The Use of Colchicine in the Rat to Investigate A Trophic Influence of the Motor Nerve

by

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ABSTRACT

Denervation of skeletal muscle fibres is known to lead to a marked alteration of their normal characteristics. The mechanism for this is not clear, but there is great interest in the possibility that the changes are the result of the cutting off of neurotrophic factors which normally continuously act on the muscle fibres. To study this question, colchicine, a drug known to interfere with neuronal transport, was injected through the epineurium of one sciatic nerve in rats, in an attempt to prevent the postulated neurotrophic factors from reaching the extensor digitorum longus (EDL) muscle, without interfering with impulse activity in the nerve, or the consequent activity of the muscle. Following this procedure, ipsilateral EDL muscle fibres were found to exhibit extra-junctional sensitivity to acetylcholine (ACh), tetrodotoxin (TTX) resistant action potentials, and lowered membrane potentials (RMP); all characteristic changes observed in denervated skeletal muscle fibres. These fibres however, were shown not to be denervated, since they displayed miniature end-plate potentials (m.e.p.p.s) and neuromuscular transmission was normal.

Unexpectedly, the contralateral EDL muscle fibres which were examined as a routine control, also displayed the features of "denervation" following an injection of colchicine into the ipsilateral sciatic nerve, and were virtually indistinguishable from the ipsilateral EDL fibres.

It thus appeared possible that nerve injections of colchicine were acting systemically, and indeed this was shown to be the case when similar denervation-like changes were caused bilaterally after intraperitoneal (I.P.) injections of the same dose of colchicine.

Although these results showed that colchicine was acting systemically, it was still possible that it was effective by blocking neuronal transport in the muscle nerves. Axoplasmic transport was therefore measured in the sciatic nerve of rats, following colchicine injections both systemically and into the contralateral nerve. At no time was there any evidence of an impairment of transport, even though both EDL muscles contained fibres which displayed extrajunctional sensitivity to ACh. It was concluded that extrajunctional sensitivity to ACh following colchicine injections was not due to an interference with axoplasmic transport.

In the injected sciatic nerve, an impairment of transport was consistently observed and this generally was associated with a detectable paresis and a small percentage (5-7%) of apparently denervated EDL muscle fibres. In addition, the indirectly evoked tetanic tensions produced by the ipsilateral EDL muscles decreased in comparison to the contralateral EDL muscles, commencing about 2 days following the subepineural injection of colchicine. From these observations, it was suggested that the demonstrable impairment of axoplasmic transport in the ipsilateral sciatic nerve caused the impairment of neuromuscular transmission on that side.

Colchicine was also chronically applied to rat sciatic nerves by the use of drug-impregnated silicone rubber nerve-cuffs. This procedure

prevented the systemic effect of colchicine, but the mechanical presence of the cuff resulted in a usually transitory and variable block of impulse conduction in the nerve. Since muscle inactivity per se also leads to changes previously believed to be due to denervation, the changes that were observed with colchicine-cuffs did not constitute unequivocal evidence supporting neurotrophic effects on the ipsilateral EDL muscle fibres.

It was concluded from the results presented in this thesis that the results of experiments involving colchicine injections or drug-impregnated nerve-cuffs do not unequivocably support the existence of a neurotrophic control of skeletal muscle fibre properties.

Finally, preliminary experiments in which colchicine was used in association with denervation indicated that the most likely mechanism of action of colchicine was a direct one on the muscle fibre membrane itself.

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ACh	acetylcholine
AMP	Ampere
AT	anterior tibialis muscle
b.w.	body weight
CHE	choline esterase
cm	centimetre
DC	direct current
EDL	extensor digitorum longus muscle
e.p.p.	end-plate potential
Hz	Hertz (cycles per second)
I.D.	inside diameter
I.M.	intramuscular
I.P.	intraperitoneal
M	molarity
mM	millimolarity
m.e.p.p.	miniature end-plate potential
msec	millisecond
mV	millivolt
nC	nanocoulomb
O.D.	outside diameter
RMP	resting membrane potential
S.E.M.	standard error of the mean
TTX	tetrodotoxin
µg	microgram
µl	microlitre
w/w	weight per weight

SUMMARY

1. The main objective of this work was to investigate the possibility that the motor neuron exerts a trophic (as opposed to impulse mediated) control over rat skeletal muscle fibres by means of factors, elaborated in the nerve cell body and transported via axoplasmic transport to the terminals, where release is assumed to occur. The major strategy used was an attempt to block the axoplasmic transport of the postulated neurotrophic factors without impairing the impulse conduction of the nerve or neuromuscular transmission.

2. Colchicine, a drug known to interfere with axoplasmic transport, was injected subepineurally into the sciatic nerve of rats, and the extensor digitorum longus (EDL) muscle was studied by conventional electrophysiological techniques. When examined 4 to 5 days following this procedure, the EDL muscle fibres on the treated side exhibited extrajunctional sensitivity to acetylcholine (ACh), tetrodotoxin (TTX) resistant action potentials, and lowered resting membrane potentials (RMP) ; all these changes are characteristic of those following denervation of skeletal muscle fibres. The muscle fibres in the colchicine experiments however were shown not to be denervated since they displayed miniature end-plate potentials (m.e.p.p.s), exhibited transmitted action potentials, and neuromuscular transmission was essentially normal.

3. Unexpectedly, the contralateral EDL muscle fibres, which were examined as a routine control, also displayed the features of "denervation" following an injection of colchicine into the ipsilateral sciatic nerve, and were virtually indistinguishable from the ipsilateral EDL fibres. It was concluded that the subepineural sciatic nerve injections of colchicine were acting systemically, and indeed this was shown to be the case when similar denervation-like changes were produced bilaterally by intraperitoneal (I.P.) injections of the same doses of the drug.

4. Although these results showed that colchicine was acting systemically, it was still possible that its mechanism of action involved blocking axoplasmic transport in the muscle nerves. To test this possibility, axoplasmic transport was measured in the sciatic nerves of rats following colchicine injections, both systemically and into the contralateral nerve. Transport was measured by injecting ^{3}H-leucine into the spinal cord and then after an appropriate time analyzing the sciatic nerve for radioactivity. Using this method, no measurable impairment of axoplasmic transport could be detected at any time in sciatic nerves contralateral to nerve injections of colchicine, or in the sciatic nerves of animals following systemic injections of the drug, even though the EDL muscles of both sides displayed extrajunctional sensitivity to ACh. It was concluded that the extrajunctional sensitivity to ACh was not caused by interference with axoplasmic transport.

5. In the ipsilaterally injected sciatic nerve, an impairment of axoplasmic transport could be observed following colchicine injections. There was also an ipsilateral slight to moderate paresis, and a small percentage (5-7%) of apparently denervated fibres in the ipsilateral EDL muscle. In addition, the indirectly evoked tetanic tensions produced by the ipsilateral EDL muscles decreased in comparison to the contralateral EDL muscles, commencing about 2 days following the subepineural injection of colchicine. From these observations it was suggested that the demonstrable impairment of axoplasmic transport in the ipsilateral sciatic nerve caused the impairment of neuromuscular transmission observed on that side.

6. Colchicine was also chronically applied to rat sciatic nerves by the use of drug-impregnated silicone rubber nerve-cuffs. This procedure prevented the systemic effect of colchicine; however the presence of the cuff on the nerve resulted in a usually transitory conduction block of varying degree, causing for example a moderate to severe paresis on the second day following cuff application, which disappeared leaving no detectable clinical abnormalities by the 5th to 8th day. Since muscle inactivity per se also leads to denervation-like changes in the presence of a normal nerve, the changes that were observed in the ipsilateral EDL muscle fibres following cuff application could not be taken as evidence that neurotrophic factors had been blocked by the action of colchicine.

7. Since the action of colchicine in producing denervation-like changes in the EDL muscle fibres was not associated with an impairment of axoplasmic transport, the possibility of a direct action of the drug on the muscle fibre itself was considered. Preliminary experiments in which colchicine was used in association with denervation indicated that this is in fact the most simple and likely explanation of the mechanism of action of colchicine in causing extrajunctional sensitvity to ACh, the appearance of TTX-resistant action potentials and a lowered RMP.

8. The present state of the evidence supporting the existence of a neurotrophic control of skeletal muscle fibre characteristics is discussed.

PART I

GENERAL INTRODUCTION

THE OBJECTIVE OF THE PRESENT STUDY

THE OBJECTIVE

Communication between cells is one of the most important elements in the development and function of complex, multicellular organisms. Among all cell types, neurons are the most highly developed to perform the role of communication in the body, continually sending and receiving information by way of electrical impulses along their processes, and chemical transmitters at the junctions (synapses) between them. This aspect of their function in the transfer of information is now relatively well understood. In addition however, there are indications that neurons may also be involved in other forms of information transfer as well, the effects of which may produce long term physiological, biochemical and even morphological changes in their target tissues.

