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# THE ACTION OF ACETYLCHOLINE AND SOME RELATED SUBSTANCES ON CONDUCTION IN MAMMALIAN NON-MYELINATED NERVE FIBRES

BY CHRISTINE J. ARMETT* AND J. M. RITCHIE

*From the Department of Pharmacology, Albert Einstein College of Medicine, Yeshiva University, New York* 61, *N.Y., U.S.A.*

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Although there is ample evidence that acetylcholine can excite the terminals of amphibian and mammalian sensory nerves, whether they are terminals of myelinated fibres (Douglas & Gray, 1953; Gray & Diamond, 1957; Diamond, 1959), or of non-myelinated fibres (Douglas & Ritchie, 1960), acetylcholine appears to be without effect on amphibian myelinated fibres, on the large non-myelinated fibres of the squid or on mammalian myelinated fibres (Lorente de Nó, 1944; Hodgkin, 1947; Straub, 1955; Diamond, 1959). However, recent experiments have shown that it acts on mammalian non-myelinated fibres to depolarize them, reduce their spike height, and enhance their spike after-positivity and slow conduction, and that this action is reduced by eserine (Armett & Ritchie, 1960).

The aim of the present experiments was to determine to what extent the action of acetylcholine on mammalian non-myelinated fibres is common to other cholinesters, to choline itself and to some alkaloids which are pharmacologically related to acetylcholine, and also to see how this action is influenced by different types of anticholinesterase and by drugs which block some of the actions of acetylcholine, such as tubocurarine, hexamethonium and atropine.

## METHODS

Desheathed vagus nerves were obtained from rabbits anaesthetized with urethane (1·6 g/kg given into a marginal ear vein as a 25% w/v solution) and mounted in the sucrose-gap apparatus originally described by Stämpfli (1954) and developed by Straub (1956, 1957). The methods were similar to those described earlier (Armett & Ritchie, 1960). Resting and action potentials of a desheathed nerve were recorded through a pair of Ag–AgCl non-polarizable cotton-wick electrodes, fed into a low grid-current cathode-follower and amplifier, displayed on a cathode-ray oscilloscope and photographed. The amplifier was directly-coupled and there was a 20% fall in its response at a frequency of about 15 kc/s with the input impedance presented by the sucrose-gap. The C potential was the

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main elevation of the compound action potential and was easily identified by its slow conduction velocity (less than 1 m/sec at 35° C). The C potentials recorded were large, sometimes nearly 20 mV. The stimuli used to excite the vagal C fibres were brief rectangular pulses of current about 0·5 msec in duration and were delivered from an RF stimulus isolation unit (Schmitt, 1948) to minimize the shock artifact. The conduction distance was about 6 mm.

The Locke's solution used to perfuse the nerve flowed at a rate of about 1·2 ml./min and its temperature was about 35° C near the point where the action potentials were recorded. The solution could be quickly replaced by a modified Locke's solution containing the drug to be tested by switching a tap, hardly interrupting the perfusion and producing little or no artifact. The effect of a rapidly acting drug such as acetylcholine appeared after an initial delay of about 20 sec, which represented the time taken to replace the Locke's solution in the 'dead space' of the perfusion apparatus by the new solution. Usually, and if not otherwise stated, an exposure of 3 min was allowed before the effect of stimulation of the nerve was tested.

The Locke's solution used in the experiments was bubbled with oxygen and its composition was (mM): NaCl, 158; KCl, 5·5; $CaCl_2$, 2·2; dextrose, 5; sodium phosphate buffer, 1 (appropriate amounts of $Na_2HPO_4$ and $NaH_2PO_4$ to bring the pH usually to about 7·0; in a few experiments the pH was brought to various values between 6·0 and 8·0). In some experiments phosphate concentrations up to 8 mM were used (the pH remaining constant at about 7·0) or the phosphate buffer was replaced by a bicarbonate–carbon-dioxide buffering system with 1·9 and 25 mM sodium bicarbonate equilibrated with a 5 % $CO_2$ + 95 % $O_2$ gas mixture; these changes are indicated in the text. The pH of all solutions was regularly checked on a sensitive pH meter.

