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Effectiveness of pyridostigmine in reversing neuromuscular blockade produced by soman

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The effects of pyridostigmine pretreatment on the neuromuscular blockade produced by soman in anaesthetized, atropinized animals have been studied on the soleus and anterior tibialis muscle (rhesus monkeys, cats and rabbits) and the gastrocnemius muscle (guinea-pigs and rats). Pyridostigmine pretreatment produced a complete recovery of neuromuscular function following blockade by soman; the rate of recovery was similar in all the species, suggesting a common mechanism of action. In the absence of pyridostigmine or if pyridostigmine was delayed until after blockade by soman, there was no recovery of neuromuscular function. Detailed studies in the guinea-pig showed that the recovery of neuromuscular function was related to the dose of soman and to the degree of carbamoylation of blood cholinesterase at the time of nerve agent challenge, i.e. to the dose of pyridostigmine and the time interval between the administration of pyridostigmine and soman. It is suggested that the effectiveness of pyridostigmine pretreatment is due to the carbamoylation of a portion of the tissue acetylcholinesterase, which protects it against irreversible inhibition by soman: after poisoning spontaneous decarbamoylation produces sufficient free acetylcholinesterase to restore normal function.

Poisoning by the organophosphate anticholinesterase compound soman does not respond to treatment with a combination of atropine and an oxime (Loomis & Salafsky, 1963; Heilbron & Tolagen, 1965), but pretreatment with certain carbamates, in conjunction with atropine therapy, confers considerable protection against soman poisoning (Berry & Davies, 1970; Gordon, Leadbeater & Maidment, 1978). The quaternary carbamate pyridostigmine, which does not readily cross the blood brain barrier (Birtley, Roberts & others, 1966), has been shown to be one of the most effective carbamates tested (Gordon & others, 1978). It is likely therefore that this compound exerts its main protective action at a peripheral site. The effect of pyridostigmine pretreatment on soman-induced neuromuscular blockade (paralysis), caused by the accumulation of acetylcholine, has therefore been investigated.

MATERIALS AND METHODS

Compounds

The following were synthesized within this Establishment; soman (1,2,2-trimethylpropyl methylphosphonofluoridate), VX(*o*-ethyl *S*-diisopropylaminoethyl methyl-phosphonothiolate), P_2S(1-methyl, 2-hydroxyiminomethyl pyridinium methanesulphonate) and pyridostigmine iodide. Atropine sulphate was purchased from BDH Limited, and BC51 [hexamethylene bis (*N*-methyl-carbaminoyl-1-methyl-3-oxypyridinium hydrochloride] was provided by Professor F. Hobbiger.

Animals

'Porton Strain' guinea-pigs (450–550 g), 'Porton Strain' rats (300 –350g), 'Old English' rabbits (2·0–2·3 kg), cats (2·0–3·0 kg) and rhesus monkeys (2·8–3·2 kg), all of mixed sexes, were used.

Recording of single twitch and tetanic contractions from striated muscle

Guinea-pigs and rhesus monkeys were anaesthetized with 3 ml kg^{-1} of chloralose urethane (2·5% chloralose, 25% urethane aqueous solution) by the intramuscular (i.m.) and intravenous (i.v.) routes respectively; rabbits with 7 ml kg^{-1} (i.v.) of a 25% aqueous solution of urethane and cats and rats with 10 ml kg^{-1} of a 1% aqueous solution of chloralose given by the intravenous and intramuscular routes respectively.

Recordings were made from the tibialis anterior and soleus muscles in rhesus monkeys, cats and rabbits and from the gastrocnemius muscle in rats and guinea-pigs using a method similar to that described by the Edinburgh Staff (1970). The hind limb was set up in a horizontal position on a myograph stand and the tendon of the muscle from which the recording was to be made was attached to a Devices 4150 isometric force transducer (linear

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up to 1·5 kg). The sciatic nerve was cut between ligatures high in the thigh and bi-polar platinum electrodes were placed on the distal part of the nerve. A small wad of cotton wool soaked in liquid paraffin was placed on the electrode-nerve assembly and the preparation was maintained as close as possible to 37° by means of a height adjustable 100 W light bulb positioned directly over the muscle.