When nerves supplying sense organs such as taste buds are cut, these sensory structures atrophy and degenerate within a few days (Guth, 1971). Similarly, within 3 to 4 days after the motor nerve is cut, muscle fibres undergo marked physiological changes such as the development of supersensitivity to acetylcholine (ACh), and eventually they atrophy (Guth, 1968; Gutmann, 1976). Conversely, when certain sensory nerves are allowed to reinnervate their original tissues, new sense organs are often induced to differentiate, as in the case of taste buds. When motor nerves reinnervate denervated muscle fibres, the physiological characteristics of the fibres frequently can return to normal (Harris, 1974).

Two main arguments may be put forward as likely explanations of these phenomena. The first is that nerve activity is required by the innervated structures for the maintenance of their physiological and structural integrity, and the changes that are observed following denervation are merely the consequences of inactivity. While this argument may be valid for the motor system in which denervation results in complete paralysis of the involved muscles, it is not so readily seen as explaining the changes observed in denervated sensory structures. The functional activity of these organs is not dependent on an intact nerve, since the impulses which are initiated conduct centrally.

The other possibility to explain these phenomena is that the neuron may, in addition to conducting impulses, also exert some kind of metabolic influence which supports and maintains its target tissue. If this relationship then is interrupted by nerve section, the deprived end-organ may undergo degenerative or "atrophic" changes. This possible effect of nerves has been termed "trophic", in the sense that the metabolism of the target tissue appears to be influenced in a nutritive, or at least long-term fashion. This somewhat vague description encompasses all "denervation-changes" in the target tissue that cannot be directly attributed to a loss of impulses. In the present work, references to a "trophic" influence of the neuron are, unless otherwise stated, to be understood as describing the effects of the neuron which are apparently not mediated by impulses.

We are confronted with two seemingly irreconcilable hypotheses: on one hand, the possibility that nerve impulse activity <u>per se</u> is

sufficient to control end-organ characteristics, on the other, that the nerve exerts a trophic (non-impulse mediated) influence on the target tissue. For the motor system in particular, nerve activity and a possible trophic effect could be difficult to dissociate in practice, since denervation simultaneously deprives a muscle both of nerve impulses and of a possible trophic effect of the nerve.

The exact mechanism by which the nerve could exert a trophic influence is unknown. It is conceivable however, that chemical factors are elaborated by the neuron, transported down its axon to the terminals and released to influence its target cells. The purpose of this present investigation was to discover if a dissociation between nerve activity and its trophic influence could be achieved by interfering with the intra-axonal transport of these substances. The model selected for this study was the rat extensor digitorum longus (EDL) muscle and its nerve. Colchicine, a potent anti-mitotic drug has been demonstrated to block intra-axonal transport of materials (Dahlstrom, 1968; Kreutzberg, 1969). It was used therefore in an attempt to block the passage of trophic factors from the nerve cell bodies to the muscle, while leaving impulse conduction along the axons unaffected. The muscle was then studied for electrophysiological changes that could for example be compared to those caused by nerve section. In this way, it was hoped that specific effects that could be attributed to a loss of trophic factors would be distinguished.

PART II

HISTORICAL INTRODUCTION TO THE PROBLEM

1. TROPHIC PHENOMENA IN THE MOTOR SYSTEM: PHYSIOLOGICAL CHANGES IN SKELETAL MUSCLE FOLLOWING DENERVATION

The concept that nerves can exert a trophic influence on their target tissues has been most clearly supported by experiments in which the relationship between sensory nerves and their target tissues have been examined (to be discussed later), since activity of the end-organ does not appear to be involved in the phenomenon. For skeletal muscles however, neurally-evoked muscle activity ceases following denervation. Thus the denervation changes in muscles therefore could be the result of a loss of motor nerve impulses, a loss of trophic factors to the muscle, or a combination of the two. The difficulty lies in properly dissociating these events. Despite this, skeletal muscle has the advantage of undergoing a number of well-defined, distinguishable physiological changes following denervation, and thus may provide a good monitor of various changes which might be happening to its nerve.

a) SENSITIVITY TO ACh

Following denervation, the sensitivity of skeletal muscle fibres to the neural transmitter substance acetylcholine (ACh) is greatly increased. Brown (1937) used "close aterial injections" of ACh to excite cat gastrocnemius muscle; 6 - 7 days following denervation, the

dose required to elicit a mechanical response was reduced to about 1/1000 of that needed for normal muscle. Nicholls (1956) showed that this increased sensitivity to ACh could not be explained in terms of changes in the electrical constants of the muscle fibre membrane. He found that the specific membrane resistance (R_m) of denervated frog muscle fibres increased to twice normal values and consequently the length constant (λ) and the time constant (τ) of the membrane increased significantly as well; however these changes could not account for the great increase in the sensitivity to ACh seen in denervated fibres. It was generally believed that some alteration in the sensitivity of ACh receptors at the end-plate was responsible.

Axelsson & Thesleff (1959) investigated this possibility with the technique of iontophoretic application of ACh (Nastuk, 1953; del Castillo & Katz, 1955). This technique consists of ejecting a small amount of ACh from a micropipette which is usually filled with a strong solution of ACh chloride, by passing an electric current pulse through it. With this method, it is possible to apply extremely small quantities of ACh to a highly localized area of a muscle fibre. The effects of the ACh can be monitored by a nearby intracellular electrode which can record changes in the resting membrane potential (RMP). If the membrane at the site of ACh application is sensitive, a depolarization of a few millivolts (mV) can be observed. In this way it is possible to map the sensitivity to ACh of the entire length of a fibre, and Axelsson & Thesleff demonstrated by such experiments that in normally innervated muscle fibres, the sensitivity to ACh was confined solely

to the end-plate region. Following denervation however, the entire length of the muscle fibre became sensitive to ACh. This large change in the ACh - receptive area of the muscle fibre occurs at the same time that supersensitivity to ACh had been observed in earlier experiments, and thus could account for "denervation supersensitivity".

b) EXTRAJUNCTIONAL ACh RECEPTORS

The "extrajunctional" ACh-sensitivity following denervation can be shown to be related to the appearance of ACh receptors in the extrajunctional membrane. By using radioautographic methods and a radioactively labelled neurotoxin, ^{125}I-α-bungarotoxin, which binds specifically and strongly to ACh receptors (Lee, Tseng & Chiu, 1967), the sites of the ACh receptors can be visualized. With this technique, Hartzell & Fambrough (1972) demonstrated binding of labelled bungarotoxin along the entire length of denervated muscle fibres, whereas in normally innervated fibres it was confined to the end-plate region. One possibility which has been considered is that ACh receptors are always present in the extrajunctional membrane, but are normally covered or concealed in some way (Blunt, Jones & Vrbova, 1975). Recently however, it was demonstrated that these extrajunctional ACh receptors are newly synthesized following denervation (Devreotes & Fambrough, 1975; Brockes, Berg & Hall, 1975). The possibility that pre-formed extrajunctional receptors may have spread from the end-plate region following denervation is made unlikely by the observation of Katz & Miledi (1964) that segments of muscle fibres separated from their

end-plate regions are also capable of developing ACh receptors.

c) RESTING MEMBRANE POTENTIAL (RMP)

Mammalian skeletal muscle fibres exhibit RMPs of about -80mV *in vivo*. Following denervation however, the RMP drops about 20mV to reach a final value of about -60mV (Ware, Bennett & MacIntyre, 1954; Locke & Solomon, 1967; Thesleff, 1974). The depolarization of muscle fibres starts at the end-plate region and may be observed as early as 2 hrs following denervation (Albuquerque, Schuh & Kauffman, 1971). Within about a day, the entire length of muscle fibre may exhibit a lowered RMP. The mechanism for this drop in RMP following denervation is unknown. It may however be related to the findings that in denervated muscle fibres there is a decreased influx of radioactive potassium ^{42}K and thus the transport or permeability of potassium ions (K^+) may be diminished (Dockry, Kernan & Tangeny, 1966).

d) FIBRILLATION

Another well known phenomenon exhibited by denervated skeletal muscles is a spontaneous, asynchronous twitching of individual muscle fibres. This activity of denervated muscle has been termed fibrillation. The exact mechanism of this activity is not fully understood, but it appears that it is due to biphasic oscillations in the membrane potential of these fibres (Thesleff & Ward, 1975), which fire off action potentials when they reach threshold, thereby causing these fibres to

twitch. Furthermore, it appears that these oscillations are initiated at the site of former end-plates (Belmar & Eyzaguirre, 1966). Previously, it had been thought that a cholinergic mechanism might be involved in fibrillation, especially in view of the fact that denervated muscles undergo a large increase in sensitivity to ACh. This explanation however is made unlikely by the findings that curare, a drug which blocks the action of ACh on muscle, has no effect on fibrillation (Rosenblueth & Luco, 1937; Belmar & Eyzaguirre, 1966).

e) RESISTANCE TO TETRODOTOXIN (TTX)

Following denervation, the spike-generating mechanism of skeletal muscle fibres becomes partially resistant to the effects of the puffer fish poison, tetrodotoxin (TTX, Redfern & Thesleff, 1971). TTX has a highly selective blocking action on the "sodium channels", the membrane changes associated with the increased conductance of sodium ions (Na^+) during the muscle membrane action potentials (Kao, 1966). Normally innervated muscle fibres are unable to generate action potentials when TTX is in the bathing fluid in concentrations of 10^{-6}M. Denervated muscle fibres however, are able to produce regenerative spikes (action potentials) under these conditions.

