 mg) is effective in reducing twitch size towards normal after sarin has exerted maximal effects.

Tetani were far less well protected, particularly at the higher frequencies. Often in these cases the effects of further dosage with PMCG were additive, increasing both the degree of depression and the non-maintenance. This reflects the action of PMCG itself (see Brimblecombe & Everett, 1970). Attempted protection of tetani of FHL (60 Hz for 0·5 s) is shown in Fig. 6.

Since there is a distinct possibility that PMCG may be protecting twitches against sarin by virtue of its weak curare-like action (Brimblecombe & Everett, 1970), the protection afforded by tubocurarine itself has been estimated and compared with that of PMCG. Fig. 1a and b includes such a comparison and muscle action potentials show the development of repetitive firing after increasing the sarin in both muscles. Following PMCG (2 mg) and tubocurarine (25 μg) repetitive firing and

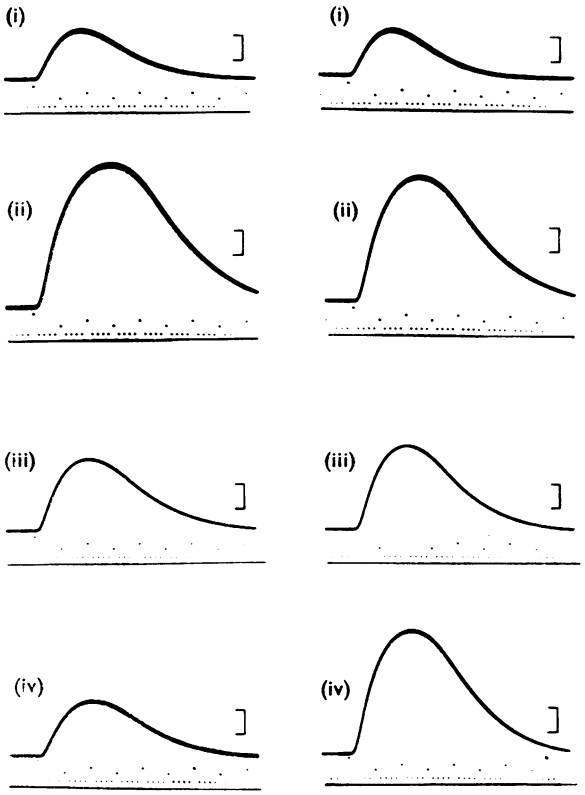

FIG. 5. Both legs. Soleus. Both records: (i) control twitches; (ii) after sarin 2·5 μg. Left hand record, (iii) 3 min after PMCG 1 mg and (iv) 30 min after PMCG (34 min after sarin). Right hand record, (iii) 3 min after (+)-tubocurarine 25 μg and (iv) 30 min after (+)-TC (34 min after sarin). Calibration: twitch tension 200 g, time base 10 ms.

potentiation after sarin (total 7·5 μg in a, 10 μg in b) are abolished but there is a very marked difference in the duration of these actions exerted by each drug. This is clearly shown for soleus (Fig. 1b). The duration of action of each drug is taken as the time for redevelopment of maximum tension and repetitive firing. After PMCG repetition of the action potential reappeared 30 min later and became maximal after some 85 minutes. Following this, tubocurarine again suppressed repetition but it reappeared within 4 min and was maximal within 22 minutes.

This difference between PMCG and tubocurarine is further illustrated in Fig. 5 in which the results of a two leg experiment are presented. Both soleus muscles were studied, control twitches before and after sarin (2·5 μg to each leg) being recorded. Roughly equipotent protective doses (as determined in other experiments) of PMCG (1 mg) and tubocurarine (25 μg) were injected, one drug to each hind leg. The time course of protection and redevelopment of the effects of sarin were followed. Although both PMCG and tubocurarine reduced the sarin-potentiated twitches to control size, this action of PMCG far outlasted that of tubocurarine. Thus 30 min after treatment, PMCG protection was still complete while the curarized muscle was completely unprotected as evidenced by its maximal potentiation.