Single twitches were elicited by stimulating the sciatic nerve with rectangular pulses of 0·2 ms duration applied at 0·25 Hz. Tetanic fused contractions were elicited at 2 min intervals by trains of 0·2 ms pulses applied for 3 s at 50 Hz in cats and at 100 Hz in other species; the voltage of the stimulus was twice that required to evoke a maximal response. The electrical stimuli were delivered by means of a Devices Digitimer and recordings were made on a Devices M19 direct writing polygraph. The drugs were dissolved in 0·9% NaCl and were administered into a cannulated femoral vein. Details of times of administration and doses are given in Results and Discussion. In some experiments where spontaneous respiration ceased, artificial respiration was applied by means of a Palmer pump connected to a tracheal cannula, in order to maintain life for the duration of the experiment.

Estimation of whole blood cholinesterase (ChE)—time activity profiles after intravenous administration of pyridostigmine and BC51

In chloralose urethane anaesthetized guinea-pigs the left jugular vein and right carotid artery were cannulated for injection of the drug and collection of serial blood samples, respectively. ChE activity was measured at 30° by the spectrophotometric method of Ellman, Courtenay & others (1961). The reaction was measured at 412 nm with a Pye-Unicam SP 500 coupled to a Weyfringe ADCP-2 digital printer. An optimal substrate concentration of 1 mM acetylthiocholine was used. The reaction was linear for 2 min and therefore no decarbamoylation was occurring during the assay. The term ChE refers to total acetylthiocholine hydrolase activity of the blood. All results are expressed as a percentage of the control values obtained from each animal. The mean value of ChE activity in whole blood of untreated guinea-pigs was 2·32 with s.d. 0·46 μmol min^{-1} ml^{-1} blood.

RESULTS AND DISCUSSION

A. *Effectiveness of pyridostigmine pretreatment in reversing neuromuscular blockade produced by soman in various species*

The protection afforded by pyridostigmine pretreatment against lethality in soman poisoning is subject to marked species variation, guinea-pigs respond better than rabbits whereas rats are relatively unresponsive (Gordon & others, 1978); it has subsequently been shown (Dirnhuber, French, Green, Leadbeater & Stratton, unpublished) that rhesus monkeys respond better than guinea-pigs. In view of this species difference the protective action of pyridostigmine against neuromuscular blockade by soman has been studied in several species.

Animals were given pyridostigmine 100 μg kg^{-1} (i.v.) and 10 min later a dose of atropine sulphate (1 mg kg^{-1}, i.v. in rhesus monkeys, 2 mg kg^{-1}, i.v. in cats and 17 mg kg^{-1}, i.v. in rabbits, guinea-pigs and rats); the atropine was given to protect against depression of the medullary respiratory centre (Douglas & Matthews, 1952) and against the muscarinic actions of accumulated acetylcholine produced by a subsequent infusion of soman. These doses of atropine were also given 5 min before soman infusion in all control experiments where pyridostigmine was not administered. Fifteen min after pretreatment with pyridostigmine sufficient soman was infused intravenously over 4 to 6 min to produce a reduction in tetanus tension of about 70% and then the infusion was switched off; in the following 2 to 3 min this reduction in tension spontaneously proceeded to a value of 85–98%. This method was used so that the degree of neuromuscular blockade could be controlled and the method also allowed the sequence of events occurring during the neuromuscular blockade to be followed.

