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A GANGLION STIMULATING ACTION OF NEOSTIGMINE

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The actions of neostigmine on transmission in the superior cervical ganglion have been investigated by means of the nictitating membrane preparation in the anaesthetized cat. Intravenous injections of neostigmine produced a rapid and often complete reversal of hexamethonium block, whereas eserine had no effect. This reversal by neostigmine was obtained regularly even after large doses of eserine or dyflos, but was sometimes brief. In ganglia perfused with heparinized plasma containing neostigmine (10^{-8} to 10^{-4}), there was no potentiation of the responses to maximal stimulation of the preganglionic nerve. Intra-arterial injections of neostigmine to both normal and preganglionically denervated ganglia produced first a potentiation of the responses to nicotine, then, with larger doses, a contraction of the nictitating membrane and, concurrently, a depression of the responses to nicotine. These results are consistent with the view that neostigmine exerts a direct stimulant action on the ganglion, which is distinct from its anticholinesterase action.

Eserine and neostigmine are both inhibitors of cholinesterase, yet their actions at the skeletal neuromuscular junction differ (Bülbring & Burn, 1942). This difference was investigated by Riker & Wescoe (1946) and Wescoe & Riker (1957), who reported that neostigmine has a direct stimulant action at the skeletal neuromuscular junction in addition to its anticholinesterase action, and that this accounts for the rapidity with which the effects due to neostigmine appear at this site, for instance, the rapid reversal of the block produced by competitive blocking agents such as tubocurarine. As eserine exerts only an anticholinesterase action at this site, its effects, such as the reversal of competitive block, are slower in onset, since acetylcholine has to accumulate at the synapse.

Anticholinesterases have been reported to exert comparatively little effect on the ganglionic synapse (Feldberg & Vartiainen, 1935 ; Brown & Feldberg, 1936 ; Cannon & Rosenblueth, 1937 ; Eccles, 1944 ; Kamijo & Koelle, 1952), since only in the presence of submaximal stimulation at low frequencies could any facilitation of transmission through the ganglion be obtained with these compounds.

Evidence is now presented that neostigmine can exert a direct stimulant action on ganglia analogous to that at the skeletal neuromuscular junction.

METHODS

All the experiments were carried out in cats anaesthetized with pentobarbitone sodium (30 to 60 mg/kg). Transmission in the superior cervical ganglion was assessed by recording the responses of the nictitating membrane. The cervical sympathetic nerve was stimulated

electrically using stimuli of 0.5 msec duration at a frequency of 10/sec sufficient to elicit a maximal response (Bell & Quilliam, 1956). Drugs were administered either intravenously through a cannula in the femoral vein, or by retrograde intra-arterial injection through a cannula in the lingual artery while the external carotid artery was clamped. Care was taken to tie all the arterial branches so that the injection was limited to the ganglion and immediately adjacent structures and did not reach a wider area.

Perfusion of the ganglion was carried out as described by Feldberg & Gaddum (1934). The perfusion medium was usually heparinized plasma obtained from the cat at the beginning of the dissection. In a few experiments Locke solution containing 6% dextran was used in place of plasma. Control experiments showed that transmission through the ganglia was well maintained with both media, provided the flow was not less than 0.15 ml./min. The constitution of the Locke solution was: NaCl 0.9 g; KCl 0.042 g; $CaCl_2$ 0.024 g; $NaHCO_3$ 0.015 g; glucose 0.1 g; and distilled water to 100 ml.

In the denervated preparations the preganglionic fibres had been divided at least 14 days before. The cats were anaesthetized with ethyl chloride and pentobarbitone sodium. Using an aseptic technique, the cervical sympathetic nerve on one side was identified, a piece 0.5 cm long was cut out low in the neck, the wound was closed, and the animal was allowed to recover. Denervation was confirmed, first, by the relaxation of the nictitating membrane and by the changed pupil diameter, and, secondly, by observation of the division of the nerve during the dissection when setting up the preparation.

