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THE PRODUCTION OF DENERVATION-LIKE CHANGES IN RAT MUSCLE BY COLCHICINE, WITHOUT INTERFERENCE WITH AXONAL TRANSPORT OR MUSCLE ACTIVITY

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SUMMARY

1. Rat extensor digitorum longus (EDL) muscles were examined after colchicine treatment of the sciatic nerve. Colchicine was applied in one of two ways: (i) a single sub-epineural injection; (ii) a chronically implanted silicone cuff.

2. After the sub-epineural injection, the entire membrane of muscle fibres became sensitive to iontophoretically applied acetylcholine and the muscle action potentials became resistant to tetrodotoxin. However, the majority of these fibres were found to be normally innervated.

3. These effects were not restricted to the EDL muscle of the colchicine injected side but were also found in the EDL muscle of the contralateral side, indicating that the action of colchicine was systemic.

4. In the treated sciatic nerve there was a partial block of axonal transport of ^{3}H-labelled proteins, which correlated with a partial paralysis of the ipsilateral leg. However, axoplasmic transport was found to be normal in the contralateral sciatic nerve and the contralateral limb was not paralysed despite the supersensitivity of the investigated muscle on that side.

5. When colchicine was applied with a silicone cuff, denervation-like changes were confined to the ipsilateral EDL muscle. However, impulse conduction block at the level of the cuff was usually observed.

6. It is concluded that (i) colchicine can produce denervation-like changes in normally active muscle without blocking axoplasmic transport, through an action probably exerted directly on the muscle membrane, and (ii) that colchicine-cuff experiments failed to provide unambiguous evidence in support of the existence of neurotrophic influences on the muscle membrane.

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INTRODUCTION

The role of inactivity in the origin of denervation-like changes in muscle such as spread of acetylcholine (ACh) sensitivity outside the junctional membrane (Axelsson & Thesleff, 1959; Miledi, 1960) has been studied with a variety of approaches. The general outcome of these investigations is that whenever the skeletal muscle is made inactive either by botulinum toxin (Thesleff, 1960) or by a chronic conduction block along the motor nerve (Lømo & Rosenthal, 1972), muscle fibres become supersensitive to ACh just as after denervation. Since chronic stimulation of denervated rat muscle prevents or strongly reduces denervation supersensitivity (Jones & Vrbová, 1970, 1971; Lømo & Rosenthal, 1972; Drachman & Witzke, 1972; Cohen & Fischbach, 1973; Purves & Sakmann, 1974*a*) it appears that activity is an important factor controlling the distribution of ACh-receptors in the extrajunctional muscle membrane.

Support for a control of ACh-sensitivity by neural factors rather than muscle activity is particularly suggested by experiments of partial denervation in the frog (Miledi, 1960) and by the importance of the length of the nerve stump in determining the time of onset of denervation-like phenomena (Luco & Eyzaguirre, 1955; Emmelin & Malm, 1965; Albuquerque, Shuh & Kauffman, 1971; Harris & Thesleff, 1972). The implied trophic factor has been postulated to be acetylcholine itself (for review see Drachman, 1974), or it could be some component of the materials continuously transported via axoplasmic flow from cell somata to nerve terminals.

We have approached the problem of the neural control of muscle by applications of colchicine to the motor nerves since this drug is known to block axoplasmic transport (Dahlström, 1968; Kreutzberg, 1969). The same approach has been utilized by several investigators (Albuquerque, Warnick, Tasse & Sansone, 1972; Hofmann & Thesleff, 1972; Fernandez & Ramirez, 1974). While some of our results are similar to those reported by these investigators, our new findings suggest that previous interpretations of the effects of colchicine may be incorrect.

Preliminary accounts of these results have been published (Cangiano, 1973; Cangiano & Fried, 1974).

METHODS

Animal treatment. All experiments were performed on male Wistar rats 145–450 g in body weight. The animals were treated with colchicine (B.D.H. Ltd, mol. wt. 399·4) following either one of three procedures. (i) During ether anaesthesia, the sciatic nerve of one side was exposed at the mid-thigh level and 1–5 μl. 0·2 M colchicine solution was injected below the epineurium, as superficially as possible, using a glass microcapillary connected by a polyethylene tubing to a 10 μl. Hamilton syringe. To avoid clogging of the solution in the injecting system, the colchicine was

dissolved in a mixture of 2 parts of ethanol and 8 parts of 0·1 M-Na phosphate buffer at pH 7·2 (Kreutzberg, 1969). Some of the animals were injected with the solvent alone as a control. (ii) 0·1–0·5 ml. 2 mM solution of colchicine in saline was injected systemically (in the lumbar muscles). The dose range of colchicine thus corresponded to that used for the nerve injection experiments (i.e. 80–400 μg per animal). (iii) Using the same surgical procedure described above, the sciatic nerve of one side was surrounded with a silicone cuff (Robert & Oester, 1970) impregnated with colchicine. To minimize diffusion of the drug to the surrounding tissues a plastic sheath was placed around the cuff and kept in place by a ligature. The cuffs had a length of about 8 mm, outside diameter (o.d.) of about 5 mm and inside diameter (i.d.) varying from 0·8 to 1·6 mm (the rats used for this group of experiments weighed 270–350 g). In order to avoid trauma of the nerve, the cuff was not only slit longitudinally on one side but also was cut almost completely on the opposite side, just leaving a thin bridge which acted as a hinge: the cuff was opened for its placement around the nerve and then gently closed. Once positioned, the cuff completely surrounded the sciatic nerve. The cuffs were prepared first carefully mixing the colchicine with the fluid silicone to obtain a concentration of 0·1–0·15% (w/w); the mixture was then placed in appropriate moulds, where it was left for several days until polymerization was completed. Two types of silicone were utilized, both produced by Dow Corning (Ann Arbor, Michigan): Silastic Medical Adhesive Silicone type A and Sylastic Medical Grade Elastomer 382. The first type is a one-part system which polymerizes by giving off acetic acid in contact with moist air, while the second is a two part system, i.e. it polymerizes by the addition of an appropriate catalyst (Dow Corning catalyst E) to the silicone monomer. Catalyst E was used at a concentration of 0·1%. In a small series of rats, cuffs impregnated with 0·10% vinblastine (Vinblastine sulphate, Lilly), instead of colchicine, were implanted.

After treatment, all rats were carefully checked (usually twice daily) for the possible development of leg paresis by examining the degree of active extension and spread of the digits, the amount of extensor tone of the foot and the strength of extensor voluntary movements. To denervate the EDL, the sciatic nerve of one side was exposed at the mid-thigh level and cut.

