The mechanisms of motor end-plate depolarization due to a cholinesterase-inhibiting drug / by W.W. Douglas and W.D.M. Paton.

[*Reprinted from the Journal of Physiology*,
1954, Vol. **124**, No. 2, p. 325.]
PRINTED IN GREAT BRITAIN

J. Physiol. (1954) 124, 325–344

THE MECHANISMS OF MOTOR END-PLATE DEPOLARIZATION DUE TO A CHOLINESTERASE-INHIBITING DRUG

By W. W. DOUGLAS and W. D. M. PATON
From the National Institute for Medical Research, Mill Hill, London, N.W.7

(*Received* 12 *November* 1953)

Many cholinesterase-inhibitors are stated to have a direct acetylcholine-like action as well as an action due to the preservation of acetylcholine normally destroyed. The widespread use of such drugs in analysing the functions of acetylcholine in the body depends for its validity on knowledge about such direct action. For such an action might be attributed erroneously to acetylcholine; and criticisms have been levelled at analyses involving the use of anticholinesterases for this reason.

In this paper we have chosen TEPP (tetraethylpyrophosphate) for study, because it is one of the strongest and most commonly used alkyl-phosphate cholinesterase inhibitors, and we have analysed its effects at the neuromuscular junction. A preliminary account of this work has already been published (Douglas & Paton, 1951).

METHODS

Cats anaesthetized with chloralose (80 mg/kg intravenously after induction with ethyl chloride and ether) were used in all experiments. The trachea was cannulated. Intravenous injections were made through a cannula in the jugular vein.

For experiments on the depolarization of the motor end-plate, the method of Burns & Paton (1951) was used. The cat's gracilis was exposed and enclosed in a paraffin bath formed from skin flaps, and its motor nerve tied and cut. The spatial distribution of electrical potential along a length of the muscle which contained suitable end-plate regions was recorded as follows: a scanning electrode was made to traverse the region chosen 15 times a minute and an indifferent electrode was placed on the tendon. The potential difference between the electrodes was applied to the Y-plates, and the movement of the scanning electrode (after conversion to a potential change) to the X-plates, of a cathode-ray oscilloscope. Supramaximal shocks of 0·5 msec duration were applied to the tied motor nerve through shielded electrodes.

For experiments on the tibialis anterior, the contractions of this muscle prepared for close arterial injections were recorded with a flat spring myograph on a smoked drum.

Samples of blood (3 ml.) for assay were withdrawn from a steel cannula tied into the carotid artery; or through a glass cannula inserted into the splenic vein, from which portal blood could be

obtained by temporary occlusion of the portal vein. Assay of these samples was made on the eviscerate cat's blood pressure. The samples were stored on ice before assay. Assay was made as soon as possible, although there was no apparent loss in acetylcholine content of a few samples in which the assay was repeated after standing for 1–2 hr.

RESULTS

The innervated gracilis muscle with tied and cut motor nerve

The effects of TEPP on this preparation depended on the dose used. A small dose (100 μg/kg) produced no depolarization of the end-plate zone at any time, but increased the magnitude and duration of the depolarization produced by repetitive stimulation of the motor nerve. For example, in one experiment 33 stimuli/sec for 4 sec, which produced in a normal muscle no detectable depolarization (less than 0·2 mV), when reapplied 1 min after 100 μg/kg TEPP produced a depolarization of 0·8 mV which took 4 sec to disappear. Twenty minutes later, the muscle responded normally.

With a larger dose (500 μg/kg), variable effects were obtained. In some animals no, or very little, depolarization was observed in the absence of motor nerve stimulation. In others, as much as 8 mV depolarization was recorded. But in the latter experiments the negativity did not rise to a maximum and fall again regularly, but after a delay of $\frac{1}{2}$–$1\frac{1}{2}$ min waxed and waned in an irregular manner for periods of 5–25 min (Fig. 1). Sometimes the rise and fall had a peculiar rhythmic character (Fig. 2). In all animals the effects of nerve stimulation after this dose were greatly increased and prolonged. Fig. 3 shows the effects of repetitive stimulation of the motor nerve, recorded immediately after the stimulation and for short intervals afterwards. This augmentation of the depolarization was still detectable, although very slight, 40–50 min after the injection of TEPP.

The fact that depolarization was entirely absent in one experiment, in which the nerve had been tied and cut several hours earlier, showed that TEPP in this dose did not itself affect the end-plates. An explanation of the waxing and waning depolarization, when it occurred, was suggested by the observation that it was greatest when recent mechanical injury to the motor nerve to gracilis had occurred, for instance when some unusually vigorous fasciculations of the muscle had stretched the nerve where it was fixed to the stimulating electrodes. It seems probable, therefore, that the fluctuating depolarization sometimes seen is due to irregular discharges originating from points of injury of the main nerve trunk, produced in tying and cutting the nerve.

With higher doses (2–20 mg/kg) the course of the depolarization is of a different character. It begins sooner after the injection, grows more rapidly, reaches its maximum value in a few seconds, fluctuates comparatively little, and soon settles to a steady or gradually declining value (Fig. 4). The duration

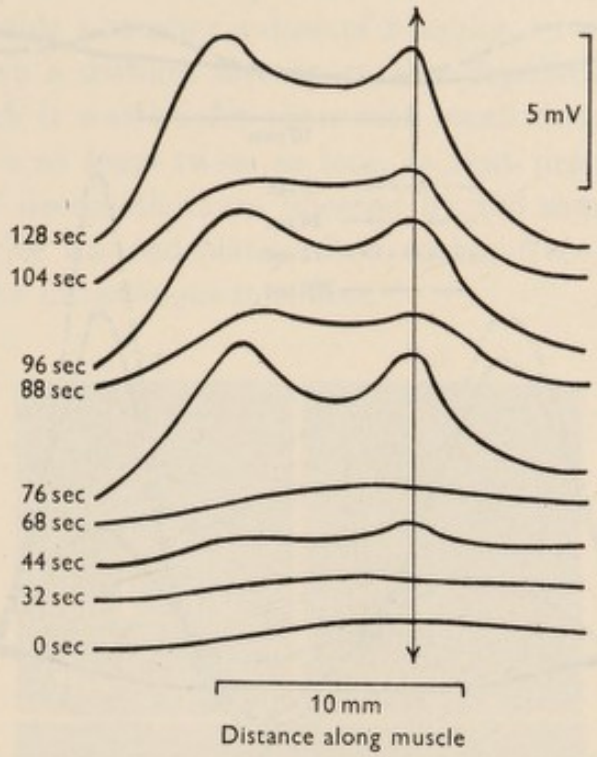

Fig. 1. Distribution of potential along cat's gracilis muscle fibre after TEPP 500 μg/kg intravenously. Nerve to gracilis tied and cut. Control tracing taken at zero time, when TEPP was injected, and subsequent records at times marked after injection (successive records have been shifted upwards to avoid overlap).

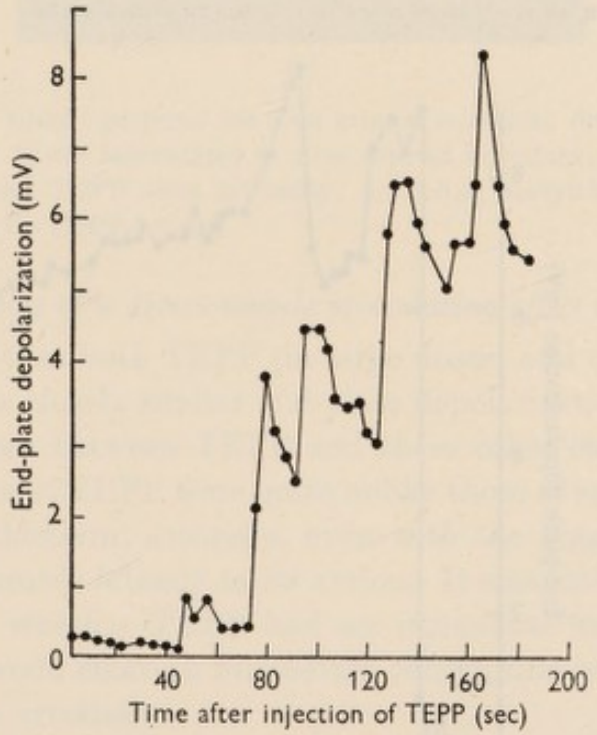

Fig. 2. Graph of depolarization of an end-plate region of cat's gracilis muscle after TEPP 500 μg/kg intravenously. Nerve to gracilis tied and cut.