RESULTS

Effects on ganglion block due to hexamethonium

In the nictitating membrane preparation subjected to continuous preganglionic stimulation sufficient to elicit a maximal response, the intravenous injection of neostigmine (40 μg/kg to 500 μg/kg) regularly reversed the ganglion block produced by a previous injection of hexamethonium bromide (0.4 to 0.8 mg). This reversal was rapid in onset and usually restored transmission almost completely, so that the contraction of the nictitating membrane was nearly as great as that seen before the hexamethonium. This reversal was seen whether or not the preparation had received atropine (1 to 2 mg/kg). The intravenous injection of 100 μg/kg eserine did not reverse the block due to a previous injection of hexamethonium bromide. When the dose of eserine was increased to 400 μg/kg, the ganglion block was temporarily increased, after which the usual rate of recovery was resumed.

Provided the preparations had been atropinized to eliminate any peripheral effects of the anticholinesterases, pretreatment with neostigmine or eserine did not produce any consistent reduction in the sensitivity of the preparations to ganglion blocking substances. Indeed, in one experiment the injection of larger doses of neostigmine (1 to 1.5 mg/kg) increased the sensitivity of the preparation to hexamethonium.

Effects of neostigmine on hexamethonium block after dyflos or eserine

These experiments were carried out in preparations which had previously received atropine and a large dose of either dyflos or eserine, considered to inhibit completely the cholinesterase in the preparation (Kamijo & Koelle, 1952).

Five experiments were carried out in the presence of atropine 1 to 2 mg/kg and dyflos 4 to 5 mg/kg. In every instance, 20 to 80 μg/kg of neostigmine intravenously reversed the block due to a previous injection of 0.4 to 0.8 mg hexamethonium (Fig. 1). However, in most preparations the reversal of the block was temporary

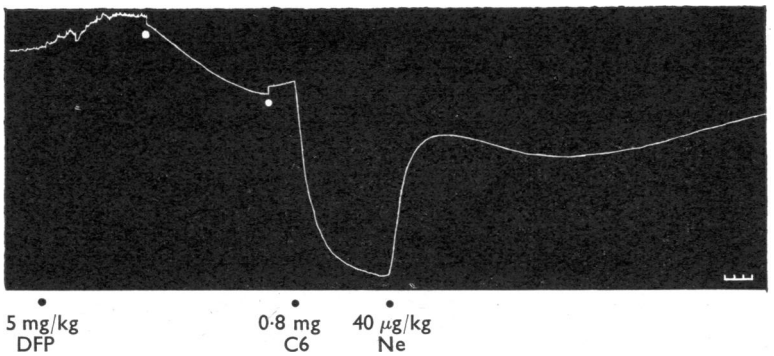

Fig. 1. Contractions of the nictitating membrane (upwards) in a cat (2.9 kg) anaesthetized with pentobarbitone. Atropine, 1 mg/kg, was injected intravenously 1 hr before. The cervical sympathetic nerve was stimulated maximally at 10/sec. throughout. Injections were made intravenously : time, 30 sec. The white dots indicate that the drum was stopped for 10 min. 40 μg/kg neostigmine (Ne) produced an immediate reversal of the block due to 0.8 mg hexamethonium (C6) in a preparation which had received 5 mg/kg dyflos (DFP) 25 min previously.

so that the membrane relaxed again after 3 to 5 min, sometimes showing greater ganglion block than before. In one preparation pretreated with atropine but which had received 4 mg/kg eserine instead of dyflos, neostigmine in a dose of 20 to 80 μg/kg was ineffective, but larger doses, 185 to 370 μg/kg, produced a temporary reversal of the hexamethonium block, lasting 2 to 4 min.

Effects on transmission under conditions of maximal stimulation

Since neostigmine reversed the ganglion block due to hexamethonium while eserine was without effect, it seemed possible that the neostigmine might potentiate the transmission through the ganglion in conditions of maximal preganglionic stimulation, although Feldberg & Vartiainen (1935) have shown that eserine was without effect. Such a potentiation would occur if the anticholinesterase produced a repetitive response in the ganglion cells for each preganglionic volley.