Micro-electrode experiments. At various times (1–8 days) after colchicine treatment, the rats were anaesthetized with pentobarbitone sodium and the EDL muscles were dissected out with their nerves and perfused in a chamber at room temperature (22–25° C). In most experiments, both the EDL muscles of the treated and of the contralateral untreated side were placed in the chamber. On the treated side, the nerve was isolated up to about 1 cm central to the site of colchicine injection. In the initial experiments with silicone cuffs the nerve–muscle preparation, including the cuff and a short segment of nerve central to it, was investigated *in vitro*. However after some time (usually about 1 hr) a conduction block developed at the level of the cuff; this was in all likelihood due to failure of oxygen to diffuse in adequate amounts to the portion of the nerve enclosed in the cuff. This procedure was therefore abandoned in later experiments and only the portion of the nerve distal to the cuff was included in the chamber. The perfusing fluid was bubbled continuously before reaching the chamber with a gas mixture of 95% O_2 and 5% CO_2 and had the following composition in mM: NaCl, 135; KCl, 5; $CaCl_2$, 2; $MgCl_2.6H_2O$, 1; $NaHCO_3$, 15; Na_2HPO_4, 1; D-glucose, 11. The perfusion rate was about 300 ml./hr.

Intracellular recordings from superficial muscle fibres of the deep surface of EDL muscles were made with 3 M-KCl micropipettes of 5–15 MΩ resistance and tip potentials generally less than 5 mV. ACh sensitivity of the junctional and extrajunctional membranes was measured by micro-iontophoresis (del Castillo & Katz,

1955) with micropipettes filled with 3 M-AChCl by the glass-fibre technique (Tasaki, Tsukahara, Ito, Wayner & Yu, 1968). A breaking current of 5×10^{-9} to 1×10^{-8} A was required with most micropipettes to prevent leakage of ACh from the tip and resultant receptor desensitization. ACh was released by positive pulses of variable intensity and duration and membrane sensitivity was measured in units according to Miledi (1960), 1 unit = 1 mV depolarization per 10^{-9} coulomb of charge passed through the pipette. The minimal sensitivity that we could measure was 10^{-2} units.

Direct excitation of muscle fibres was done by applying cathodal pulses through a 2 M-K citrate micropipette inserted into the muscle fibre at about 100 μm distance from the recording pipette. A d.c. hyperpolarizing current was passed through the 'current' electrode to obtain a steady membrane potential of 90–100 mV (Redfern & Thesleff, 1971*a*). The action of tetrodotoxin (TTX, Sankyo, Tokyo) on the spike generating mechanism was assessed by adding the toxin to the bathing fluid to obtain a concentration of 10^{-6} M.

Axoplasmic transport measurements. Axonal transport of tritium-labelled material was measured with a technique already described by others (Ochs & Burger, 1958; Ochs, 1972). Measurements were made in a series of rats 3–51 hr after colchicine injection in one sciatic nerve, either on the ipsilateral or on the contralateral side or both. Animals were anaesthetized with pentobarbitone sodium and a laminectomy was performed over three vertebrae of the lumbar enlargement of the spinal cord. The precursor, L-[4,5-^{3}H,N]leucine, was supplied by New England Nuclear Co. and had a specific activity of 41·2 c/m-mole in a 0·01 N-HCl solution at a concentration of 1 mc/ml. One ml. of solution was desiccated to dryness and resuspended in 200 μl. isotonic saline: thus the final concentration was 5 mc/ml. Once the dura was opened, five injections were made in the selected half of the spinal cord, 4 μl. each and 1·8 mm apart in a rostrocaudal direction, at depths of 1·2–1·6 mm. Injections were made using Dow Corning glass microsampling pipettes (50 μl.), graduated by us in 4 μl. segments and pulled to have tips to about 30 μm in diameter. Accurate placement of the injections was usually signalled by strong twitches and movements of the ipsilateral leg. The skin flaps were then closed and the animals kept warm to maintain rectal temperatures of 36·5–37·5° C. This was important because of the dependence of the rate of axoplasmic transport on temperature (Ochs & Smith, 1971). Pentobarbitone was administered as required.

After the desired flow time of the labelled material had elapsed (up to 6·25 hr) the animals were decapitated, sciatic nerves and corresponding ventral roots (usually two roots) were isolated and the dorsal root ganglia dissected away. Nerves were partially dried, at their *in vivo* length; they were then divided into 3 mm segments each of which was placed in a scintillation vial, re-hydrated with a droplet of distilled water and solubilized by adding 0·5 ml. Protosol (New England Nuclear) and heated at 50° C for a few hours. After solubilization, each vial was analysed for 10 min in a Beckman counter following the addition of 10 ml. scintillation fluid. No correction was made for quenching.

Muscle contraction measurements. Isometric recordings of the EDL muscle contraction were performed *in vivo*, during pentobarbitone anaesthesia. The distal tendon was sectioned and the distal half of the muscle belly freed from the surrounding tissues. The tendon was then connected to a Grass FT.03 Transducer. The knee was rigidly fixed with a small screw connected to a bar and the foot held in a clamp. Single and repetitive supramaximal electrical pulses (up to 100/sec) were applied to the sciatic nerve both proximal and distal to the site of colchicine application (injection or cuff). To avoid contamination due to the evoked contraction of other muscles, the posterior tibial nerve was transected and the tendons of all muscles supplied by the common peroneal nerve were sectioned at the level of the ankle.

Histology. Nerves were removed from the animal, fixed in buffered 3% glutaraldehyde and post-fixed in Dalton fixative (Dalton, 1955). The nerves were then embedded in Spurr epoxy resin. Sections (6–7 μm) were cut and stained with toluidine blue for light microscopy.

RESULTS

Colchicine-injection experiments

Sensitivity to ACh. Micro-iontophoretic application of ACh revealed that a few days after subepineural injection of colchicine into one sciatic nerve, the ipsilateral EDL muscle fibres had become sensitive to the cholinergic transmitter over their entire surface (Fig. 1). In contrast, we found no

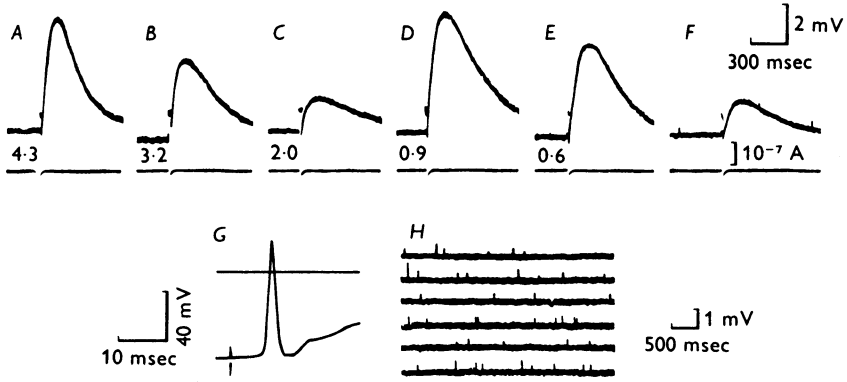

Fig. 1. Extrajunctional sensitivity to micro-iontophoretically applied acetylcholine (ACh) in a fibre of the extensor digitorum longus (EDL) muscle ipsilateral to a sciatic nerve, injected 108 hr earlier with 152 μg colchicine (rat 163 g). *A*, tendon region; *F*, end-plate region. Distances from the end-plate (in mm) are indicated below *A–E*. Iontophoretic pulse duration is 20 msec in *B* and *C* and 10 msec in the remaining records. The low amplitude of the 'ACh-potential' at the end-plate is due to the low resting membrane potential after multiple penetrations and to poor positioning of the ACh electrode. *G*, indirect spike recorded at the tendon region of the same fibre, soon after the ACh-potential shown in *A* was evoked; after the spike the muscle twitch dislodged the electrode. *H*, miniature end-plate potentials (min. e.p.p.s) recorded at position *F*. The average sensitivity at the tendon region was 8·2 ± 2·5 units (mean ± S.E. of mean, nine fibres). Six out of the nine fibres were excitable by nerve stimulation.

