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DEPOLARIZING ACTION OF NEOSTIGMINE AT AN AUTONOMIC GANGLION

BY

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The actions of neostigmine have been examined using electrical recording from normal and denervated superior cervical ganglia from rat and kitten. Neostigmine produced a rapid and reversible depolarization of the ganglion cells, both *in vitro* and *in vivo*. This depolarization could be antagonized by hexamethonium, and was not related to an anticholinesterase action. Eserine blocked transmission and only produced a small depolarization of slow onset and development. This depolarization was reversible, but could not be antagonized by hexamethonium.

In experiments using mechanical recording of the contractions of the nictitating membrane in the cat, evidence has previously been obtained suggesting that neostigmine could exert a stimulating action on the superior cervical ganglion (Mason, 1962). It was concluded that neostigmine acted directly on the ganglion cells, and the action was neither a reflexion of an anticholinesterase effect nor a peripheral effect. This paper reports experiments, using electrical recording from isolated and intact superior cervical ganglia, which were performed to elucidate the mode of action of neostigmine at the ganglion.

The experiments were of three main types. First, those conducted on isolated rat ganglia mounted vertically and using a moving fluid electrode in the manner of Pascoe (1956) to determine the depolarization or hyperpolarization which followed the application of drugs to the preparation. Later this technique was modified slightly for kitten ganglia, since rat ganglia are comparatively insensitive to ganglion blocking agents. Second, experiments were made on isolated kitten ganglia mounted horizontally to study the effects of these agents on transmission. Third, *in vivo* experiments were carried out in anaesthetized kittens using a modified form of the technique described by Paton & Perry (1953). Using this technique, the long-term depolarization or hyperpolarization could be studied as well as the effects on transmission. Lastly, experiments of the first and third types were carried out using ganglia which had been preganglionically denervated at least 14 days before.

METHODS

The apparatus used in the first type of experiment is shown in Fig. 1. The ganglia were suspended by means of silk threads, tied to the post- and pre-ganglionic nerves, and attached

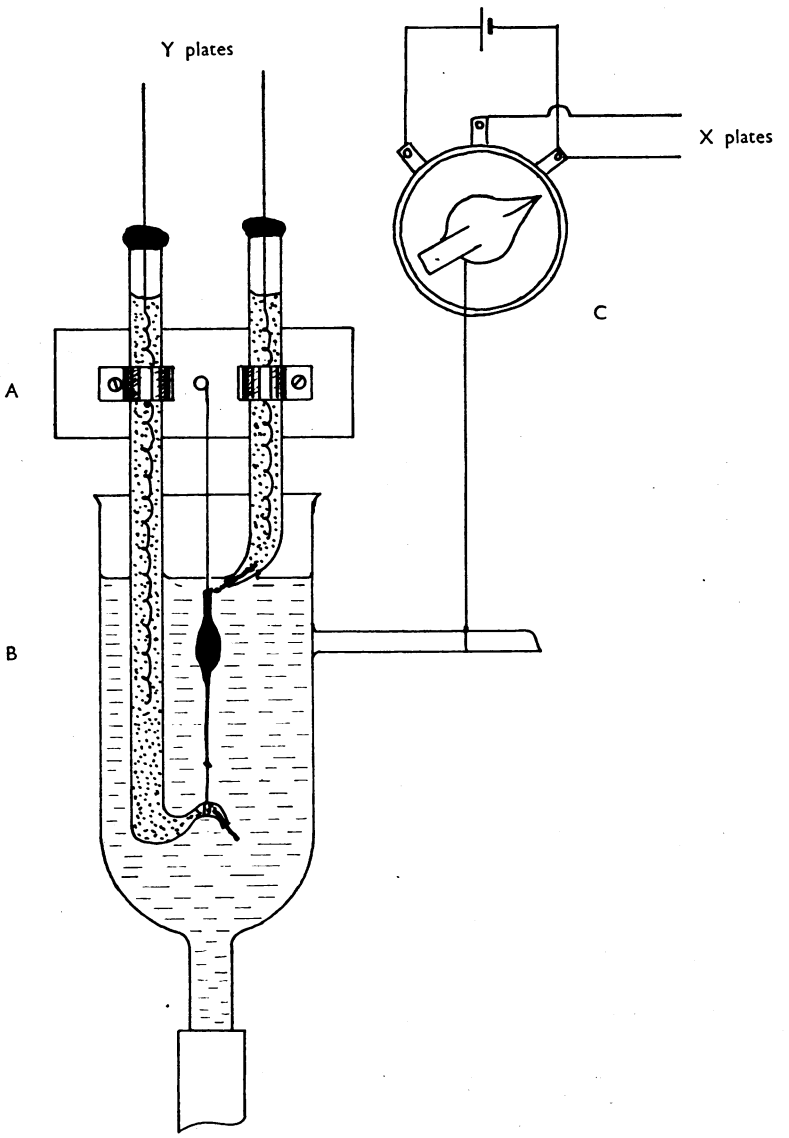

Fig. 1. Diagram of apparatus for moving fluid electrode experiments. The ganglion was suspended between the lower non-polarizable electrode and the insulated electrode holder, A. The upper non-polarizable electrode was in contact with the severed postganglionic nerve. The bath, B, was free to move up and down so that the meniscus of the Krebs solution traversed the surface of the ganglion. Due to the high conductivity of the Krebs solution, the meniscus formed the point from which the lower electrode effectively recorded. The movement of the bath was controlled by a flexible connexion from the shaft of the potentiometer, C, which provided the X deflexion of the oscilloscope. Thus the potentials from the various points on the ganglion always appeared at the same point on the record.

to the plastic electrode holder, A, and to the lower electrode. The upper electrode was in contact with the severed post-ganglionic nerve. The electrodes were silver/silver chloride in saline/agar and contained in glass tubes with a cotton wick closing the end. The electrodes and ganglion, with the electrode holder, A, were fixed. Due to its low resistance, the bath fluid acted as an extension to the lower electrode. Therefore, the electrical responses were measured between the electrode on the severed postganglionic nerve and the meniscus of the fluid in the bath (Fig. 1). The bath, B, was free to move vertically so that the meniscus of the Krebs solution traversed the surface of the ganglion. The movement of the bath was controlled by a flexible link from the shaft of the potentiometer, C. This potentiometer, with its battery, provided the X deflection on the cathode ray oscilloscope so that the potential from any point on the ganglion always appeared at the same place in the record and successive records could be superimposed.