In the nictitating membrane preparation, stimulation of the preganglionic fibres at frequencies of 0.1 to 10/sec for a period of 2 min produced contractions of the membrane whose size was related to the frequency of stimulation. The frequency/response curve was steepest at frequencies of stimulation of 0.5 to 2.0/sec. In this range even " double " firing of the cells, as distinct from " multiple " firing, would be expected to produce a considerable increase in the response of the membrane. This was confirmed in a control experiment where a frequency/response curve was constructed and then compared with the curve obtained when pairs of stimuli, separated by intervals ranging from 10 to 80 msec, were substituted for each single stimulus. For example, it was found that the response to stimulation with pairs of stimuli at a rate of 2/sec produced a response identical to that produced by evenly spaced stimuli at a rate of 4/sec.

In some experiments the compounds were administered intravenously. However, this method could only be used with small or very large doses, since in the intermediate range muscular fasciculation precluded recording. Therefore in the majority

of the experiments the ganglia were perfused with heparinized plasma and the anticholinesterase added to the perfusion. The principal limitation of this method was the small amount of plasma available.

The concentrations of neostigmine employed ranged from 10^{-8} to 10^{-4}. In none of the experiments was any change recorded in the shape or position of the frequency/response curve which might have indicated repetitive firing of the ganglion cells. Higher concentrations, 10^{-5} to 10^{-4}, decreased the response (Figs. 2 and 3). In two experiments the responses to very low rates of stimulation (0.01 to 0.1/sec) were very slightly potentiated, but this was less than would have been expected with "double" firing. Since it seemed possible that the repetitive firing might occur for only a short time out of each period of stimulation, the responses to stimulation at 2/sec were recorded on a fast kymograph. Any periods of repetitive firing would then be seen as a change in the speed of onset, height, or duration of the response. No such changes were observed.

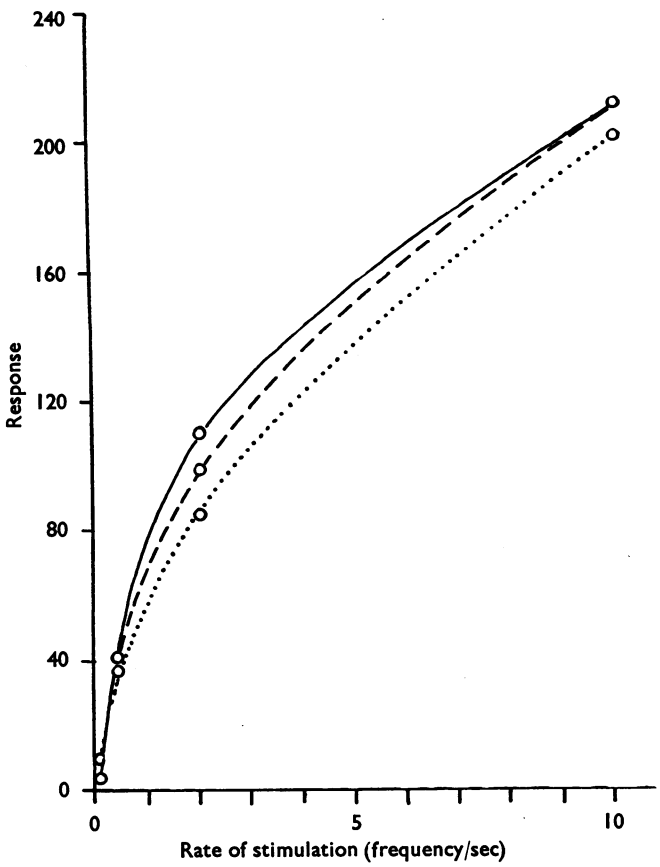

Fig. 2. Graph showing the relationship between the size of contraction of the nictitating membrane in arbitrary units and frequency of maximal stimulation of the cervical sympathetic nerve, when the superior cervical ganglion was perfused with heparinized plasma, alone (solid line) or containing 10^{-8} or 10^{-6} neostigmine (broken and dotted lines respectively).