extrajunctional sensitivity to ACh in EDL muscles from normal rats confirming previous reports (Miledi & Zelená, 1966; Albuquerque & Thesleff, 1968). In the colchicine treated rats miniature end-plate potentials (min.e.p.p.s) were present in the majority of the supersensitive fibres and stimulation of the nerve evoked action potentials which could be recorded

both at the end-plate and near the tendon of these fibres (Fig. 1*G*). The extrajunctional ACh-sensitivity could therefore not be attributed to denervation or to failure of action potential conduction along the muscle fibres.

To test for the possibility that colchicine might diffuse away from the site of injection and affect the muscle systemically, we examined the ACh-sensitivity of EDL muscle fibres from the opposite leg. Unexpectedly, these fibres were also found to be supersensitive to ACh (Fig. 2). Indeed, 4–5 days after injection of 152 μg colchicine into one sciatic nerve of eight animals (weight range, 145–170 g; dose range, 90–105 μg/100 g body wt.)

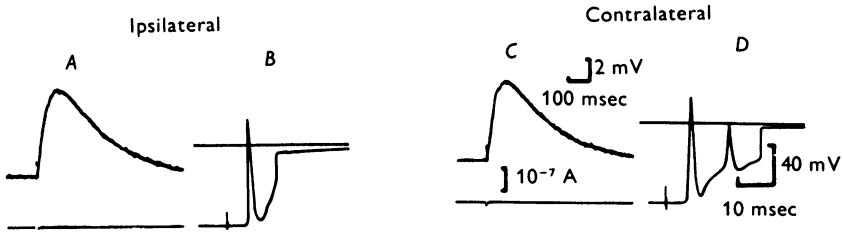

Fig. 2. Extrajunctional ACh-sensitivity (tendon region) of two normally innervated EDL muscle fibres, one ipsilateral and the other contralateral to the sciatic nerve injected 75 hr before with 152 μg colchicine (rat, 166 g). *A* and *C*, ACh-potentials elicited with a 5 msec pulse. *B* and *D*, spikes evoked in the same fibres with single shock nerve stimulation. The average ACh-sensitivity at the tendon region (fourteen fibres for each side) was $10{\cdot}9 \pm 2{\cdot}9$ (S.E. of mean) units ipsilaterally and $6{\cdot}8 \pm 1{\cdot}3$ units contralaterally; the difference was not statistically significant ($P > 0{\cdot}1$). Indirect spikes were obtained in eleven ipsilateral and in all contralateral fibres.

the average sensitivity at the tendon region of innervated fibres was $6{\cdot}7 \pm 0{\cdot}9$ units (mean $\pm$ S.E.) ipsilaterally (ninety-seven fibres) and $5{\cdot}8 \pm 0{\cdot}6$ contralaterally (101 fibres); these values are not significantly different ($P > 0{\cdot}1$). This indicated that colchicine acts systemically, a conclusion further supported by the finding that a deliberate systemic injection of similar doses of colchicine (about 100 μg/100 g body wt.) into the lumbar musculature produced supersensitivity of equal magnitude ($6{\cdot}9 \pm 0{\cdot}7$ units, seventy-one fibres, two pairs of EDL muscles). Thus, colchicine has the same effect whether it is injected into the nerve of one leg, or systemically into some distant musculature. In both cases a bilateral supersensitivity to ACh appears in the EDL muscles.

The threshold dose for causing ACh-supersensitivity was approximately 40–50 μg/100 g body wt. Thus, 4 days after inection of this dose into one sciatic nerve only four out of thirty-five ipsilateral and two out of fifty contralateral muscle fibres showed any ACh-supersensitivity (six animals).

Doubling the dose to 80–100 μg/100 g body wt. induced supersensitivity in nearly all ipsilateral (83 % of 126) and contralateral (84 % of 156) fibres (fourteen animals). The result was the same whether large (320–440 g, six animals) or small rats (145–170 g, eight animals) were used. In each case the supersensitivity opposite the side of injection was approximately the same as on the side of the injection.

The time course of the systemic effect of colchicine is shown in Fig. 3. The supersensitivity increased rapidly between the third and the fifth day after the injection and then declined rapidly. The effect was somewhat

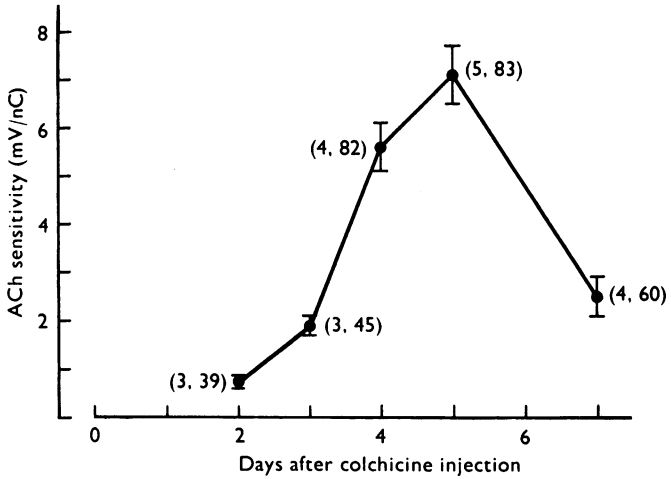

Fig. 3. Time course of extrajunctional acetylcholine sensitivity changes in EDL muscles (tendon region) due to the systemic action of colchicine. Dose range was 90–105 μg/100 g body wt. Bars represent S.E. of the mean. Beside each bar: first number indicates number of animals, second number the number of fibres.

smaller than that obtained after denervation. The maximal values measured at the tendon region usually ranged from 5 to 10 units for colchicine, in contrast to those measured at the same region 4–5 days after denervation which were around 50 units.

The distribution of ACh-sensitivity along single muscle fibres resembled that seen in denervated fibres (Albuquerque & McIsaac, 1970). It was highest near the end-plate and the myotendinous junction and lowest in the intermediate region (Fig. 4).

