J. Physiol. (1959) 145, 611–629

THE EFFECTS OF INJECTING ACETYLCHOLINE INTO NORMAL AND REGENERATING NERVES

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(*Received* 1 *October* 1958)

There is ample evidence that some part of the peripheral ending of a receptor nerve fibre is sensitive to acetylcholine and related substances, whilst the rest of the fibre is not (Gray & Diamond, 1957). The reason for this peculiar pharmacological property of receptor nerve terminals is unknown, but it is unlikely that acetylcholine plays a direct part in the initiation of impulses at these endings during normal activity (Diamond, 1955). The present investigations were made to determine whether or not the endings of *regenerating* nerves show a similar sensitivity to acetylcholine, and to compare the results from such growing endings with those obtained from the normal terminals of motor and receptor nerves. The results suggest that the terminals of regenerating receptor nerves do in fact behave like the normal terminals of such nerves in being excitable by acetylcholine. Though it is not conclusive, evidence is presented suggesting that the endings of both normal and regenerating motor nerves are relatively insensitive to acetylcholine. During this work the incidental observation was made that in the normal sural nerve trunk of rabbits, or in tissue closely applied to it, there are nerve endings which are excitable both mechanically and by acetylcholine. A preliminary report of a part of this work has been published (Diamond, 1957).

METHODS

Application of drugs. Injections were made, using the 'close arterial' technique, into the sural, and in some experiments the lateral and medial popliteal, nerves of rabbits anaesthetized with urethane (1·8 g/kg). Fig. 1 indicates the anatomy of the popliteal region; the branch of the popliteal artery supplying this region was clamped at *A* during an injection, and blood flow was restored by releasing the clamp between injections. These were made into the lateral branch, *B*, which was cannulated with a hypodermic syringe needle. Before injections were begun the animal was given heparin intravenously (1000 i.u./kg). All the accessible side branches of the artery were ligated close to their origins except *C*; this was ligated distal to the point where it invariably gave origin to a vessel supplying the adjacent sural and popliteal nerves. Further, fine vessels supplying the sural nerve, which is usually closely applied to the main artery, were

recognized under the dissecting microscope and preserved. The distribution of injected material depended on the volume injected and on the point at which the peripheral end of the main artery was clamped; although many points have been used, most injections were made with a clamp applied either just distal to the origin of branch *C*, the 'high' position, *H*, or some 3–4 cm peripheral to this at the 'low' position, *L*. When the lateral and medial popliteal nerves were used, the high clamp was applied and a small artery which supplies them, having its origin from the cannulated artery, *B*, was left intact. The lateral popliteal nerve was ligated and cut just distal to the point of entry of this artery, and the medial popliteal was similarly treated close to the gastrocnemius muscle. When the sural nerve only was used, the popliteal nerves, in addition to being divided at the gastrocnemius level, were also divided at a point some 2–4 cm above the muscle. The perfused region was displayed by injecting Indian ink at the end of most experiments; its approximate extent is indicated in Fig. 1 by brackets, the letters *H* and *L* indicating the use of the high clamp (injection volume, 0·1–0·3 ml.) and low clamp (0·3–0·4 ml.), respectively. In the majority of experiments the most rapid staining of the nerve occurred when the high arterial clamp was applied.

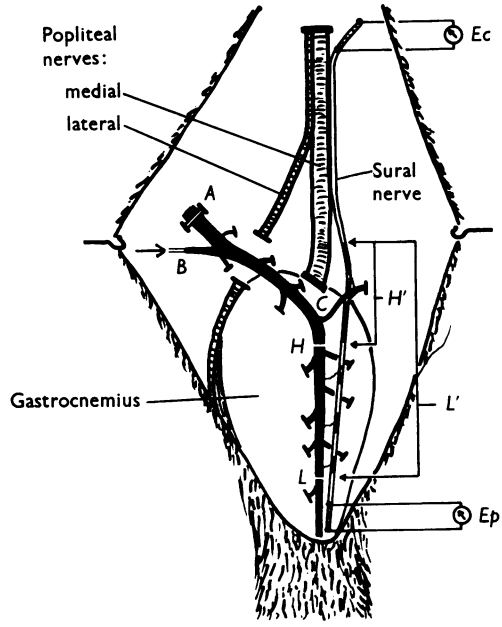

Fig. 1. Diagram of popliteal region, left hind leg of rabbit, prepared for experiment on sural nerve. *A*, clamp on main artery; *B*, cannulated side branch of artery; *C*, small branch artery to sural nerve; *H*, position of high clamp; *H'* plus bracket = perfused region when high clamp applied; *L*, position of low clamp; *L'* plus bracket = perfused region when low clamp applied; *Ec*, central recording electrodes on sural nerve; *Ep*, peripheral recording electrodes on sural nerve. Arteries were accompanied by corresponding veins (not indicated in diagram).

Drugs were dissolved in Ringer–Locke's solution, of the following composition (mM). NaCl 154, KCl 5·6, $CaCl_2$ 1·8, $NaHCO_3$ 1·8, glucose 5·6.

Recording. When receptor nerve fibres (sural) were investigated, impulses were recorded from the sural nerve at points some 6 cm above, and in a number of experiments some 5 cm below, the level of the high arterial clamp (Fig. 1, *Ec* and *Ep*). The whole nerve was cut at both points. When motor fibres (lateral and medial popliteals) were investigated, impulses were recorded from lumbosacral ventral roots; in a few experiments simultaneous records were obtained from receptor

nerve fibres in the corresponding dorsal roots. The electrodes used were platinum wires, and impulses were amplified and displayed on a double-beam cathode ray oscilloscope, and photographed on moving paper. Injections were usually signalled on the second beam of the oscilloscope, the signals indicating the duration of contact of the finger with the syringe plunger; this system probably gave an over-estimate of the actual duration of the injection.