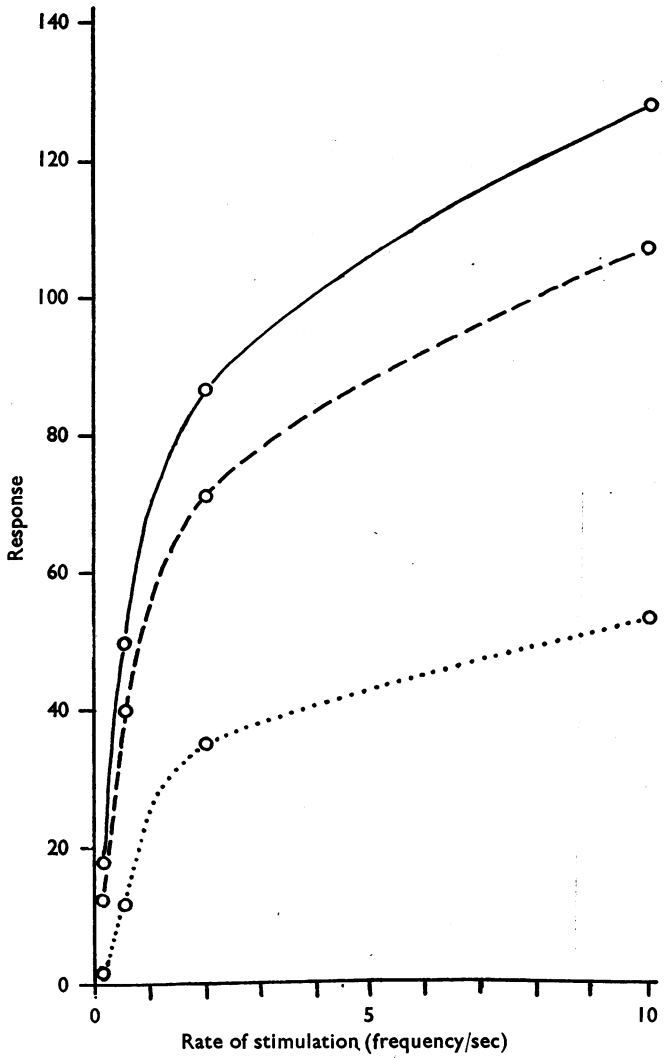

Fig. 3. Graph showing the relationship between the size of contraction of the nictitating membrane and the frequency of stimulation of the cervical sympathetic nerve, when the superior cervical ganglion was perfused with heparinized plasma, alone (solid line) or containing 10^{-5} or 10^{-4} neostigmine (broken and dotted lines respectively).

When ganglia were perfused with eserine (10^{-7} to 10^{-4}), again no changes were observed in the responses which might have indicated repetitive firing in the ganglion cells, but high concentrations, 10^{-5} to 10^{-4}, produced a decrease in the height of the responses.

Responses to close-arterial injections of neostigmine and eserine

From the results reported above it seemed possible that neostigmine might cause a direct stimulation of the ganglion cells. Therefore experiments were carried out

to examine the responses of the ganglion to close-arterial injections of neostigmine and, for comparison, eserine. Since very large doses of neostigmine had to be injected to obtain certain of the responses in normal ganglia, the majority of the experiments were carried out in preparations where the cervical sympathetic had been divided not less than fourteen days before.