The drugs used were acetylcholine chloride, methacholine chloride (acetyl-β-methylcholine chloride), carbachol (carbamoyl-aminocholine chloride), bethanechol (β-methylcholine chloride urethane), arecoline chloride, pilocarpine nitrate, nicotine hydrogen tartrate, eserine (physostigmine) salicylate, prostigmine (neostigmine) bromide or methyl sulphate, di-*iso*-propylfluorophosphonate (DFP), tubocurarine chloride, hexamethonium chloride, atropine sulphate, tetramethylammonium chloride (TMA) and tetraethylammonium chloride (TEA).

## RESULTS

### *Choline and its esters, TMA, nicotine, arecoline and pilocarpine*

Figure 1 shows the effect of various choline esters, of choline itself and of nicotine on the action potential of the non-myelinated nerve fibres. Of the choline esters studied, acetylcholine and carbachol were about equally effective in reducing the size of the spike, whereas methacholine and bethanechol were relatively ineffective; choline had a relatively weak action, as was described previously (Armett & Ritchie, 1960). The threshold concentration of carbachol required just to produce a fall in spike height was of the same order as that for acetylcholine, $3 \times 10^{-5}$–$3 \times 10^{-4}$ g/ml. Although methacholine had little or no effect on spike height it did occasionally enhance the positive after-potential, as is illustrated in Fig. 1*b*. Tetramethylammonium chloride ($1\frac{1}{2}$–5 mM) also reduced the action potential of the vagal C fibres.

Of the alkaloids tested nicotine was the most potent in reducing the spike height. As is shown in Fig. 1*e*, a concentration of 0·6 mM was sufficient to produce a decrease of about 15 %. Arecoline and pilocarpine

were effective, but only in larger concentrations. Thus, as is shown in Fig. 2, a concentration of arecoline 5·2 mM and of pilocarpine 3·7 mM caused smaller reductions than that of acetylcholine 1·7 mM. Arecoline, unlike pilocarpine, enhanced the positive after-potential.

The effect of a given drug was not always compared with the response to acetylcholine in the same molar concentration. The probable response of the nerve to acetylcholine in the concentration at which the given drug was tested, however, could be predicted, since the logarithmic dose–response curve for acetylcholine was comparatively straight. In six experiments the effects of several concentrations of acetylcholine up to 50 mM

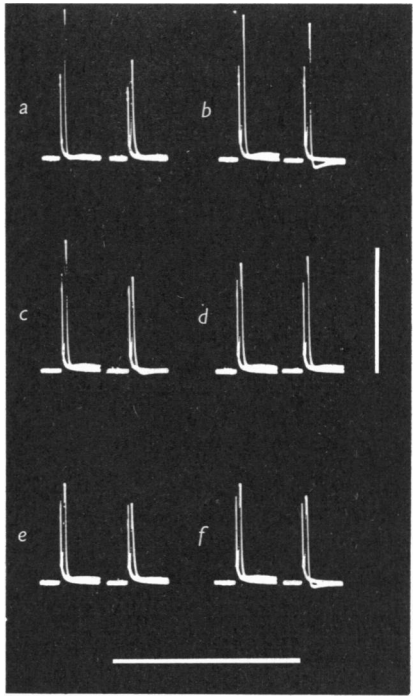


Fig. 1. Records of the compound action potential of a rabbit's vagus nerve. In these and subsequent records the first, more rapid, deflexion is the A and B potentials of the myelinated fibres together with some stimulus artifact. The second and main deflexion is the C potential of the non-myelinated fibres. In each pair of records the left-hand record was taken before and the right-hand record was taken 3 min after exposure to the following drugs (as chlorides, except for nicotine hydrogen tartrate): *a*, acetylcholine, $3 \times 10^{-4}$ g/ml. (1·7 mM); *b*, methacholine, $10^{-3}$ g/ml. (5·1 mM); *c*, carbachol, $3 \times 10^{-4}$ g/ml. (1·6 mM); *d*, bethanechol, $10^{-3}$ g/ml. (5·0 mM); *e*, nicotine, $3 \times 10^{-4}$ g/ml. (0·6 mM); *f*, choline, 17 mM. The drugs were tested successively on the same preparation at intervals of about 20 min. The vertical bar represents 10 mV and the horizontal bar 400 msec.

were examined on the same preparation. A tenfold increase in acetylcholine concentration resulted in a further reduction of $27{\cdot}6 \pm 3{\cdot}0\,\%$ (S.D.) of the spike height.