The arrangement of the ganglion and electrodes was similar for the second and third types of experiment. On the preganglionic nerve were platinum stimulating electrodes and non-polarizable earthing and recording electrodes. A second recording electrode was in contact with the body of the ganglion near the preganglionic pole, and a third was in contact with the cut end of the postganglionic nerve. Records between the first and second electrodes showed the action potentials in the preganglionic nerve, while records between the second and third electrodes showed the ganglion action potentials as well as the slower potential changes produced by drugs.

For the *in vitro* experiments the drugs were dissolved in the bathing fluid. For the *in vivo* experiments the drugs were injected through a cannula in the lingual artery, while the external carotid artery was clamped.

The non-polarizable electrodes were connected via a calibrator to a direct coupled amplifier and the potentials displayed on a double-beam cathode-ray oscilloscope and photographed. The second beam of the oscilloscope was used both for time-marking and as a reference point when long-lasting potentials deflected the recording beam. When required, maximal stimuli were applied to the preganglionic nerve from an electronic stimulator (derived by P. M. G. Bell, from Attree, 1950) synchronized with the time base of the oscilloscope.

Isolated ganglia were obtained from rats of 100 to 200 g body weight, or kittens, preferably of 150 to 300 g, but occasionally up to 1,000 g body weight. The animals were anaesthetized with urethane intraperitoneally. After removal, the ganglia were placed in Krebs solution having the composition: sodium chloride, 0.69 g; potassium chloride, 0.035 g; calcium chloride, 0.028 g; sodium bicarbonate, 0.21 g; potassium dihydrogen phosphate, 0.016 g; magnesium sulphate heptahydrate, 0.029 g; glucose, 0.2 g; and water to 100 ml. Adjacent tissues and the sheath were stripped from the ganglion and nerves, and the preparation mounted in the bath. The experiments were conducted at room temperature (18 to 20° C). Under these conditions the preparation gave repeated responses over periods of 8 hr or more. The experiments using kitten ganglia *in vivo* were prepared in the manner described by Paton & Perry (1953) for cat ganglia.

Preganglionically denervated ganglia were prepared in rats (150 g) or kittens (7 to 10 days old). Anaesthesia was produced by intraperitoneal injections of pentobarbitone sodium (30 mg/kg) supplemented where necessary by ethyl chloride on a mask. Using an aseptic technique, the cervical sympathetic trunk was exposed and a piece 0.5 cm long cut out. The incision was closed and the animal allowed to recover. After a period of 14 days or more, these preganglionically denervated preparations were used in one or other of the experimental procedures described above. In every instance the denervation was confirmed, first by the relaxation of the nictitating membrane and the changed diameter of the pupil, and, second, when the preparation was set up the division of the nerve was confirmed by direct inspection.

The compounds employed were: acetylcholine chloride; nicotine hydrogen tartrate; tetramethylammonium bromide; choline chloride; hexamethonium bromide; neostigmine methylsulphate; edrophonium chloride; eserine sulphate; and dyflos (di*iso*propylfluorophosphonate). The doses throughout refer to the salts. Concentrations are expressed as g/ml.

RESULTS

Depolarization of isolated ganglia

On setting up the preparation the postganglionic nerve was negative with respect to the ganglion, due to the injury potential. As this potential difference slowly declined during the experiment, each response was measured with respect to a control record taken immediately before the administration of the drug. The greatest depolarization always occurred near the preganglionic pole of the ganglion. Therefore, when the response was plotted against time, the electrical change was always measured at the same point on the surface of the ganglion, chosen by inspection of the records, and usually one-quarter to one-third of the distance from the preganglionic pole.

Control experiments using rat ganglia. Pascoe (1956) showed that normal rat ganglia could be depolarized with acetylcholine (approximately 1.8×10^{-5}), but only after the preparation had been sensitized with an anticholinesterase. In the present experiments depolarization was readily obtained, in the absence of anticholinesterases, provided the concentration of acetylcholine was of the order of 10^{-4}. Sometimes depolarization was seen with concentrations as low as 4×10^{-5}. This depolarization reached a maximum within 2 min and was proportional to the concentration of acetylcholine. On washing in normal Krebs solution, depolarization was rapidly replaced by hyperpolarization, which in its turn subsided within 4 min.

Nicotine, in concentrations of 2×10^{-6} and greater, produced a similar depolarization which reached a maximum in 2 to 4 min (Fig. 2). On washing, the effect subsided slowly over a period of 8 or more min, and was sometimes followed by a small hyperpolarization which slowly subsided.

Rat ganglia which had been preganglionically denervated for at least 14 days were also depolarized by acetylcholine. The preparations were more sensitive to acetylcholine, but the time to maximum effect and the hyperpolarization on washing out were all similar to those obtained in normal ganglia.

Control experiments using kitten ganglia. In the absence of anticholinesterase normal kitten ganglia did not respond to acetylcholine unless the concentration exceeded 2.5×10^{-4}. Addition of low concentrations of anticholinesterase to the bath fluid considerably increased the sensitivity of the ganglion to acetylcholine, and responses were seen similar to those in the rat ganglion. The depolarization commenced within 15 to 30 sec and reached a maximum within 2 to 4 min. On washing, the effect rapidly subsided and was replaced by hyperpolarization lasting up to 8 min.