Regeneration experiments. Nerve lesions were made, under aseptic conditions, at times which varied from a few hours to a few months before the acute experiment. The animal was anaesthetized with Nembutal or Pentothal (pentobarbitone or thiopentone; Abbott Laboratories: 40–50 mg/kg) and ether, and the sciatic nerve was exposed in the thigh and popliteal region. Three types of lesion have been made:

(*a*) crushing with smooth-tipped forceps or modified artery forcept for 20–30 sec;

(*b*) cutting, with subsequent apposition of the cut ends; the apposition was maintained either with fine thread sutures through the epineurium, or by simply restoring the cut ends to their original site within the investing connective tissue sheath, in which a slit had been made to extract the nerve initially;

(*c*) removal of about 6 mm or more of nerve, which resulted in the formation of an 'end-neuroma' on the proximal stump.

The site of the nerve lesion varied in different animals from just below the level of the trochanter to the popliteal region, and for sural nerves to the mid-calf region; *most lesions were made above the upper limit of the perfused length of nerve shown in Fig.* 1.

RESULTS

Normal nerves

Sural nerve fibres

The close arterial injection of acetylcholine (10^{-5}–10^{-4} g/ml.) excited impulses which were recorded at the electrodes placed central to the perfused region of the nerve (*Ec*, Fig. 1). The discharge was, in part, due to mechanical stimulation by the pressure of the injection, but the magnitude of the response to acetylcholine always exceeded that due to a control injection of Locke's solution (Figs. 2, 3) or blood. The number of fibres which appeared to be excited depended on the length of nerve perfused. When this was short, i.e. when the high clamp was applied (*H*, Fig. 1), the effect of an injection was absent or slight, usually involving a few small fibres, as evidenced by the spike heights (Fig. 2*a*, *b*). When the greater length of nerve was perfused (*L*, Fig. 1) many more, and often larger, spikes were initiated by the drug and usually also by the mechanical stimulus, though the latter effect did not occur in the experiment illustrated (Fig. 2*c*, *d*). The largest response of all occurred when the artery was not clamped at all, so that the injected fluid passed peripherally to the skin innervated by the sural nerve; when this happened the response to acetylcholine often outlasted the injection by many seconds (Fig. 2*f*). This long discharge was not obtained after the nerve was cut at the level of the low clamp; the responses to acetylcholine while the clamps were applied were unaltered or only slightly diminished after nerve section at this level. It was clear from a comparison with records of nerve activity following mechanical stimulation of the skin that the effects of the injections into the artery supplying the nerve trunk were limited to a small fraction of

the total fibres available. Moreover, injections of KCl-enriched Locke's solution excited many more fibres than did acetylcholine.

Nicotine was used in doses similar to those which were effective for acetylcholine, but was always less effective, and sometimes without effect, on the normal nerve.

Site of nerve excitation. If the region of the nerve excited by acetylcholine were remote from the end of the nerve, then it would be expected that impulses would travel in both directions from the point of their initiation. This possibility was investigated by placing electrodes on the whole sural nerve at a

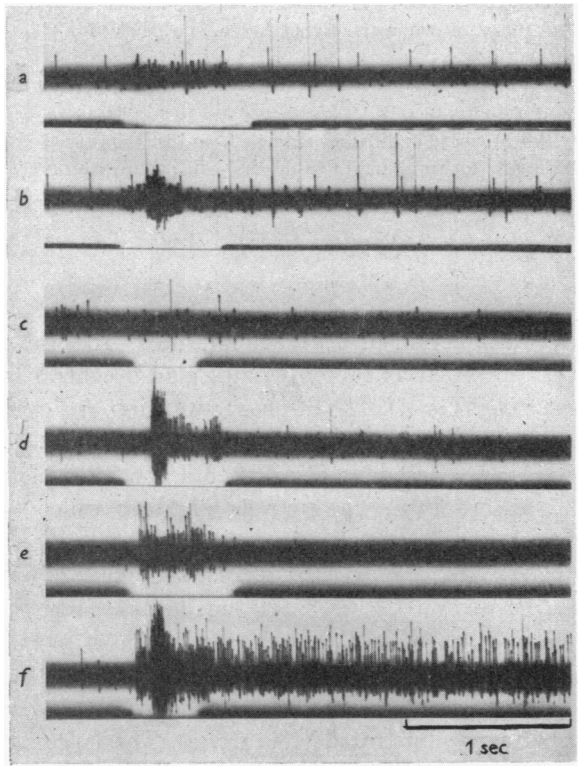

Fig. 2. Activity in twig of normal sural nerve. In this experiment the nerve was not cut off from the skin. In this and subsequent figures, each pair of records shows impulses (above) and duration of injection (below); *HC* = high arterial clamp applied; *LC* = low arterial clamp applied; *NC* = no arterial clamp applied; ACh = acetylcholine; unless otherwise stated, impulses in the nerve were recorded from the central electrodes (*Ec* in Fig. 1). (*a*) *HC*, 0·25 ml. Locke's solution; (*b*) *HC*, 0·25 ml. ACh 10^{-4} g/ml.; (*c*) *LC*, 0·4 ml. Locke's solution; (*d*) *LC*, 0·4 ml. ACh 10^{-4} g/ml.; (*e*) *NC*, 0·4 ml. Locke's solution; (*f*) *NC*, 0·4 ml. ACh 10^{-4} g/ml. All records, particularly the first four, include 'spontaneous' impulses arising at peripheral receptors in the skin; they were unrelated to the injection and were abolished by cutting the nerve from the skin. See text for explanation of the long-lasting discharge in (*f*).