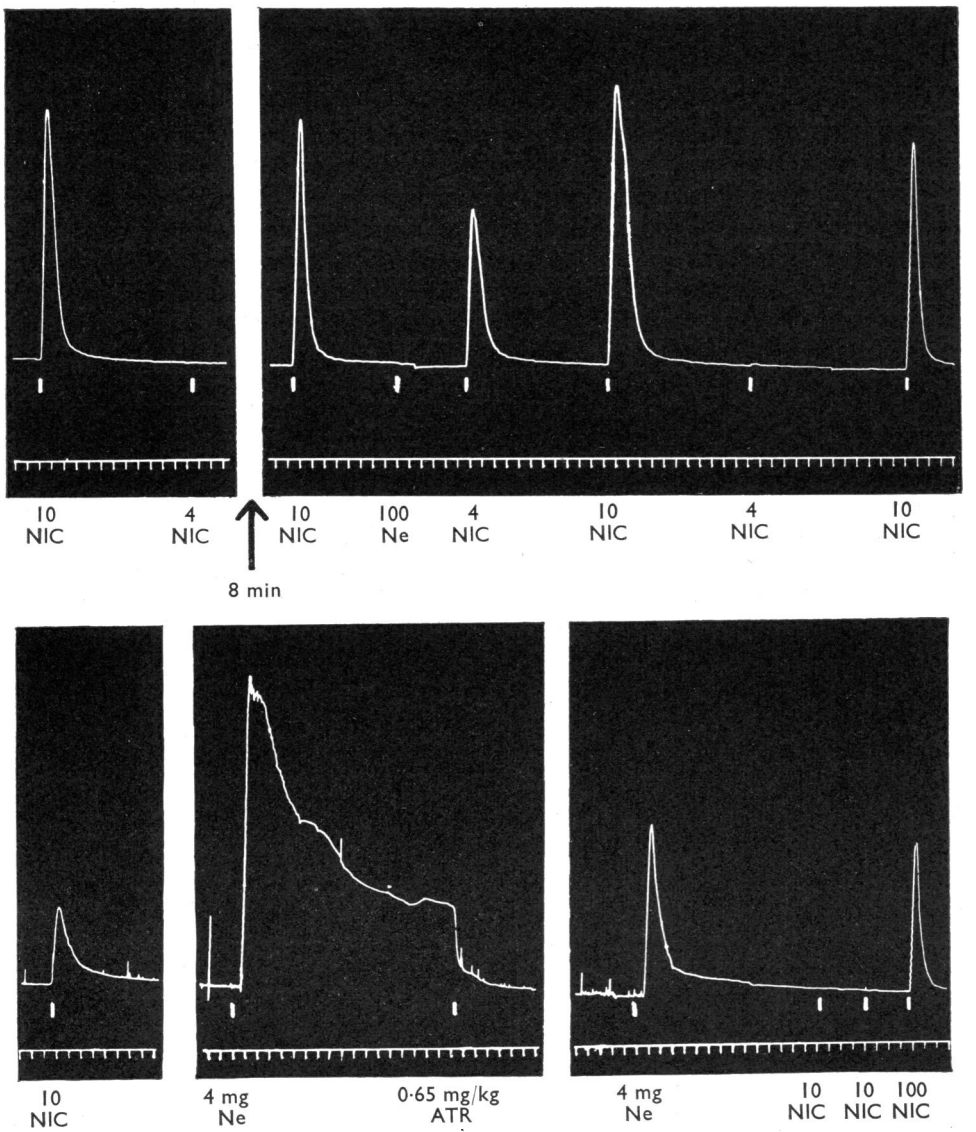

Fig. 4. Contraction of the nictitating membrane (upwards) in a normal cat (4.0 kg) anaesthetized with pentobarbitone. Nicotine (NIC) and neostigmine (Ne) (doses in μg except where otherwise indicated) were injected retrogradely through the lingual artery with a clamp on the external carotid artery. Atropine 0.65 mg/kg (ATR) was injected intravenously: time, 30 sec. There was an interval of approximately 30 min between upper and lower sections of the figure.

The close-arterial injection to the normal ganglion of 100 to 400 μg of neostigmine did not produce any direct effect on the membrane, but there was a potentiation of the response to a subsequent injection of nicotine. In two experiments the injection of 1.0 mg and 4.0 mg neostigmine was followed by a rapid contraction of the membrane which commenced before the injection was complete, and lasted for 2 to 4 min (Fig. 4). Although the responses declined with repeated injection, they could still be elicited after the intravenous injection of atropine, but not after the postganglionic nerves had been divided. Following these large doses of neostigmine, the responses to nicotine were reduced or eliminated.

Intra-arterial injections of neostigmine were made into seven denervated preparations. Doses of 40 μg or greater produced a potentiation of the subsequent responses to injections of nicotine. This potentiation lasted for 10 to 15 min, but the duration of the individual responses to nicotine was not appreciably modified. This potentiation increased with increasing doses of neostigmine, but when very large doses of neostigmine were injected the subsequent responses to nicotine were depressed. Doses of 400 to 1,000 μg neostigmine produced an immediate rapid contraction of the membrane. The delay between the completion of the injection and the appearance of the response was comparable to that after nicotine, and was of the order of 3 to 10 sec. These responses could be elicited repeatedly in each preparation, but each response was smaller than those preceding it. The larger the dose necessary to produce the effect, the more rapidly the succeeding responses declined. While extended rest periods between doses did reduce the decline, it could not be prevented (Fig. 5).