Nicotine, choline and tetramethylammonium also produced a rapid depolarization, which usually reached a maximum in 4 to 8 min but which subsided only slowly on washing. The previous addition to the bath of hexamethonium (10^{-5}) prevented the responses to these compounds.

Kitten ganglia which had been denervated preganglionically at least 14 days previously tended to come from older animals than the normal ganglia. Nevertheless, these denervated preparations regularly showed a depolarization to concentrations of acetylcholine which were ineffective in normal ganglia. The onset of

the effect, the time to maximum response and the hyperpolarization on washing out were all similar to those seen in normal ganglia.

The effects of neostigmine. The effects of the anticholinesterase compounds were examined in three ways—the potentiation of acetylcholine, the inhibition of the

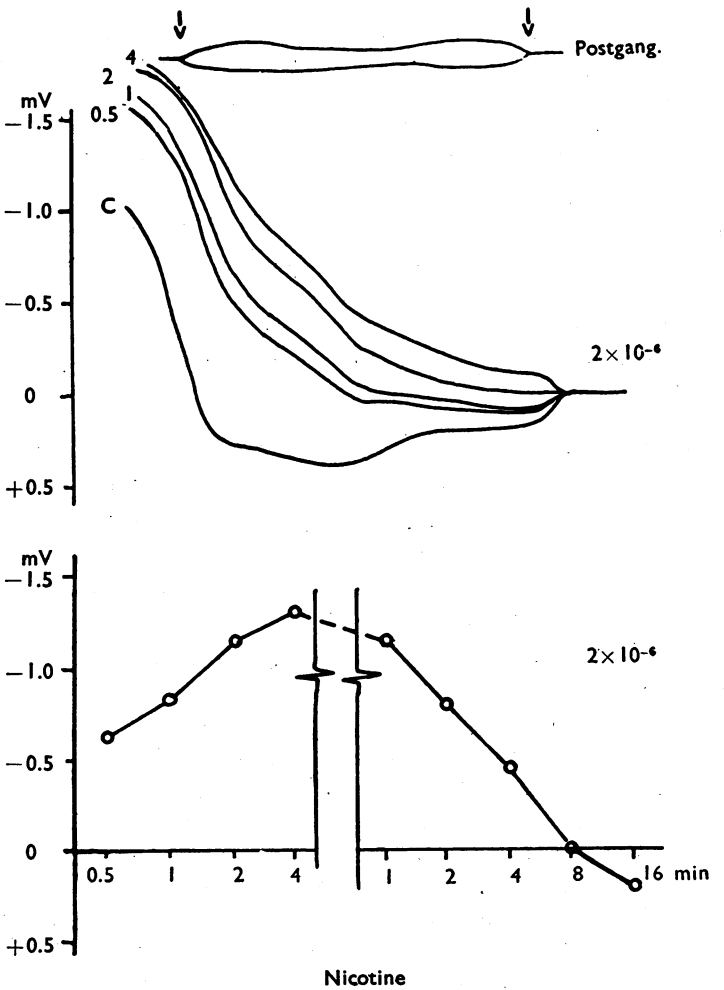

Fig. 2. Isolated rat superior cervical ganglion. The family of curves in the upper part of the illustration shows the distribution of the electrical potential over the surface of the ganglion, measured by a moving fluid electrode with respect to the severed postganglionic nerve, the common origin of all the curves to the right. Diagram above indicates the physical relation of the ganglion to the depolarization records. The lower graph relates the change of potential with time at one selected point on the ganglion. Time scale is logarithmic to show more clearly the early stages. The broken vertical lines indicate a wash. The upper curves show the control record (C), and those obtained 0.5, 1, 2, and 4 min after the addition of 2×10^{-6} nicotine. Depolarization is shown by an upward displacement of the record over the surface of the ganglion. The lower graph shows the development of the potential due to 2×10^{-6} nicotine at a selected point on the ganglion, and also the decay after washing.

response to acetylcholine, and the direct effect of the anticholinesterase on the ganglion.

Addition of neostigmine in concentrations of 10^{-7}, or less, had no effect on the responses of normal rat ganglia to acetylcholine. The sensitivity to acetylcholine increased with increasing concentrations of the anticholinesterase to reach a maximum at 10^{-5} neostigmine. Under these conditions 3×10^{-7} acetylcholine produced as large a depolarization as 3×10^{-5} acetylcholine before exposure to neostigmine. Increasing the concentration of neostigmine to 10^{-4} did not further increase the sensitivity to acetylcholine, while a concentration of 10^{-3} neostigmine caused a fall in sensitivity (Fig. 3).

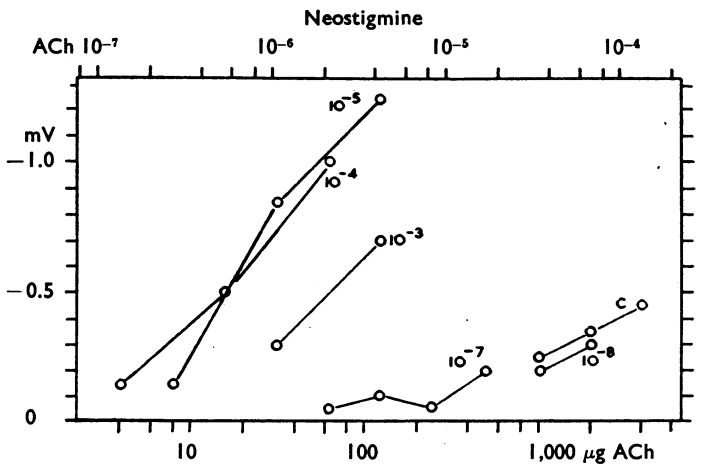

Fig. 3. Normal rat superior cervical ganglion. Potentiation of acetylcholine response by neostigmine. The ganglion was exposed to various concentrations of acetylcholine for periods of 2 min. The maximum depolarization was plotted against the concentration of acetylcholine. The control curve (C) was with acetylcholine alone. The remaining curves were plotted after exposing the ganglion to neostigmine in the concentration indicated for at least 30 min.