The results from soleus muscle in the rhesus monkey are illustrated in Figs 1 and 2. Treatment with 100 μg kg^{-1} pyridostigmine produced no effect on single twitch or tetanus tension or the degree of single twitch post-tetanic potentiation observed before treatment. When the soman was infused, a typical anticholinesterase action was observed (Hobbiger, 1976), i.e. single twitch tension increased and tetanus tension declined (tetanus fade) with a transient decrease in single twitch tension following the tetanus (Fig. 1). With pyridostigmine pretreatment full recovery of tetanus tension, together with post-tetanic single twitch potentiation, occurred within 20 to 30 min after poisoning (Figs 1 and 2) whereas there was no recovery when pyridostigmine was given after soman poisoning (Fig. 2) or without pyridostigmine pretreatment. Pyridostigmine pretreatment showed no marked effect in enhancing soman-induced decrease in tetanus tension.

The recordings of single twitch tension demon-

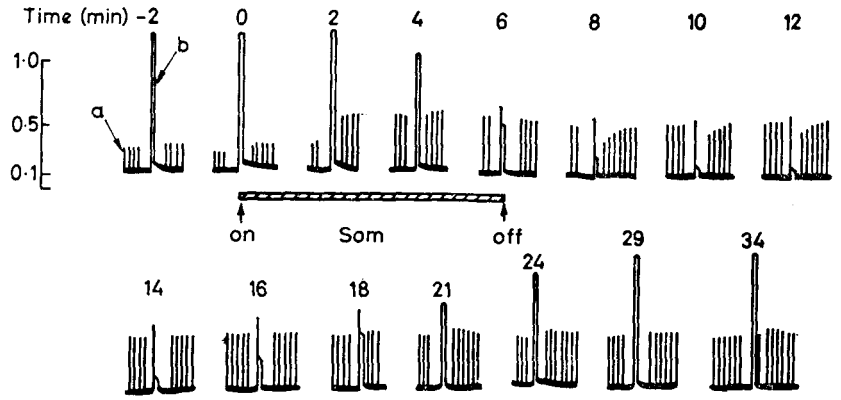

FIG. 1. Rhesus monkey soleus muscle. Experimental record (traced) showing effect of pyridostigmine pretreatment (100 μg kg^{-1}, i.v. given 15 min before soman infusion Som, 2 μg kg^{-1} min^{-1}) in restoring soman depressed tetanus tension. a: single twitch; b: tetanus. Scale represents kg.

strated the anticholinesterase action of soman. However, quantitative studies on the decline of tetanus tension are more relevant than those on single twitch tension in terms of relating recovery from neuromuscular blockade with survival since in the conscious animal the frequency of impulses in motor neurons (e.g. those controlling respiratory muscles) during muscular activity ranges from 9 to 110 Hz, with a mean of 50 Hz (Krnjevic & Miledi, 1958; Hobbiger, 1976). Thus the effectiveness of pyridostigmine in restoring soman-depressed tetanic responses elicited by high frequency stimulation (50 or 100 Hz) and not effects on single twitch tension,

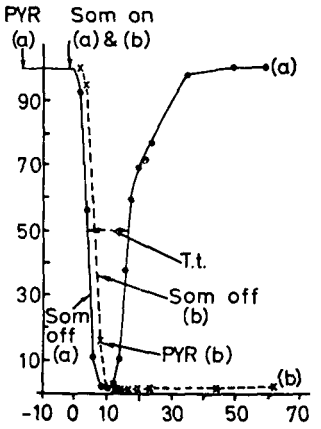

FIG. 2. Rhesus monkey soleus muscle. Effectiveness of pyridostigmine pretreatment (100 μg kg^{-1}, i.v.) in restoring soman depressed tetanus tension. (a) ●—●, pyridostigmine (PYR) given 15 min before infusion of soman (14 μg kg^{-1}, i.v.); (b) × – – ×, PYR given 7 min after infusion of Som (14 μg kg^{-1}, i.v.). Som: Soman. T.t.: tetanus tension recovery time. Ordinate: Tetanus tension (% of maximum). Abscissa: Time (min).

has been determined as a measure of recovery from soman-induced neuromuscular blockade.