2. EXPERIMENTS SUPPORTING A POSSIBLE NEUROTROPHIC CONTROL OF SKELETAL MUSCLE FIBRES

a) EFFECT OF NERVE STUMP LENGTH

One of the most compelling pieces of evidence in favour of a neurotrophic control of skeletal muscle fibres has been the observation that the time to the appearance of changes in skeletal muscle after denervation is dependent on the length of the distal nerve stump left after the nerve has been sectioned. When motor nerves are severed close to their muscles, denervation changes occur sooner than if the nerve is sectioned farther away. It is interesting however, that this kind of phenomenon, and its likely explanation was first demonstrated in the sensory system (see later). Luco & Eyzaguirre (1955) found that the onset of fibrillation in the cat tenuissimus muscle and of its supersensitivity to intra-arterially injected ACh, was dependent on the length of the nerve stump left connected to the muscle. Emmelin & Malm (1965) and Salafsky, Bell & Prewitt (1968) corroborated these observations. In the cat tongue, Emmelin & Malm (1965) noted an earlier onset of supersensitivity to intra-arterially injected ACh with short nerve stumps than with long, and similarly Salafsky, Bell & Prewitt (1968) demonstrated that fibrillation potentials in rat muscles appeared sooner with a short nerve stump than with a long one. The range of values for nerve lengths and times were studied by

Harris & Thesleff (1972) who observed in rat muscle that there was a 2 hr delay in the appearance of the phenomenon of the appearance of TTX-resistant action potentials for each additional 1 cm of nerve stump left. This relationship between nerve stump length and the time of onset of denervation changes suggested that substances required by the muscle may be flowing down the distal stump. From their results, Harris & Thesleff suggested that substances required by the muscle may be flowing at a rate of about 120 mm per day in the nerve.

Miledi and Slater (1970) examined the relationship between nerve stump length and the time to failure of neuromuscular transmission in the rat diaphragm. Following section of the phrenic nerve, failure was complete at about 20 hrs. The time to failure could be delayed about 45 minutes for every 1 cm of increased nerve stump left intact. This observation also suggested a flow of materials down the nerve stump, and in this experiment a rate of 320 mm per day could be calculated. Neuromuscular transmission failure was accompanied by cessation of miniature end-plate potentials (m.e.p.p.s). The functional failure of the nerve terminal was closely paralelled by ultrastructural changes. Mitochondria in the denervated nerve terminals began to assume spherical shapes instead of their normal elongated ones within a few hours after denervation. Vesicles in the terminals were disrupted, and the terminal Schwann cell started to become phagocytic and engulf the nerve ending.

Locke & Solomon (1967) observed that not only was the time of onset of depolarization of muscle fibres dependent on the stump

length, but also its rate of fall as well. Albuquerque, Schuh & Kauffman (1971) also examined this relationship. As early as 2 hrs following a nerve crush at its point of entry in the rat EDL muscle, they observed a drop in the RMP of about 6 to 8 mV at the end-plate of muscle fibres. This drop occurred at a time when neuromuscular transmission, as evidenced by the presence of m.e.p.p.s, was still normal. The onset of depolarization of the muscle fibres which began at the end-plate region was dependent on the intra-muscular nerve stump length; those muscle fibres with shorter intra-muscular stumps began to depolarize sooner than those with longer ones.

b) PARTIALLY DENERVATED MUSCLE FIBRES

A particularly important piece of evidence in favour of trophic actions of the motor nerve on skeletal muscle was obtained by Miledi (1960) on frog sartorius muscle. The fibres in this muscle are dually innervated, affording Miledi the opportunity to obtain partially denervated muscle fibres. The partially denervated fibres were presumably still normally active since they retained innervation through the one remaining end-plate. Around the site of the denervated end-plate, Miledi observed an increase in the amount of extrajunctional sensitivity to ACh. This was interpreted to be the result of a lack of trophic control of the muscle fibre membrane at this region rather than a result of inactivity of the muscle fibres.

c) CROSS REINNERVATION STUDIES

Cross reinnervation studies of "fast" and "slow" skeletal muscles were initially interpreted as a demonstration of a trophic effect of the motor nerve (Buller, Eccles & Eccles, 1960). Skeletal muscles can be placed in two categories based on their speed of contraction. One group is characterized by a relatively slow response to a stimulus and these muscles are called slow twitch muscles. Typically, in the cat, the time to peak tension (TP) for a single twitch is about 75 msec. The other group contains fast twitch muscles; such a muscle, in the cat, may have a TP of about 18 msec. Representative of slow and fast twitch muscles are the soleus (SOL) and the flexor hallucis longus (FHL) respectively. When a nerve originally going to a fast twitch muscle in the cat (kitten) is cut and then made to reinnervate a denervated slow twitch muscle, the slow muscle acquires characteristics of the fast one (Buller, Eccles & Eccles, 1960). The TP for the slow muscle significantly shortens. Similarly, in the reverse experiment, the TP for the fast muscle lengthens.

Though these results were believed to be due to a trophic effect of the nerve, the possibility that the pattern of nerve impulses arriving at the muscle could account for this phenomenon was not excluded. Indeed, it was shown (Eccles, Eccles & Lundberg, 1959) that nerves going to slow muscles had a much lower discharge rate (10-30 Hz) than nerves innervating fast muscles (30-60 Hz). The

concept that patterns of nerve impulses may account for some dynamic properties of muscles has had a lot of support (Eccles, Eccles & Kozak, 1962; Salmons & Vrbova, 1969; Lømo, Westgaard & Dahl, 1974). The latter workers demonstrated that the dynamic properties of denervated skeletal muscles could be altered simply by altering the frequency at which the muscles were stimulated by chronically implanted electrodes. Denervated rat soleus stimulated at a high frequency (100 Hz) had a TP of 16 msec in comparison to 35 msec for the normally innervated soleus, and 41 msec for denervated unstimulated muscles. Denervated soleus muscles stimulated at a low frequency (10 Hz) had a TP of about 33 msec, in the range of normally innervated soleus muscles. It thus appears that the discharge rate of the motor neuron may play a significant role in determining the dynamic properties of skeletal muscle fibres.

d) THE ROLE OF DEGENERATING NERVE TERMINALS

One possible explanation for the early denervation changes seen in skeletal muscles is that they are triggered by the degeneration of the motor nerve terminals in the muscle (Jones & Vrbova, 1974; Lømo & Westgaard, 1976). As yet, there is no solid evidence for such a view, but there is some circumstantial evidence which must be taken into consideration. Direct electrical stimulation of denervated muscles (see later) can often prevent the development of denervation changes, and even reverse them if they have been allowed to occur (Lømo & Westgaard, 1976). However, if the frequency of stimulation

is relatively low (10 Hz), it cannot prevent an early transient increase in extrajunctional sensitivity to ACh, although if it is continued for long periods (about 10 days) this level of stimulation is adequate to reverse all denervation changes (Lømo & Westgaard, 1975). The early transient increase in extrajunctional sensitivity to ACh has been attributed to the presence of degenerating nerve terminals. Lømo & Westgaard (1976) obtained soleus muscles which had been functionally hyper-innervated by the fibular nerve; they found that after cutting this foreign nerve there was an increase in sensitivity to ACh at the region of the degenerating nerve terminals.