Potentiation of the responses to acetylcholine was also seen when chronically denervated preparations were exposed to neostigmine.

Neostigmine alone also produced a depolarization of the ganglia. Depolarization was observed in 2 out of 6 normal rat ganglia exposed to concentrations of neostigmine as low as 10^{-5}. Increasing the concentration to 10^{-4} regularly produced a response. The depolarization commenced after a delay of 30 sec to 2 min with low concentrations of neostigmine (Fig. 4) but within 15 to 30 sec with higher concentrations. The effect usually reached a maximum in 2 to 8 min and then remained constant, but in a few preparations the response to high concentrations declined and disappeared.

On washing, the neostigmine depolarization subsided over a period of 10 to 30 min. No hyperpolarization was seen.

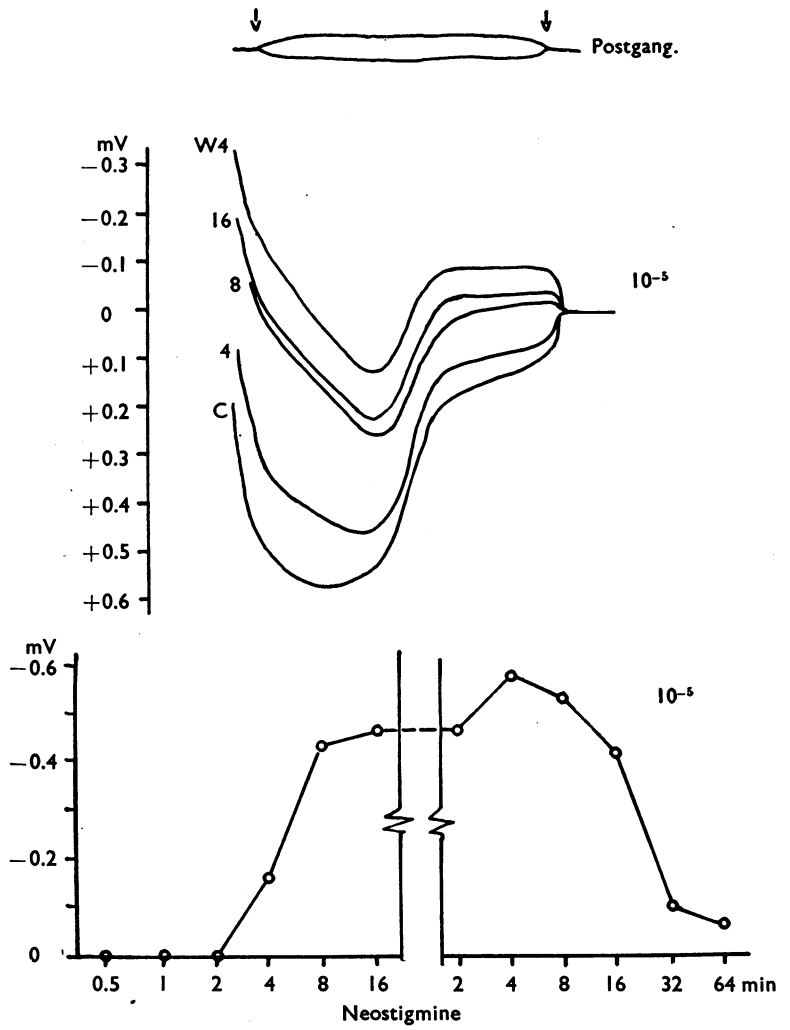

Fig. 4. Normal rat ganglion. Depolarization by neostigmine 10^{-5}. Details as for Fig. 2. Family of curves in upper part show the records obtained at different times omitting records taken before 2 min, which were identical with the control record. This was the minimal effective concentration and effect only appeared after a delay. With higher concentrations no delay was seen.

Normal kitten ganglia were also depolarized by neostigmine (18 experiments), but the magnitude of the response was more variable. Concentrations as low as 10^{-5} produced a response in a few preparations, but 5×10^{-5} regularly produced a depolarization which commenced within 15 sec and reached a maximum within 8 min (Fig. 5). On washing the preparation, the effect declined slowly.

Denervated rat and kitten ganglia also showed a rapid depolarization, which reached a maximum in 2 min, when exposed to neostigmine in concentrations of 2×10^{-5} or greater.

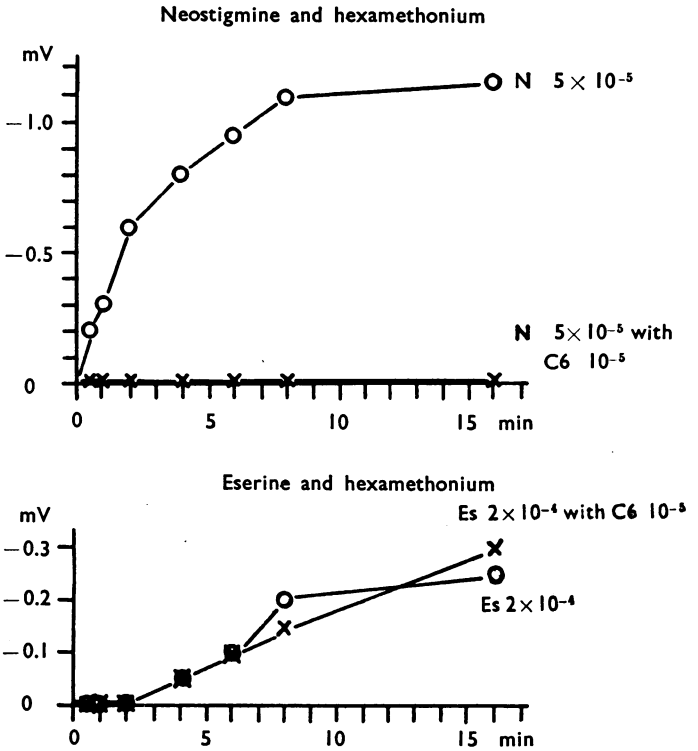

Fig. 5. Normal kitten ganglion. Effect of hexamethonium on responses to neostigmine and eserine. Details as for Fig. 2. Linear time scale. In each instance the hexamethonium bromide (C6) was added to the bath 5 min before the neostigmine (N) or the eserine (Es). It prevented the depolarization due to neostigmine but not that due to eserine.