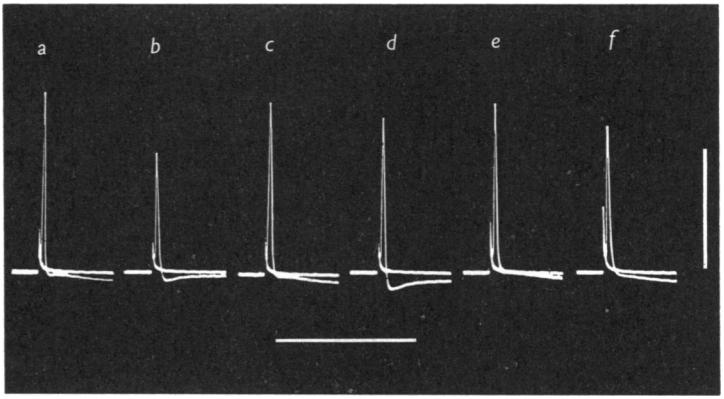


Fig. 2. A comparison of the effects of acetylcholine, arecoline and pilocarpine on conduction in mammalian non-myelinated nerve fibres. Records *a*, *c* and *e* are records of the C potential of the rabbit's vagal non-myelinated fibres taken with the desheathed nerve in Locke's solution. The other records are the corresponding records taken 3 min after perfusing the preparation with: *b*, acetylcholine, $3 \times 10^{-4}$ g/ml. (1·7 mM); *d*, arecoline, $10^{-3}$ g/ml. (5·2 mM); *f*, pilocarpine, $10^{-3}$ g/ml. (3·7 mM). The vertical bar represents 10 mV and the horizontal bar represents 200 msec.

### *Modification of the action of acetylcholine*

*Anticholinesterases.* In the absence of acetylcholine eserine, when tested in a concentration $10^{-5}$–$3 \times 10^{-5}$ g/ml., and prostigmine, when tested in a concentration of $10^{-4}$–$10^{-3}$ g/ml., did not affect spike height. With DFP a slight reduction was obtained with a concentration of $10^{-3}$ g/ml. but not with weaker concentrations.

The anticholinesterases reversibly attenuated the action of acetylcholine on the spike potential. Eserine had been investigated previously but for only one concentration of acetylcholine (Armett & Ritchie, 1960). The effect of eserine $3 \times 10^{-5}$ g/ml. on various concentrations of acetylcholine is shown in Fig. 3. The line drawn through the solid points gives the dose–response curve of the effect of acetylcholine in reducing spike height in the absence of eserine; the crosses give the dose–response curve in its presence. It can be seen that all concentrations of acetylcholine are affected by eserine and that the reduction in spike height produced by acetylcholine becomes small. When near-threshold concentrations of acetylcholine were used, eserine often no longer antagonized the effect of acetylcholine but potentiated it.

The effect of DFP in varying concentrations on the response to a constant concentration of acetylcholine (1·7 mM) is shown in Fig. 4: the effect on the action potential obtained with acetylcholine in the presence of DFP is expressed as a percentage of the effect obtained in its absence. The open circles represent the results obtained on 3 min exposure to acetylcholine. It can be seen that the stronger concentrations of DFP attenuate the reduction produced by acetylcholine on the action potential,