The results obtained with all the species tested are summarized in Table 1. Recovery time from the soman-induced neuromuscular blockade was assessed by measuring the time interval between 50% reduction in tetanus tension to 50% tetanus tension recovery (see Fig. 2). The 50% levels were chosen since at these points the decline and recovery rate of tetanus tension was highest and the time intervals between these points were highly reproducible. The recovery time was constant in all types of muscle (e.g. slow-contracting soleus and fast contracting anterior tibialis) and in all the species tested. The dose of soman required to reduce tetanus tension varied with the species and it appeared to be related to the lethal toxicity of soman in the same species. These results suggest that the mechanism by which pyridostigmine reverses the neuromuscular blockade by soman is the same in all species tested.

In rhesus monkeys, guinea-pigs and rabbits, it was observed that in the absence of pyridostigmine pretreatment, respiratory arrest occurred when the soman-induced decrease in tetanus tension fell below 85% and artificial respiration had to be given to support life even though the animals were pretreated with atropine to protect the respiratory centre (Table 1). Assuming that the intercostal and diaphragm muscles, which maintain respiratory movement, are affected by soman to a similar extent as the hind limb muscles from which records were made in the present experiments, it may be expected that where this decrease in tetanus tension occurred, respiratory muscle paralysis would concurrently be produced leading to death. In pyridostigmine-pretreated

Table 1. *Effectiveness of pyridostigmine (Pyr) pretreatment (100 μg kg⁻¹) in restoring tetanus tension (T.t. = recovery time in min between 50% decline and 50% recovery) depressed by infusion of soman (Som. μg kg⁻¹, i.v. reducing tetanus 85–98%) in atropinized animals.*

Species	Exp. No.	Muscle	Som.	T.t.	Time of arrest of spont. resp. (min after start of infusion)
Rhesus monkey	1 (Con. No pyr.)	SOL	14	∞*	8
		TIB		∞*	
	2	SOL	14	13	No arrest
		TIB		14	
	3	SOL	14	14	No arrest
		TIB		12	
Cat	1 (Con.)	SOL	50	∞	No arrest
		TIB		∞	
	2	SOL	45	13	No arrest
		TIB		9	
	3	SOL	55	16	No arrest
		TIB		13	
Rabbit	1 (Con.)	SOL	30	∞*	6
		TIB		∞*	
	2	SOL	20	15	No arrest
		TIB		11	
	3	SOL	30	12	No arrest
		TIB		11	
Guinea-pig	1 (Con.)	GAS	18	∞*	10
	2	GAS	20	6	No arrest
	3	GAS	22	8	No arrest
	4	GAS	26	8	No arrest
Rat	1 (Con.)	GAS	288	∞*	3
	2	GAS	176	13*	1
	3	GAS	155	12*	2

TIB = Tibialis anterior; SOL = Soleus; GAS = Gastrocnemius; * Artificial respiration applied.

monkeys, guinea-pigs and rabbits, tetanus tension was decreased by more than 85% but respiration remained intact. However, the decrease in tetanus tension below the 85% level was short lived (Fig. 1) and the rate of recovery was probably sufficient to maintain respiratory muscle function. Although the depth of anaesthesia will to a certain extent affect the time of arrest of respiration during soman infusion, the results obtained with these species do suggest that the protective effective of pyridostigmine at the neuromuscular junction may play a major role in survival.

In the absence of pyridostigmine no arrest of respiration was observed in the cat where tetanus tension was decreased by 85 to 98%, but in the rat respiration ceased at doses of soman that produced only 50 to 80% reduction in tetanus tension. This marked respiratory effect of soman in the rat was also observed in pyridostigmine-pretreated rats (Table 1) where recovery of tetanus tension could only be demonstrated by applying artificial respiration for the duration of the experiment. This result supports a previous suggestion (Meeter & Wolthuis, 1968) that the lethal action of soman in the rat is due to a very predominant central action; this may possibly account for the ineffectiveness of pyridostigmine pretreatment in protecting against the lethal effects of soman in the rat (Gordon & others, 1978).