Other denervation-like changes. After denervation, the spike-generating mechanism of skeletal muscle fibres becomes partially resistant to concentrations of TTX that completely block action potentials in normal muscles (Redfern & Thesleff, 1971*b*). We found the same to be true after colchicine

injection; similar results have been obtained by Hofmann & Thesleff (1972) in the EDL muscle ipsilateral to the colchicine-injected sciatic nerve. In addition, we found that TTX-resistant action potentials also developed in the contralateral EDL muscle and after systemic injections of colchicine (around 100 μg/100 g body wt.). Fig. 5 illustrates one such case. In the twelve out of fourteen fibres of the 'colchicine muscle' which showed this phenomenon in this particular experiment, min.e.p.p.s were present.

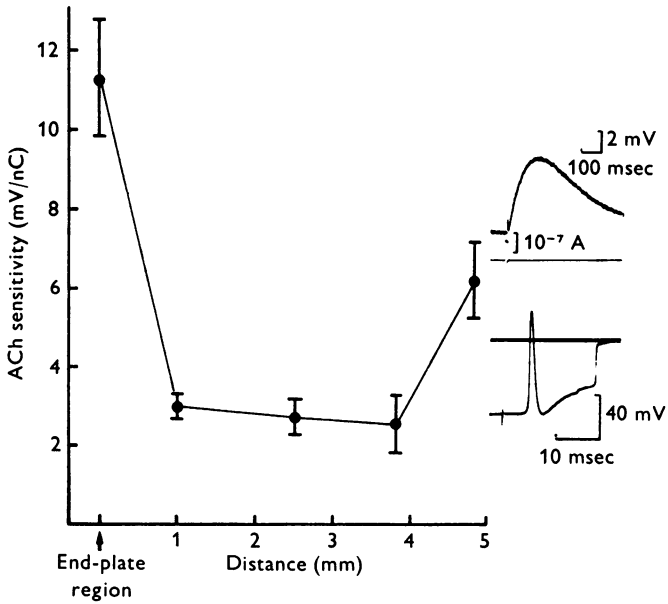

Fig. 4. Profile of ACh-sensitivity from the tendon to the end-plate region in fifteen adjacent fibres of the EDL muscle *contralateral* to a sciatic nerve injected 100 hr earlier with colchicine (98 μg/100 g body wt.). All these fibres exhibited an action potential following nerve stimulation. Inset shows records from one fibre at the tendon region (5 msec iontophoretic pulse of ACh). *Normal* EDL fibres have no detectable ACh-sensitivity outside the end-plate region, even at the tendon region.

Muscle fibres of the colchicine-treated animals showed a decrease in the resting membrane potential (cf. Lømo, 1974) similar to that observed after denervation (Locke & Solomon, 1967). This effect, like the ACh-supersensitivity and TTX-resistant action potentials was mediated by a systemic action of the drug. The magnitude of the membrane depolarization after colchicine was, however, rather smaller than after denervation. The average values were as follows: normal muscle fibres, $74{\cdot}7 \pm 0{\cdot}7$ mV (mean $\pm$ S.E. of mean, forty-two fibres); 4- to 5-day-denervated fibres, $54{\cdot}6 \pm 0{\cdot}9$ mV (fifty fibres); 'colchicine fibres', $67{\cdot}5 \pm 0{\cdot}9$ mV (156 fibres,

either after systemic injection or contralateral to the injected side). It therefore appears that the systemic action of colchicine is less effective than denervation in producing membrane depolarization and ACh-supersensitivity.

We did not usually detect fibrillation in the 'colchicine' muscles by visual inspection of the muscle surface *in vivo* and this may indicate that the systemic action of colchicine affects the various mechanisms which are influenced by denervation differentially. However, before concluding that

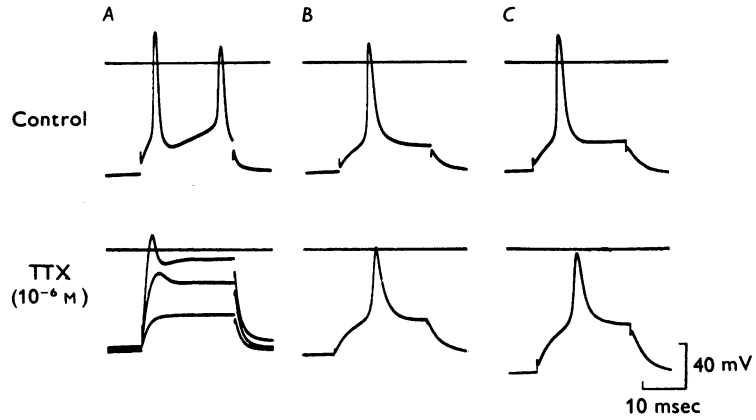

Fig. 5. *C*, partial resistance to tetrodotoxin (TTX) of the directly evoked action potential in EDL muscle 4 days after systemic intramuscular injection of colchicine (106 μg/100 g body wt.). *A*, for comparison, examples from a normal and, *B*, 4-day-denervated EDL muscle are shown. In the normal muscle the action potential was blocked by TTX in eleven tested fibres, whereas in the denervated muscle seven out of nine fibres were resistant to the poison. In each record, the on and off artifacts of the depolarizing current pulse are visible before and after the evoked spike, respectively. (In the normal control fibre, the depolarizing current pulse was strong enough to evoke two consecutive action potentials; in the normal fibre after TTX, three depolarizing pulses of increasing amplitude are superimposed, none of them being able to evoke an action potential.)

colchicine does not affect the mechanism that produces fibrillation, intracellular recordings in the *in vivo* preparation need to be done in order to exclude the possible presence of subthreshold fibrillatory potentials (Purves & Sakmann, 1974*b*).

Effects on the nerve. Several findings indicated that colchicine also affected the nerve. Following injections of colchicine (80–100 μg/100 g body wt.) into one sciatic nerve some supersensitive EDL fibres on the injected side failed to respond to nerve stimulation or showed only subthreshold end-plate potentials. Many of the fibres were also without

min.e.p.p.s. In contrast, virtually all supersensitive fibres in the contralateral EDL responded to nerve stimulation with action potentials, using this dose of colchicine. The percentage of ipsilateral fibres not responding with an action potential to stimulation of the nerve was higher (64 % of sixty-seven fibres) in the larger rats which received the largest amount of colchicine (300–320 μg) into the nerve than in the smaller rats (37 % of 127 fibres) which received the lower dose (152 μg). With the larger dose an ipsilateral leg paresis was also clearly detectable.