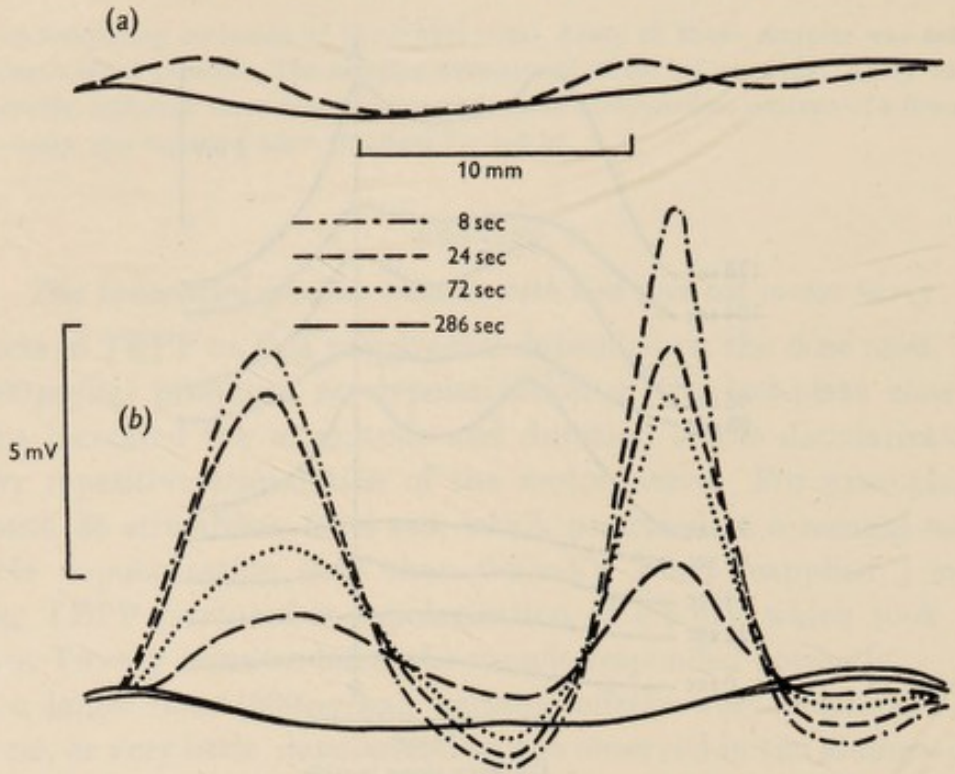

Fig. 3. Tracings of end-plate depolarization produced by repetitive motor nerve stimulation (*a*) before and (*b*) after TEPP 500 μg/kg. (*a*) Before TEPP: continuous line, control record; dotted line, peak depolarization 8 sec after motor nerve stimulation (33/sec for 5 sec). The animal had had TEPP 40 min previously, and there is a slight residual effect apparent. (*b*) Effect of TEPP: lower continuous line, control record before TEPP; upper continuous line, record 104 sec after TEPP (no significant effect); interrupted lines, records taken 8, 24, 72 and 286 sec (from above down) after motor nerve stimulation.

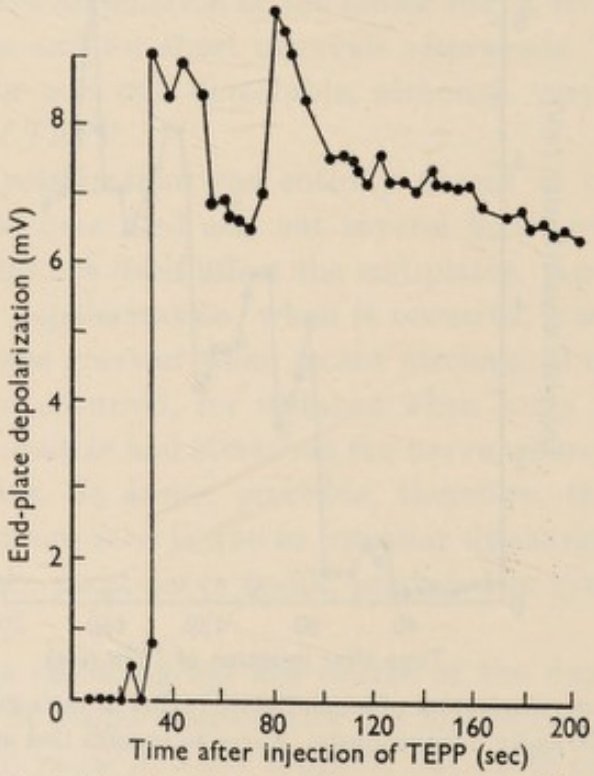

Fig. 4. Graph of depolarization of end-plate region of cat's gracilis muscle after TEPP 2 mg/kg intravenously. Nerve to gracilis tied and cut.

of the depolarization is considerably increased with these larger doses, and it may still be detectable 1 hr after a dose of 2 mg/kg.

There was always a distinct latency in this depolarizing action of large doses, and although it was briefer than with small doses, it was never less than 26 sec. This is at least twice as long as that preceding the action of acetylcholine or of decamethonium injected by the same route. These two substances depolarize the end-plates often within 6 sec and always within 12 sec of making the intravenous injection.

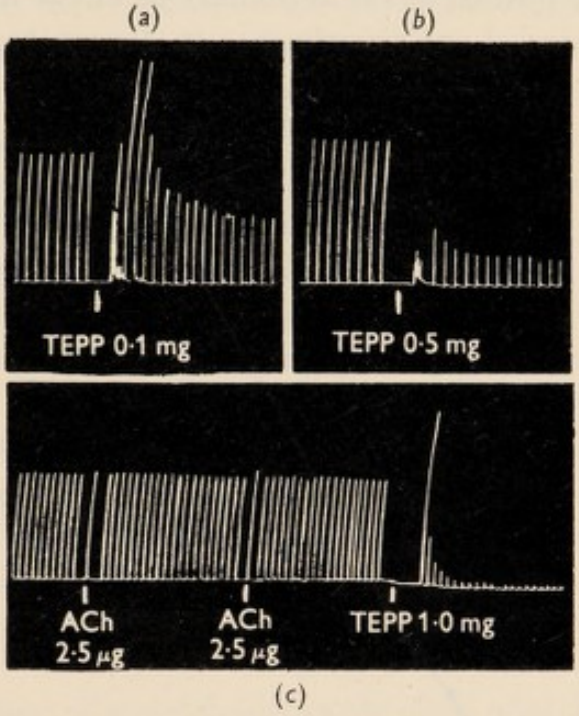

Fig. 5. Cat, chloralose, tibialis prepared for close arterial injection. Supramaximal stimulus to sciatic nerve every 10 sec, intermitted at close arterial injections: (*a*) 0·1 mg TEPP close arterially; (*b*) 0·5 mg TEPP close arterially; (*c*) 2·5 μg acetylcholine given twice close arterially, followed by TEPP 1·0 mg.

The absence of a direct muscle stimulating effect of TEPP

Despite the fact that both TEPP (in large doses) and directly acting compounds can produce closely similar end-plate depolarizations, there were two important differences between TEPP and these other substances: first, the effects of small doses of TEPP were quite unlike those of small doses of acetylcholine or decamethonium; secondly, even with the biggest doses of TEPP there was a considerable latency in its action. It was therefore important to test more directly whether TEPP had an immediate stimulant action, by trying whether it could elicit an immediate contraction of the tibialis muscle when injected close arterially.

The results of two experiments are shown in Fig. 5. Doses of TEPP from 0·1 to 1 mg, injected close arterially, never elicited a twitch, although the muscle responded vigorously to as little as 2·5 μg acetylcholine. Soon after

the injection of the TEPP, the usual effects of injection of an anticholinesterase into this preparation were observed, i.e. fasciculation and potentiation of the twitch succeeded by partial or complete paralysis according to the dose.