B. *Mode of action studies in guinea-pigs*

Studies relating inhibition of functional acetylcholinesterase (AChE) at the neuromuscular junction with tetanus tension fade (Hobbiger, 1976) have shown that tetanus fade only occurs when more than 70% of AChE is inhibited. Furthermore the difference between the level of inhibition at which tetanus fade was just perceptible and that at which tetanus fade was complete was relatively narrow (e.g. 70% inhibition perceptible and 78% inhibition complete, at a stimulation frequency of 100 Hz).

It has been suggested (Berry & Davies, 1970) that the effectiveness of carbamate pretreatment in organophosphate poisoning may be explained on the basis of partial inhibition of AChE by carbamoylation; this partial inhibition would prevent complete phosphonylation of AChE by the organophosphate. The carbamoylated AChE would then undergo spontaneous decarbamoylation and provided that the rate of this enzyme regeneration was less than the rate of loss of free agent from the nervous system sufficient free AChE, which may be related to the fraction of carbamoylated AChE at the time of nerve agent challenge, would become available to maintain life. The following experiments were carried out, using the gastrocnemius muscle in guinea-pigs, to evaluate the above hypothesis. In all experiments atropine sulphate (17 mg kg⁻¹, i.v.) was given 5 min before injection of soman; tetanus tension recovery time was assessed by measuring the time interval between 50% decline to 50% recovery of tetanus tension (Fig. 2).

(a) *Relation between recovery time of tetanus tension and dose of soman administered.* Animals were pretreated with pyridostigmine (100 μg kg⁻¹, i.v.) 15 min before infusion of soman, the soman being infused over a constant time of 6 min (0·1 ml min⁻¹); the concentration of soman was varied. There was a linear relation between tetanus recovery time and log dose of soman (Fig. 3).

(b) *Relationship between dose of pyridostigmine and tetanus tension recovery time.* The results are summarized in Table 2. Doses between 1 and 100 μg kg⁻¹ (i.v.) given 15 min before infusion of soman effectively restored tetanus tension and the higher

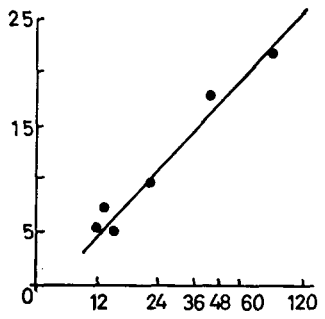

FIG. 3. Guinea-pig gastrocnemius muscle. Relationship between tetanus tension recovery time (min) (ordinate) and dose of soman infused (μg kg^{-1}, i.v.) (abscissa).

the dose the faster was the recovery time. Tetanus tension in animals pretreated with pyridostigmine, 1 μg kg^{-1}, recovered only 3 times slower than in animals pretreated with 100 μg kg^{-1}. At doses below 1 μg kg^{-1} tetanus tension failed to recover. In these experiments the degree of carbamoylation of whole blood cholinesterase (ChE) was estimated immediately before infusion of soman. Above the 5% carbamoylation level there appeared to be an inverse relation between recovery time of tetanus tension and degree of carbamoylation of blood ChE. The limit of the blood ChE assay procedure did not permit estimates of enzyme inhibition below 5%, and determinations of blood ChE activity produced by doses of pyridostigmine (0·5 and 1·0 μg kg^{-1}) that would be expected to give less than 5% inhibition were, therefore, not made. The blood ChE inhibition figures represent inhibition of both AChE and butyrylcholinesterase but 80 to 90% of the total acetylthiocholine hydrolase activity in guinea-pig blood is due to the AChE (French, Sellers & Wilkinson, 1977).