The related compound, edrophonium, was also examined using normal rat ganglia. Concentrations of 10^{-6} to 10^{-4} produced depolarizations, whose onset and time-course were similar to those produced by neostigmine in denervated ganglia.

In one experiment using a normal kitten ganglion, neostigmine 5×10^{-5} still produced a depolarization in the presence of eserine 5×10^{-5}. In a second experiment neostigmine 10^{-5} produced a depolarization in a ganglion which had been first exposed to dyflos 10^{-5} for 30 min, then to dyflos 10^{-4} for 30 min.

Neostigmine 5×10^{-5} was added to 6 normal kitten ganglia which had been treated with hexamethonium bromide 10^{-5} (or, in one experiment, 10^{-4}). In two of these experiments the neostigmine responses were markedly reduced, while in the remaining four the depolarization was completely eliminated (Fig. 5). When the hexamethonium was washed out, the response to neostigmine could again be elicited.

The effects of eserine. Concentrations of 10^{-7} eserine or less had no effect on the responses of normal rat ganglia to acetylcholine. Increasing the concentration of eserine produced an increased sensitivity to acetylcholine which reached a maximum at 10^{-5} eserine. Further increases to 10^{-4} eserine did not produce a

corresponding increase in sensitivity to acetylcholine, while still further increases to 10^{-3} eserine caused a sharp fall in the sensitivity to acetylcholine. These effects (Fig. 6) were very similar to those observed with neostigmine. In the same manner increasing concentrations of eserine first potentiated then blocked the responses of chronically denervated rat ganglia to acetylcholine.

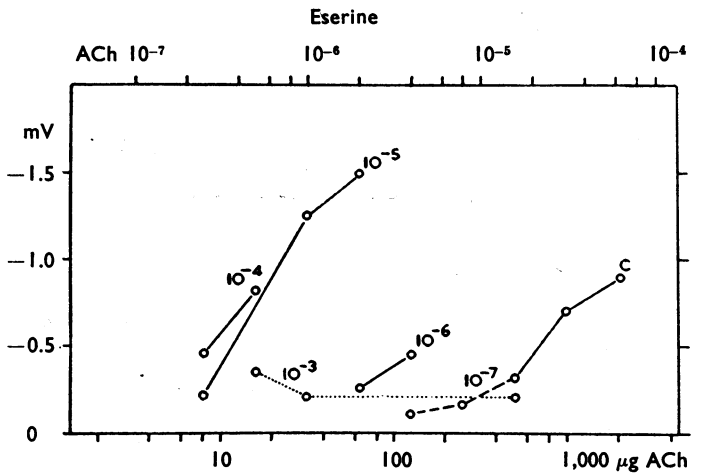

Fig. 6. Normal rat ganglion. Potentiation of acetylcholine responses by eserine. Experimental details as for Fig. 3.

Eserine in concentrations of 5×10^{-5} and greater exerted a direct depolarizing effect on the rat ganglion, similar in magnitude and time-course in both normal and denervated ganglia. In contrast to the response due to neostigmine, the depolarization due to eserine was slow in onset. It was often only just detectable when the drug had been in the bath for 4 min. In no experiment did the effect reach a maximum in less than 8 min, and in several it continued to increase as long as the eserine was in the bath. The magnitude of the depolarization was usually less than that seen with neostigmine. After washing the response declined only slowly.

When normal or denervated kitten ganglia were used no depolarization was obtained with concentrations less than 2×10^{-4} eserine. In some preparations concentrations as high as 10^{-3} or 2×10^{-3} had to be used to obtain depolarizations which were small and slow in onset, only appearing after 4 to 8 min and often continuing to develop as long as the eserine was in the bath (Fig. 5). Since the effect was small and slow in onset, it seemed possible that it might be confused with the slow decay of the injury potential at the cut end of the postganglionic nerve. It was found that the depolarization due to eserine was always greater than the decay of the injury potential in a similar period, and, further, the eserine depolarization was abolished by washing the preparation. Hexamethonium bromide in concentrations of 10^{-5} to 10^{-4} did not prevent the depolarization produced by eserine (Fig. 5). The actions of neostigmine and eserine on these preparations are compared in Table 1.

TABLE 1
COMPARISON OF EFFECTS OF NEOSTIGMINE AND ESERINE ON ISOLATED RAT AND KITTEN GANGLIA

	Neostigmine	Eserine
Concentration for maximum potentiation of acetylcholine depolarization	10^{-5}	10^{-5}
Minimum effective concentration for depolarization by anticholinesterase alone	10^{-4} (rat) 5×10^{-5} (kitten)	5×10^{-5} (rat) $>10^{-4}$ (kitten)
Speed of onset	Rapid 15 to 30 sec	Slow 2 to 4 min
Time to maximum	2 to 8 min	>8 min
Effect of previous eserine or dyflos	None	
Effect of previous hexamethonium	Antagonized	None

Transmission in isolated ganglia

The block of the response to acetylcholine produced by high concentrations of neostigmine and eserine could correspond to a block of transmission. This was examined using isolated kitten ganglia, applying maximal stimuli to the preganglionic nerve, and recording both conduction in the preganglionic nerve and the compound action potential seen with the electrodes on the body of the ganglion and the severed postganglionic trunk. This compound potential consisted of a spike potential followed in order by an afternegativity and an afterpositivity. The relative magnitudes of the component parts of this complex varied considerably from one preparation to another, but in the absence of drugs they remained constant throughout each individual experiment.