The concept that degenerating nerve terminals can trigger the early denervation changes could be used to explain the relationship observed between the length of the distal nerve stump and the onset of denervation changes. Miledi & Slater (1970) have shown that the viability of the motor nerve terminal in the rat diaphragm is dependent on the length of its distal nerve stump. Thus with a short nerve stump, the motor nerve terminals would begin to degenerate sooner and trigger denervation changes in the muscle at an earlier time than with a longer nerve stump.

e) A POSSIBLE NEUROTROPHIC CONTROL OF SKELETAL MUSCLE CHOLINE ESTERASE ACTIVITY (ChE)

Following denervation, levels of choline esterase (ChE) activity at skeletal muscle fibre end-plates rapidly decreases (reviewed by Guth, 1968). The reason for this is not known but could be related

to a loss of a possible trophic influence of the nerve. Filogamo & Gabella (1966) demonstrated that in the chicken, an intact nerve supply is required for the maintenance of end-plate ChE. Treating the animals with difluorophosphate (DFP) which is an irreversible inhibitor of ChE, and using histochemical techniques, they showed that ChE activity gradually reappeared at end-plates in normally innervated muscles but did not reappear in muscles that had been denervated at the time of DFP treatment. Thus it appeared that the nerve was required for the re-establishment of normal ChE levels. In organ culture studies of newt skeletal muscle, Lentz (1971) demonstrated that the consequent loss of ChE from the muscles could be significantly retarded or even reversed by addition of newt sensory ganglia or nerve homogenates to the culture environment. This suggested that some diffusable factor from the nervous tissue could influence the ChE levels in the explanted muscle. Rathbone, Beresford & Yacoob (1974) confirmed these observations and extended them by demonstrating that 10 day old chick brain homogenates could also maintain ChE levels in newt muscle explants. Furthermore, they showed that the active factor in these homogenates was heat labile and sensitive to dialysis and trypsin digestion. It was thus concluded that the factor was, or at least contained in part as an active agent, a peptide. Though these studies look extremely promising, it still remains to be directly shown that a specific neurotrophic substance is in fact controlling the level of end-plate ChE in skeletal muscle.

3. THE IMPORTANCE OF MUSCLE ACTIVITY IN CONTROL OF MUSCLE FIBRE CHARACTERISTICS

One approach in the study of the motor nerve's influence on skeletal muscle has been an attempt to dissociate the possible effects of nerve impulses from a trophic influence of the nerve. The former (i.e. nerve impulses) has proven to be more accessible to experimental manipulation than the latter (trophic influences) and thus most strategies to date have involved examining the effect of altering motor nerve impulses going to skeletal muscle.

a) DIRECT ELECTRICAL STIMULATION OF DENERVATED MUSCLES: REPLACEMENT OF NERVE ACTIVITY

One experimental technique which has had considerable success in explaining why skeletal muscles undergo changes following denervation is direct electrical stimulation of denervated muscles. Both nerve impulses and a possible trophic influence of the nerve have been simultaneously removed from such muscles. By stimulating the muscles directly however, an attempt is made to mimic the normal excitatory influence of the nerve, and perhaps then reveal the residual effects due to the absence of a neurotrophic influence.

In every report published to date, direct electrical stimulation has significantly diminished or completely reversed the usual denervation

changes, and it now seems clear that activity per se of skeletal muscles may be a very important factor in the control and expression of its properties. Drachman & Witzke (1972) chronically stimulated denervated rat diaphragm in vivo for a period of 4 days. At the end of this time, they demonstrated that the extrajunctional regions of muscle fibres in the stimulated muscles were much less sensitive to iontophoretically applied ACh than usually observed in denervated muscle fibres. The stimulated denervated muscle fibres exhibited average extrajunctional sensitivities of about 4 units (see Methods for full description of how this is measured) compared to about 50 units in unstimulated ones. Similarly, Lømo & Rosenthal (1972) demonstrated that chronic electrical stimulation of denervated rat soleus and EDL muscles for 5 to 8 days could markedly lower the level of extrajunctional sensitivity to ACh. Furthermore, electrical stimulation could largely prevent the onset of ACh sensitivity when stimulation was begun immediately following denervation. This result was confirmed by Jones & Vrbova (1974) who used the rat soleus muscle. In the latter experiments however, they found that stimulation could never eliminate all extrajunctional sensitivity to ACh, even though its level was markedly reduced. Purves & Sakmann (1974) similarly showed that stimulation of denervated rat diaphragm strips in vitro for up to 7-8 days significantly reduced the level of extrajunctional sensitivity to ACh. Furthermore, the stimulation of the diaphragm strips prevented these muscles from fibrillating for periods up to several days.

One intriguing question with respect to fibrillation had been: if muscle fibre activity per se is the factor which controls muscle fibre characteristics, why is it that denervation changes are expressed at all in denervated fibres which are known to be highly active in the form of fibrillation? A possible answer to this was provided by Purves & Sakmann (1974), who demonstrated that only 1/4 to 1/3 of the total number of muscle fibres in the denervated rat diaphragm were fibrillating at any one time, and that these fibres would fibrillate for periods of about 20 hrs and then remain quiescent for the next 2 to 3 days. This period of inactivity, it can be argued, allows for the expression of denervation changes, and the ensuing 20 hrs of activity is not adequate to eliminate them.

b) THE EFFECT OF CONDUCTION BLOCK IN THE NERVE

Several techniques have been used to deprive skeletal muscles of nerve impulse activity without otherwise interrupting their nerve supply. Lømo & Rosenthal (1972) paralyzed rat EDL and soleus muscles by placing silicone cuffs impregnated with local anesthetics around the sciatic nerve. This procedure prevented nerve impulses from arriving at the muscles, but it did not otherwise appear to produce any ill effects. Seven days after the application of the anesthetic cuffs, the paralyzed EDL and soleus muscles exhibited high levels of extrajunctional sensitivity to ACh, similar to those seen in denervated muscles. They also observed fibrillation of the muscle fibres. Robert & Oester (1970), who had previously introduced the cuff

technique, failed to observe any changes in rabbit anterior tibialis muscle even after two weeks of continuous blockade of impulses in the sciatic nerve. At the moment, there is no good explanation for the discrepancy between these two results.

One possible criticism of these blocking experiments is that the local anesthetics (Lidocaine, bupivicaine) may at the same time block axoplasmic transport of trophic factors to the muscles (Fink, Kennedy & Hendrickson, 1971; Drachman, 1974; Bisby, 1975). To overcome this possibility, Cangiano (Cangiano, Lutzemberger & Zorub, 1975) produced nerve conduction block by applying a mildly compressive silicone cuff, which did not contain any drug; impulse conduction was blocked merely by the slight compression of the nerve. In preliminary experiments, he demonstrated that EDL muscles paralyzed in this fashion exhibited changes such as lowered RMP, TTX-resistant action potentials and fibrillatory activity. Interestingly, these changes were less pronounced than similar denervation changes in muscles that had been denervated for the same length of time. Similarly, Drachman (1976) blocked nerve impulse conduction in the rat sciatic nerve by nerve injections of TTX. He demonstrated that this procedure did not interfere with axoplasmic transport. The muscles paralyzed in this fashion exhibited an increase in the number of extrajunctional ACh receptors. It therefore appears that muscle activity per se is very important, at least in part, in the control of skeletal muscle fibre characteristics.

c) BLOCK OF NEUROMUSCULAR TRANSMISSION

Another manner in which inactivity of skeletal muscle can be produced is by blocking neuromuscular transmission. This can be achieved with pharmacological agents such as botulinum toxin which prevents the release of ACh from the nerve terminals (Brooks, 1956) or with agents like curare, which bind to ACh receptors and so hinder or totally prevent ACh from producing the usual excitation of the muscle fibres (Taylor, 1959). In every experiment to date, muscles which have been paralyzed as a result of neuromuscular transmission block have exhibited denervation-like changes. Thesleff (1960) demonstrated that the injection of botulinum toxin into the hindlimb of cats led to the appearance of extrajunctional sensitivity to iontophoretically applied ACh in the tenuissimus muscle, at about the same time as this takes to appear after denervation. Drachman (1974) demonstrated that neuromuscular block by botulinum toxin, curare and hemicholinium in the chick embryo, produced atrophy, degeneration and fatty replacement of skeletal muscle, identical to the effects observed after denervation. Hemicholinium produces a block by interfering with the uptake of choline by the nerve terminal, leading to the depletion of ACh inside, and failure of release of ACh from the nerve terminals. Even though the three blocking agents have different mechanisms of action, their results were the same. This indicated that normal cholinergic transmission is therefore required by chick embryo skeletal

muscles for proper maintenance.

Berg & Hall (1975) paralyzed rats for up to 3 days by chronic administration of the postsynaptic blocking agents D-tubocurarine (curare), succinylcholine or α-bungarotoxin. These agents do not affect the release of ACh from the nerve terminal. After 3 days of paralysis an increase was observed in the sensitivity to iontophoretically applied ACh of the extrajunctional membrane of the diaphragm fibres. This was associated with increased binding of radioactively labelled α-bungarotoxin to the extrajunctional membrane. The muscle fibres also exhibited TTX-resistant action potentials, as do denervated muscle fibres. Similarly, Chang, Chuang & Huang (1975) paralyzed rats for 7 days using hemicholinium and bungarotoxin. At the end of this period, they observed an increase in the binding of labelled bungarotoxin to the extrajunctional areas of rat diaphragm muscle fibres.