Table 2. *Guinea-pig gastrocnemius muscle.* Relation between tetanus recovery time, dose of pyridostigmine and whole blood ChE carbamoylation at time of challenge with soman. (Soman infused (24 to 28 μg kg^{-1} in 4 to 6 min) to produce 85 to 98% decrease in tetanus tension). I Dose of pyridostigmine given intravenously 15 min before the start of soman infusion. II Tetanus tension recovery time (min). Mean time between 50% decline to 50% recovery. n = 3. III % blood ChE carbamoylation at time of challenge with soman. n = 2. Limits of experimental variation in brackets.

I	II	III
100	5·5 (4·0–7·5)	48·0 (45–51)
20	7·6 (6·0–9·1)	30·0 (26–34)
4	11·0 (9·5–14·0)	5·5 (5–6)
1	15·5 (14·0–17·5)	—
0·5	∞	—

(c) *Duration of effectiveness of pyridostigmine pretreatment in restoring tetanus tension.* Animals were pretreated with pyridostigmine (20 μg kg^{-1}, i.v.) and soman was infused at various times afterwards. The results are summarized in Table 3. Tetanus tension recovered in 7, 7 and 11 min after soman challenge when the latter was given 15 min, 1 h and 2 h respectively after administration of pyridostigmine. During this 15 min to 2 h period blood ChE carbamoylation was more than 18%. Four h after pretreatment when blood ChE carbamoylation was about 10%, tetanus tension recovery time was extended to 32 min.

Table 3. *Guinea-pig gastrocnemius muscle.* Duration of effectiveness of pyridostigmine (20 μg kg^{-1}, i.v.) pretreatment in restoring soman depressed tetanus tension. (Soman infused (24 to 28 μg kg^{-1}, i.v. in 4 to 6 min) to produce 85 to 98% decrease in tetanus tension.) I Time after pyridostigmine administration (h). II Tetanus tension recovery time (min). Time between 50% decline to 50% recovery. n = 3. III % blood ChE carbamoylation at time of challenge with soman. n = 2. Limits of experimental variation in brackets.

I	II	III
0·25	7·6 (6·0– 9·1)	30 (26–34)
1·0	7·0 (6·5– 7·6)	30 (29–31)
2·0	11·0 (10·2–14·0)	18 (15–21)
4·0	32 (20·0–50·0)	10 (5–15)

The results of the experiment described in (a) to (c) above are consistent with the hypothesis that recovery of neuromuscular function after pretreatment with pyridostigmine is related to spontaneous regeneration of free AChE.

According to this hypothesis any excess soman still present will phosphonylate any spontaneously reactivated AChE and reduce the rate of recovery of tetanus tension. Recovery of tetanus tension should therefore be related to the dose of soman administered; such a relation was found.

The amount of spontaneously reactivated AChE will be proportional to the amount of enzyme carbamoylated. When the dose of pyridostigmine was varied and the duration of effectiveness of pyridostigmine determined there appeared to be a relationship between degree of whole blood ChE carbamoylation and recovery of tetanus tension.

To conform with this hypothesis the amount of spontaneously reactivated AChE should also depend on the rate of decarbamoylation as well as the amount of enzyme carbamoylated. Evidence for this was obtained by showing that pretreatment of guinea-pigs with BC51, a bis-quaternary carbamate with a long duration of action associated with a very slow rate of spontaneous reactivation of BC51 carbamoylated AChE (Herzfeld, Kraupp & others, 1957), gave a slower and less complete recovery from soman depressed tetanus tension than pretreatment with pyridostigmine. A blood profile of ChE activity in guinea-pigs (Fig. 4) showed that with 36 μg kg^{-1} (i.v.) BC51, the maximum degree of carbamoylation (about 56%) occurred 60 to 90 min after dosing and no measurable decarbamoylation occurred over 3 h; with 100 μg kg^{-1} pyridostigmine the maximum degree of carbamoylation (about 55%) occurred 30 to 40 min after dosing but the degree of inhibition then declined to 14% after 4 h. When animals were pretreated with 36 μg kg^{-1} (i.v.) BC51 and infused