Thus, TEPP must be supposed to lack any significant direct muscle-stimulant action; if it exists it is not greater than $\frac{1}{1000}$th that of acetylcholine. But it remains to be explained how TEPP produces a depolarization of the unstimulated muscle in some respects so deceptively similar to that of a directly acting drug. The most obvious explanation was that it acts by preserving acetylcholine formed at the terminations of the motor nerve. To test this possibility, we carried out experiments on denervated muscles.

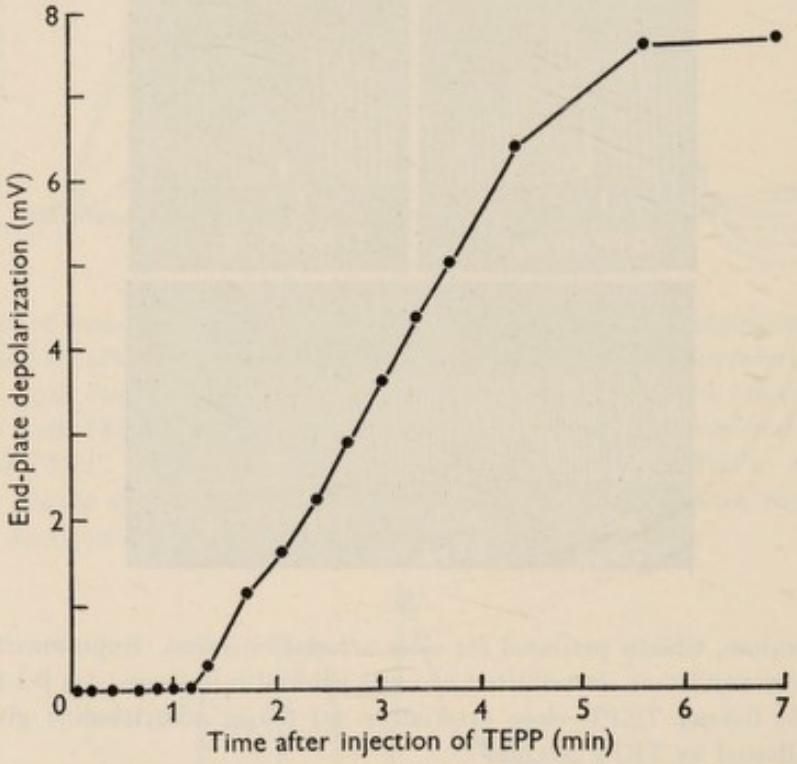

Fig. 6. Graph of depolarization of end-plate region of cat's denervated gracilis muscle after TEPP 10 mg/kg intravenously.

The denervated gracilis muscle

In two experiments, the effect of 500 μg/kg of TEPP on a muscle denervated 4 and 6 days previously was hardly detectable. Stimulation of the peripheral stump of the nerve to the muscle, or of the muscle itself in the neighbourhood of the end-plate zone, in both experiments failed to cause any depolarization and confirmed that significant acetylcholine release from nerve elements in the muscle was absent. But in these animals and in each of two others, depolarization occurred with large doses of TEPP (10–20 mg/kg). This depolarization was much slower both in onset and in rate of rise, and smaller than in the innervated preparation (Fig. 6) and in degree was roughly comparable to that produced by 1 mg/kg TEPP in the normal muscle. It was clear

that large doses of TEPP could cause depolarization of the muscle even in the absence of motor nerve fibres as local sources of acetylcholine. The absence of any fluctuation in the course of the depolarization in these denervated muscles supported our belief that the fluctuating component with the normally innervated muscle was due to motor nerve activity.

There is good reason to believe that any changes at the end-plate due to denervation would favour the demonstration of a direct action by TEPP, since denervation is known to sensitize a muscle to acetylcholine. Nevertheless, we did a further experiment, on a 2-day denervated muscle, in the hope of obtaining a preparation with functionless motor nerve, in which any change in sensitivity produced by denervation would be as small as possible. We found, however, that although no obvious contraction resulted on nerve stimulation, this preparation was not completely denervated, since repetitive stimulation of the motor nerve at 33 shocks per second produced a transient depolarization, which in the presence of TEPP amounted to as much as 4 mV. It seems, therefore, that actions independent of functional motor nerves cannot be obtained much earlier than with the 4-day denervation we have studied.

These experiments, then, showed that the effect of smaller doses of TEPP ($<500\,\mu$g/kg) requires the integrity of the motor nerve fibres, but that with larger doses (10–20 mg/kg) an additional process may come into play, detectable with denervated muscle. Since it is in this additional mechanism alone that any direct action of TEPP must reside, it was analysed further.

Presence of acetylcholine in the blood after TEPP

What was most striking about the response of the denervated muscle was the delay in onset of the depolarization and its slow, smooth, steady rise. This seemed most unlike a direct action; rather it suggested to us the course of events seen if an isolated tissue such as the intestine is exposed to anticholinesterase, when endogenous acetylcholine gradually exerts its effect as it progressively accumulates.

Since local sources of acetylcholine could be discounted in the denervated preparation, this acetylcholine could accumulate only from other sources and should therefore appear in the blood. Estimates of the acetylcholine equivalent of the blood after TEPP were made in six experiments. Blood (1 ml.) before injection of TEPP, had no depressor action whatever on the blood pressure of a second cat. But shortly after injection of TEPP, and for some time later, the blood of the poisoned animal possessed considerable depressor activity. This closely resembled that produced by acetylcholine, and was abolished by small doses (0·05 mg) of atropine injected into the assay cat. It could not be ascribed to the presence of TEPP in the blood because at the time when the TEPP concentration would be at its peak, i.e. immediately after the injection,

no depressor activity was present, and it only appeared after a distinct latency (see below). Further experiments to establish that the depressor substance was in fact acetylcholine seemed unnecessary in view of these facts and of the conditions of its appearance (i.e. after the injection of an anticholinesterase). The assay of the acetylcholine equivalent of the blood was complicated by the presence of atropine (about 100 μg/kg) which had to be given to the

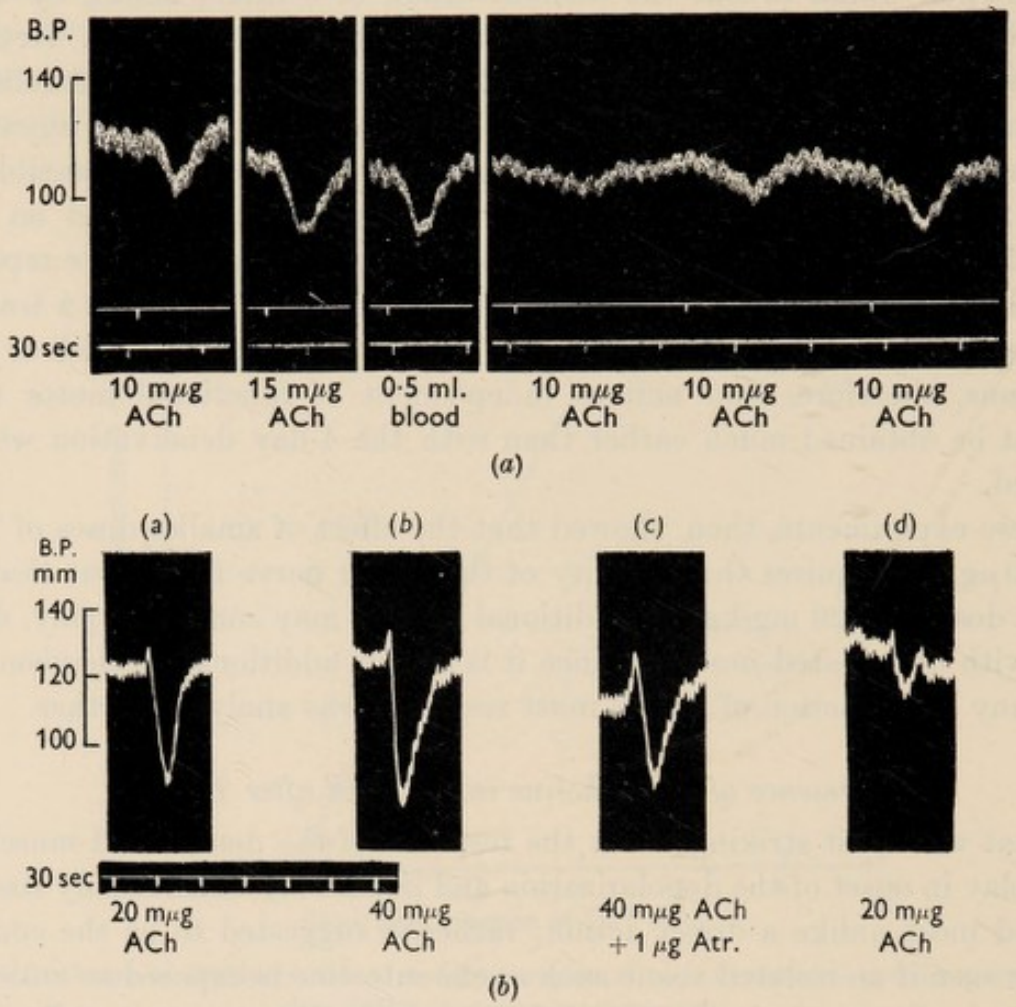

Fig. 7. Cat, chloralose, blood pressure. (*a*) Effect of injection of 0·5 ml. blood drawn from cat treated with TEPP 10 mg/kg and atropine 0·1 mg/kg on blood pressure showing immediate depressor response and antagonism to subsequent test doses of acetylcholine. (*b*) Effect of a dose of atropine, which antagonizes a subsequent response to acetylcholine as in (*a*), on the response to acetylcholine given simultaneously.