It therefore can be concluded that an interference with neuromuscular transmission can result in the appearance of denervation changes in the skeletal muscle fibres.

d) LIMB IMMOBILIZATION

Several investigators have tried to assess the importance of mechanical activity of the muscle in the control of its characteristics by immobilizing limbs. This can be achieved in the hindlimb of an animal by surgically placing pins through knee and ankle joints. Generally, these types of studies have demonstrated muscle atrophy; however this is accompanied by very little other changes. Fischbach &

Robbins (1971) pinned the knee and ankle joints in the hindlimb of rats and examined the soleus muscle in the consequently disused limb 3 days to 6 weeks following this procedure. They observed only a very small increase in extrajunctional sensitivity to iontophoretically applied ACh 8-10 days after the onset of disuse. Solandt, Partridge & Hunter (1943) had previously used this technique and reported an increase in sensitivity to intra-arterially injected ACh in the rat gastrocnemius muscle following 2 weeks of disuse. Generally however, very little denervation-like changes occur in immobilized muscles and it could be argued that this is an indication of the maintained trophic influence of the nerve. However, although these muscles are mechanically fixed, their motor nerves are not necessarily quiescent. Fischbach & Robbins (1968) demonstrated that although the activity of the motor nerve is greatly diminished in immobilized limbs, there is still 5 to 15% of the normal EMG activity present. This suggests that a low level of nerve activity may be adequate to influence the immobilized muscle, and largely prevent the appearance of denervation changes (cf. Lømo & Westgaard, 1975).

4. EFFECTS OF THE MOTOR NERVE DURING DEVELOPMENT

During primary development, rat skeletal muscle fibres are sensitive to ACh along their entire length (Ginetzinsky & Shamarina, 1942; Diamond & Miledi, 1962) as they are in denervated muscles.

However, following the establishment of neuromuscular activity, the area sensitive to ACh becomes progressively reduced, and within a few weeks of birth only the end-plate region of the muscle fibre is sensitive. This observation has generally been interpreted to indicate a trophic influence of the nerve, but the possibility that the ACh sensitivity became localized as a result of muscle activity was not excluded.

Speculation concerning the reason why developing and denervated muscle fibres are sensitive to ACh along their entire length has raised the possibility (Katz & Miledi, 1964) that this phenomenon constitutes a "signal" to developing or regenerating nerves to guide them and induce them to form synapses. Whether this is the case or not is unresolved, but it has been demonstrated that blocking ACh receptors in developing or denervated muscles with curare (Cohen, 1972) or with α-bungarotoxin (Jansen & Van Essen, 1975) does not prevent the formation of functional synapses on the muscles. Thus it appears that end-plates can be formed even if the ACh receptors are pharmacologically blocked.

The induction of sites of high sensitivity to ACh (hot spots) on developing muscle fibres in vitro, co-cultured with nervous tissue, has also been thought to be due to a trophic influence of the motor nerve (Steinbach, Harris, Patrick, Schubert & Heinemann, 1973). Such an influence of the nerve on the distribution of ACh receptor sites would be especially important in relation to the formation of end-plates during synaptogenesis. In tissue culture, dissociated neurons will extend axonal and dendritic processes to form synaptic

connections with other neural cells (Varon & Raiborn, 1969; Crain & Bornstein, 1972) and with dissociated myoblasts (Shimada & Fischman, 1973). The point of nerve and muscle cell contact *in vitro* is associated with an increased ACh-sensitivity on the muscle (Steinbach, 1974), reminiscent of the localized ACh-sensitivity found *in vivo*. This localization of sensitivity to ACh does not appear to be dependent on muscle fibre activity *per se* since it can occur in a tissue culture environment in which muscle fibre activity has been blocked by high K^+ in the medium (Steinbach, 1974).

Although the localization of ACh-sensitivity on developing muscle fibres *in vitro* may be an example of a trophic effect of the nerve, there is some suggestion that "Hot spots" may develop in chick myotubes that had not yet been exposed to nerves. Berkoff & Betz (1976) mapped the sensitivity of chick myotubes to iontophoretically applied ACh. The myotubes were obtained by culturing myoblasts from chick hindlimb buds, removed at a time before innervation had occurred. In these myotubes, Berkoff & Betz observed "hot spots" and they concluded that the development of these sites of high sensitivity to ACh is not dependent on prior contact with nerve processes. Similarly, Prives, Silamn & Amsterdam (1976), demonstrated with ^{125}1-α-bugarotoxin labelling and autoradiographic techniques that the appearance, condensation into clusters and gradual depletion of ACh receptors in cultured chick myotubes appear to be sequential events in the differentiation of skeletal muscle which can occur in the absence of a direct neuronal influence.

Generally, it is unclear whether or not the appearance of extrajunctional ACh receptors following denervation of skeletal muscles plays any significant role in their reinnervation. Similarly, in primary development, the role of the nerve in establishing the adult pattern of ACh-sensitivity is not resolved.

5. TROPHIC PHENOMENA IN THE SENSORY SYSTEM

The concept that nerves can exert a trophic influence on their target tissues not only has arisen from, but is still most clearly supported by experiments in which the relationship between sensory nerves and their target tissues have been examined. In many instances, when a sensory nerve is cut, its end-organ suffers marked atrophy and degenerative physiological changes.

a) TASTE BUDS

Following section of the gustatory nerve, taste buds begin to degenerate and eventually disappear in fish and mammals (Olmsted, 1920; May, 1925; Parker, 1932). Parker (1932) found that after the nerve was allowed to regenerate and reinnervate its target tissue, taste buds could once again be observed. The presence of intact innervation thus seems to be necessary to support the existence of taste buds. In addition, Parker (1932) also observed that the latent period between the cutting of the nerve and the onset of atrophic changes in the taste buds was roughly proportional to the length of the distal nerve stump.

After cutting the lateral line nerve of the catfish, degeneration of the sense organs (similar to taste buds) along the lateral line spread proximo-distally from the cut at a rate of about 2 cm per day (Parker, 1932; Parker & Paine, 1934). These observations suggested that the latent period might be due to gradual depletion from the peripheral stump of the cut nerve of substances required for the sustenance of the taste buds and lateral line sense organs. When these substances were used up, which would take longer the greater the length of the nerve stump, the taste buds would be affected. Electrical stimulation of the distal nerve stump was reported by the same workers to hasten the disappearance of the taste buds, a phenomenon which could possibly be due to acceleration of movement or metabolism of substances in the nerve (Parker & Paine, 1934).

The simplest interpretation of these observations is that the nerve cell elaborates and releases from its terminals a hormone-like substance that is required to maintain the structural integrity of the sense organ (Olmsted, 1920). The cell population in taste buds has a high turnover rate (Beidler & Smallman, 1965). It is thus likely that as cells in the taste bud die, they are continuously replaced by adjacent epithelial cells which differentiate as a result of a neural influence. When the tissue is denervated, taste bud cells are no longer induced to differentiate and as a consequence of normal cellular turnover, the taste buds disappear. It is well known that the presence of nerve fibres always precedes the appearance of taste buds (Landacre, 1907; Olmsted, 1920b); thus it seems possible that

in fish and mammals at least, the role of the nerve is to induce the differentiation of epithelial cells to replace old taste cells, rather than to maintain the structural integrity of pre-formed taste bud cells.

b) MUSCLE SPINDLES

The muscle spindle, a sensory organ of skeletal muscles, is also highly dependent on its sensory innervation, but this dependence is apparent only during a certain stage of development. If the innervation of muscle by sensory fibres is prevented in developing rat muscle by intra-uterine severance of the sciatic nerve in fetal rats, no spindles are formed in the denervated muscles (Zelena, 1957; Zelena & Hnik, 1960 ab). If the muscle is denervated by crushing the sciatic nerve at birth, the half-formed spindles degenerate within 10 days and on reinnervation, only a few are found to have developed again, some 5 months later. If the same experiment is performed on 14 day old rats, when spindle development is almost complete, there is little, if any spindle degeneration, and they can be successfully reinnervated. It seems that the sensory nerve is required for the initial differentiation of these sensory structures, but after they mature they are no longer dependent on these nerves.

c) OTHER MECHANORECEPTORS

Certain other mechanoreceptors have also been shown to be dependent on sensory nerves for their development and maintenance. In primary development, these mechanoreceptors differentiate only after

sensory nerves have invaded the tissues from which they arise (Jacobson, 1970). Following denervation, the same mechanoreceptors atrophy or degenerate completely, indicating also a long term dependence on the nerve.

d) HERBST AND GRANDRY CORPUSCLES

The histogenesis of Herbst and Grandry corpuscles, mechanoreceptors which have been especially studied on the beak and tongue of the duck, is completely dependent on the presence of sensory nerve endings (Saxod, 1972). Only somatosensory nerves are able to have this effect, and sympathetic and motor fibres cannot induce these sensory structures to differentiate (Saxod, 1972). Herbst and Grandry corpuscles furthermore begin to degenerate when their nerve, the opthalmic branch of the trigeminal nerve, is crushed (Quilliam, 1962). While the Grandry corpuscles degenerate almost completely after about 48 days, the Herbst corpuscles in contrast atrophies but maintains a recognizable structure. Upon reinnervation, Grandry corpuscles reappear and Herbst corpuscles reassume their normal appearance.