point peripheral, as well as at one central, to the region perfused (*Ep*, Fig. 1) and repeating the injections. It can be seen from Fig. 3*c* that even when the longer nerve perfusion was used (clamp *L*, Fig. 1), neither the pressure stimulus nor acetylcholine excited impulses which were conducted as far peripherally as the electrodes. The drug was certainly reaching nerve fibres, for a similar injection of KCl-enriched Locke's solution excited a discharge of impulses which were recorded at both pairs of electrodes (only the peripheral record is shown in Fig. 3). An injection of Indian ink in such experiments showed that

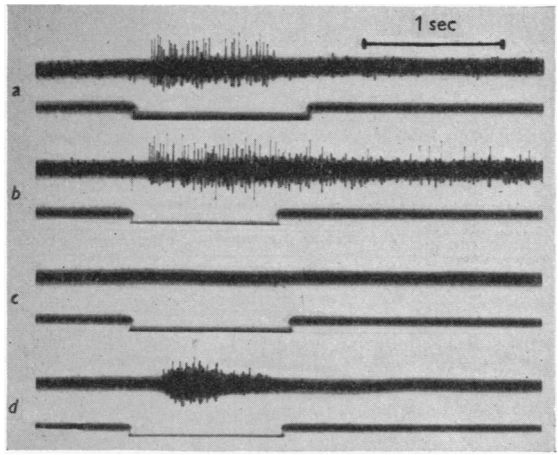

Fig. 3. Activity in twig of normal sural nerve. (*a*) (*b*) recorded with central electrodes (*Ec*, Fig. 1), *LC*, (*a*) 0·3 ml. Locke's solution; (*b*) 0·3 ml. ACh 10^{-4} g/ml.: (*c*), (*d*) recorded with peripheral electrodes (*Ep*, Fig. 1). *LC* (*c*) 0·3 ml. ACh 10^{-4} g/ml.; (*d*) 0·3 ml. KCl solution. In this and subsequent figures, KCl solution refers to KCl 1·2% in Locke's solution.

when the 'short' perfusion was adopted the region perfused included only the nerve, the adjacent artery and its branch to the nerve, plus a small amount of adherent connective tissue. With the 'long' perfusion, the length of artery and nerve involved was extended peripherally, and often a small amount of gastrocnemius muscle immediately below the artery was also stained with the Indian ink. There was, however, never a detectable muscle response during an injection of acetylcholine or KCl-enriched Locke's solution with the high clamp applied, and rarely one with the low clamp. The simplest explanation of these results (which occurred in the presence of atropine) is that the acetylcholine was exciting fibres which ended in the nerve trunk itself, the artery or local connective tissue; presumably the impulses were initiated at or near the terminals, and were then conducted centrally in the sural nerve, as is illustrated schematically in Fig. 4.

Motor fibres in the popliteal nerves

Two types of experiment were done on motor fibres. In the first, the medial popliteal nerve was tied and cut just above the gastrocnemius muscle, and the muscle arteries were ligated; the high clamp was applied to the main artery (see Methods, and Fig. 1), and thereafter injected fluids could reach only the nerves. In the second type, the low arterial clamp was applied and sufficient side arteries were left patent to allow injected fluid to pass into the gastrocnemius muscle, as well as into the popliteal nerves above. Recording electrodes were applied to lumbosacral ventral roots, the correct roots being identified by stimulating them and noting muscle movements, and by stimulating the popliteal nerves in the thigh and noting the appearance of an antidromic volley in the roots, corresponding with muscle activity below the knee.

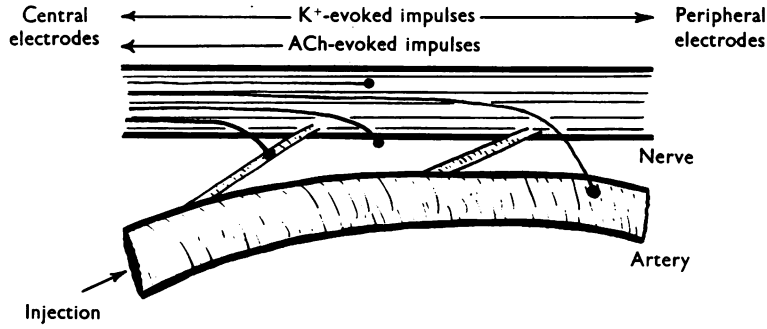

Fig. 4. Schematic representation of normal sural nerve and adjacent artery, showing postulated nerve end organs excited by ACh or by a mechanical stimulus (see text).

In the first type of experiment, in which injections reached nerve only, neither the pressure stimulus nor acetylcholine excited impulses which could be detected in the corresponding ventral roots. That the drug was reaching the nerve fibres was indicated by the positive response to an injection of KCl-enriched Locke's solution, and by the visible staining inside the nerve trunk after Indian ink was injected.

The results from the second type of experiment, in which injections certainly reached the vicinity of the motor nerve endings in gastrocnemius muscle, differed to some extent from the findings of Masland & Wigton (1940), who did similar experiments. In the present experiments an injection of acetylcholine always evoked the twitch-like response of gastrocnemius muscle; an associated discharge of antidromically conducted impulses in the ventral roots occurred only occasionally, however. Typically, of the ventral roots known to include motor fibres to gastrocnemius, one, and rarely two, were found which included but one, two or three fibres activated by the acetylcholine injection. The effect was repeatable only for a few injections, and then

could not be elicited although the muscle was still visibly twitching when each injection of acetylcholine was made. In some experiments in which the muscle responded vigorously to acetylcholine, there was no detectable back-firing in motor fibres. In all experiments the muscle responded to electrical stimulaion of the ventral roots used. Indian ink injections made at the end of each experiment revealed an extensive perfusion of gastrocnemius muscle.