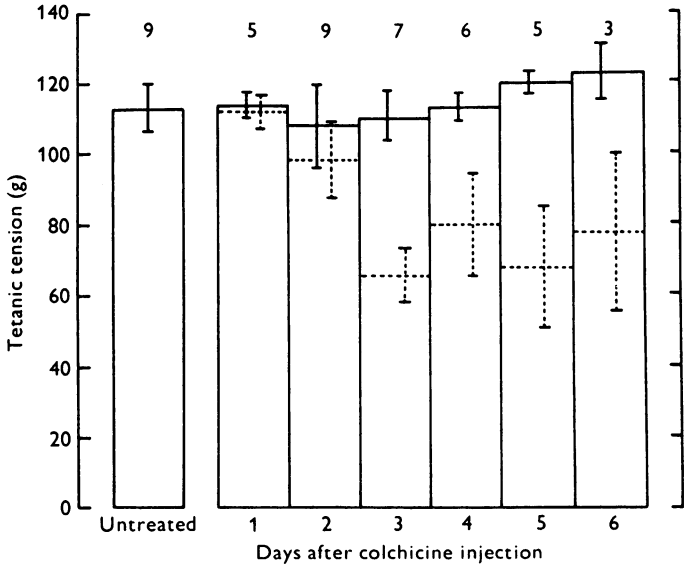

Fig. 6. Indirect tetanic tension of ipsilateral (columns with dashed lines) and contralateral EDL muscles (columns with continuous lines) in a series of rats, as a function of time after a unilateral sciatic nerve injection of colchicine (152 μg). The figures above the columns indicate the number of rats examined and the bars represent the S.E. of the mean.

A more complete study of this ipsilateral effect of colchicine was carried out by measuring the amount of tetanic contraction elicited in the EDL muscle by sciatic nerve stimulation at 100 pulses/sec. Measurements were made, both ipsi- and contralaterally, at various times after a unilateral neural injection of colchicine (152 μg) in a series of rats (weight range 145 –180 g; dose range 85–105 μg/100 g body wt.). The results of the experiment are presented in Fig. 6. It can be seen that while the average tetanic tension of contralateral muscles (columns with continuous lines) remained at all tested times similar to the control value, the average tension of the ipsilateral muscles (columns with dashed lines) showed a drop beginning

on the third day. No drop in tension occurred in the EDL muscle of three rats whose ipsilateral sciatic nerves were injected 3–5 days earlier with the solvent alone (not shown). This ipsilateral muscle paresis was not due to conduction block at the site of colchicine injection, since nerve stimulation proximal and distal to this site evoked EDL muscle tensions of the same magnitude. Histological examination of nerves distal to the colchicine-injected region (two rats, 5 days after injection) did reveal signs of partial nerve damage; fragmented axis cylinders and myelin sheaths were found intermingled with normal axons. On the other hand no histological alterations were detected in the contralateral sciatic nerves as well as in a sciatic nerve injected 5 days earlier with the solvent alone.

It therefore appears from these findings that the subepineural injection of the above indicated dose of colchicine produces a partial paralysis of the ipsilateral muscle, attributable to degeneration of some of the axons in the nerve.

Dependence of effects on colchicine dose. When doses of colchicine higher than 100 μg/100 g body wt. were injected, no further increase in supersensitivity occurred but within 1 day the rats started to appear sick and showed paresis of both hind limbs (Ferguson, 1952). The paresis was most pronounced during the second day and then regressed in the surviving animals so that by the fourth to the fifth day it was no longer noticeable. Since the paresis was both bilateral and transient, it seemed to be related to the systemic effect of colchicine and, possibly, was caused by a different mechanism from the axonal degeneration just described which was restricted to the injected nerve. Furthermore, it seemed possible that lower doses of colchicine also might cause a transient bilateral paresis which would be too small to be detected clinically. If so, the supersensitivity to ACh after lower doses of colchicine might, at least in part, be attributed to muscle inactivity (Lømo & Rosenthal, 1972; Lømo & Westgaard, 1975). To study these questions further we injected systemically a series of rats with doses of colchicine ranging from 85 to 150 μg/100 g body wt. and 2 days later measured tetanic tensions developed by EDL muscles in response to nerve stimulation at 100 impulses/sec. The results of the experiment are shown in Fig. 7. It can be seen that it is only with doses in excess of 105 μg/100 g body wt. that the average tetanic tension starts to drop in respect of the tension of the untreated group. This progressive reduction in evoked tension was paralleled by an increasing behavioural paresis of the limbs. ACh-supersensitivity, however, appeared after doses below threshold for causing any reduction in tetanic tension or any signs of behavioural paresis. Thus, in four rats which received colchicine (85–95 μg/100 g body wt.) into the lumbar musculature, 84 % of the tested fibres showed spread of ACh-sensitivity with an average of $4{\cdot}1 \pm 0{\cdot}5$ units at the

tendon region (eighty-six fibres, fourth day after injection). Larger doses (130–150 μg/100 g body wt.), which produced clear behavioural paresis from the second day, did not induce any higher extrajunctional ACh-sensitivity. If anything, the sensitivity appeared to be smaller ($2{\cdot}1 \pm 0{\cdot}3$ units in forty-six fibres of three rats). In two rats showing a practically complete immobilization of the hind limbs on the second day after injection of a toxic dose of colchicine (150 μg/100 g body wt.) many EDL muscle fibres (forty-seven) were impaled at the end-plate region. All of

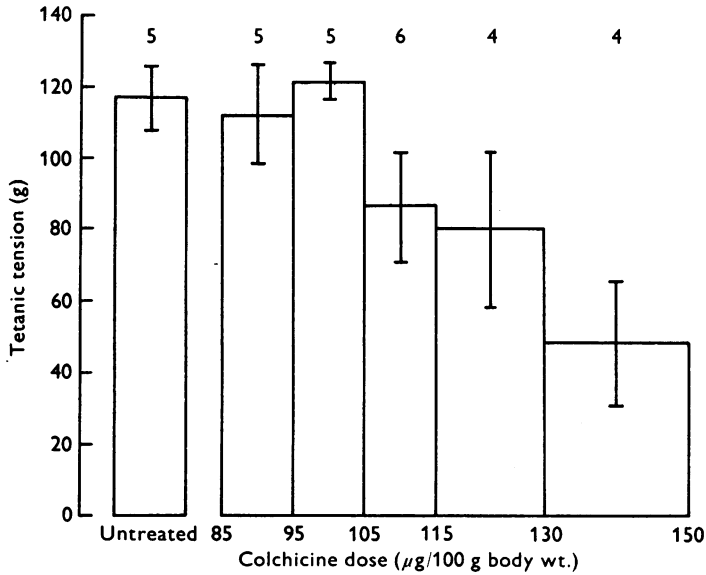

Fig. 7. Tetanic tension evoked in the EDL muscle by nerve stimulation at 100 pulses/sec, as a function of the dose of colchicine injected systemically 2 days earlier. Left-hand column shows results of control experiments. The figures above each column indicate the number of rats examined and the bars represent the S.E. of the mean.

these fibres had spontaneous min.e.p.p.s; however, the large majority of them (83 %) responded only with subthreshold end-plate potentials (e.p.p.s) and not with action potentials to stimulation of the nerve (cf. Katz, 1972). Thus, systemic injections of toxic doses of colchicine impaired neuromuscular transmission but differed from intraneural injections which completely blocked transmission in some fibres as a result of axonal degeneration.

It may be concluded that interrupted nerve impulse conduction or transmission cannot alone account for the generalized ACh-supersensitivity induced by colchicine. The two phenomena develop with different doses and appear to be independent effects of colchicine.