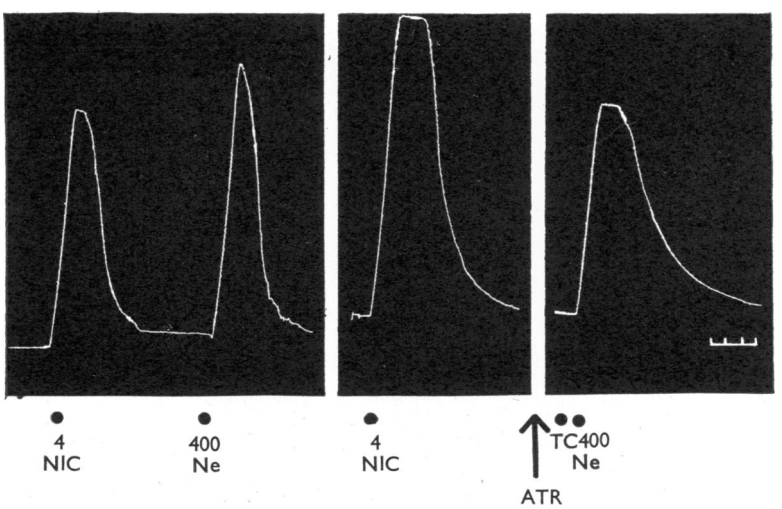

Fig. 5. Contraction of the nictitating membrane (upwards) in a cat (3.5 kg) under pentobarbitone anaesthesia. The cervical sympathetic nerve was divided 23 days before. Nicotine (NIC) and neostigmine (Ne) (doses in μg) were injected retrogradely through the lingual artery with a clamp on the external carotid artery. Tubocurarine 100 μg (TC) was injected through the lingual artery but without a clamp on the external carotid, so that the drug reached the orbit in high concentration. Atropine 0.8 mg/kg (ATR) was injected intravenously: time, 30 sec.

Paton (1954) has shown that in a few preparations the close-arterial injection of compounds to the ganglion may cause a contraction of the nictitating membrane by a peripheral action, instead of, or in addition to, an action on the ganglion. This may occur despite a clamp on the external carotid artery and the tying of adjacent arterial branches. Apparently these agents may escape through small aberrant arterial connexions, perhaps embedded in the postganglionic nerve, and reach the arterial supply to the orbit. There they may produce a contraction by a direct action on the smooth muscle of the nictitating membrane, as could acetylcholine, or on the striated muscle of the orbit, as could suxamethonium. Although such actions were not demonstrable in any of the preparations in the present work, an attempt was made to eliminate this possibility by studying the effects of various blocking drugs on the responses to neostigmine.

In one normal preparation, and in five denervated preparations, it was found that atropine, injected in doses of 0.5 to 2 mg/kg, either at the beginning of the experiment or immediately before the neostigmine, did not prevent the direct response to neostigmine (Figs. 4 and 5).

In one denervated preparation 100 μg of tubocurarine was injected through the lingual artery, but without a clamp on the external carotid, so that it reached the orbit in high concentration. Neostigmine still produced a contracture of the membrane (Fig. 5). Dimethyl-tubocurarine was administered in a similar manner in two other denervated preparations. Small doses of this agent sufficient to prevent any response to suxamethonium, also injected into the external carotid artery, did not prevent the contraction due to neostigmine.

Hexamethonium did not modify the response to neostigmine in one denervated preparation, but Perry & Reinert (1954) have shown that hexamethonium does not block the response to acetylcholine in the denervated ganglion. In one normal preparation it was not possible to elicit a response to neostigmine after hexamethonium. However, since it was necessary to use doses of 4.0 mg of neostigmine in this preparation, the response may have failed due to the rapid spontaneous decline with such a dose as described previously.