Addition of neostigmine to the bath in concentrations up to 5×10^{-5} did not modify the preganglionic potential but reduced the size of the spike and the afternegativity. Higher concentrations (10^{-4}) eliminated all the ganglion potentials without modifying the preganglionic potential. Washing rapidly restored transmission and the action potential resumed its original form.

Eserine blocked transmission in concentrations of 2×10^{-4} or greater. In addition, the action potential recorded from the preganglionic nerve was partially reduced, suggesting that this effect of eserine was not confined to the synapse. Lower concentrations, which reduced but did not block transmission, did not modify the shape of the response recorded from the ganglion, since all the components of the complex were reduced equally. The block due to eserine was rapidly removed by washing.

In these experiments the slow changes in potential due to depolarization or hyperpolarization were not recorded, since they were observed more readily in the *in vivo* preparations.

Transmission in intact ganglia

The recording conditions in this preparation were similar to those in the isolated preparation. Drugs were injected close-arterially. As before, a maximal preganglionic stimulus produced at the ganglion a negative spike potential, followed in order by an afternegativity and an afterpositivity. In addition records were made

of slow potential changes indicative of depolarization or hyperpolarization of the ganglion cells, and also records of conduction in the preganglionic nerve.

Substances which modify ganglionic transmission may be divided into two groups according to the changes produced in the electrical potentials recorded at the ganglion (Paton & Perry, 1953). In the present experiments the first group was represented by acetylcholine, tetramethylammonium, choline and nicotine, all of

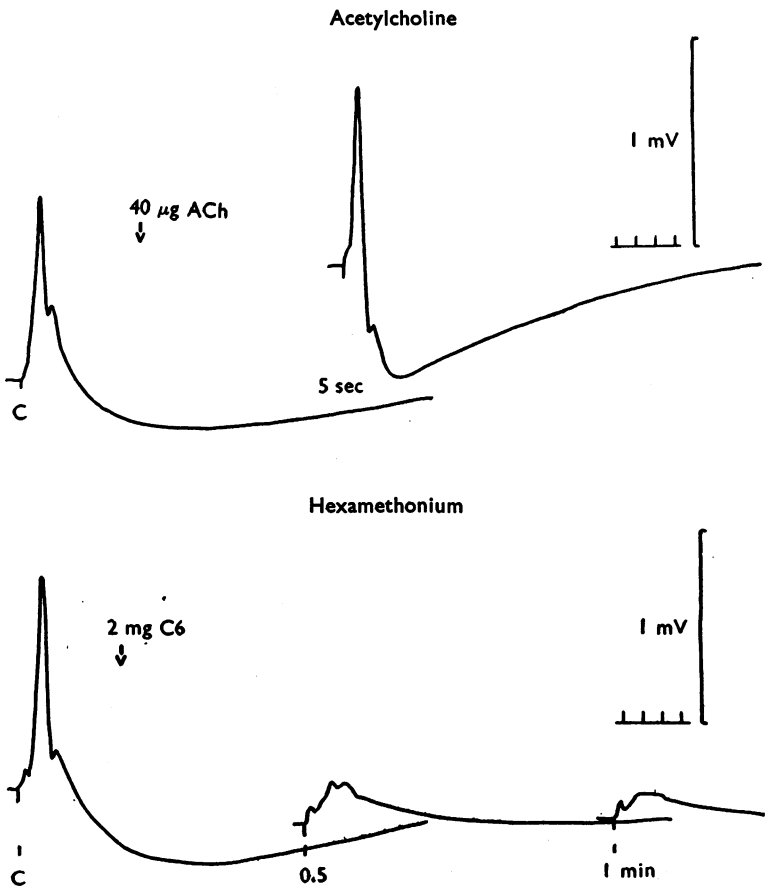

Fig. 7. Effects of acetylcholine and hexamethonium on ganglionic transmission *in vivo* in the kitten. The responses to single preganglionic stimuli were recorded between the severed postganglionic nerve and the body of the ganglion. Increased negativity of the ganglion (depolarization) caused the record to be displaced upwards. The scales indicate 1 mV and 20 msec intervals. The control records are indicated (C). The times under subsequent records indicate the time that has elapsed since the intra-arterial injection of the drug. The preganglionic stimuli were applied at 5 sec intervals. *Upper panel:* This shows the depolarization (upward displacement) and change in shape of the complex following 40 μg acetylcholine injected intra-arterially. *Lower panel:* This shows the ganglion block produced by 2 mg hexamethonium bromide (C6). There was some hyperpolarization (downward displacement of the record) after this large dose of hexamethonium, a phenomenon seen several times.

which produced similar initial changes and only differed in duration of action. The close-arterial injection of 40 μg acetylcholine (second record in Fig. 7a) produced a rapid depolarization of the ganglion cells (shown by an upward displacement of the whole record) and a reduction in the negative afterpotential. The spike potential was not reduced. Larger doses of acetylcholine produced greater depolarization and blocked transmission, as shown by the elimination of all ganglion action potentials, although the preganglionic action potential was not modified. The response to a small dose of acetylcholine was usually complete within 30 sec, and was followed by a hyperpolarization.

Hexamethonium bromide represented the second group of substances. This compound did not depolarize the ganglion, nor did it selectively alter any constituent part of the potential complex recorded at the ganglion, but reduced all the potentials as ganglion block appeared (Fig. 7b).