poisoned animal to prevent complete circulatory collapse. This atropine was recognized in the assay sample by its causing a temporary reduction of the effects of subsequent test doses of acetylcholine (Fig. 7*a*), and an attempt was made to estimate how far it was reducing the depressor activity of the blood samples containing it. We found that quite small quantities of atropine (0·1–1 μg), which produced a significant reduction of the effect of *subsequent* doses of acetylcholine, could exert a considerable antagonism to the depressor effect when given *simultaneously* with the acetylcholine (Fig. 7*b*). It did not

prove possible, however, from experiments of this sort, to estimate how much atropine was present in a given blood sample, and hence its true acetylcholine content, with anything like the required accuracy. We have, therefore, given the apparent acetylcholine content of the blood samples as though they contained no atropine, and consequently our values are underestimates.

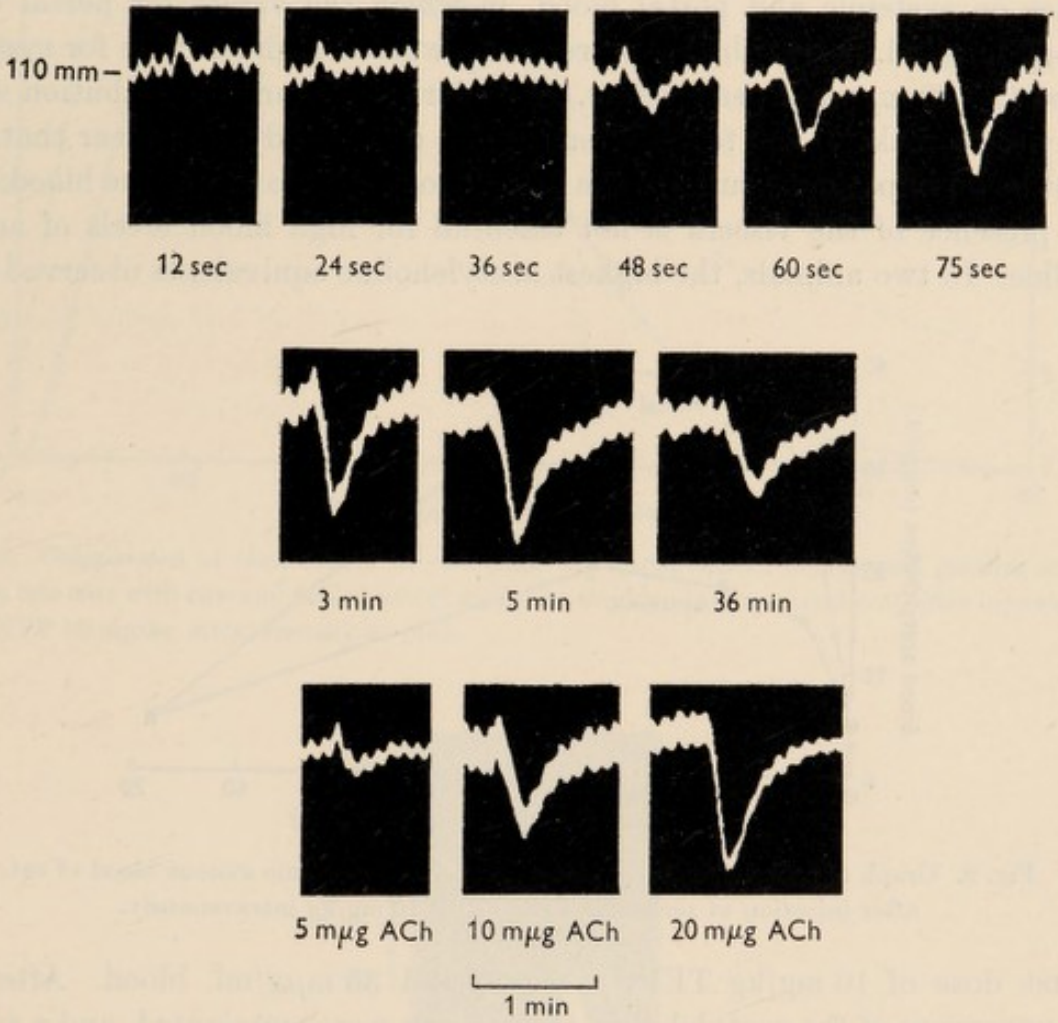

Fig. 8. Cat, chloralose, blood pressure. Depressor activity of blood samples from a donor cat given 10 mg/kg TEPP intravenously. Samples taken at various times after injection, as marked. All samples 1 ml. volume, except at 5 min, when it was 0·5 ml. For comparison, responses to 5, 10 and 20 mμg acetylcholine are shown.

In each of the six experiments carried out in three animals, acetylcholine was detected in the blood soon after the injection of 10 mg/kg TEPP. Measurable amounts could not be found until 48 sec had elapsed; but thereafter the acetylcholine content of the blood rose rapidly, and then more slowly, to reach a peak value of 35 mμg/ml. in 5 min (Fig. 8). Subsequently the acetylcholine diminished until none, or a trace only, was detectable an hour after the injection of TEPP. The train of events could be quite closely reproduced by injecting a second dose of TEPP.

A few experiments were made on the source of the acetylcholine. Repetitive stimulation of the vagus nerve or of the sciatic nerves failed to produce significant increases in the blood acetylcholine level. Donomae (1934) found that, after eserine, small amounts of acetylcholine (5–50 mμg/ml.) could be demonstrated in the portal blood, and traces in the systemic blood. We have done two similar experiments (one shown in Fig. 9) with simultaneous estimates on systemic and portal blood, in which the values for portal blood (40–60 mμg/ml. peak value) were roughly twice as high as those for systemic blood (20–30 mμg/ml. peak value). Considering the large contribution which the viscera make to the total venous return of the body, it is clear that they must be an important source of the acetylcholine appearing in the blood. But the presence of the viscera is not essential for high blood levels of acetylcholine. In two animals, the highest acetylcholine equivalents observed after

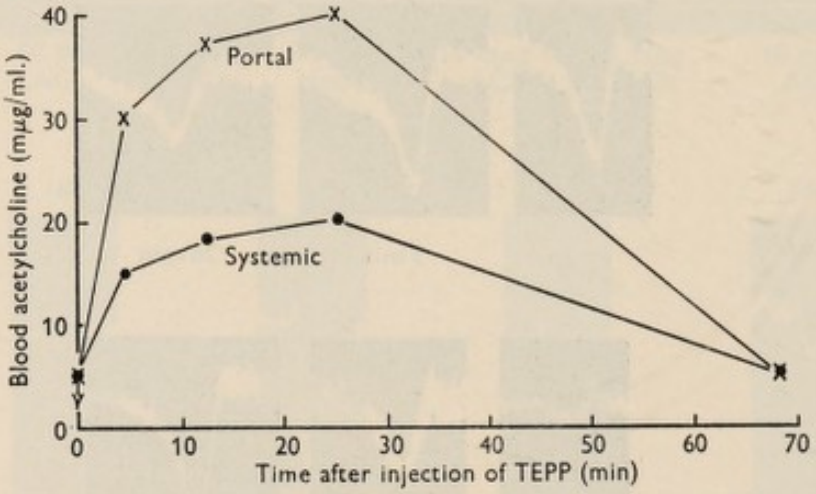

Fig. 9. Graph of acetylcholine content of portal and systemic venous blood of cat, after injection at zero time with TEPP 10 mg/kg intravenously.

a first dose of 10 mg/kg TEPP were 20 and 35 mμg/ml. blood. After the disappearance of the acetylcholine the animals were eviscerated, and a second dose of TEPP given following which acetylcholine reappeared in the blood in comparable amounts (up to 40 and 36 mμg/ml.).