Although the nerve appears to cause the appearance of these end-organs, the actual type which differentiates appears to be a characteristic of the epithelium rather than that of the nerve. This was demonstrated by Saxod & Sengel (1968), who transplanted skin from the beak of duck embryos to the chorioallantoic membrane or to other parts of the body of chick and duck embryos. In the absence of innervation, no sensory corpuscles developed. In transplanted skin that

became innervated, the corpuscles which developed were those characteristic of the skin even when their innervation was by "foreign" sensory nerves. Thus even though sensory nerves appear to trigger and support the differentiation of Herbst and Grandry corpuscles, it is the nature of the epithelium however that determines what type of sensory receptors will be formed.

e) PACINIAN CORPUSCLES

Pacinian corpuscles, another type of mechanoreceptor widely distributed in mammals, also requires the presence of a sensory nerve for induction and long term maintenance. Zelena (1976) observed that when the sciatic nerve in newborn rats is cut, developing Pacinian corpuscles in the interosseous membrane disintegrate and disappear within 10 days. Without the nerve supply, no Pacinian corpuscles were observed to form. In the cat, the mature Pacinian corpuscles, like the Herbst corpuscle, does not disappear when it is denervated, although it undergoes degenerative changes within 6 to 12 hrs following nerve section (Chouchkov, 1976). When foreign sensory nerves such as the femoral or saphenous are made to innervate the cat mesentery, they too are capable of inducing the formation of Pacinian corpuscles (Chalisova & Ilyinsky, 1976). This result is similar to the developmental aspects of the Herbst and Grandry corpuscles mentioned above, in that the specificity of the sensory receptor is determined by the tissues, but the stimulus of a sensory nerve is required to initiate differentiation.

f) MERKEL CELLS

Merkel cells are cutaneous mechanoreceptors. When the nerve supplying the tactile pads in adult cats is cut, the Merkel cells eventually disappear (Iggo, 1963; Kasprzak, Tapper & Craig, 1970). Upon nerve regeneration into the pad, the specialized Merkel cells reappear. Palmer (1965), after ligating the infra-orbital nerve in the opossum, reported that Merkel cells could not be identified by electron microscopy, 72 hrs later. In the cat and the opossum therefore, the sensory nerve is required to maintain the Merkel cell. In the rat however, Merkel cells appear to be less dependent on the nerve. Even for periods as long as 3 months, Merkel cells persist following nerve degeneration according to Kasprzak, Tapper & Craig (1970). Smith (1967) similarly demonstrated a lack of dependence of the Merkel cell on the sensory nerve in the rat. He removed dorsal root ganglia, and even when there was a complete absence of axon terminals, no change in the structure of Merkel cells was observed up to 90 days after the operation. Similar findings to these have been reported for Merkel cells in the salamander skin. Cooper, Scott & Diamond (1976) observed that Merkel cells did not disappear from salamander skin even after 6 months of denervation. Furthermore, they observed that these specialized cells appeared in the regenerating skin of denervated limbs of adult animals, suggesting that in this case, a nerve influence was not required for their induction.

g) SENSORY NERVE FIELDS IN SALAMANDERS

The establishment of peripheral sensory nerve fields may also be under a trophic control by the sensory neuron (Aguilar, Bisby, Cooper & Diamond; 1973; Diamond, Cooper, Turner & MacIntyre, 1976). By applying colchicine to a peripheral nerve in salamander hindlimb, Diamond and his colleagues demonstrated that the adjacent sensory nerves enlarged their terminal fields, presumably by sprouting of their endings. These results were interpreted as suggesting that a trophic factor released by the sensory nerve was involved in the regulation of sensory nerve fields. This work appears to be additional powerful evidence indicating the presence and importance of a non-impulse mediated influence of the neuron, at least in the sensory system.

h) SUMMARY

The presence of the sensory nerve seems crucial for the primary development of many sensory end-organs. However the role of the nerve in the long term maintenance of these structures is less clear. Perhaps only those end-organs which are comprised of cell populations which have high turnover rates such as taste buds, are totally dependent on the sensory nerve, suggesting that the influence of the sensory nerve is most pronounced on undifferentiated cells, and diminishes with their specialization.

6. THE TRANSPORT OF MATERIALS IN NERVES

A possible mechanism by which nerves could achieve a trophic control of their target tissues might involve the elaboration of trophic factors by the nerve cell body, transport of these factors to the nerve terminals and the consequent release of these factors in the region of the target cells.

a) OBSERVATIONS SUGGESTING FLOW OF MATERIALS IN NERVES

The concept that substances flow in nerves is ancient, but evidence supporting such a flow has only recently been obtained. Scott (1906) was one of the first to conduct experiments which suggested that there was a passage of materials from the cell body to the nerve terminals. He demonstrated that prolonged intensive stimulation of the central end of a frog dorsal root which had been sectioned to exclude the ganglion, resulted in irreversible failure of reflex activity at a much earlier time than in the case of a root in which the connection with the ganglion was intact. Furthermore, roots connected to their ganglia could, after a period of rest, recover their ability to evoke reflex responses to stimulation in contrast to cut roots. Scott thus demonstrated that the nerve cell body was an important factor in the maintenance of the reflex function of the axon. The mechanism for this he suggested, was a passage of substances from the nerve cell body to

the nerve endings.

The existence of such a mechanism received strong support from the experiments on taste buds, as mentioned before, in which the onset of degeneration depended on the length of the remaining distal nerve stump. The most likely explanation for the phenomenon appeared to be that the nerve released substances required to support taste buds and that these factors could be passing down the nerve axon from the cell bodies (Parker, 1932; Parker & Paine, 1934).

b) SLOW AXOPLASMIC FLOW

Weiss & Hiscoe (1948) were the first to provide direct evidence for the existence of a proximo-distal flow of materials in nerve axons. They placed partially constricting cuffs around nerves, and observed that proximal to the cuffs, there was a swelling of the nerve. They concluded that a proximo-distal flow of axoplasm was being dammed up. When the obstruction on the nerve was released, the axoplasm which had accumulated began to move at rates estimated at 1 to 3 mm per day. This movement of axonal material has been called slow axoplasmic flow.

The study of the intra-axonal movement of substances became more refined with the advent of autoradiograhic and radioactive tracer techniques. Droz & Leblond (1963), were able to demonstrate that following injections of ^{32}P- and ^{3}H-labelled compounds in mice, radioactive material appeared to have moved through the interiors of nerve cells and axons at a rate of about 3 mm per day. Following injections of ^{32}P- into the spinal cord of cats in the vicinity of spinal motor neurons,

Ochs and his colleagues (Ochs & Burger, 1958 ; Ochs, Dalrymple & Richards, 1962) were able to demonstrate that radioactively labelled material was moving in the sciatic nerve towards the periphery at a rate of about 4.5 mm per day.

Although the mechanism of slow transport is not clear, it appears that there must be a continuity between the cell body and its axon for the process to continue. In rabbit vagus nerves, Frizell, Mclean & Sjostrand (1975) demonstrated that ligatures placed on the nerve stopped slow transport of ^{3}H-labelled protein in the distal portion of the nerve whereas the transport in the proximal section was left intact.