Regenerating nerves

Site of the regenerating terminals. The region of the nerve trunk where impulses were most readily elicited by mechanical stimulation (tapping the nerve directly with a light stylus) was taken as the site of the regenerating endings. With practice, it proved possible to define within 1–2 mm the most peripheral point where the applied stimulus was effective. In most experiments crushing the nerve even 2–3 mm peripheral to this point elicited no impulses, but crushing at or central to it was always effective. In view of the findings of Gutmann, Guttmann, Medawar & Young (1942) it seems probable that the method adopted here defines the site of the furthest-grown fibre terminals to within about 5 mm or less. The scatter along the nerve of the regenerating terminals depended on how much time had elapsed since the lesion was made. No systematic study has been made of growth rates, but in general the rates of regeneration of the fastest growing fibres agree closely with the figures of Gutmann *et al.* (1942); such fibres usually gave rise to large and medium sized spikes; the most slowly regenerating fibres were usually very small, as suggested by their spike heights. The largest spikes were usually elicited by mechanical stimulation of the nerve at a point which corresponded to a regeneration distance which was less than that of the fastest growing fibres.

Sural nerve fibres

Provided that the growing ends of some of the regenerating fibres lay in the perfused region of the nerve (see above), an injection of acetylcholine (0·1–0·2 ml., 10^{-5}–10^{-4} g/ml.) always excited impulses which were conducted to the central, but not to the peripheral, electrodes. Nicotine was as effective as acetylcholine in similar doses; examples of responses evoked by both drugs are shown in Fig. 5. The differences between the effects in normal and regenerating nerves are discussed in a later section. If spike heights are taken as an indicator of fibre size, the results show that large and small diameter fibres responded to acetylcholine and to nicotine; in most experiments the smallest effective doses of the drugs excited only the smallest fibres; a thickening of the baseline in some instances suggested that unmyelinated fibres, or the smallest myelinated ones, were participating in the response. In some preparations in which acetylcholine excited impulses, there was no response excited mechanically by the injection (Fig. 6). In these experiments there was evidence that the perfusion of the nerve was inefficient (e.g. following an injection of KCl-enriched Locke's solution, which often would fail to excite impulses, there was a profound block of the terminals to direct mechanical stimulation for 20–60 min after the blood flow was restored).

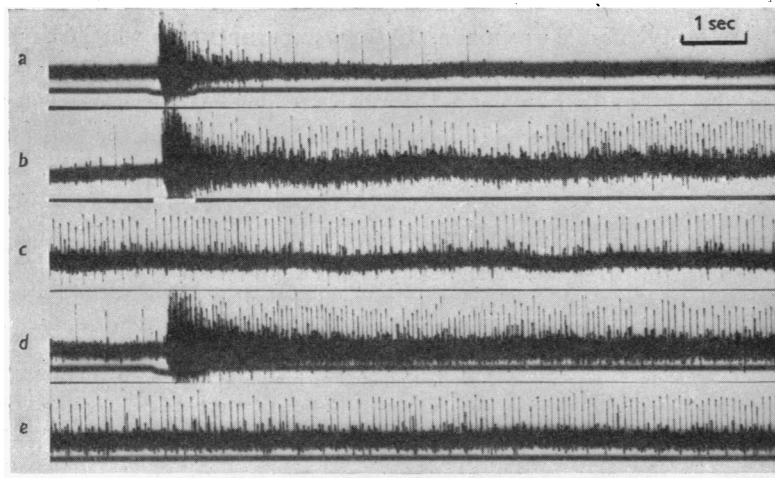

Fig. 5. Activity in twig of regenerating sural nerve (crushed 16 days previously); *HC* for all records. (*a*) 0·25 ml. Locke's solution; (*b*) 0·25 ml. ACh 10^{-4} g/ml. (break in lower trace indicates injection interval); (*c*) continued from (*b*) after break of 20 sec; (*d*) 0·25 ml. nicotine 10^{-4} g/ml.; (*e*) continued from (*d*) after break of 20 sec.

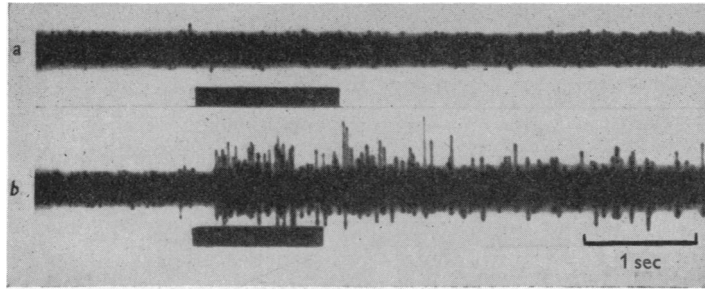

Fig. 6. Activity in twig of regenerating sural nerve (cut 17 days previously). (*a*) *HC*, 0·3 ml. Locke's solution; (*b*) *HC*, 0·3 ml. ACh 10^{-4} g/ml. In this experiment an injection of KCl failed to excite impulses.

Site of action of drugs

It is possible that the mechano-sensitive endings of the regenerating fibres were excited secondarily, as a result of a direct action of the drugs on (*a*) local vascular smooth muscle, or (*b*) local skeletal muscle underlying the nerve. The latter includes the possibilities of excitation due to both mechanical and electrical muscle activity.

Vasomotor effects. It is unlikely that these contributed to the responses for the following reasons:

(1) Atropine (up to 10 mg/kg injected intravenously before the popliteal region was opened up) did not prevent the stimulating action of acetylcholine.

(2) Injections of the vasomotor substances adrenaline, noradrenaline and sodium nitrate, in doses known to cause vasomotor changes, were no more effective in eliciting impulses than control injections of Locke's solution, the animals' own blood, or 0·9% NaCl.

(3) The effects of both acetylcholine and nicotine were prevented by a previous injection of hexamethonium (see below), which does not normally block the direct action of acetylcholine on vascular smooth muscle.

(4) Regenerating *motor* nerve endings, which are also sensitive to mechanical stimulation, were not excited by injections of acetylcholine which evoked a response in regenerating receptor nerves (see below).