The time-course of the appearance of acetylcholine in the blood was similar to that of the depolarization of the end-plate region in denervated muscle. We did not make simultaneous measurements of blood acetylcholine and end-plate depolarization, but the likeness in the time-course can be seen in Fig. 10. Further, as already described, both depolarization and the appearance of acetylcholine have distinct latent periods of 30 sec or more (cf. Figs. 6 and 8). In some experiments, it was observed that the cardiovascular effects of a large dose of TEPP also occurred only after a distinct latency, although the depressor effect when it came was rapid and profound (Fig. 11).

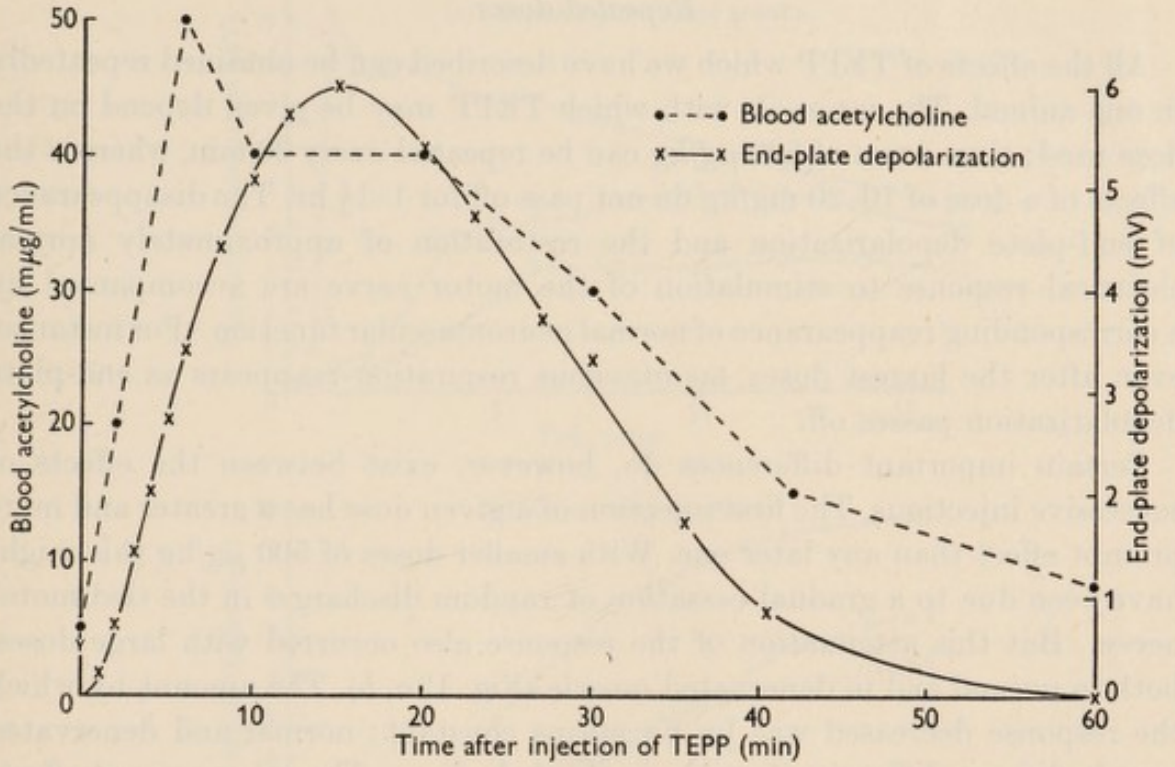

Fig. 10. Comparison of time-course of end-plate depolarization of denervated gracilis muscle (in one cat) with rise and fall of acetylcholine in the blood (in a second cat) after injection of TEPP 10 mg/kg intravenously to each.

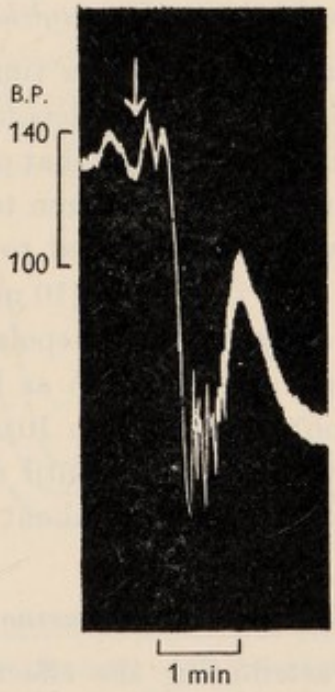

Fig. 11. Cat, chloralose, blood pressure. Effect of 10 mg/kg TEPP on blood pressure, showing a latency of 30 sec before depressor response.

Repeated doses

All the effects of TEPP which we have described can be obtained repeatedly in one animal. The intervals with which TEPP may be given depend on the dose used: thus doses of 500 μg/kg can be repeated every 30 min, whereas the effects of a dose of 10–20 mg/kg do not pass off for 1–1$\frac{1}{2}$ hr. The disappearance of end-plate depolarization and the restoration of approximately normal electrical response to stimulation of the motor nerve are accompanied by a corresponding reappearance of normal neuromuscular function. For instance, even after the largest doses, spontaneous respiration reappears as end-plate depolarization passes off.

Certain important differences do, however, exist between the effects of successive injections. The first injection of a given dose has a greater and more prompt effect than any later one. With smaller doses of 500 μg/kg this might have been due to a gradual cessation of random discharges in the tied motor nerve. But this attenuation of the response also occurred with large doses, both in normal and in denervated muscle (Fig. 12*a*, *b*). The amount by which the response decreased was by no means constant; normal and denervated muscle did not differ significantly in this behaviour. The other general effects of TEPP poisoning, such as convulsive movements of the animal and fasciculation of its muscles, also diminish with repeated doses.

Depolarization caused by injected acetylcholine after TEPP

If acetylcholine be given after TEPP, at a time when the depolarization due to the TEPP itself has partly subsided, depolarization re-occurs which is indistinguishable in spatial distribution from that originally seen due to TEPP alone (Fig. 13). The opportunity was also taken to determine the amount of acetylcholine, injected intravenously, required to produce depolarization of the end-plate after a large dose of TEPP (10 mg/kg). It was found that 10 μg/kg acetylcholine gave a considerable depolarization (Fig. 13); smaller doses gave smaller effects and a response to as little as 1 μg/kg was easily detectable. The depolarization resulting from 10 μg/kg began 6 sec after the injection, but did not reach its maximum until 80 sec later; after this, the depolarization only waned over a period of about 10 min.