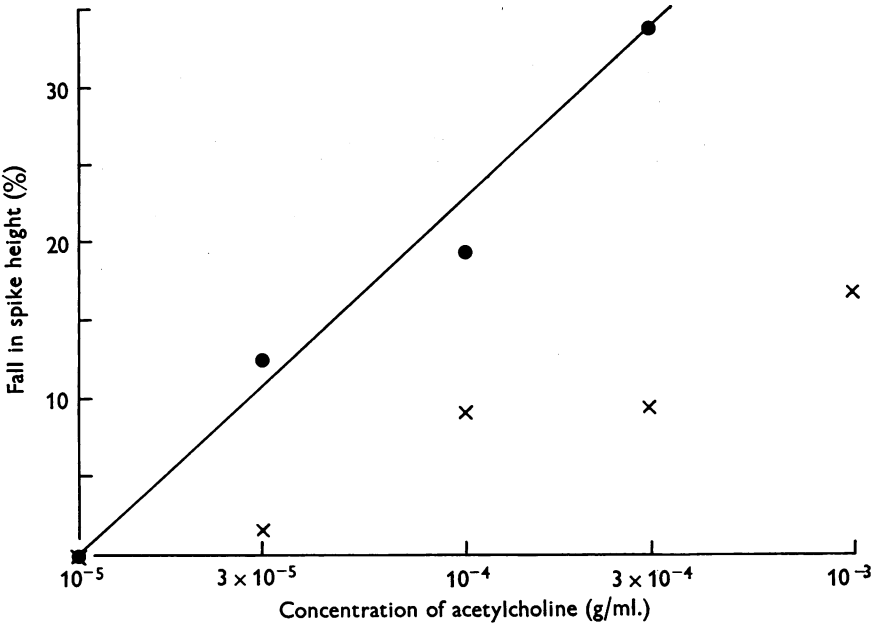


Fig. 3. Reduction in amplitude of the compound action potential of the C fibres in a desheathed rabbit's vagus nerve produced by 3 min exposures to acetylcholine in various concentrations in the absence (●) and in the presence (×) of eserine $3 \times 10^{-5}$ g/ml. (0·07 mM). The ordinate is the reduction in spike amplitude expressed as a percentage of the original spike amplitude. Semi-log scale.

but that weak concentrations have the opposite effect. The potentiating effect was regularly obtained, but was particularly pronounced when near-threshold concentrations of acetylcholine were used. This is shown by the experiment of Fig. 5, where the reduction produced by acetylcholine alone was only about 10% as indicated by the horizontal line; but in the presence of weak concentrations of DFP the reduction increased more than threefold.

Another method by which this potentiating effect became pronounced was by reducing the time of exposure to acetylcholine. In previous experiments (Armett & Ritchie, 1960) it was found that the intensity of

the effect of acetylcholine depended on the time of the nerve's exposure to it; during the first minute after switching to perfusion with acetylcholine the effect was often greater than after 3 min. The solid circles in Fig. 4 show the effect of DFP tested after ½–1 min exposure to acetylcholine, when the effect of acetylcholine appeared to be maximal. It will be seen that the potentiation by weak concentrations of DFP was more pronounced than when tested with 3 min exposures to acetylcholine.

Prostigmine was investigated in relatively high concentrations. These reduced or abolished the effect of acetylcholine on spike potential. The result obtained with a concentration of $10^{-3}$ g/ml. is illustrated in Fig. 6.

Edrophonium, a drug which possesses some anticholinesterase activity, was as effective as prostigmine in reducing the effect of acetylcholine.