Effect of colchicine injections on axonal transport

Systemic action. We have shown that injected colchicine produces ACh-supersensitivity by way of a systemic action. The question then arises whether this effect is mediated through an impairment of axoplasmic transport. To answer this, measurements of transport of ^{3}H-labelled material were made along the motor fibres of sciatic nerves in a series of rats. Twenty animals served as a control group. The experimental group

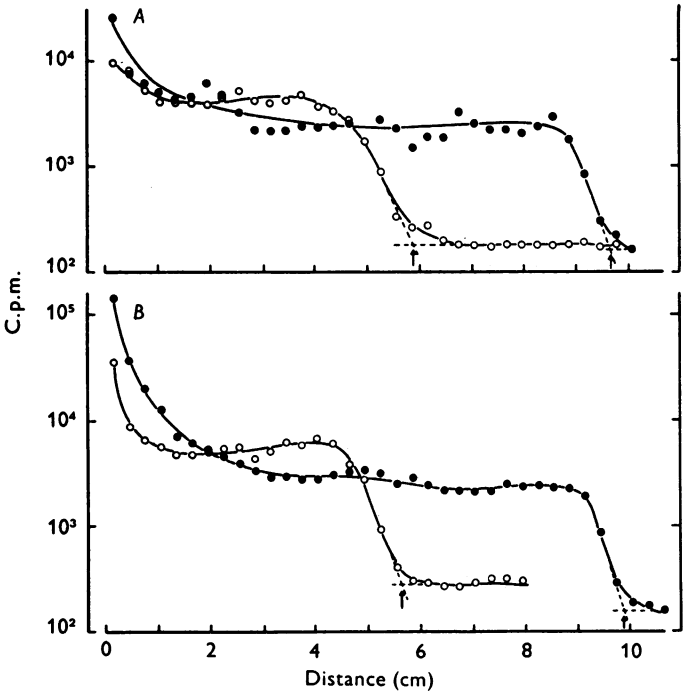

Fig. 8. Wave-fronts of ^{3}H-labelled material 3·25 hr (open circles) and 6·25 hr (filled circles) after spinal cord injection of ^{3}H-leucine: *A*, two control rats; *B*, two rats injected about 20 hr earlier in the contralateral sciatic nerve with colchicine in a dose (100 μg/100 g body wt.) which routinely caused supersensitivity to ACh in muscles of both sides. Each data point represents the amount of radioactivity in a 3 mm segment of nerve. The abscissa indicates the distance, in cm, along ventral roots and sciatic nerves; the zero point was the portion of ventral root emerging from the spinal cord segment injected with [^{3}H]leucine. Flow rate was determined by dividing the distance covered by the wave-front into the flow time, the distance being measured between the spinal cord (zero point on abscissa) and the foot of the wave-front (indicated by the arrow). Wave-front amplitude was measured between the background level and the top of the wave-front.

consisted of forty-five animals, which were examined between 3 and 51 hr after colchicine treatment. Part of this group (twenty-eight) had been injected with the drug in the contralateral sciatic nerve and the remainder (seventeen) in the lumbar muscles. The dose was between 100 and 150 μg/100 g body wt., which was equal to or greater than that adequate to produce full ACh-supersensitivity. In the control group a wave-front of radioactivity appeared in the sciatic nerve, having an amplitude of

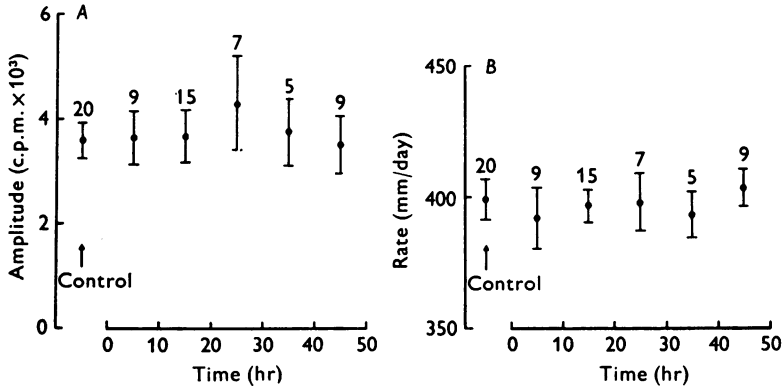

Fig. 9. *A*, amplitude and *B*, rate of movement of the wave-front of ^{3}H-labelled material in a series of rats, as a function of time after colchicine injection either in the contralateral nerve or systemically (intramuscular). Values for untreated rats are shown to the left in both graphs. Bars represent the S.E. of the mean. The number of rats examined is shown above each bar.

3,582 ± 344 c.p.m. (mean ± S.E. of mean) and a calculated rate of transport of 399 ± 7·6 mm/day, in agreement with previous findings (Ochs, 1972). As regards the experimental group, should the systemically acting colchicine impair axoplasmic flow, one would expect either a reduction in the amplitude of the wave-front of ^{3}H-labelled transported material or a reduction in flow rate, or both. This however was not the case. Fig. 8 shows examples of the lack of influence on the amount and rate of flow of ^{3}H-labelled material of doses of colchicine which consistently produced muscle ACh-supersensitivity. Indeed, at no time during the tested period after colchicine treatment was the wave-front of radioactivity reduced in amplitude or in rate of transport, in respect to the values of untreated rats (Fig. 9). Since the ACh-supersensitivity induced with colchicine is already detectable by the end of the second day, it can be concluded that it is not preceded by, or associated with, a demonstrable impairment of axoplasmic transport.

Action on the treated nerve. The effect of colchicine on the treated sciatic

nerve was also investigated. Fig. 10*A* (filled circles) shows the disruption of the wave-front and the large accumulation of labelled material occurring just central to the site of sub-epineural injection of a high dose of colchicine. For comparison, the position of the wave-front along the contralateral,