Effect of D-*tubocurarine*

If we argue correctly in attributing the effects of TEPP at the neuromuscular junction to acetylcholine, then these effects should be opposed by D-tubocurarine. We have tested the action of D-tubocurarine on the depolarization which results from TEPP alone or from motor-nerve stimulation in the presence of TEPP. The effect of D-tubocurarine (1 mg/kg) on the end-plate

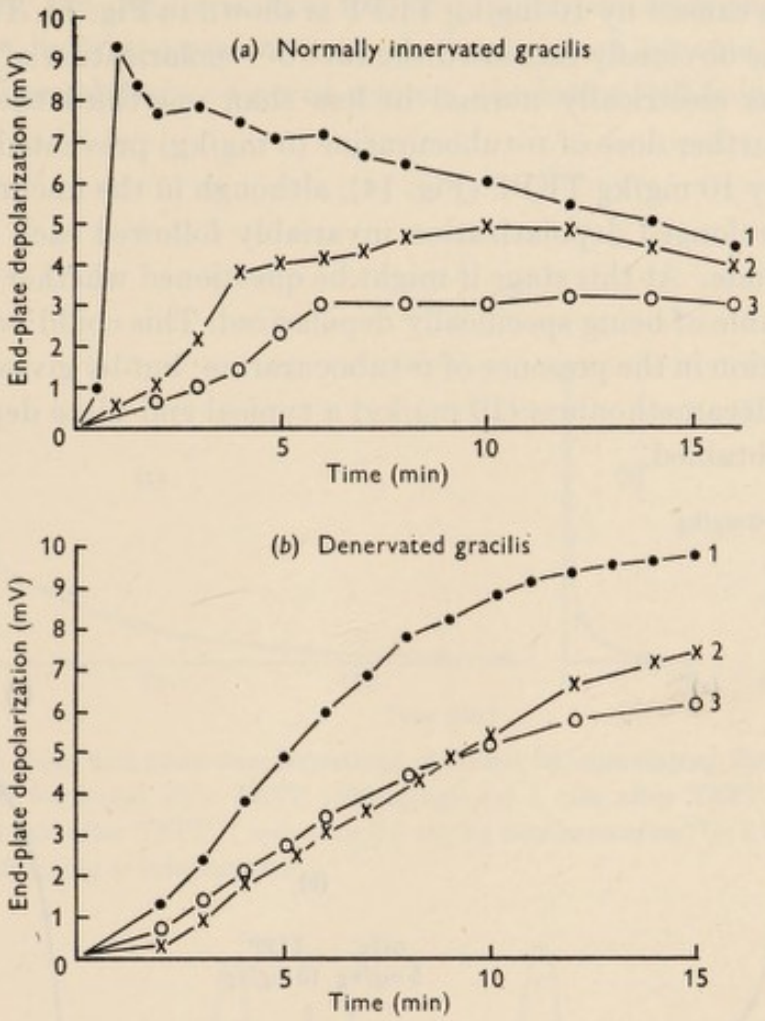

Fig. 12. End-plate depolarizations with successive doses of TEPP (10 mg/kg repeated at 1–1¼ hr intervals). (*a*) Three successive responses occurring in a gracilis muscle, the nerve to which had been tied shortly before recording. (*b*) Three successive responses occurring in a gracilis muscle denervated 4 days prior to recording.

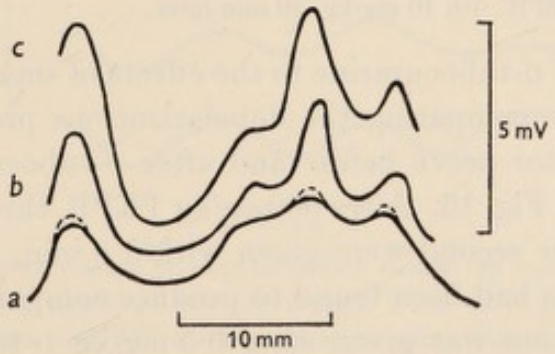

Fig. 13. Cat, chloralose. Distribution of potential in gracilis denervated 4 days previously. TEPP 10 mg/kg given 13 min previously, and approximately steady state of depolarization reached (*a*). Superimposed on this are effects obtained with acetylcholine. The first response, to 1 μg/kg acetylcholine, is shown by the dotted lines superposed on (*a*). The second response, 2 min later, to 10 μg/kg acetylcholine, is shown by (*b*) and (*c*), 8 and 80 sec after the acetylcholine injection.

depolarization caused by 10 mg/kg TEPP is shown in Fig. 14. The injection of D-tubocurarine obviously increased the rate of repolarization of the end-plate, so that it was electrically normal in less than one-third the time usually required. A further dose of D-tubocurarine (5 mg/kg) prevented any depolarization at all by 10 mg/kg TEPP (Fig. 14), although in the uncurarized animal, a large and prolonged depolarization invariably followed each dose of TEPP of this magnitude. At this stage it might be questioned whether the end-plates were still capable of being specifically depolarized. This could not be tested by nerve stimulation in the presence of D-tubocurarine; but by giving a sufficiently large dose of decamethonium (10 mg/kg) a typical end-plate depolarization of 8·5 mV was obtained.

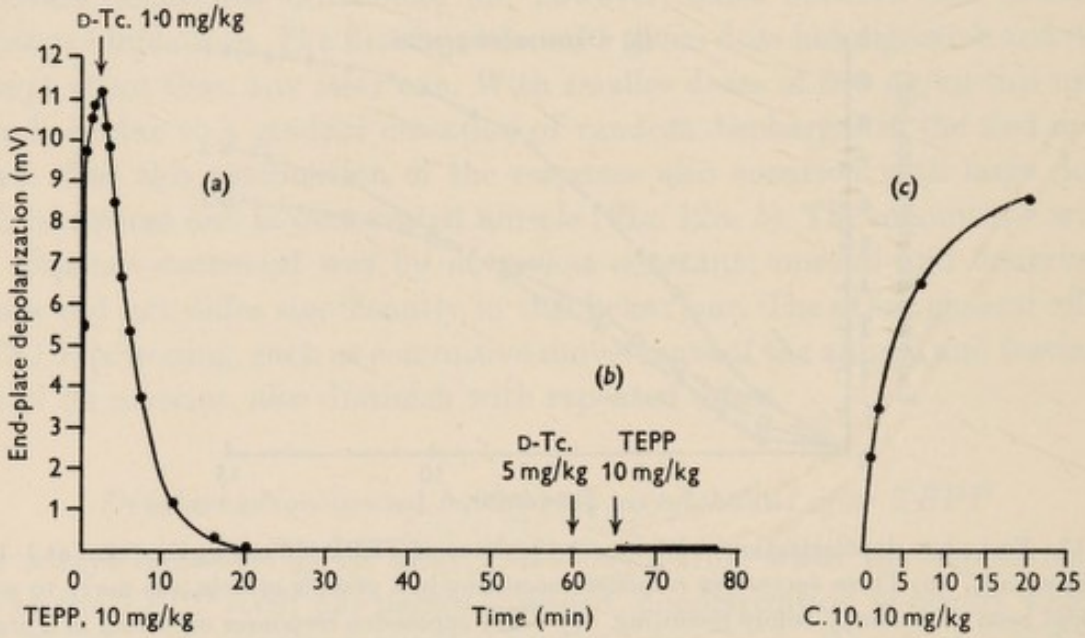

Fig. 14. Graph of end-plate depolarization produced (*a*) by TEPP 10 mg/kg, followed by D-tubocurarine 1·0 mg/kg; (*b*) by TEPP 10 mg/kg, after 5 mg/kg D-tubocurarine, 50 min later; (*c*) by decamethonium (C. 10) 10 mg/kg, 30 min later.

The antagonism of D-tubocurarine to the effects of small doses of TEPP was seen most clearly by comparing the depolarizations produced by repetitive stimulation of a motor nerve before and after D-tubocurarine. One experiment is illustrated in Fig. 15. After 500 μg/kg TEPP, three periods of stimulation at 33 shocks per second were given within 8 min, a time within which successive excitations had been found to produce comparable depolarizations. The second stimulation was given after 0·5 mg/kg D-tubocurarine, and the third after a total of 2·0 mg/kg D-tubocurarine. The depolarization from the second stimulation had a peak value little more than half that of the control, and a total duration less than a third as long. The depolarization after the third stimulation was still further reduced both in magnitude and duration.