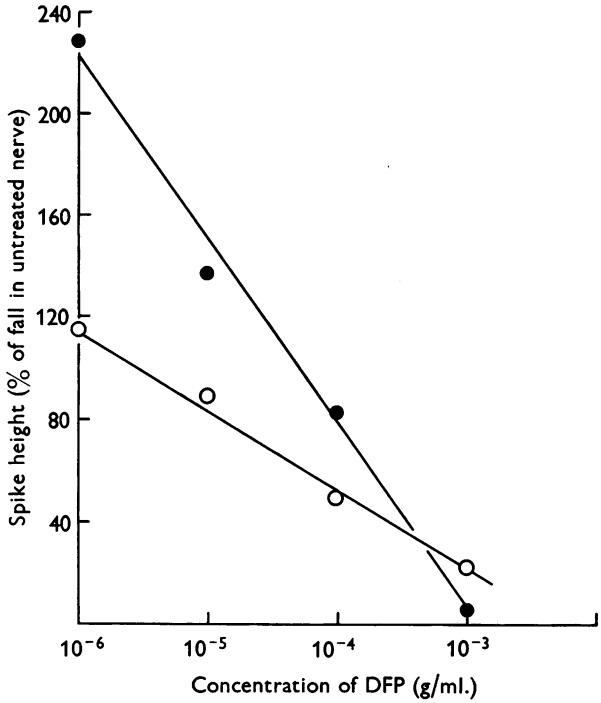


Fig. 4. The effect of different concentrations of DFP on the action of acetylcholine on the non-myelinated nerve fibres of desheathed rabbit's vagus nerves. Each point gives the average of the responses to acetylcholine in about five experiments. The reduction in spike amplitude by acetylcholine in the presence of DFP is expressed as a percentage of the response to acetylcholine in the absence of DFP. The results were obtained from experiments on 22 different nerves. ○ = 3 min, ● = ½–1 min exposures to acetylcholine (see text). In the absence of DFP the average falls in spike height with 3 min and with ½–1 min exposures were 24·8 and 26·1 respectively. Semi-log. scale.

The anticholinesterases had the same attenuating effect on the reduction of spike potential produced by carbachol as on that produced by acetylcholine. This is shown for prostigmine in Fig. 6.

*Antagonists of acetylcholine.* Neither tubocurarine nor hexamethonium reduced spike height, even with long exposures (15–60 min) to concentrations of $10^{-2}$ g/ml., whereas atropine in a concentration of $10^{-3}$ g/ml. (but not in a concentration of $10^{-4}$ g/ml.) reduced it slightly. All three substances tested in a concentration of $10^{-3}$ g/ml. reduced the action of acetylcholine (Fig. 7); sometimes they were effective in a concentration of $10^{-4}$ g/ml.

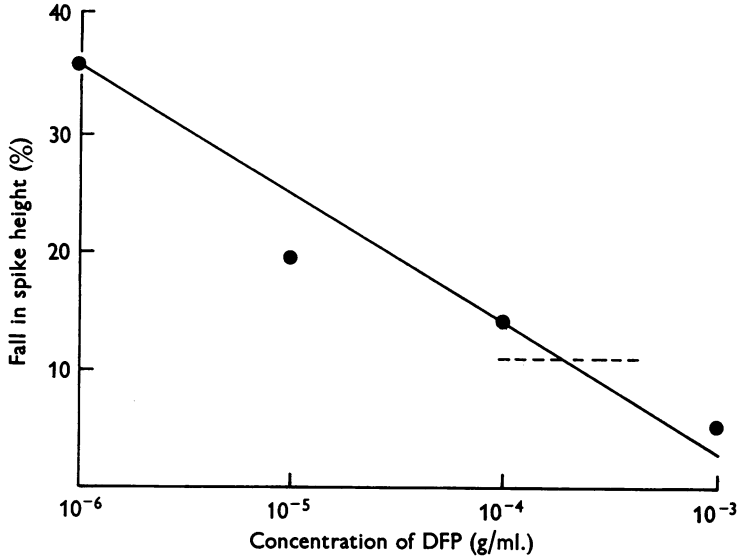


Fig. 5. The potentiating effect of DFP on the action of a weak concentration ($10^{-4}$ g/ml.) of acetylcholine. The ordinate is the percentage reduction in the amplitude of the action potential of the non-myelinated fibres in a desheathed rabbit's vagus nerve in the presence of various concentrations of DFP. The horizontal broken line represents the response in the untreated nerve. Semi-log. scale.

Tetraethylammonium chloride was tested in concentrations of 5 and 15 mM: it also reduced the action of acetylcholine, but did not by itself affect spike amplitude.

*Phosphate.* Increasing the concentration of sodium phosphate in the Locke's solution greatly reduced the effect of acetylcholine. In one experiment, for example, acetylcholine (1·7 mM), when tested in a phosphate concentration of 1 mM, caused a reduction of spike height of 23% and a depolarization of 2·1 mV. When retested in a phosphate concentration of 4 mM the reduction amounted to only 12% of spike height and

the depolarization only 1 mV. Figure 8 shows that the reduction in spike height produced by a given concentration of acetylcholine (1·7 mM) was roughly proportional to the negative logarithm of the phosphate concentration of the medium. Glucose-6-phosphate and sodium pyrophosphate had the same effect as sodium phosphate, but glucose-1-phosphate had hardly any effect.