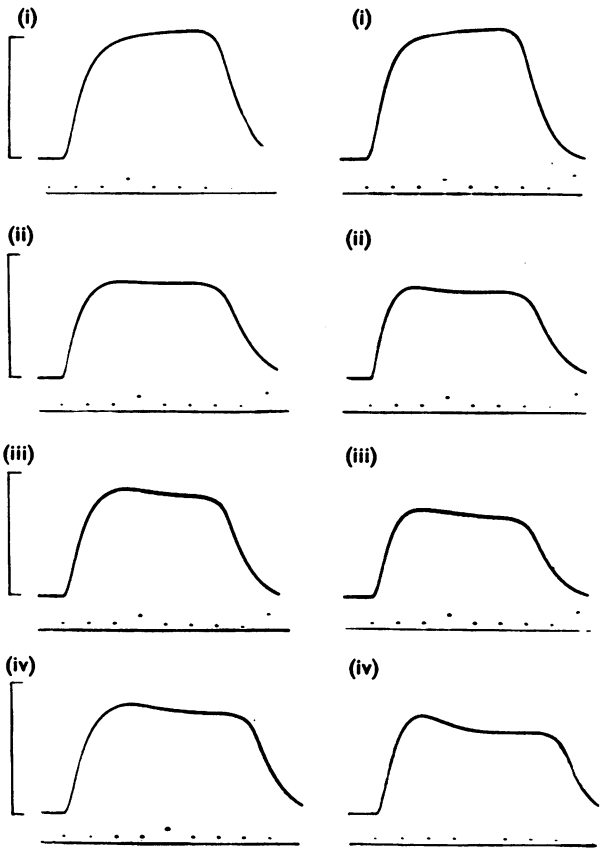

FIG. 6. Both legs. Soleus (same cat as in Fig. 5). Both records: (i) control tetani at 60 Hz; (ii) after sarin 2·5 μg. Left hand record, (iii) 4 min after PMCG 1 mg and (iv) 35 min after PMCG (39 min after sarin). Right hand record, (iii) 4 min after (+)-tubocurarine 25 μg and (iv) 35 min after (+)-TC (39 min after sarin). Calibration: tetanic tension 1 kg, time base 100 ms.

In the experiment with these soleus muscles, tetani (60 Hz for 0·5 s) were also studied (Fig. 6). At this frequency and duration the effect of sarin (2·5 μg) was evident but not particularly pronounced. However, in the circumstances, the small improvement resulting from PMCG administration is shown by increase in maximum tetanic tension (over the sarin depressed tension). In contrast, tubocurarine appeared to be without beneficial action.

Blood cholinesterase

Blood cholinesterase levels were determined during experiments in which PMCG was given both before and after sarin. No change in the development of cholinesterase inhibition by sarin could be demonstrated even though twitches were protected by PMCG.

Atropine

Finally, atropine itself was tested for protective ability in these muscles and, confirming reports of other workers, was found to be very poor in this respect.

Discussion

The results of these experiments yield no direct information concerning the relationship between cholinesterase levels in the muscles studied and the phenomena described, but according to Rump, Kaliszan & Edelwejn (1968) there is little discrepancy between the inhibited cholinesterase levels in erythrocyte, plasma and muscle 2 min after intravenous injection of sarin in the rat. Barnes & Duff (1953) found that blood and muscle levels of cholinesterase fell with similar time courses after paraoxon providing dosage was not too high ; with high dosage erythrocyte levels fell far more rapidly. Our results indicated that potentiation of twitches could occur before even blood levels were noticeably changed, and that enhancement was still present with inhibition up to 60%. Barnes & Duff (1953) observed enhancement of rat diaphragm twitches with inhibition of muscle cholinesterase from 50% down to 10%. Barstad (1960) found that enhancement of the rat diaphragm twitches after dyflos commenced at cholinesterase levels of 30% normal, but that increases of 46% occurred at 26% cholinesterase. Inhibition of twitches was observed at levels below 8%. Our results, therefore, suggest that either these workers were not measuring cholinesterase levels soon enough after observation of twitch enhancement or that, contrary to other reports, muscle cholinesterase in our cats was more inhibited than that in the blood. Our blood samples were immediately frozen in solid carbon dioxide to prevent further interaction between sarin and the enzymes.

Further discrepancies occur in the literature over the extent of cholinesterase inhibition required for abolition of tetanic responses. Our results are in general agreement with those of Rump *et al.* (1968), who found that tetani of FHL and soleus were much depressed when cholinesterase levels were still 30–40% control. Barnes & Duff (1953), using the rat diaphragm, observed the ability to maintain tetani with levels as low as 10% and possibly with only 5% pseudo- and zero true cholinesterase present. A most important factor here is the frequency of tetanic stimulation, the higher it is the more marked the depression of tension observed

by the different workers. Barstad (1960) demonstrated this when he showed that tetani at 60 Hz and 120 Hz were sustained when cholinesterase was 10–15% and 15–20% of control respectively.