Skeletal muscle effects. These too may be excluded as being responsible for the effects described above, because:

(1) There was no visible muscle contraction during responses evoked by acetylcholine or nicotine.

(2) When the 'short' perfusion technique was used, it was possible to prevent the nerve response to drugs by clamping the side artery *C* (Fig. 1), thus diverting the injected fluid into muscle arteries which in these experiments had been deliberately left patent; when the artery *C* was unclamped, injections of acetylcholine again evoked impulses in the nerve.

(3) When the 'short' perfusion technique was used, in most preparations an injection of Indian ink revealed that no muscle tissue was infiltrated.

(4) The close arterial injection of acetylcholine into the gastrocnemius muscle caused a visible muscle twitch, but only evoked impulses in the nerve in a few experiments in which the regenerating endings had grown as far as the middle of the calf; even then the nerve response was brief and did not extend beyond the injection period.

(5) Spontaneous muscle twitching and fasciculation, which readily occurs after an intravenous injection of eserine, never caused impulses to appear in the sural nerve even when the muscle directly underlying the nerve was visibly active.

These observations exclude the two possibilities that the nerve endings were excited mechanically, and that they were excited by muscle action potentials.

Type of fibre excited

It may be concluded from the above that acetylcholine and nicotine acted directly on fibres in the sural nerve. There is one important possibility which must be considered before it can be concluded that it was *regenerating* fibres which were excited. The results previously described for the normal sural nerve suggest that this nerve may contain endings which are functional end-organs. It is possible that during regeneration these endings become functional again, and that it is on these that acetylcholine acts, which would be in

keeping with what is already known of the pharmacology of normal receptor nerve terminals. This possibility has not been excluded, but the following findings suggest that it does not explain all the effects which have been observed.

(1) When the results from all experiments were pooled, a rough correlation was obtained between the number of fibres which appeared to be active during the response to acetylcholine, and the number of regenerating fibres which ended in the perfused region of the nerve (determined as described previously). The evoked response was greatest when most of the fibres ended in the region of the nerve included in the bracketed length *H* in Fig. 1, and when the high rather than the low arterial clamp was applied. When the fastest growing fibres had reached only the upper limit of the perfused region of the nerve, then the drugs excited only a few fibres; their spike heights were similar to those elicited by mechanical stimulation of the nerve at this upper limit. In an animal in which re-innervation of the skin appeared to be complete, the findings resembled those in a normal control nerve, except for a tendency for the responses to outlast the injection by a second or two. In a few of such animals acetylcholine or nicotine evoked a typical response in very small fibres. Summing up all these results, one can describe the time course of the drug-sensitivity of the short perfused length of nerve as beginning at zero (when the lesion was made), becoming slight (a few medium-sized spikes), then reaching a maximum (many spikes, wide range of amplitudes), maintaining this maximum (many smaller to very small spikes), and then diminishing to a minimum (short burst of a few small spikes). Clearly such a time course also reflects the pattern of regeneration in the sural nerve (see earlier), ending in the restoration of the normal nerve picture.

(2) The duration of typical responses to acetylcholine or to nicotine, even when obtained with the short length perfusion, was considerably greater than any obtained from the normal nerve. The responses in regenerating nerves were comparable, both in terms of number of active fibres and duration of discharge, with those obtained from receptor nerve endings in the skin (Fig. 2).

(3) A proximal stump end neuroma (see Methods) lying at the upper limit of the perfusion area gave results typical of those obtained from experiments in which conventional nerve regeneration was allowed.

It is difficult, without making unjustifiable assumptions, to explain these findings on the basis of the refunctioning of the supposed end organs in normal nerve. A fourth point, though perhaps of less significance, is the finding that nicotine was always as effective as acetylcholine in the regenerating nerve, but was always much less effective than acetylcholine on the normal nerve.

It might be argued that the differences between the results from the normal

and the operated animals depended on the interference with the local vasculature in the latter; this might cause a hold-up of an injected solution, thus allowing drugs to reach naturally occurring endings which were normally inaccessible, and to have a prolonged stimulating action. This is improbable for the following reasons.

(1) The results on regenerating fibres in the popliteal region were typical even when the site of the nerve lesion was high in the thigh, and when there had been no initial exposure of the region to be subsequently perfused.

(2) The results from the normal sural nerves of control animals were typical even when the popliteal region had been exposed at a previous operation, and the medial popliteal nerve cut, with deliberate interference to the local blood supply to the sural.

(3) In many experiments there was visible evidence under the dissecting microscope that the nerve perfusion was adequate, and similar to that in 'control' experiments; for vessels on the sural nerves blanched as injections were made, and reddened rapidly when the blood flow was restored.

It is concluded that impulses were set up in regenerating fibres in the sural nerve by acetylcholine and by nicotine. It is clear from what has been said above that the drugs acted not everywhere on the axon, but somewhere peripherally, presumably at or near the growing tip. A further indication of this was given by the finding that crushing the nerve at the region of greatest mechanical sensitivity, which must have been the site of most of the growing ends in those experiments in which the regeneration time was only a few days, markedly reduced or abolished the response to acetylcholine and to mechanical stimulation; there was, however, a much smaller reduction in the response to an injection of KCl-enriched Locke's solution, which is unlikely to act only at the nerve endings. Moreover, the early onset of the sensitivity to acetylcholine (see below) and the time course of its development point to the terminal region of the regenerating fibre as being the site of action of the drug.