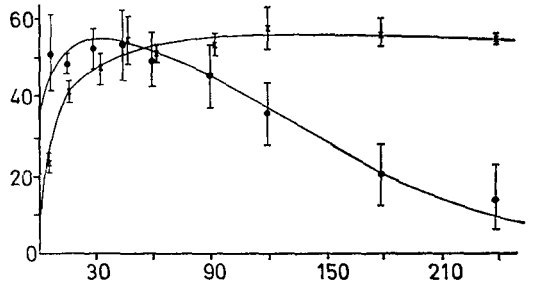

FIG. 4. Anaesthetized guinea-pig. Inhibition of blood ChE after intravenous administration of 100 μg kg^{-1} pyridostigmine (●—●) and 36 μg kg^{-1} BC51 (×——×). Bars show limit of experimental variation (n = 2). Ordinate: % inhibition of whole blood ChE. Abscissa: Time (min).

with soman 1 h later (i.e. corresponding to the time of maximal carbamoylation) tetanus tension was only slowly and partially restored, the degree of restoration depending on the dose of soman (range 23–90 μg kg^{-1}, i.v.) administered (Fig. 5). In contrast, when animals were pretreated with 100 μg kg^{-1}, i.v. pyridostigmine and infused with soman 30 min later, complete recovery of tetanus tension occurred in the dose range 20–150μg kg^{-1}, i.v. soman (Fig. 5). According to these results pyridostigmine would be expected to be more effective than BC51 in protecting against the lethal effects of soman. This is indeed the case; it has been shown (Green & Inns, unpublished) that pretreatment of guinea-pigs with BC51, supported by atropine/P_2S/diazepam therapy,

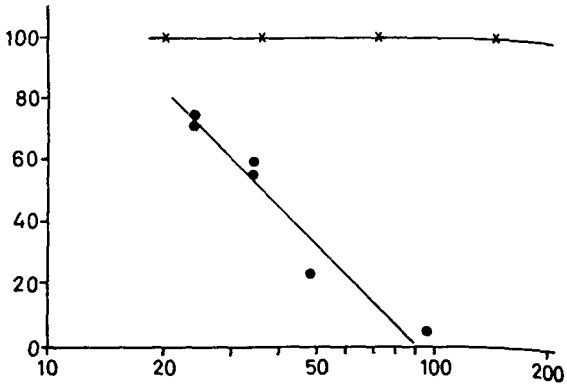

FIG. 5. Guinea-pig gastrocnemius muscle. Comparison between effectiveness of pretreatment with pyridostigmine (100 μg kg^{-1}, i.v.) (×——×) and 36 μg kg^{-1}, i.v. BC51 (●—●) in restoring tetanus tension after challenge with various doses of soman. Ordinate: % recovery of tetanus tension 1 h after soman in fusion. Abscissa: Dose of soman infused (μg kg^{-1}, i.v.).

protects against only 4 LD50s soman compared with a protection against 14 LD50s by pyridostigmine pretreatment supported by the same therapy.

There remains a possibility that pyridostigmine may be exerting its protective action by a direct effect unrelated to spontaneous reactivation of AChE. Evidence against such a direct action was the finding that pyridostigmine was ineffective when given after poisoning. Further evidence for the spontaneous reactivation hypothesis and against a