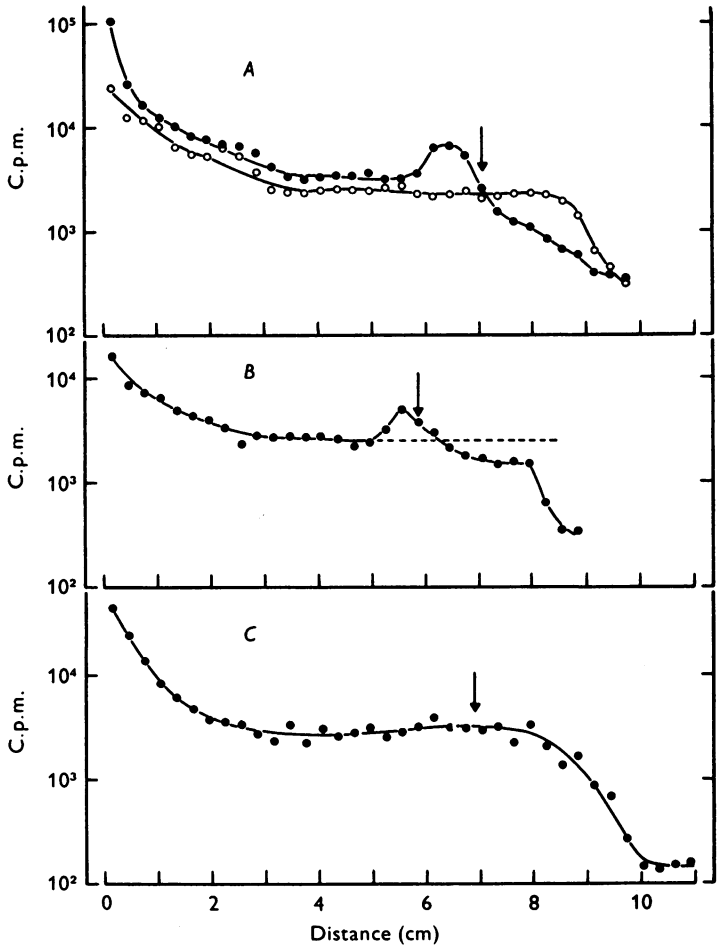

Fig. 10. Block of axonal transport of ^{3}H-labelled material, occurring along the sciatic nerve injected (at arrow) with colchicine: *A*, 48 hr after sub-epineural injection of 400 μg colchicine in 5 μl. solvent; *B*, 18 hr after injection of 152 μg colchicine; *C*, 44 hr after injection of 5 μl. solvent.

non-treated, sciatic nerve is also displayed (open circles). The solvent alone was ineffective (Fig. 10*C*). Furthermore, the possibility that the accumulation was due to blood-borne [^{3}H]leucine rather than to labelled material transported along the nerve, was discarded: no accumulation occurred at

the site of colchicine injection in three rats in which [^{3}H]leucine was injected systemically or in an inappropriate region of the spinal cord (not illustrated).

While Fig. 10*A* shows a major block of axonal transport due to a large dose of colchicine, Fig. 10*B* is an example of the partial block of transport which usually occurred with sub-epineural injections of a smaller dose of the drug (152 μg, in rats of 150–170 g). As can be seen, an accumulation still occurs but the wave-front can be recognized beyond the blocked region. In fact, such a wave-front proceeded through this region without any detectable reduction in rate, though there was a significant reduction of the amplitude. Similar results were obtained in eight additional rats injected with the smaller dose: a moderate damming up of the label was present in all of them, with one exception. It should be noted that this dose (152 μg) also produces a moderate paresis of the ipsilateral EDL muscle (Fig. 6), attributable to partial nerve damage, thus suggesting that a close correlation exists between block of axonal transport and axonal degeneration (cf. Angevine, 1957; Singer & Steinberg, 1972; Perisic & Cuénod, 1972).

Colchicine-cuff experiments

In an attempt to avoid the systemic effect associated with injection of colchicine we applied colchicine or vinblastine-impregnated silicone cuffs around the sciatic nerve as done by others (Albuquerque *et al.* 1972). In this case, no supersensitive fibres were found in the contralateral EDL muscle 5–8 days after application of the cuff, confirming the results of Albuquerque *et al.* and showing that the systemic effect had in fact been avoided. On the side of the cuff, however, many innervated fibres were found supersensitive to ACh and it became essential to establish whether muscle inactivity resulting, for example, from a local conduction block at the level of the cuff could be responsible for this effect.

Cuffs having an internal diameter of 1·6 and 1·25 mm caused paresis of the hind limb in many animals. The paresis was frequently transient being present from day 1–2 to day 5–7 after the application of the cuff. The larger the colchicine dose or the smaller the internal diameter of the central hole, the more pronounced was the paresis (Table 1). Different silicone rubbers (Silastic Medical Adhesive and Silastic Medical Elastomer) produced similar paresis. Also control cuffs devoid of colchicine produced paresis but less frequently and to a lesser degree (Table 1). Vinblastine-cuffs also caused paresis (Table 1). The paresis appeared in part to be due to a local conduction block at the site of the cuff. Thus, twitch and tetanic isometric contractions were smaller when the nerve was stimulated proximal to the cuff than distal to it (Fig. 11).

TABLE 1. Incidence of paresis of the leg, with nerve cuffs (Silastic Medical Adhesive Silicone type A) of two different internal diameters (i.d.). Under the column 'paresis' are included all the rats which were affected, irrespective of its degree which ranged from mild to complete. Weight range of the animals was 270–350 g. Note that results similar to those with colchicine were obtained with vinblastine-cuffs. Cuffs made with Silastic Medical Grade Elastomer 382 (colchicine, 0·1 %) produced similar paresis (not shown)

	1·6 mm i.d.		1·25 mm i.d.	
Type of cuff	Total no. of rats	No. and % with paresis	Total no. of rats	No. and % with paresis
Control	30	7 (23 %)	15	13 (87 %)
Colchicine (0·10 %)	23	13 (57 %)	9	9 (100 %)
Colchicine (0·15 %)	24	20 (83 %)	9	9 (100 %)
Vinblastine (0·10 %)	24	16 (67 %)	—	—

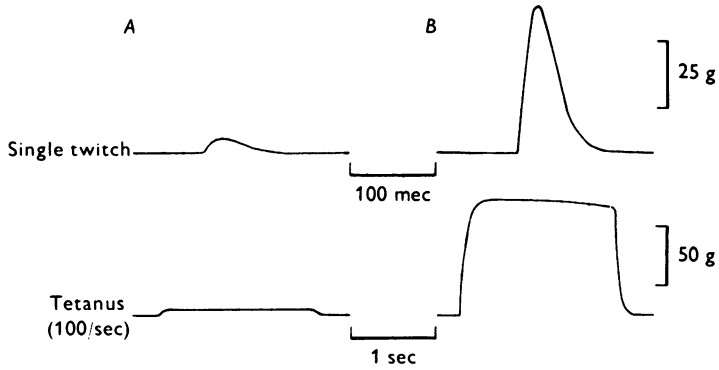

Fig. 11. Conduction block of the sciatic nerve at the site of application of a colchicine cuff (1·6 mm i.d., 0·15 %), implanted 4 days earlier. A subtotal paresis of the cuffed leg had appeared about 20 hr after application of the cuff. *A*, isometric contractions of EDL muscles are shown caused by supramaximal stimulation proximal and, *B*, distal to the cuff *in vivo*. Tetanic tension of the contralateral normal EDL muscle was 192 g.