Neostigmine. When neostigmine was injected close-arterially to the ganglion in doses of about 0.25 mg, there was a depolarization of the ganglion accompanied by similar changes in the form of the ganglion potential to those seen after acetylcholine. The afternegativity was considerably reduced so that the positive afterpotential appeared much greater than in the control record (Fig. 8a). These effects appeared very rapidly and were fully developed before the first stimulus after the injection, that is, within 5 sec. The rapid depolarization following neostigmine could still be elicited though not so readily, after large doses (2 to 8 mg) of eserine, provided the immediate effects of the eserine had worn off. With increasing doses of neostigmine a block of transmission developed and was complete with doses of 1 mg or more. The duration of the block varied from 4 to 15 min. When the immediate effects of the neostigmine had disappeared, the preparation was very much more sensitive to acetylcholine.

The previous injection of hexamethonium bromide in a dose sufficient to block transmission prevented the depolarization due to neostigmine (Fig. 8b).

Eserine. The close-arterial injection of 0.4 to 8.0 mg eserine to the ganglion did not produce any immediate depolarization (Fig. 9). In several experiments a slow depolarization appeared after a delay of 2 to 3 min, but this may have been due to an accumulation of acetylcholine since the preganglionic nerve was stimulated once every 5 sec.

Doses of 0.4 mg eserine produced a partial block of transmission. This increased with increasing dosage, until it was complete with doses of 2.0 mg. Block was maximal in 30 to 60 sec and lasted for 2 to 20 min.

Eserine did not selectively alter any constituent part of the ganglionic response to preganglionic stimulation, but reduced all the components as ganglion block developed. Provided care was taken to ensure that no acetylcholine could accumulate, by taking records rapidly after the injections or by withholding intermediate preganglionic stimuli, then there was no depression of the negative afterpotential such as that seen following neostigmine. When the immediate effect of the eserine had disappeared, the preparation was more sensitive to acetylcholine.

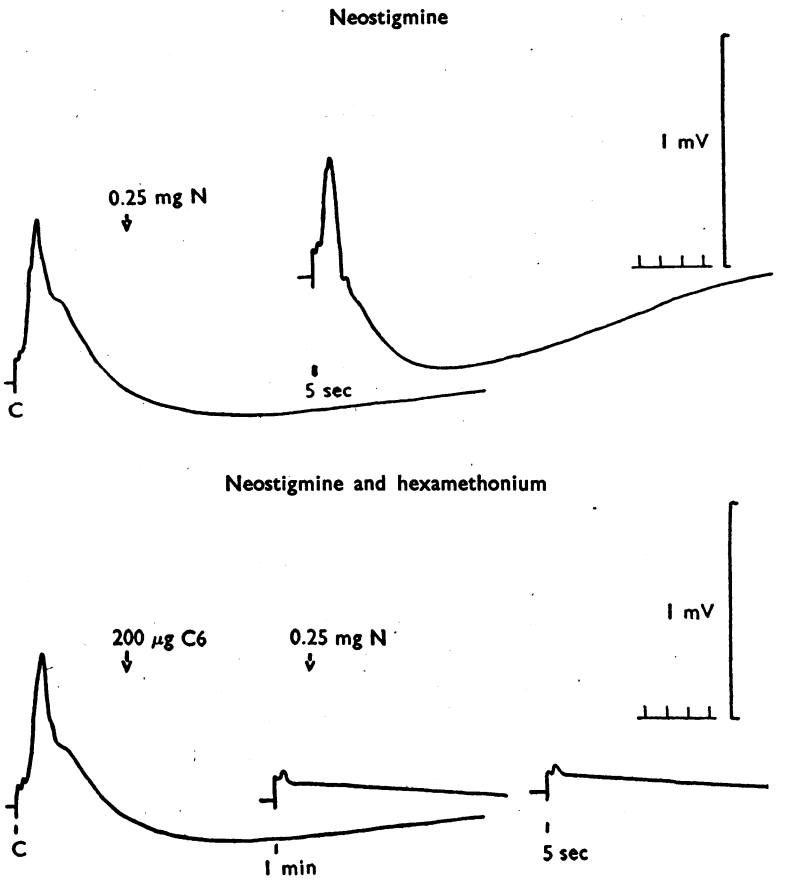

Fig. 8. The effect of neostigmine and hexamethonium on ganglionic transmission *in vivo* in the kitten. The experimental details are the same as for Fig. 7. *Upper panel:* 0.25 mg of neostigmine (N) produced a depolarization, a depression of the afternegativity and a partial block of transmission. *Lower panel:* Injection of 200 μg hexamethonium bromide (C6) produced a ganglion block within 1 min. Now, the injection of 0.25 mg neostigmine did not depolarize the ganglion.

Depolarization of denervated ganglia in vivo

These experiments were similar to those described with normal ganglia *in vivo* except that the cervical sympathetic nerve had been divided at least 14 days before. Since transmission could not be recorded, the resting potential was recorded continuously and not intermittently as before. All injections were made intra-arterially to the ganglion.

The injection of acetylcholine, tetramethylammonium, choline or nicotine produced a rapid depolarization of the ganglion, which usually commenced before the injection was complete and reached a maximum within 2 to 5 sec. The depolarization due to acetylcholine subsided rapidly (within 10 sec) and was often followed by a hyperpolarization lasting 10 to 15 sec. This hyperpolarization was greater