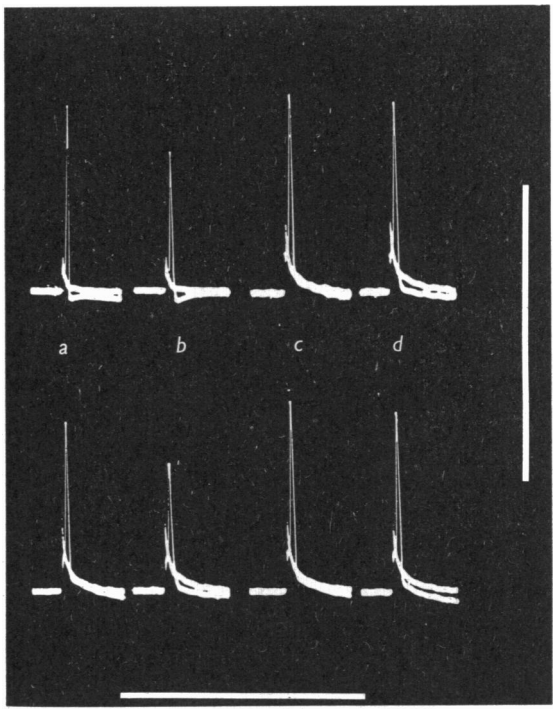


Fig. 6. The effect of prostigmine on the action of acetylcholine and carbachol on the non-myelinated nerve fibres in a desheathed rabbit's vagus nerve. The two records of the C potential in *a* show the responses of a nerve before, and those in *b* the two similar response after, the preparation has been exposed for 3 min to $3 \times 10^{-4}$ g/ml. (1·7 mM) acetylcholine (upper record) and $3 \times 10^{-4}$ g/ml. (1·6 mM) carbachol (lower record). The records in *c* and *d* show the corresponding responses in the presence of prostigmine $10^{-3}$ g/ml. (3·0 mM). The vertical bar represents 10 mV and the horizontal bar 300 msec.

*Bicarbonate.* The action of acetylcholine on the spike potential was also influenced by the bicarbonate content of phosphate-free Locke's solution (equilibrated with a 5 % $CO_2$, 95 % $O_2$ gas mixture). In a few experiments when the bicarbonate concentration was 1·9 mM a 3 min exposure to acetylcholine 1·7 mM reduced the spike height by 20–30 %; when retested in 25 mM bicarbonate, the reduction was 8–15 % of the spike height.