The fate of the proteins transported in axons is unclear. The major portion of the material is undoubtedly destined to be used by the axon itself; however, some of it may be transferred across synapses to other neurons. Grafstein (1971) demonstrated that following injections of ^{3}H-proline into the posterior eye chamber in mice, a significant amount of radioactivity could be observed in the visual cortex, suggesting that a transneuronal transfer of labelled material had occurred. Such a transfer of material could be the basis of the mechanism of nerve trophic action.

c) FAST AXOPLASMIC TRANSPORT

In addition to the slow rate of flow described above, there is a much faster transport system in nerve axons. Ochs and coworkers (Ochs & Ranish, 1969 ; Ochs & Johnson, 1969) demonstrated that when a labelled amino acid such as ^{3}H-leucine is injected into a dorsal root

ganglion in the cat, a wave of radioactivity can be found travelling outwards from the cell body and down the sensory axons at rates of approximately 400 mm per day. Similar rates were obtained in motor nerve fibres when the ^{3}H-leucine was injected into the ventral portion of the spinal cord. The ^{3}H-leucine injected into a dorsal root ganglion or the spinal cord is very rapidly taken up by the cell bodies of the neurons, incorporated into proteins and then transported out into the axons (Ochs & Johnson, 1969). If the protein synthesis inhibitors puromycin or cycloheximide were injected into a ganglion prior to the injection of ^{3}H-leucine, no out-flow of labelled material was observed (Ochs, Sabri & Ranish, 1970). If the injection of inhibitor was made 5 minutes after the injection of ^{3}H-leucine it was no longer as effective and some outflow occurred. An injection 20 minutes after that of ^{3}H-leucine produced almost no impairment at all. Furthermore, biochemical analysis of the nerve following an injection of ^{3}H-leucine showed that most of the radioactivity was in the protein fraction, and that very little radioactivity was due to free leucine in the nerve.

d) INVOLVEMENT OF MICROTUBULES

The precise mechanism of fast axoplasmic transport is not yet clear, but evidence suggests that the microtubules found in nerves may be involved (cf Schmitt & Samson, 1968), perhaps by providing a "track" along which substances may be transported. These linearly organized structures are also observed in other cellular systems in which movement occurs, such as in the mitotic spindle and in cilia and flagella.

Agents such as the anti-mitotic drug colchicine, which impair movement in these processes, apparently by interacting and binding to microtubles, also block axoplasmic transport (reviewed by McClure, 1972).

Though microtubules are thus strongly implicated as being an important component in the mechanism of fast axoplasmic transport, there are some observations which are apparently not so clearly indicative of their role. Axoplasmic transport has been blocked by colchicine injections in the crayfish without evidence of microtubule disruption (Fernandez, Huneeus & Davison, 1971). Byers (1974) similarly demonstrated block of axoplasmic transport with colchicine application to rabbit vagus nerves, without significant damage to microtubules. Furthermore, garfish olfactory nerves appear to have only 2 or 3 microtubules per fibre (Gross & Beidler, 1973), but axoplasmic transport in these nerves is similar to that in nerves containing far greater numbers of these structures. It has been suggested (Byers, 1975) that the endoplasmic reticulum and the axolemma may be involved, and that microtubules play mainly a structural role. Thus at the present, the real nature of the mechanism of fast axoplasmic transport remains vague. Whatever the mechanism however, the fact remains that anti-mitotic drugs such as colchicine and vinblastine are able to block this process. Kreutzberg (1969) demonstrated that proximal to sites of colchicine injections in rat sciatic nerves, large accumulations of acetylcholine esterase (AChE) could be observed. This was interpreted as being due to a block of axoplasmic transport by the drug. Similarly, Dahlstrom (1968) demonstrated that the passage of amine storage

granules in nerves could be blocked by colchcine.

e) ENERGETICS OF FAST AXOPLASMIC TRANSPORT

Fast axoplasmic transport in nerve fibres is independent of the cell body and normal rates of flow can be observed in nerve segments distal to ligatures (Ochs, 1971). Even isolated segments of axons in vitro exhibit normal rates of transport. The mechanism for fast axoplasmic transport appears to be present along the entire length of the axon. It is also highly dependent on oxidative metabolism (Ochs, 1971). Axoplasmic transport will not occur through an area made anoxic or an area poisoned by inhibitors of oxidative metabolism like cyanide (CN) or dinitrophenol (DNP , Ochs, 1971).

PART III

THE GENERAL STRATEGY USED IN THIS STUDY

THE GENERAL STRATEGY

The experiments described in this thesis were undertaken in order to obtain a better understanding of the way by which the motor nerve might exert a trophic influence on skeletal muscle. Denervated skeletal muscle, as has been described earlier, undergoes a large number of physiological changes. However, the question is still not resolved -- are these the consequences of a loss of trophic influences of the nerve, or the loss of impulses which normally keep the muscle active?

The following report describes experiments designed to detect a possible trophic influence that the nerve might have on skeletal muscle, by attempting to interfere with neuronal transport while leaving nerve impulse activity intact. To achieve this, colchicine was used to block axoplasmic transport in the sciatic nerve of rats. The assumption was made that if trophic factors were involved, they would most likely be synthesized in the cell bodies and then carried to the terminals via axoplasmic transport. Colchicine was injected subepineurally into the sciatic nerve of rats and subsequently the EDL muscle was studied to observe whether or not "denervation-like" changes had occurred. If such changes could be observed in the muscle without any impairment of nerve impulse activity, then a case could be made for the participation in the control of skeletal muscle fibre properties of trophic factors supplied by the motor neuron.

PART IV

METHODS

1. ANIMALS

The animals used in the experiments described in this thesis were male Wistar rats procured from a local supplier (Woodlyn Laboratories Ltd., Guelph, Ontario). Animals weighed from 140 g to 450 g.

2. DRUG APPLICATION

Animals were treated with colchicine in one of three ways: (1) a solution of colchicine was injected through the epineurium of the sciatic nerve, (2) a solution of colchicine was injected systemically and (3) colchicine was applied chronically by means of a silicone rubber implant, impregnated with the drug. Vinblastine sulphate (Eli Lilly) was also used in a few nerve-cuff experiments. The colchicine was obtained from BDH Chemicals Ltd. (Toronto). The vinblastine sulphate was obtained from Eli Lilly & Co. Ltd. (Toronto). The doses of colchicine injected ranged from 40 to 150 µg per 100 grams of animal body weight (b.w.).

a) SUBEPINEURAL INJECTIONS OF COLCHICINE

During ether anaesthesia, the sciatic nerve of one side was exposed at mid-thigh level and 1 to 5 µl of a 0.2 M colchicine solution was injected through the epineurium, as superficially as possible. The injection was made 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 ethanol and 8 parts sodium phosphate buffer (pH 7.2) as described by Kreutzberg (1969). As a control, animals were also injected with this solvent alone (1 to 5 µl).

b) SYSTEMIC INJECTIONS OF COLCHICINE

Colchicine was administered systemically by injecting 0.1 to 0.5 ml of a 2 mM solution of the drug dissolved in saline (0.9% sodium chloride) intraperitoneally (I.P.) or intramuscularly (I.M.) into the lumbar muscles.

c) CHRONIC APPLICATION OF COLCHICINE TO THE SCIATIC NERVE

Under ether anaesthesia, one sciatic nerve was exposed at mid-thigh level and enclosed by a silicone-rubber nerve-cuff, impregnated with colchicine. This procedure was similar to that of Robert & Oester (1970), who applied nerve-cuffs impregnated with local anaesthetics to the sciatic nerve in rabbits. To minimize diffusion of the drug out of the cuff 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 an inside diameter (I.D.) that varied from 0.8 to 1.6 mm in different experiments. The rats used in this series 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 by carefully mixing colchicine with the fluid silicone monomer to obtain a concentration of 0.10 - 0.15% w/w. This mixture was then placed in appropriate molds where it was left for several days until polymerization was completed. Two types of silicone rubber were used, 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 on contact with moist air, giving off acetic acid in the process. The second is a two part system, requiring the addition of an appropriate catalyst (Dow Corning catalyst E) to polymerize the silicone monomer. Catalyst E was used at a concentration of 0.10%. In a few rats, cuffs impregnated with vinblastine (0.10% w/w) were implanted.

3. DENERVATION PROCEDURE

To denervate the EDL muscle, the sciatic nerve of one side was exposed at the mid-thigh level and cut.

4. CLINICAL EXAMINATION OF ANIMALS

After treatment, all rats were carefully checked (usually twice a day) for possible neuromuscular impairment. The appearance of leg paresis was ascertained by visual and manual examination. The degree of active extension and spread of the hindlimb digits, the amount

of extensor tone of the foot and the strength of extensor voluntary movements were noted. Rats were picked up by the back of the neck and held in a vertical position. Under these conditions, the hindlimb digits in normal rats are spread (extended) and remain so for as long as the animals are maintained in such a position. In animals that had developed even a mild paresis, the extension of the hindlimb digits was obviously affected. The foot was also lightly stroked and a finger placed under the foot to test the degree of extensor tone and extensor voluntary movements.