To avoid the complications arising from a partial and reversible conduction block, we selected rats without detectable paresis at any time after cuff application and examined the ACh-sensitivity of the EDL muscles (fifteen rats, day 5–8; colchicine cuffs). In these muscles only a small percentage of the innervated fibres was supersensitive to ACh (fifty-seven of 168 examined, i.e. 34 %; average value of sensitivity for the affected fibres, 17·8 units $\pm$ 1·7 S.E. of mean), the rest being insensitive. Furthermore, of the fifty-seven supersensitive fibres only eight responded with an action potential to stimulation of the nerve. The inexcitable fibres however had min.e.p.p.s and evoked end-plate potentials, as also reported by Albuquerque *et al.* (1972). In contrast, insensitive fibres in these same

muscles invariably responded with action potentials to stimulation of the nerve. The inexcitable fibres also had low resting membrane potentials (62·6 mV ± 0·7 S.E. of mean, as compared to 77·8 ± 0·5 for the excitable fibres) and this might account, at least in part, for the less effective transmission. It is impossible to know, however, whether or not the failing transmission as demonstrated here *in vitro* had also been present *in vivo*. Similar results have been obtained with vinblastine-cuffs.

DISCUSSION

In several recent studies local application of colchicine to a motor nerve has been shown to produce ACh-supersensitivity and other denervation-like changes in innervated muscle fibres (Albuquerque *et al.* 1972; Hofmann & Thesleff, 1972; Cangiano, 1973; Cangiano & Fried, 1974; Fernandez & Ramirez, 1974). These effects have generally been attributed to the ability of colchicine to block axonal transport and have been taken to support the concept that muscle membrane properties are regulated, at least in part, by neurotrophic substances carried by axonal transport (Albuquerque *et al.* 1972; Hofmann & Thesleff, 1972; Fernandez & Ramirez, 1974). The present experiments, however, favour a different interpretation.

Systemic effects of colchicine

The results clearly show that colchicine injected either into the nerve or into some distant musculature has systemic effects on muscle. In either case, EDL muscles ipsi- and contralaterally to the injections became equally supersensitive to ACh. It is possible that colchicine acted systemically to block the axonal transport of peripheral nerves in general. However, in these experiments supersensitivity developed in the absence of any demonstrable effect on axonal transport. Furthermore, Lømo (1974) has shown that colchicine causes supersensitivity in denervated stimulated soleus muscles which are insensitive in the absence of colchicine. These results strongly indicate that colchicine causes supersensitivity by some mechanism which is independent of the effects of the drug on nerve impulse activity or axonal transport. Probably, the effect is directly on the muscle fibre membrane.

Effects on the nerve

When colchicine was injected into the sciatic nerve, it blocked axonal transport in that nerve in a dose-dependent manner. If this block also interrupted the transport of trophic substances controlling ACh-sensitivity, one would expect this effect to sum with the moderate systemic effect of colchicine to produce a more pronounced supersensitivity to ACh on the injected side. Instead, however, the supersensitivity was the same in the

innervated fibres of both legs. The doses used for these experiments (150 μg) produced a moderate though definite block of axoplasmic transport (Fig. 10 *B*), in agreement with the results of Kreutzberg (1969) who found impairment of axonal flow of cholinesterase in rats' sciatic nerves, with a threshold dose of about 100 μg. In our experiments, intraneural injections of 150 μg usually resulted in some muscle fibres on the side of the injection having no min.e.p.p.s and failing to respond to stimulation of the nerve. Measurements of muscle contractions following nerve stimulation showed on this side a paresis which was of moderate degree (Fig. 6), in good correlation with the effect on axonal transport. With increasing doses (300–400 μg) not only did the block of axonal transport become greater but at the same time neuromuscular transmission was more severely affected. It seems possible, therefore, that the partial interruption of axonal transport obtained with the lower doses reflects not so much a partial impairment of flow in each axon as a more complete interruption in some axons which then degenerate.

In addition to causing axonal degeneration colchicine may have other effects on the nerve. When given systemically in toxic doses, it caused a transient bilateral paresis associated with signs of ineffective neuromuscular transmission but without signs of overt degeneration. These effects were not accompanied by any demonstrable effect on axonal transport and their mechanism is unknown.

From these results it seems clear that colchicine may affect nerves and muscles by various mechanisms: first, it may act systemically directly on the muscle, inducing ACh-supersensitivity; secondly, with higher doses it may again act systemically impairing reversibly neuromuscular transmission; thirdly, it may block axonal transport at the site of application on the nerve, producing axonal degeneration and muscle denervation.

Colchicine-containing cuffs

With colchicine-containing cuffs around the sciatic nerve the systemic effect was avoided and the supersensitivity was restricted to the treated side. In this case the effect was probably on the nerve and not directly on the muscle – for example, by local diffusing of colchicine. The evidence for this is, first, that the supersensitive fibres were intermingled among a large number of insensitive ones, in contrast to the supersensitivity affecting virtually all fibres in muscles exposed to the systemic action of the drug. Yet, the sensitivity was higher in the affected fibres of the cuffed preparations than with the systemic action. Secondly, Kauffman, Warnick & Albuquerque (1974) have shown with cuffs containing radioactive colchicine that the amount of drug reaching the ipsilateral muscles by diffusion is smaller than that necessary to produce ACh-supersensitivity by the

systemic action. This result could be taken as evidence for neurotrophic control (Albuquerque *et al.* 1972). However, in our hands, the cuffs frequently caused a transient paresis which in acute experiments could be related to a local conduction block at the level of the cuff or failing neuromuscular transmission. Albuquerque *et al.* (1972) using similar sized rats and cuffs of the same material and colchicine content to ours reported innervated supersensitive muscle fibres in the absence of any demonstrable conduction block. In this work they used cuffs with smaller internal diameter (0·8 mm) than ours (1·25 and 1·6 mm) and it is difficult to see how a major conduction block could be avoided in these circumstances. We had to abandon the use of cuffs with internal diameters of 1 mm or less, because of the extensive paresis and conduction block that followed. These results are therefore difficult to reconcile with ours. In an attempt to avoid the complications due to conduction block, one can select animals with no detectable paresis. It is important to know, however, the percentage of muscle fibres which are supersensitive to ACh, information not given by Albuquerque *et al.* (1972). In fact, if the percentage of supersensitive fibres is small, as we found in such preparations, it becomes very difficult to rule out that they had not been subject to conduction block between the application of the cuff and the acute experiment. Indeed, the paresis may be transient and also difficult to detect if the block involves only a few nerve fibres. Furthermore, interruption of muscle activity for only 2 days is sufficient to cause a generalized supersensitivity to ACh of some days' duration (T. Lømo & C. R. Slater, personal communication).

Control cuffs containing no colchicine resulted in scattered muscle fibres which were innervated and yet supersensitive to ACh, in some of the muscles. Similar results have been reported by others (Lømo & Rosenthal, 1972). In general, however, the internal diameter of the control cuffs had to be somewhat smaller to produce the same effect as the colchicine-containing cuffs, possibly because colchicine causes the nerve to swell and so to become more compressed inside the cuff. Indeed, compression of the nerve seems a likely cause of the observed conduction block as compression is known to cause a local conduction block of variable duration, with structurally and functionally intact axons below the compressed region (Fowler, Danta & Gilliatt, 1972).

In conclusion, it has not been possible for us with the use of colchicine to find unequivocal evidence for the existence of neurotrophic substances being carried by axonal transport to regulate the extrajunctional membrane properties of the innervated muscle fibres.

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