One interesting observation was made in an experiment in which a depolarization produced by TEPP had been reinforced by acetylcholine (500 μg). In

this experiment, the depolarization not only collapsed after the injection of D-tubocurarine, but for a period the end-plate regions became positive with respect to the immediately adjacent regions, the latter still being negative to

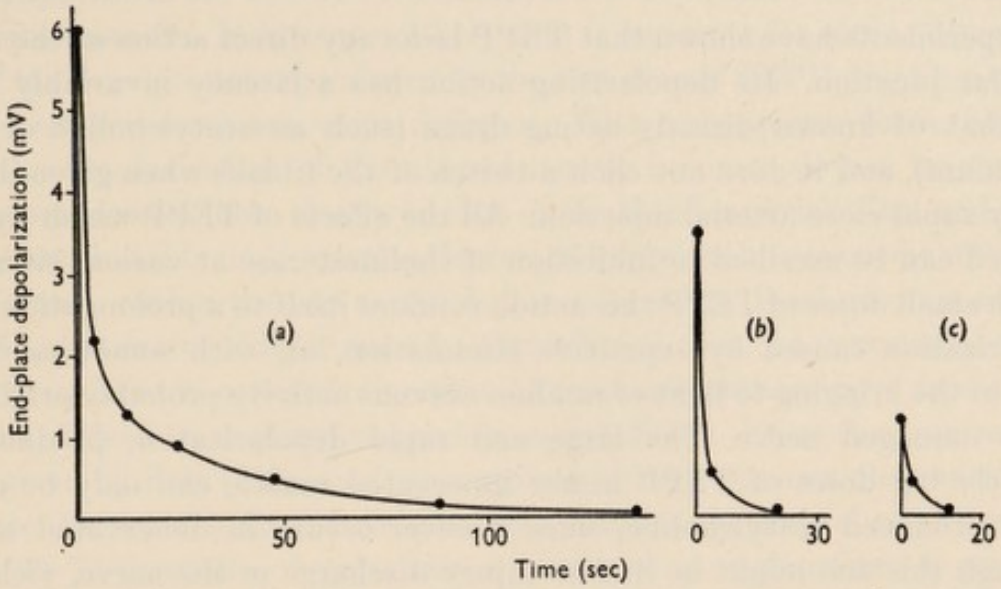

Fig. 15. Graph of three end-plate depolarizations obtained by stimulating the motor nerve to gracilis (33/sec for 5 sec) after TEPP (500 μg/kg): (*a*) 1 min after TEPP, before D-tubocurarine; (*b*) 4 min after TEPP, 1 min after 0·5 mg/kg D-tubocurarine; (*c*) 9 min after TEPP 2 min after 1·5 mg/kg D-tubocurarine.

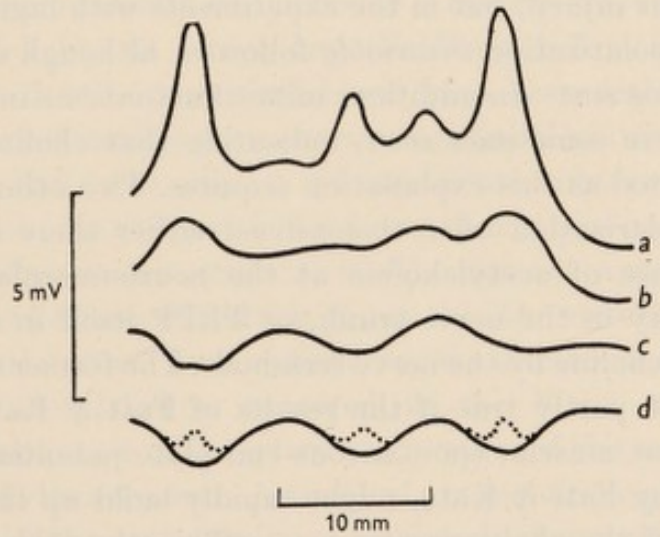

Fig. 16. The effect of D-tubocurarine on depolarization caused by acetylcholine (500 μg) in the presence of TEPP (10 mg/kg given 5 min previously). Denervated gracilis. (*a*) Depolarization caused by acetylcholine and TEPP. Immediately after this D-tubocurarine (5 mg) was injected. (*b*), (*c*) and (*d*) show the effects 116, 328 and 388 sec after D-tubocurarine. The dotted line superimposed on (*d*) is the effect of injecting a further dose of acetylcholine (500 μg).

the reference electrode (Fig. 16). There was no doubt that these now relatively positive regions corresponded to the end-plate zones since a further dose of acetylcholine elicited a typical depolarization centred on the points previously

of maximum positivity. This observation is exactly analogous to that made with D-tubocurarine given in the presence of a depolarization by decamethonium (Burns & Paton, 1951).

DISCUSSION

Our experiments have shown that TEPP lacks any direct action at the neuromuscular junction. Its depolarizing action has a latency invariably longer than that of known directly acting drugs (such as acetylcholine or decamethonium), and it does not elicit a twitch of the tibialis when given in large dose by rapid close arterial injection. All the effects of TEPP which we have observed can be ascribed to inhibition of cholinesterase at various sites.

With small doses of TEPP this action confines itself to a prolongation of the depolarization caused by repetitive stimulation, or, with somewhat higher doses, to the bringing to light of random nervous activity probably originating in the damaged nerve. The large and rapid depolarization produced by relatively big doses of TEPP in the innervated muscle can only be due to locally produced acetylcholine, since it never occurs in denervated muscle. Although this too might be due to injury discharge in the nerve, yielding a prolonged and unfluctuating depolarization because of an intense degree of cholinesterase inhibition, this explanation does not seem likely. We have already pointed out that fluctuating depolarization after moderate doses of TEPP was observed only in some experiments where the nerve was the object of relatively recent injury; but in the experiments with higher doses, prompt and sustained depolarization *invariably* followed, although care was taken to avoid injury of this sort. In addition, minor fluctuations in the high level of depolarization were sometimes seen, indicating that cholinesterase was not completely inhibited as this explanation requires. Two other explanations of this intense depolarization offer themselves: either there is a considerable spontaneous release of acetylcholine at the neuromuscular junction independent of activity in the nerve trunk, or TEPP itself in some way causes a release of acetylcholine by the nerve terminals. The former explanation must indeed be at least partly true if the results of Fatt & Katz (1952) on frog muscle hold for cat muscle; spontaneous end-plate potentials of 1% normal size as observed by Fatt & Katz, might rapidly build up the local depolarization described if the cholinesterase was sufficiently depleted. With regard to the latter explanation we have no evidence excluding the possibility that TEPP provokes a slow output of acetylcholine from the motor nerve, but the absence of a twtich on close arterial injection shows that it does not produce any substantial instantaneous release.

The effects observed on denervated muscle cannot be due to any of the mechanisms just discussed, and cannot result from local acetylcholine production. But they are still attributable to the cholinesterase-inhibiting properties of TEPP, for the concentrations of acetylcholine circulating in the

blood seem quite adequate to account for the depolarization of denervated muscle which we observed.

The question arises whether after the injection of TEPP sufficient acetylcholine appears in the blood to contribute to the depolarization of the normally innervated muscle. It is very unlikely that it accounts for more than a small part of the depolarization produced by the first dose of TEPP; the time-courses of the two processes are quite different. With subsequent doses, however, when the depolarization of the normal muscle becomes slower, its course in time approximates more closely to that of the blood acetylcholine, and it may well be attributable, in increasing degree, to acetylcholine carried through the blood stream rather than to acetylcholine released by local nervous activity. On the quantitative side, it must be remembered that the denervated muscle is more sensitive to acetylcholine than the normal, perhaps by a factor of 10, in terms of the dose required to produce equal effects. From the dose-response curve given by Fatt (1950) on frog muscle, and from our own observations, we estimate that a given dose of acetylcholine would in the normal muscle produce about 60–70% of the depolarization obtained in a denervated one. This magnitude of depolarization would not be far from that obtained in the normal muscle after several injections of TEPP.

Sources of circulating acetylcholine

The most obvious source of acetylcholine is the viscera, which are known to synthesize acetylcholine at a high rate (Feldberg & Lin, 1950); and in fact amounts between 5 and 50 mμg/ml. blood have been detected in the portal blood of cats treated with eserine or neostigmine (Donomae, 1934). In our experiments, no doubt the viscera did contribute substantially to the high acetylcholine levels, for the portal blood contained twice as high a concentration of acetylcholine as the systemic blood. But the appearance of acetylcholine in the blood after evisceration shows that the viscera do not constitute the only important source. In the absence of the intestines, a total of approximately 7–10μg acetylcholine appeared in the blood within 10–15 min. Although we cannot state with certainty the origin of this acetylcholine, there are at least two obvious sources. Activity of autonomic ganglia could certainly account for some of the acetylcholine, particularly if compensatory vasomotor activity through the sympathetic ganglia was vigorous because of the low blood pressure. If the acetylcholine output of the bulk of the ganglia is comparable to that of the perfused superior cervical ganglion, it could well be sufficiently great to account for the whole of the acetylcholine appearing in the blood after an injection of TEPP. Secondly, there is no doubt that TEPP initially causes an extensive motor nerve discharge of central origin, and this, too, must contribute to the rise of acetylcholine in the blood, especially if the motor nerve activity is maintained by C.N.S. convulsive action even though

masked by peripheral neuromuscular block (cf. Wescoe, Green, Macnamara & Krop, 1948). Whatever the actual details of the origin of the acetylcholine, its presence in the blood in the concentrations we have found makes it necessary to be cautious in interpreting the local effects resulting from large doses of cholinesterase-inhibitors given to the whole animal. One cannot confidently ascribe a given effect of an inhibitor to local action on an organ, if that organ is simultaneously exposed to high concentrations of active substances in the circulation.