Other pharmacological properties

A preceding injection of eserine (10^{-5} g/ml.) reduced the minimum concentrations of acetylcholine necessary to evoke a response, but did not affect the sensitivity to nicotine detectably. The response both to acetylcholine and to nicotine was prevented by a previous injection of hexamethonium (e.g. 1–5 mg/kg intravenously, or 0·1–0·2 ml. of 10^{-3} g/ml. intra-arterially); an example of this is shown in Fig. 7. It has not been possible to reverse the effect of hexamethonium; the extent to which effective perfusion could be continued was limited, apparently by the occurrence of visible oedema. However, even when excitation by acetylcholine or nicotine was prevented by hexamethonium, there was no obvious reduction in the response of the endings to mechanical stimulation (e.g. Fig. 7).

Onset of sensitivity

Two difficulties impeded the determination of the minimum interval between the production of the nerve lesion and the onset of the sensitivity to acetylcholine. First, it was necessary to make the initial lesion actually in the length of the nerve which was subsequently to be perfused. Because of this there was always a possibility that injected drugs would act on endings already

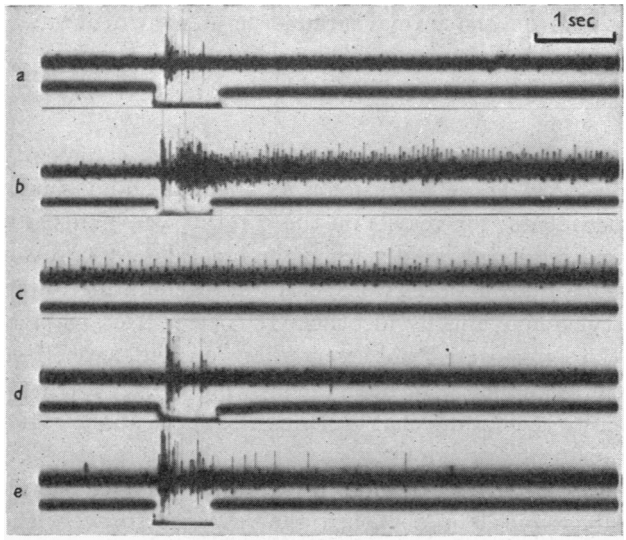

Fig. 7. Activity in twig of regenerating sural nerve (crushed 18 days previously). *HC* all records. (*a*) 0·25 ml. Locke's solution; (*b*) 0·25 ml. ACh 10^{-4} g/ml.; (*c*) continued from (*b*) after 10 sec break. (*d*) 0·25 ml. ACh 10^{-4} g/ml.; (*e*) 0·25 ml. ACh 10^{-3} g/ml. Between (*c*) and (*d*), 1 mg hexamethonium was injected intra-arterially. (A few large spikes in (*a*) and (*b*) retouched.)

present above the lesion, and unrelated to any regeneration which might occur. Secondly, the successful perfusion of the region of the nerve lesion required that the local blood supply should be re-established. Responses have been accepted as probably occurring in regenerating fibres if they satisfied all the following:

(*a*) They were evoked by nicotine as well as by acetylcholine.
(*b*) They outlasted the injection by more than 3–4 sec.
(*c*) They occurred when the high arterial clamp was applied.

On this basis there have been unequivocal effects at 3 or more days after the initial lesion was produced; fairly convincing responses occurred (though fewer fibres were active) after an interval of 1–2 days. In experiments made 12 hr after the initial lesion excitation occurred particularly in very small

fibres, as indicated by spike heights; shorter intervals have been used, and positive effects obtained (e.g. at 6 hr), but no reliance can be placed on these experiments. Certainly the larger fibres were not involved in the responses obtained less than 2 days after making the lesion. It is clear that the most satisfactory effects were obtained in preparations in which the regenerating fibres can be assumed to have penetrated the scar tissue and entered the distal portion of the nerve trunk (Cajal, 1928).

Motor fibres in the popliteal nerves

The perfusion of the popliteal nerves was poor compared with that of the sural nerve. Often after only one or two injections had been made, an injection of KCl-enriched Locke's solution failed either to excite impulses which could be detected in the dorsal or ventral roots, or temporarily to prevent their excitation by a mechanical stimulus applied to the region of the nerve endings. This was taken to indicate the failure of a significant quantity of the injected fluid to reach the nerve fibres; when such evidence was obtained the experiment was abandoned. This criterion of a successful nerve perfusion seemed justified by results obtained from experiments with the sural nerve.

In ten experiments, acetylcholine, and when it was tried nicotine also, failed to excite any impulses (in excess of those excited by a 'control' injection) which were detected in any of the ventral roots supplying the popliteal nerves (Fig. 8). In one experiment only, an injection of acetylcholine, though not of nicotine, excited a brief burst of impulses which outlasted the injection by a few seconds; the impulses clearly occurred in a single fibre. A burst of impulses of varying spike amplitude was excited in all experiments by a mechanical stimulus applied to the region of the regenerating nerve endings, and in most experiments by an injection of KCl-enriched Locke's solution (e.g. Fig. 8). In a smaller number of experiments in which the mechanical stimulus of an injection was usually ineffective, the effect of the K^+ was to prevent the response to a subsequent direct mechanical stimulus to the nerve, though it did not itself elicit impulses. It is relevant to note here that in many of the sural nerve experiments in which this occurred acetylcholine nevertheless excited the regenerating fibres. In the experiment from which the records of Fig. 9 were taken, an injection of KCl excited very few fibres in the ventral roots; there was, however, a subsequent profound block to mechanical stimulation of the nerve.