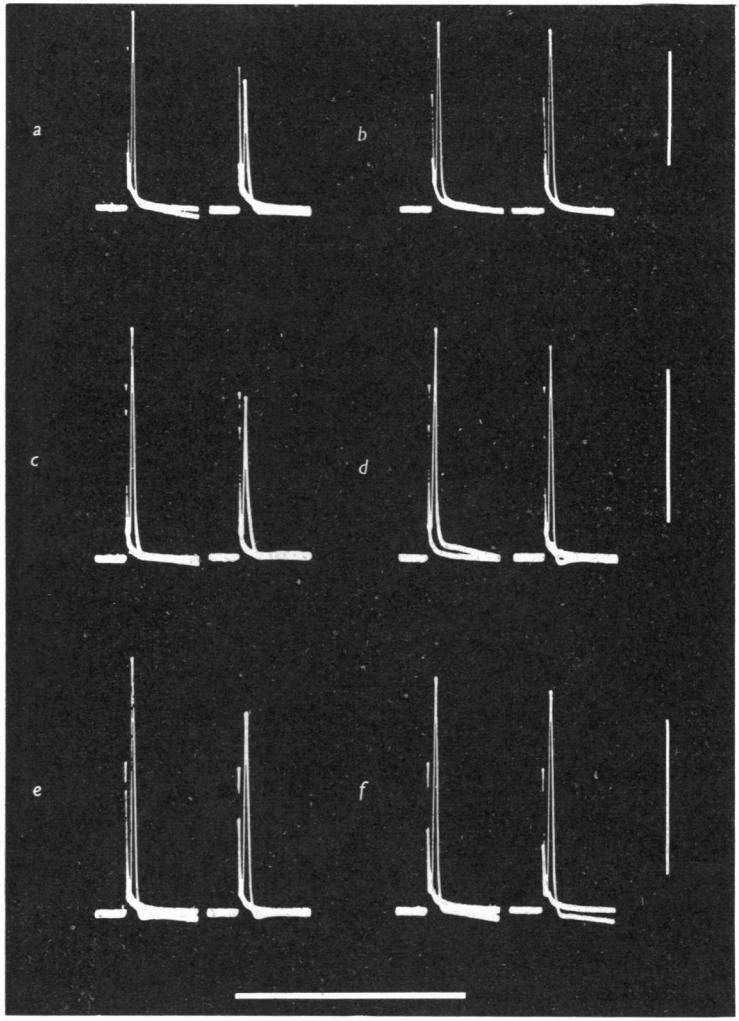


**Fig. 7.** The effect of tubocurarine, hexamethonium and atropine on the action of acetylcholine on the non-myelinated nerve fibres in desheathed rabbit's vagus nerves. The pair of records of the C potential in *a* show the responses before (left-hand record) and after (right-hand record) a 3 min exposure of the preparation to acetylcholine $3 \times 10^{-4}$ g/ml. (1·7 mM). The records in *b* are a similar pair taken in the presence of tubocurarine $10^{-3}$ g/ml. (1·4 mM). The other records illustrate the corresponding effects of hexamethonium $10^{-4}$ g/ml. (0·37 mM) (*c* and *d*) and of atropine $10^{-4}$ g/ml. (0·14 mM) (*e* and *f*). The vertical bars represent 5 mV and the horizontal bar 250 msec.

Although changing the concentration of the bicarbonate necessarily changed the pH of the solution, the effect of bicarbonate seems unlikely to have been dependent on a change in pH, for when the pH was changed by using different phosphate buffers (the total phosphate concentration being kept constant) increasing the pH did not produce any consistent reduction in the response to acetylcholine.

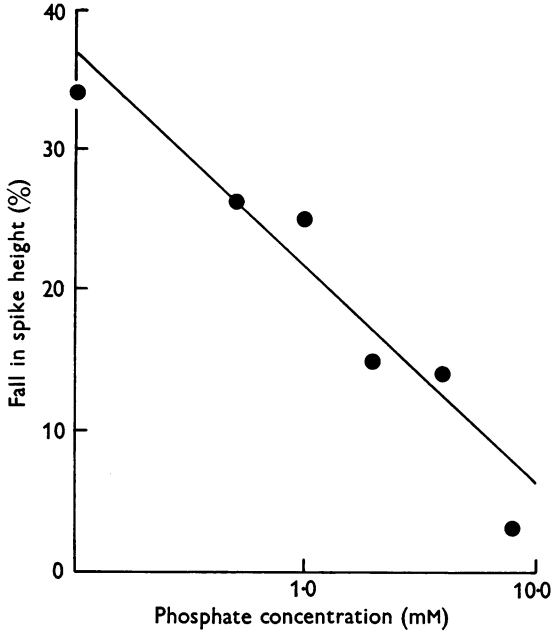


Fig. 8. The relationship between the action of acetylcholine and the phosphate concentration in the bathing solution. The ordinate is the percentage reduction in the action potential of the non-myelinated fibres in a desheathed rabbit's vagus nerve produced by a 3 min exposure to acetylcholine $3 \times 10^{-4}$ g/ml. The phosphate concentration has been plotted on a logarithmic scale. The reduction in phosphate-free Locke's solution, not plotted in the figure, was about 36 %.

## DISCUSSION

The present experiments indicate the type of receptor which may be involved in the action of acetylcholine on mammalian non-myelinated fibres described previously (Armett & Ritchie, 1960). The action of acetylcholine on spike height is shared by carbachol, by TMA and by nicotine, drugs which are known to have strong nicotinic actions, but to a lesser extent or not at all by arecoline, pilocarpine, methacholine and bethanechol, all of which have strong muscarinic actions. It is therefore tempting to assume that the acetylcholine-sensitive receptors of the nerve fibres have common features with those receptors of other structures involved in the

nicotinic actions of acetylcholine, such as those of autonomic ganglion cells or of the neuromuscular junction of skeletal muscle. This view is consistent with the finding that tubocurarine, hexamethonium and tetraethylammonium chloride, which are known to block the nicotinic actions of acetylcholine, also antagonize the action of acetylcholine on nerve fibres. The finding that atropine blocks the action of acetylcholine on C fibres is not incompatible with this view, because atropine in high concentration antagonizes nicotinic properties of acetylcholine (see Goodman & Gilman, 1955).