The effect of D-tubocurarine on the end-plate depolarization after a dose of TEPP corresponds to its effect on the end-plate depolarized by decamethonium. In particular, the fact that D-tubocurarine produced a more rapid repolarization of the centre of the end-plate region than of the adjacent muscle membrane, shows that this action, already described for decamethonium, is not a pharmacological peculiarity of decamethonium but reflects either a specific ability of D-tubocurarine actively to repolarize the depolarized end-plate region, or a difference in behaviour between the membrane of the end-plate and of the remainder of the muscle membrane, when a depolarizing influence is removed.

From early *in vitro* studies (Brauer, 1948) it was thought that TEPP permanently rendered cholinesterase inactive. Recent work (Grob, 1950; Grob, Garlick & Harvey, 1950; Hobbiger, 1951) has shown that return of cholinesterase activity may be observed *in vitro* although this occurs relatively slowly. However, signs of TEPP poisoning in the intact animal are of fairly short duration. Douglas & Matthews (1952) found that recovery from both the central and neuromuscular paralysing actions of a high dose of TEPP took place within an hour.

The tests used in the present series of experiments are capable of detecting lesser degrees of cholinesterase inactivation at the end-plate than could be revealed by tests of neuromuscular transmission: depolarizations of 0·5 mV could be detected although 5–10 times this would be needed to affect transmission. Complete recovery of the normal behaviour of the muscle end-plate in its resting potential and in its response to nerve stimulation or to acetylcholine, was observed to occur about 1–2 hr after ten to twenty lethal doses of TEPP. The fact that TEPP lacks direct end-plate stimulant action, together with the complete disappearance of its effects after 1–2 hr, make it a very useful tool in the study of neuromuscular transmission.

The reversibility of the effects of TEPP has allowed us to study the effects of repeated doses in the one animal. This has brought to light the curious fact that successive doses produce progressively smaller and slower end-plate depolarizations. In the innervated muscle, in which the rapidly rising phase of the initial response to TEPP involves nervous elements, the reduction of subsequent responses may be due to diminished nervous activity, representing

either the normal consequence of nerve section or an action of TEPP itself on nerve trunk or terminations. But since the effect was also observed in the denervated muscle, and since blood acetylcholine levels with successive doses of TEPP did not decline in the same way, it seems probable that this feature of the action of TEPP represents at least in part an accommodation by the end-plate region to the action of acetylcholine. Burns & Paton (1951) made a similar observation with decamethonium in which the action of an anti-cholinesterase is not involved. This suggests a further function of cholin-esterase, whose importance is already recognized in allowing repeated impulses to be independently effective, and in preventing neuromuscular block by acetylcholine accumulation: if persisting acetylcholine produces an ac-commodation to its action, it is clearly important for the maintenance of continuous neuromuscular activity that acetylcholine should be removed as quickly as possible between successive discharges.

SUMMARY

1. A study has been made of the mechanisms of end-plate depolarization caused by the cholinesterase-inhibiting drug tetraethylpyrophosphate (TEPP) in the gracilis muscle of cats anaesthetized by choralose, the nerve to gracilis being tied and cut in every experiment.

2. Doses of 100 μg/kg TEPP intravenously caused no depolarization of the end-plate regions in the absence of nerve stimulation, but potentiated and prolonged the depolarization caused by repetitive nerve stimulation.

3. With 500 μg/kg TEPP the effect in some cats was similar to that of 100 μg/kg, but in others end-plate depolarization occurred in the absence of nerve stimulation, particularly if the nerve had been recently tied. This depolarization fluctuated in magnitude and did not occur in the denervated muscle. It is ascribed to the release of acetylcholine by the endings of the nerve either spontaneously or by injury discharge.

4. With 2–20 mg/kg TEPP a rapid, large unfluctuating depolarization always occurred about 30 sec after injection and in the absence of nerve stimulation. After denervation, this response to TEPP was reduced, and it is therefore attributed, in part at least, to activity of nerve endings in the muscle.

5. The residual depolarization produced by TEPP in the denervated gracilis, although substantial, developed only slowly, reaching its maximum in about 15 min, and declining thereafter. This effect can be accounted for by the accumulation of acetylcholine in the blood.

6. Acetylcholine could be detected in the blood about a minute after injection of large doses of TEPP, reached a maximum concentration of about 30–40 mμg/ml. in about 15 min and disappeared about an hour after injection. The viscera produced much of this acetylcholine but considerable amounts were also obtained in the blood of the eviscerate cat.

7. Even with the biggest dosage of TEPP its latency of action was much greater than that of the directly acting depolarizing substances, acetylcholine and decamethonium. Moreover, unlike them, TEPP did not cause a twitch of the tibialis when injected close arterially in amounts sufficient to be followed by complete and prolonged neuromuscular block.

8. It is concluded that the effects of TEPP at the motor end-plate are all attributable to acetylcholine accumulating there from local or distant sources, and that TEPP lacks a direct action at the end-plate.

9. The effects of TEPP were reversible, and complete cycles of depolarization and repolarization could be obtained at approximately hourly intervals, even with the largest doses. The magnitude of the end-plate depolarization, however, diminished with successive doses.

10. D-Tubocurarine opposed the depolarization of the motor end-plates by TEPP, both preventing its appearance and hastening its disappearance. Given after end-plate depolarization by TEPP and acetylcholine, D-tubocurarine repolarized the end-plate faster than the adjacent depolarized muscle membrane, so that the end-plate region became temporarily positive to the adjacent membrane.

REFERENCES

BRAUER, R. W. (1948). Inhibition of the cholinesterase activity of human blood plasma and erythrocyte stromata by alkylated phosphorus compounds. *J. Pharmacol.* **92**, 162–172.

BURNS, B. D. & PATON, W. D. M. (1951). Depolarization of the motor end-plate by decamethonium and acetylcholine. *J. Physiol.* **115**, 41–73.

DONOMAE, I. (1934). Das Auftreten eines acetylcholinartigen Stoffes im Pfortaderblut der Katze. *Pflüg. Arch. ges. Physiol.* **234**, 318–324.

DOUGLAS, W. W. & MATTHEWS, P. B. C. (1952). Acute tetraethylpyrophosphate poisoning in cats and its modification by atropine or hyoscine. *J. Physiol.* **116**, 202–218.

DOUGLAS, W. W. & PATON, W. D. M. (1951). The mode of action of tetraethylpyrophosphate at the cat's neuromuscular junction. *J. Physiol.* **115**, 71–72*P*.

FATT, P. (1950). The electromotive action of acetylcholine at the motor end-plate. *J. Physiol.* **111**, 408–422.

FATT, P. & KATZ, B. (1952). Spontaneous subthreshold activity at motor nerve endings. *J. Physiol.* **117**, 109–128.

FELDBERG, W. & LIN, R. C. Y. (1950). Synthesis of acetylcholine in the wall of the digestive tract. *J. Physiol.* **111**, 96–118.

GROB, D. (1950). The anticholinesterase activity *in vitro* of the insecticide parathion (p-nitrophenyl diethyl thionophosphate). *Johns Hopk. Hosp. Bull.* **87**, 95–105.

GROB, D., GARLICK, W. L. & HARVEY, A. M. (1950). The toxic effects in man of the anticholinesterase insecticide parathion (p-nitrophenyl diethyl thionophosphate). *Johns Hopk. Hosp. Bull.* **87**, 106–129.

HOBBIGER, F. (1951). Inhibition of cholinesterases by irreversible inhibitors *in vitro* and *in vivo*. *Brit. J. Pharmacol.* **6**, 21–30.

WESCOE, W. C., GREEN, R. E., MCNAMARA, B. P. & KROP, S. (1948). The influence of atropine and scopolamine on the central effects of DFP. *J. Pharmacol.* **92**, 63–72.