In some of the above experiments the furthest-grown endings had reached only the upper limit of the perfused portion of the nerve; in these experiments therefore the positive effect of K^+ indicated that the injected fluid was almost certainly reaching the region of the nerve endings. Other evidence that the failure of acetylcholine to excite regenerating motor fibres was not due to their

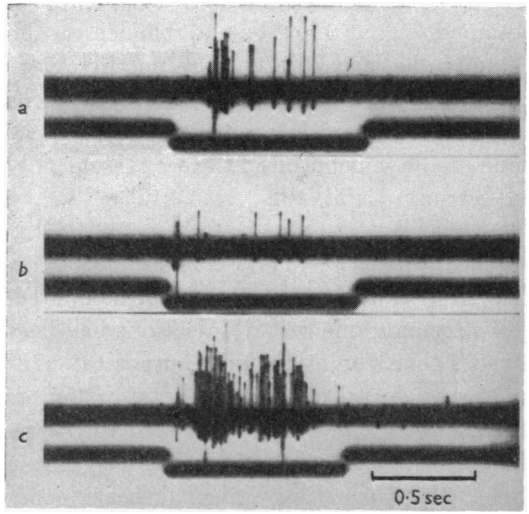

Fig. 8. Activity in ventral roots supplying popliteal nerves (lateral and medial popliteal nerves crushed 19 days previously). Perfusion as described in Methods. (*a*) 0·20 ml. Locke's solution; (*b*) 0·20 ml. ACh 10^{-4} g/ml.; (*c*) 0·20 ml. KCl solution.

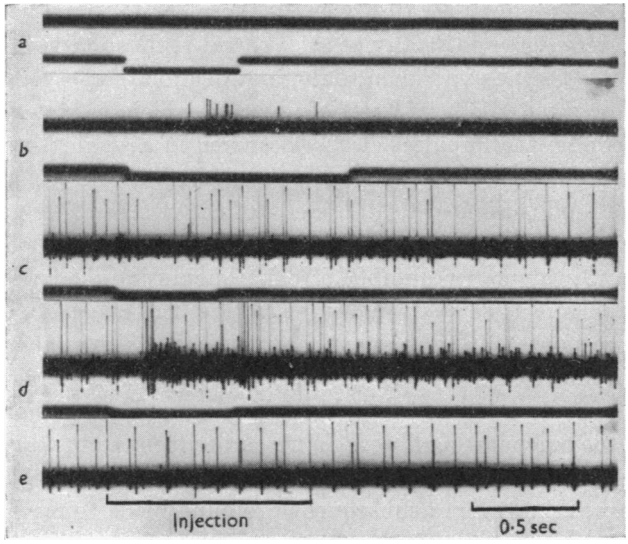

Fig. 9. Activity in ventral root (*VR*) and dorsal rootlet (*DR*) supplying popliteal nerves (lateral and medial popliteal nerves crushed 24 days previously). Perfusion as described in Methods. (*a*) *VR*, 0·15 ml. ACh 5×10^{-4} g/ml.; (*b*) *VR*, 0·15 ml. KCl solution; (*c*) *DR*, 0·15 ml. Locke's solution; (*d*) *DR*, 0·15 ml. ACh 5×10^{-4} g/ml.; (*e*) *DR*, 0·15 ml. ACh 5×10^{-3} g/ml. (injection signal inserted below). Between (*d*) and (*e*) the main popliteal trunk was cut in the thigh, above the perfused region; thus record (*e*) indicates the 'background' discharge of impulses arriving at the particular dorsal rootlet used for obtaining records (*c*) (*d*) and (*e*).

inaccessibility was given by recordings made from corresponding dorsal and ventral roots. In five such experiments the injection of acetylcholine into the region of the regenerating endings excited an obvious discharge of impulses in fine twigs of the dorsal roots (Fig. 9); in two of these experiments, however, only small receptor nerve fibres were excited, as judged by the spike heights. In all five experiments acetylcholine was ineffective in exciting impulses which were detectable in the ventral roots. It is possible, of course, that different rates of regeneration of motor and receptor nerves would result in differences in the sites of the growing ends; in the present experiments the furthest-grown fibres were always those supplying dorsal roots, an observation which supports the possibility that motor fibres regenerate more slowly (cf. Gutmann *et al.* 1942). Such experiments therefore do not necessarily provide proof that the acetylcholine did reach the motor nerve terminals.

These results, though not conclusive, do suggest that regenerating motor nerves, in contrast to receptor nerves, are relatively insensitive to acetylcholine and nicotine.

DISCUSSION

Normal nerves

The experiments have shown that an injection of any fluid into the arterial supply of the sural nerve of a rabbit evokes a discharge of impulses which are conducted only in a centripetal direction. This must be due to the excitation of mechanosensitive fibres in the nerve trunk or in the walls of the vessels supplying it. If the injected fluid contains acetylcholine the discharge of impulses is increased and prolonged. The simplest and most likely explanation of these results is that the sural nerve trunk and its adjacent tissues, in particular the artery, contain mechanosensitive nerve endings, and that these, in common with other receptor nerve terminals, are sensitive to acetylcholine. There is no evidence that the acetylcholine excited nerve fibres which did not end in the perfused region, but passed through it. This *can* be done by injecting KCl solution through the same route; this provokes a response which differs from that produced by acetylcholine in that impulses are conducted both centripetally and centrifugally.

A consequence of the above hypothesis is that the fibre population in the sural nerve central to the perfused region should exceed that lying peripherally. The results of Quilliam (1956) do not support this; however, his measurements would not reveal a difference of less than $3\frac{1}{2}\%$ of the total numbers, i.e. of about 50–60 myelinated fibres. In the present experiments the number of fibres which seemed to be excited by the pressure of an injection, or by acetylcholine, was only a small fraction of those activated by KCl or by mechanical stimulation of the skin. These results are not therefore incompatible with those of Quilliam.