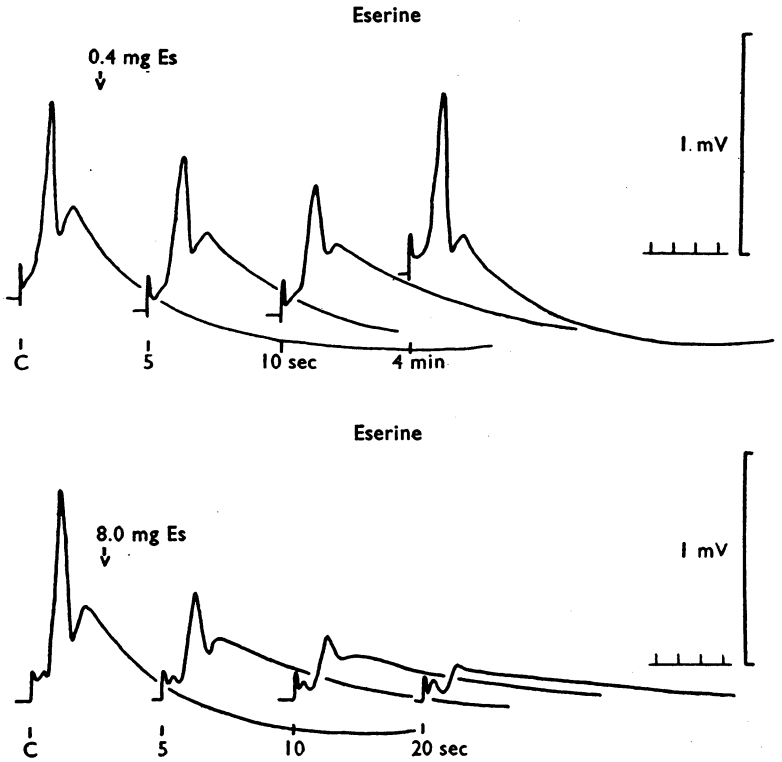

Fig. 9. The effects of eserine on ganglionic transmission *in vivo* in the kitten. The experimental details are the same as Fig. 7. *Upper panel:* 0.4 mg of eserine (Es) produced first a partial block of transmission without any depolarization or change in shape of the complex. Stimulation was continued (1/5 sec), and after 4 min a small depolarization and depression of the after-negativity developed. *Lower panel:* 8.0 mg of eserine (Es) produced a ganglion block without any depolarization.

after large depolarizations than after small. The responses to other depolarizing substances subsided slowly and some depolarization could often be detected after 60 to 90 sec. Subsequent hyperpolarization was not recorded.

Neostigmine in doses of 0.1 to 1.0 mg produced a small but rapid depolarization, which reached a maximum within 5 sec and lasted for a period which varied from a few sec to 1 or 2 min. Although the response usually subsided slowly, sometimes it was rapidly replaced by a hyperpolarization. After the smaller doses of neostigmine, the responses to acetylcholine were greatly increased both in magnitude and duration. After the larger doses of neostigmine the responses to acetylcholine were temporarily reduced, then increased.

Doses of eserine ranging from 0.2 to 4.0 mg did not depolarize or hyperpolarize the ganglion. The responses to acetylcholine immediately after the eserine were sometimes reduced or even prevented, but if this did not occur, or once the immediate inhibition had disappeared, the responses were both potentiated and prolonged.

DISCUSSION

Pascoe (1956) stated that neostigmine depolarized isolated rat ganglia. In the present study, the application of neostigmine to isolated rat and kitten ganglia produced a depolarization which developed rapidly, as did the depolarization produced by acetylcholine, tetramethylammonium and nicotine, and in contrast to that due to eserine, which was smaller and always slow in onset. This depolarization due to neostigmine could have arisen in two ways: either by a direct action on the ganglion cells, or by the preservation of acetylcholine released from the preganglionic nerve endings. The release may have been a spontaneous one such as occurs at the motor end plate or due to an action of neostigmine on the preganglionic endings similar to that exerted by potassium ions (Brown & Feldberg, 1936).

The minimal concentration of neostigmine to produce a depolarization corresponded to that which produced a maximal potentiation of the response to added acetylcholine, that is, the concentration which probably produced a complete inhibition of cholinesterase. The depolarization increased with increasing concentration of neostigmine beyond this value, and therefore could not have been related to an anticholinesterase action and the potentiation of acetylcholine from the preganglionic nerve endings. It was also seen in the presence of high concentrations of eserine and dyflos. Further, the stimulant action of neostigmine was seen in preparations where the preganglionic nerves had been divided and allowed to degenerate so that no release of acetylcholine could occur, either spontaneously or due to an action of neostigmine on the nerve endings. These chronically denervated preparations did not show any decreased sensitivity to the depolarizing action of neostigmine, but rather the reverse Lastly, if the depolarizing action was the result of an anticholinesterase effect preserving spontaneously released acetylcholine, eserine would be expected to produce a similar effect, whereas the response to eserine was much smaller and was always slower in onset.

Thus, the depolarization due to neostigmine must be, at least in part, the result of a direct action of the compound on the ganglion cells.

The points discussed above do not rule out the possibility that in normal ganglia neostigmine causes a release of acetylcholine from the preganglionic nerve endings in addition to the direct action on the ganglion cells. However, Emmelin & MacIntosh (1956) used neostigmine in place of eserine when collecting and assaying the output of acetylcholine from the perfused superior cervical ganglion, but did not report any increase in the output with neostigmine although the concentrations used (1.7×10^{-5}) were of a similar order to those now found to produce a small depolarization of the ganglion.

The depolarizing effect of neostigmine was seen also when injected close-arterially to the ganglion *in vivo*. Using this type of preparation, Paton & Perry (1953) described two phenomena following the injection of stimulant substances. First, the ganglion was depolarized, and, second, the action potential complex was modified in a characteristic manner since there was a selective depression of the after-negativity, providing ganglion block had not supervened. Both these effects were observed within 5 sec of the injection of neostigmine and in the absence of intervening stimulation, so that no acetylcholine could have accumulated. The block of

transmission without depolarization seen after eserine is in agreement with the observation of Paton & Perry (1953).

In all the experiments where normal ganglia were used, it was possible to prevent the depolarization due to neostigmine by the prior administration of hexamethonium. This suggests that the neostigmine may have been acting on acetylcholine receptors.

The results obtained in these experiments confirm the direct stimulant action of neostigmine suggested previously (Mason, 1962).

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