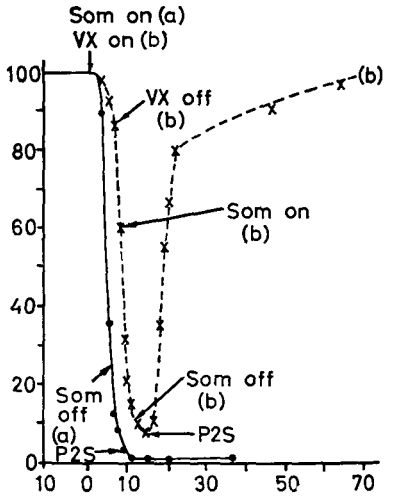

FIG. 6. Guinea-pig gastrocnemius muscle. Effectiveness of VX followed by P2S in restoring soman depressed tetanus tension. (a) ●—●, soman 4 μg kg^{-1} min^{-1}, (i.v.) for 6 min followed by 15 mg kg^{-1} (i.v.) P_2S (b) × – – ×, VX 2 μg kg^{-1} min^{-1} (i.v.) for 6 min followed by soman 4 μg kg^{-1} min^{-1} (i.v.) for 6 min followed by 15 mg kg^{-1} P_2S. Som: Soman. Ordinate: Tetanus tension (% of maximum). Abscissa: Time (min).

direct action was provided by demonstrating that it was possible to reverse soman-depressed tetanus tension by regenerating AChE, previously inhibited by the 'oxime sensitive' organophosphate VX (Gordon & Leadbeater, 1977). This was achieved by infusing for 6 min a total of 12 μg kg^{-1} of VX, followed 30 s later by a 6 min infusion of 24 μg kg^{-1} soman to produce about 85% final reduction in tetanus tension. Termination of this infusion was immediately followed by intravenous injection of 15 mg kg^{-1} of the oxime P_2S. The results are shown in Fig. 6. Tetanus tension recovered completely at a similar rate to that which occurred in animals pretreated with 100 μg kg^{-1}, (i.v.) pyridostigmine. When soman was infused alone subsequent injection of P_2S produced no recovery of tetanus tension.

To provide more definite evidence for the hypothesis, relationships between recovery of neuromuscular function and levels of inhibition and free functional AChE at the motor end plate region should be determined, since degree of blood ChE carbamoylation is not a reliable index to the level of inhibition at the neuromuscular junction.

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REFERENCES

BERRY, W. K. & DAVIES, D. R. (1970). *Biochem. Pharmac.*, **19**, 927–934.

BIRTLEY, R. D. M., ROBERTS, J. B., THOMAS, B. H. & WILSON, A. (1966). *Br. J. Pharmac.*, **26**, 393–402.

DOUGLAS, W. W. & MATTHEWS, P. B. C. (1952). *J. Physiol., Lond.*, **116**, 202–218.

EDINBURGH STAFF (1970). *Pharmacological experiments in intact preparations*, p. 42. Edinburgh and London: E. & S. Livingstone.

ELLMAN, G. L., COURTENAY, K. D., ANDREWS, V. & FEATHERSTONE, H. M. (1961). *Biochem. Pharmac.*, **7**, 88–95.

FRENCH, M. C., SELLERS, D. J. & WILKINSON, R. G. (1977). *Biochem. Pharmac.*, **26**, 1263–1266.

GORDON, J. J., LEADBEATER, L. & MAIDMENT, M. P. (1978). *Toxic. appl. Pharmac.* In the press.

GORDON, J. J. & LEADBEATER, L. (1977). *Ibid.*, **40**, 109–114.

HEILBRON, E. & TOLAGEN, B. (1965). *Biochem. Pharmac.*, **19**, 927–934.

HERZFELD, E., KRAUPP, O., PATEISKY, K. & STUMPH, C. (1957). *Wein. Klin. Wochenschr.*, **69**, 245–248.

HOBBIGER, F. (1976). *Handbuch der experimentellen Pharmakologie*. New Series, **42**, pp. 487–581. Editor: Zaimis, E. Berlin, Heidelberg, New York: Springer.

KRNJEVIC, K. & MILEDI, R. (1958). *J. Physiol., Lond.*, **140**, 440–461.

LOOMIS, T. A. & SALAFSKY, B. (1963). *Toxic. appl. Pharmac.*, **5**, 685–701.

MEETER, E. & WOLTHUIS, O. L. (1968). *Eur. J. Pharmac.*, **2**, 377–386.