Eserine was administered by close-arterial injection to five preganglionically denervated preparations in doses ranging from 100 μg to 5.0 mg. Doses of 100 to 200 μg had no effect, but doses in excess of this reduced or sometimes eliminated the response to close-arterial injections of nicotine, for periods of 10 to 60 min or more, or occasionally to the end of the experiment. This inhibition was not modified by the presence of atropine (1 to 2 mg/kg). None of the injections of eserine produced a contraction of the membrane such as that seen after neostigmine.

DISCUSSION

By analogy with pharmacological effects at the skeletal neuromuscular junction, it might be expected that the anticholinesterase compounds would produce some reversal of the effects of competitive ganglion blocking agents. Reports of previous experiments to investigate this possibility are not in agreement. Koppanyi, Dille & Linegar (1936) reported that eserine shortened the ganglion block produced by curare and also that produced by nicotine, a substance which is not usually included

with the competitive blocking agents. Chou & De Elío (1948) confirmed the reversal of curare block by eserine, but could not demonstrate any such effect with neostigmine. Similarly, Grob & Harvey (1950) using neostigmine were unable to reverse the fall in blood pressure produced in man by pentamethonium or tetraethylammonium. On the other hand, Cannon & Rosenblueth (1937) were able to reverse the effects of curare on the cat ganglion, using neostigmine, and Reardon, Marzoni & Hendrix (1947) were able to reverse the effects of tetraethylammonium on the blood pressure in both dogs and man.

In the present experiments where atropine was used to eliminate any possibility of a peripheral action, neither pretreatment with eserine nor with neostigmine produced any material or regular reduction in the sensitivity of the preparations to the competitive blocking agent, hexamethonium. However, the injection of neostigmine, when the ganglion block due to hexamethonium had reached a maximum, always produced a rapid reversal of the block, whereas eserine had no effect. This reversal may arise from several causes.

The first possibility is that the neostigmine had a strong anticholinesterase action which was very rapid in onset and short in duration. The reversal only needed to last until the ganglion had recovered naturally from the effect of the blocking drug. In some experiments the reversal was seen to be of shorter duration than the block, since the block tended to reappear. However, even this brief reversal by neostigmine was unlikely to be due to an anticholinesterase action, since it was also seen when the compound was injected into preparations that had received dyflos or eserine in doses considered large enough to inhibit the cholinesterase present (Kamijo & Koelle, 1952).

The second possibility was that neostigmine caused repetitive firing of those cells where transmission was still intact. Investigation of the effects of the anticholinesterases on the responses of the nictitating membrane preparation to maximal preganglionic stimulation at various frequencies failed to produce unequivocal evidence that repetitive firing occurred. After neostigmine the responses to low rates of stimulation were potentiated slightly. Although this was not sufficient to suggest even double firing of the cells it did agree with a report by Cannon & Rosenblueth (1937) that this compound potentiated the contraction of the nictitating membrane to single maximal stimuli in the atropinized cat. However, in the present experiments the perfusion was always established for some minutes before recording commenced. Initial effects were therefore not recorded, while the reversal of hexamethonium block by neostigmine was a phenomenon of short duration which occurred immediately after injection. A brief effect such as this might be consistent with a direct stimulant action on the ganglion cells.

As Riker & Wescoe (1946) and Wescoe & Riker (1957) have described a direct stimulant action of neostigmine at the skeletal neuromuscular junction, evidence for such an action on the ganglion was sought.

In both normal and denervated preparations the close-arterial injection of small doses of neostigmine produced a potentiation of the responses to subsequent doses of nicotine. Since nicotine is not destroyed by cholinesterase, this potentiation

could not be due to an anticholinesterase action but is readily explicable if the compound has a direct stimulant effect.

Larger doses of neostigmine caused an immediate contraction of the membrane. By the concurrent use of atropine and neuromuscular blocking drugs, it was possible to eliminate any action by neostigmine on the smooth or striated muscle in the orbit. This and the speed of onset of the response indicated that the neostigmine was acting directly on the ganglion. Brucke (1937), Sawyer & Hollingshead (1945) and Koelle (1951) have studied the cholinesterase in the ganglion and shown that the true cholinesterase almost entirely disappears following chronic preganglionic denervation. That which remains is associated with certain postganglionic cholinergic neurones. Therefore the stimulant action seen in the denervated preparations in the present experiments could not have been a result of an anticholinesterase action.