In contrast with its effect on the sural nerve, which does not contain fibres to skeletal muscle, acetylcholine evoked no response from motor fibres, as judged by recordings made from the ventral roots. Occasional small discharges, however, were recorded when the injected acetylcholine was allowed to reach and excite the gastrocnemius muscle. The present results were by no means as regular and convincing as those quoted by Masland & Wigton (1940) in similar experiments. These authors reported that the arterial injection of acetylcholine in cats gave rise to a shower of antidromically conducted impulses in the motor nerves from gastrocnemius, associated with slow and irregular muscle contractions. However, their interpretation of such results as indicating a sensitivity of motor fibre endings to acetylcholine may be questioned. Lloyd (1942) attributes the antidromic discharge of impulses in motor fibres that follows the synchronous twitch of a muscle to the excitation of the motor endings by the action potential of the contracting muscle; the effect disappears when the muscle is curarized. This may well be the explanation of the present results and those of Masland & Wigton, but curarization cannot be used as a means of deciding the question of chemical sensitivity of motor fibres, since curare would be likely to interfere with a direct effect on them of acetylcholine. It is also possible that motor nerve endings might occasionally be excited mechanically during the muscle response to acetylcholine, or as a result of an increased K^+ leakage from the end-plate region of the muscle following its excitation by acetylcholine.

The evidence from the present experiments strongly suggests that the terminals of normal motor fibres do not show the sensitivity to acetylcholine that distinguishes the endings of receptor nerves.

Regenerating nerves

The excitation of regenerating receptor nerves by acetylcholine or nicotine has been shown to result from a direct action on nerve fibres, and not to be secondary to effects on local smooth or skeletal muscle. It is extremely improbable, on the basis of the present evidence, that the drugs could have produced all the observed effects by acting on re-functioning end organs, which may normally exist in the sural nerve. It is concluded that the ends of regenerating receptor nerves are excitable by acetylcholine, and have the pharmacological properties of normal receptor nerve endings and of autonomic ganglia. The early onset of their sensitivity to drugs following the nerve lesion is not incompatible with this conclusion, for nerve sprouting following experimental nerve crushes occurs within 6–12 hr (Perroncito, quoted and confirmed by Cajal, 1928). In contrast with regenerating receptor nerves, regenerating motor fibres appear to be relatively insensitive to acetylcholine, though the evidence for this is not conclusive. It may be that, even when injected, acetylcholine reaches the terminals, adaptation occurs too rapidly for impulses

to be initiated. Such a possibility could not be excluded by using the present experimental technique.

The present results offer no evidence for a possible role for acetylcholine in the nerve, although the pharmacological structures acted on by the externally applied drug must be very similar to those at sites where acetylcholine does have a physiological function. Presumably these structures either form at an early stage of regeneration and are then pushed ahead by growth behind, or they are continually formed during regeneration, becoming 'masked' or modified as maturation to the normal fibre structure proceeds. In either case the excitable region is likely to be similar, or closely related, to that occurring at the mature receptor nerve terminal. As with normal mechanoreceptors, the effect of hexamethonium on regenerating endings is to prevent excitation by acetylcholine but not that due to mechanical stimulation. There may, of course, always be some growth occurring at normal receptor nerve endings; however, even if terminal growth and regeneration were identical, it would seem unlikely, in view of the apparent insensitivity of regenerating motor terminals, that the effects of acetylcholine are related to nerve fibre growth *per se*. The results probably indicate that the regenerating terminal is already possessed of some of the characteristics of the normal ending, though full maturation may depend upon peripheral influences operating at the region which was originally innervated.

The present results might throw some light on the mechanism of causalgia. A current explanation invokes the possibility of an 'artificial synapse' involving current flow from active sympathetic fibres to receptor nerves at the site of a nerve lesion (Barnes, 1954). There are two further possibilities: (*a*) that acetylcholine released in the vicinity (e.g. from cholinergic nerve fibres, Lissak, 1939; Chang, Hsieh, Li & Lim, 1939) excites nerve sprouts from damaged receptor fibres, and (*b*) a rise of blood pressure (which is very likely to occur during conditions which are associated with causalgic pain) leads to mechanical stimulation of receptor nerve sprouts as a result of the increased pulse pressure in adjacent arteries. The relief of the pain of causalgia by the ganglion-blocking agent tetraethyl ammonium is compatible with both these possibilities, for it would probably block a direct effect of acetylcholine on the nerve endings, and would also prevent a rise of blood pressure, which might mechanically stimulate the endings.

SUMMARY

1. Close arterial injections of acetylcholine and other drugs have been made into both normal and regenerating nerves of rabbits.

2. The injection of fluids into normal sural nerves excited a brief burst of impulses which were conducted centrally but not peripherally from the perfused region of the nerve; acetylcholine increased this response. The impulses

appeared to be initiated at mechanoreceptors occurring in the nerve trunk or the adjacent artery.

3. The injection of acetylcholine or nicotine into the region of regenerating sural nerve endings always excited a burst of impulses which outlasted the injection by many seconds. The effects of acetylcholine were potentiated by eserine, and blocked (as were those of nicotine) by hexamethonium.

4. Acetylcholine and nicotine have been shown to act directly on the terminal region of the regenerating fibres.

5. The sensitivity of the regenerating fibres to acetylcholine was usually apparent within 3 days of making the original lesion; some fibres may have been sensitive within 12 hr.

6. The injection of acetylcholine into normal popliteal nerves failed to excite impulses detectable in the ventral roots unless the drug caused a contraction of gastrocnemius muscle, when occasionally a few motor fibres were excited.

7. Acetylcholine, injected into regenerating popliteal nerves, normally failed to excite impulses detectable in the ventral roots; impulses evoked by the drug could be detected in the corresponding dorsal roots of a number of these preparations.

8. It is concluded that the ends of regenerating receptor nerves resemble normal receptor nerve terminals pharmacologically. The evidence from regenerating motor fibres is not conclusive, but they appear to be relatively insensitive to acetylcholine and nicotine, and in this respect to resemble normal motor nerves.

9. The possible relation between these results and the mechanism of causalgia is mentioned.

I wish to thank Sir Lindor Brown for his support of this work; also Professor B. Katz, F.R.S., and Drs J. A. B. Gray and R. Miledi who read and criticized the manuscript.

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