The results described establish that protection against sarin intoxication can indeed be demonstrated in skeletal muscle. Protection of the response to single stimuli and abolition of established sarin potentiation and repetitive firing by PMCG, in doses which themselves do comparatively little, are striking. Protection of tetani, especially at the higher frequencies, is much less obvious but at physiological rates of stimulation the effects may be beneficial.

The mechanism of this action of PMCG, and for that matter, other effective atropine-like compounds, in skeletal muscle is not obvious, but several possibilities may be considered. Before discussing these it should be said that the blood cholinesterase determinations rule out any possibility of regeneration of inhibited cholinesterase by PMCG.

It is accepted that atropine itself contains some so-called " curare-like " action (Bülbring, 1946 ; Beranek & Vyskocil, 1967, 1968). PMCG probably has some such activity too, since lower doses are necessary to block the effects of indirect than direct stimulation of FHL and soleus muscles (Brimblecombe & Everett, 1969a, 1970). Not unexpectedly, tubocurarine and gallamine increase the rate of return of the twitch height towards normal (Kunkel *et al.*, 1956 ; Rump *et al.*, 1968) and shorten recovery time of tetani (Axelsson, Gjone & Naess, 1957 ; Rump & Kaliszan, 1968a, b) after sarin and other anticholinesterases. However, the former authors question the usefulness of tubocurarine in this respect because of the critical nature of the correct dosage and also the considerable variation in response dependent on the anticholinesterase and the muscle under study. With PMCG, however, there was no evidence of curare-like activity at the doses used, but it should be remembered that only subthreshold doses of tubocurarine are required to abolish repetitive firing, as shown in these experiments and by others (Werner, 1961 ; Kojima & Takagi, 1969). In addition, while atropine has some curare-like activity it is only a poor antagonist to sarin and other anticholinesterases in skeletal muscle. On this basis it seems unlikely that any weak curare-like component of the activity of PMCG could account for the effects observed. In any case PMCG shows considerable superiority over curare in terms of duration of action.

Another point to be considered is that of the properties of local anaesthetics in relation to anticholinesterase activity. Procaine, in particular, is effective in abolishing repetitive firing and normalizing muscle twitches after administration of anticholinesterases (Harvey, 1939a ; Jaco & Wood, 1944 ; Werner & Kuperman, 1963). Relevant to this, Straughan (1961) has shown that procaine reduces acetylcholine output during tetanic nerve stimulation of the rat diaphragm preparation *in vitro*. More recently, Rump (1966) has demonstrated that cinchocaine normalizes the sarin-potentiated twitches of the rat anterior tibial muscle but is without effect on the blocked response to repetitive stimulation at 50 Hz. Since PMCG also possesses quite potent local anaesthetic properties, in fact more so than procaine (Brimblecombe, in preparation), it is not unreasonable to offer this action as a candidate for the mechanism of action of PMCG against sarin in cat muscles. Atropine is a weak local anaesthetic in comparison with PMCG and those local anaesthetics quoted above.

Furthermore, there is a remarkable similarity between the properties in skeletal muscle of quinine (Harvey, 1939b ; Ravin, 1940) and PMCG, both from the point of view of individual actions and interactions with an anticholinesterase.

Finally, it is most interesting that Ca^{2+} and Mg^{2+} are effective in suppressing muscle repetitive firing after dyflos (van der Meer & Meeter, 1956). Since PMCG and related compounds most probably exert many of their actions by modifications of normal calcium transport within membranes (Abood, 1968) it can be suggested that this effect alone may be responsible for the marked protective action of PMCG in muscles exposed to sarin.

It is difficult to relate the contribution of this effect of PMCG in sarin-affected cat skeletal muscles to the observed protection in whole animals. Undoubtedly, other factors such as antagonism of the central and peripheral muscarinic actions of acetylcholine—that is atropine-like actions—play a large part in the whole animal. However, it is suggested that the potency of PMCG, additional to that expected from comparison with atropine on other systems, may be the result of further antagonism within skeletal muscles, of which the most important would be those involved in respiration.

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