All the results reported here are consistent with the view that neostigmine has a direct stimulant action on the ganglion. This action is of short duration and may produce a reversal of hexamethonium block with doses as small as 40 μg/kg. The stimulant action is not related to its anticholinesterase action. No stimulant action was found with eserine.

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REFERENCES

BELL, P. M. G. & QUILLIAM, J. P. (1956). Stimulator control unit to provide single and multiple shocks in a predetermined order. *Proceedings of the XX International Physiological Congress*, p. 1008.

BROWN, G. L. & FELDBERG, W. (1936). The acetylcholine metabolism of a sympathetic ganglion. *J. Physiol. (Lond.)*, **88**, 265–283.

BRUCKE, F. Th. v. (1937). The cholinesterase in sympathetic ganglia. *J. Physiol. (Lond.)*, **89**, 429–437.

BÜLBRING, E. & BURN, J. H. (1942). The interrelation of prostigmine, adrenaline and ephedrine in skeletal muscle. *J. Physiol. (Lond.)*, **101**, 224–235.

CANNON, W. B. & ROSENBLUETH, A. (1937). The transmission of impulses through a sympathetic ganglion. *Amer. J. Physiol.*, **119**, 221–235.

CHOU, T. C. & DE ELÍO, F. J. (1948). The anti-curare activity of eserine on the superior cervical ganglion of the cat. *Brit. J. Pharmacol.*, **3**, 113–115.

ECCLES, J. C. (1944). The nature of synaptic transmission in a sympathetic ganglion. *J. Physiol. (Lond.)*, **103**, 27–54.

FELDBERG, W. & GADDUM, J. H. (1934). The chemical transmitter at synapses in a sympathetic ganglion. *J. Physiol. (Lond.)*, **81**, 305–319.

FELDBERG, W. & VARTIAINEN, A. (1935). Further observations on the physiology and pharmacology of a sympathetic ganglion. *J. Physiol. (Lond.)*, **83**, 103–128.

GROB, D. & HARVEY, A. M. (1950). Observations on the effects of the autonomic blocking agent, bistrimethylammonium pentane dibromide (C5) in normal subjects and in patients with peripheral vascular disease and hypertension, and comparison with TEAC. *Bull. Johns Hopkins Hosp.*, **87**, 616.

KAMIJO, K. & KOELLE, G. B. (1952). The relationship between cholinesterase inhibition and ganglionic transmission. *J. Pharmacol. exp. Ther.*, **105**, 349–357.

KOELLE, G. B. (1951). The elimination of enzymatic diffusion artifacts in the histochemical localization of cholinesterases and a survey of their cellular distributions. *J. Pharmacol. exp. Ther.*, **103**, 153–171.

KOPPANYI, T., DILLE, J. M. & LINEGAR C. R. (1936). Studies on the synergism and antagonism of drugs. II, The action of physostigmine on autonomic ganglia. *J. Pharmacol. exp. Ther.*, **58**, 105–110.

PATON, W. D. M. (1954). Types of pharmacological action at autonomic ganglia. *Arch. int. pharmacodyn.*, **97**, 267–281.

PERRY, W. L. M. & REINERT, H. (1954). The effects of preganglionic denervation on the reactions of ganglion cells. *J. Physiol. (Lond.)*, **126**, 101–115.

REARDON, M. J., MARZONI, S. A. & HENDRIX, J. P. (1947). The effects of neostigmine on the actions of TEA in dogs and man. *Fed. Proc.*, **6**, 364.

RIKER, W. F., Jr. & WESCOE, W. C. (1946). The direct action of prostigmine on skeletal muscle ; its relationship to the choline esters. *J. Pharmacol. exp. Ther.*, **88**, 58–66.

SAWYER, C. H. & HOLLINGSHEAD, W. H. (1945). Cholinesterase in sympathetic fibres and ganglia. *J. Neurophysiol.*, **8**, 137–153.

WESCOE, W. C. & RIKER, W. F. (1957). The pharmacology of anti-curare agents. *Ann. New York Acad. Sci.*, **54**, 438.