The present results on mammalian non-myelinated fibres differ from those obtained by Dettbarn (1960) in frog's myelinated fibres. He found that tubocurarine as well as anticholinesterases blocked nerve conduction in concentrations smaller than those found to be ineffective on mammalian non-myelinated fibres in the present experiments. Dettbarn explained his results on the hypothesis that the production and hydrolysis of acetylcholine is essential to the conduction of the nerve impulse (see Nachmansohn, 1959). It would be difficult to explain the results of the present experiments on this hypothesis. The demonstration that a large concentration of acetylcholine affects the resting and action potentials is not by itself sufficient to prove that acetylcholine plays some physiological role in the conduction of the nerve impulse. The finding that the concentrations of anticholinesterases used in the present experiments did not affect conduction in fact suggests the contrary, for they were certainly sufficient for at least partial penetration of the nerve, since they were able to antagonize the action of acetylcholine on these structures. A similar argument applies to the present results obtained with tubocurarine, hexamethonium and TEA, which did not affect the spike potential, but nevertheless blocked the action of applied acetylcholine.

The anticholinesterases are unlikely to act by allowing excessive amounts of the applied acetylcholine to build up in the region of the receptors so as to render them refractory to acetylcholine, for they also block the action of carbachol, which is not hydrolysed by cholinesterase. The idea that the anticholinesterases allow the concentration of endogenous acetylcholine to rise to such an extent as competitively to inhibit the carbachol is not supported by the fact that the anticholinesterases did not by themselves affect spike height, as might have been expected if endogenous acetylcholine were piling up. The action of anticholinesterases in inhibiting the effects of applied acetylcholine and carbachol on nerve fibres is most likely the same as the inhibitory action seen with high concentrations of anticholinesterases at motor end-plates and at sympathetic ganglia (Eccles & MacFarlane, 1949; Fatt, 1950; Paton & Perry, 1955; del Castillo & Katz, 1957).

The findings with the anticholinesterases, with the choline esters and with the antagonists of acetylcholine suggest that it may well be that the receptor mechanism so extensively studied at the end-plate and at ganglionic synapses is also present, perhaps in an attenuated form, in the membrane of nerve fibres; what its function there might be remains, however, an open question. There seems no reason to infer from the present experimental findings that it plays a role in conduction along the nerve membrane similar to its well established role in neuromuscular and synaptic transmission.

## SUMMARY

1. Isolated preparations of non-myelinated fibres of the desheathed rabbit's vagus nerve were mounted in the sucrose-gap apparatus, and the effect of drugs on the action potential of the C fibres studied.

2. Acetylcholine depolarizes the membrane and reduces the size of the spike potential.

3. The effects of acetylcholine are shared by the nicotine-like compound carbachol, by TMA, and by nicotine itself. They are shared to only a small extent by arecoline and by pilocarpine, and not at all by methacholine and bethanechol, compounds which characteristically possess muscarine-like properties.

4. The actions of acetylcholine and carbachol are inhibited by the anticholinesterases eserine ($3 \times 10^{-5}$ g/ml.), prostigmine ($10^{-4}$–$10^{-3}$ g/ml.) and DFP ($10^{-5}$–$10^{-3}$ g/ml.).

5. Under certain conditions the actions of acetylcholine are potentiated by the anticholinesterases, namely when near-threshold concentrations of acetylcholine are used, or when weak concentrations of the anticholinesterase are used or when the time of exposure to acetylcholine is shortened.

6. The action of acetylcholine is reduced or abolished by tubocurarine, hexamethonium and atropine ($10^{-4}$–$10^{-3}$ g/ml.). It was similarly reduced by tetraethylammonium chloride (5–15 mM).

7. Increasing the phosphate or bicarbonate concentration of the Locke's solution reduces the action of acetylcholine.

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