5. MICROELECTRODE EXPERIMENTS

At various times (1 to 8 days) after colchicine treatment, the rats were anaesthetized with sodium pentobarbital and the EDL muscles were dissected out with their nerves (see Fig. 1) and perfused in a chamber at room temperature (22 - 25 ^{o}C). 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: NaCl 135 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgCl_2 \cdot 6H_2O$ 1 mM, $NaHCO_3$ 15 mM, Na_2HPO_4 1 mM, D-glucose 11 mM. The perfusion rate was about 300 ml per hour. In most experiments, both the EDL muscles of the treated and of the contralateral, nontreated side were placed in the chamber.

a) INTRACELLULAR RECORDING

Intracellular recordings from the superficial muscle fibres of the deep surface of EDL muscles were made with microelectrodes filled

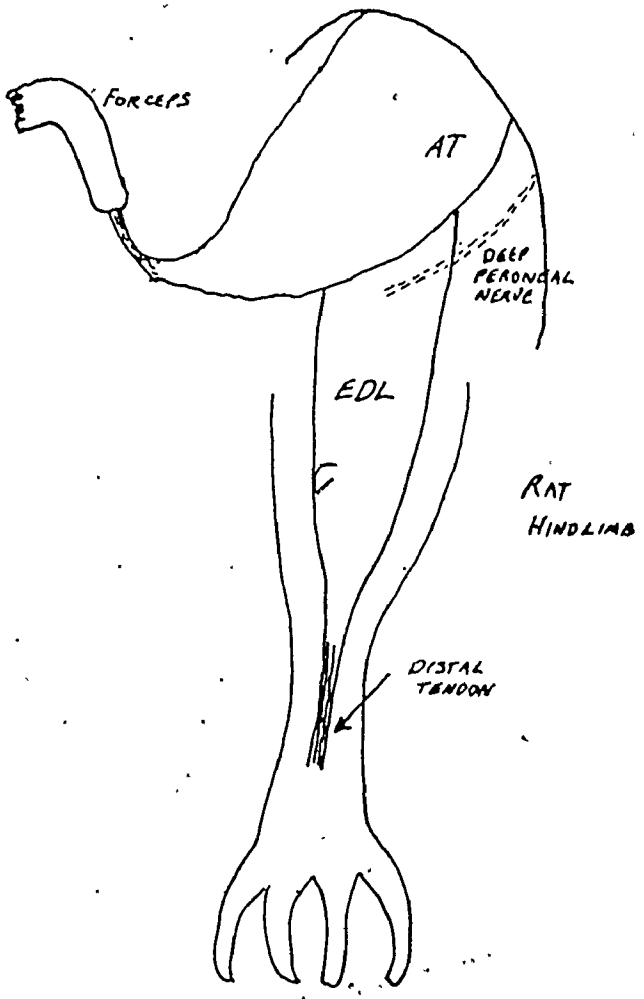
FORCEPS
AT
DEEP PERONEAL NERVE
EDL
RAT HINDLIMB
DISTAL TENDON

FIGURE 1.

FIGURE 1. Diagrammatic representation of the anatomical location of the EDL muscle. It arises from the lateral epicondyle of the femur. The muscle divides into four parts; the tendons pass under the annular ligament lateral to the tendon of the tibialis anterior (AT) and insert on the base of the third phalanx of digits two and five. The EDL muscle is innervated by the deep peroneal nerve.

Surgical Procedure: With the rat lying on its back, an incision through the skin was made in the ventral aspect of its leg and thigh. The distal tendon of the tibialis anterior (AT) was cut and the muscle was pulled up and freed of its connections to the adjacent tissues. The extensor digitorum longus (EDL) muscle is located directly subjacent to the AT. Its distal and proximal tendons were freed from the surrounding tissues and its nerve (the deep peroneal) was dissected back to the sciatic nerve and cut. The nerve-muscle preparation was then ready to be mounted in the bath.

with 3 M KCl. These electrodes had resistances of 5-15 MΩ and had tip potentials generally less than 3 - 5 mV. The electrodes were pulled from borosilicate, melting point capillary tubing glass of 1.6 - 1.8 mm diameter (Kimble Products, USA) on a David Kopf (USA) or Narashige (Japan) microelectrode puller. All microelectrodes in these experiments were "back filled" by using the glass fibre technique (Tasaki, Tsukahara, Ito, Wayner & Yu, 1968). This consisted of placing 4 to 6 fine glass fibres obtained from filter type glass wool into a capillary tube before pulling it into a microelectrode. The microelectrode resulting from this procedure could then be filled by simply introducing the desired solution into the shank of the tube. The solution would then very rapidly fill the tip by capillary action of the glass fibres and the electrode would be ready for immediate use.

b) IONTOPHORETIC APPLICATION OF ACETYLCHOLINE (ACh)

Pulses of ACh were applied to highly localized areas of EDL muscle fibres by microiontophoresis (del Castillo & Katz, 1955). The basic circuit is illustrated in Fig. 2. This technique takes advantage of the fact that ACh in aqueous solution is a positive ion. A positive charge applied to a pipette containing ACh results in the ejection of ACh^+ ions, in a charge dependent fashion. The ACh micropipettes in these experiments had resistances of 30 - 40 MΩ, and tip sizes < 0.5 μ. Since electrodes of this size "leak" a small amount of ACh from the tip, a "braking current" of 5×10^{-9} to 1×10^{-8} Amps had to be applied to prevent this leakage of ACh and to avoid consequent

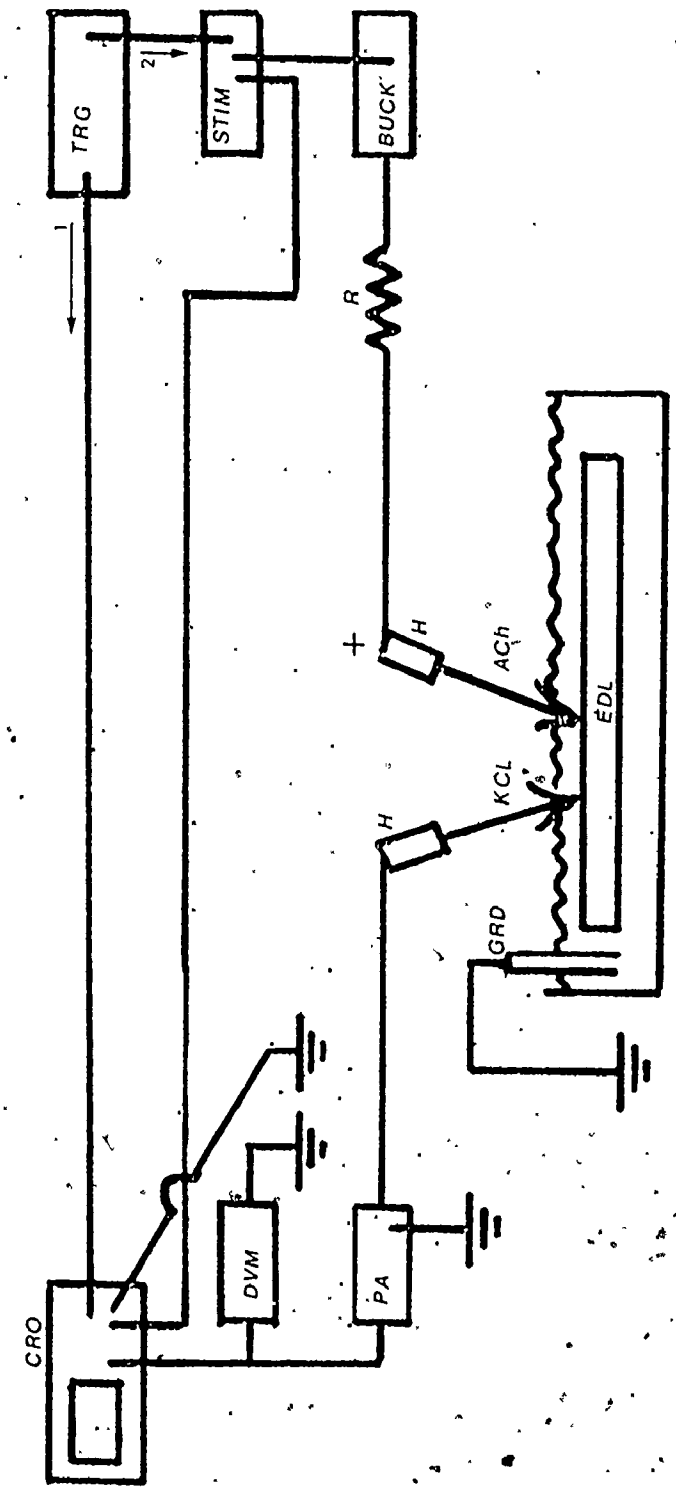

FIGURE 2.

FIGURE 2. The basic circuit diagram for the electrophysiological investigations. A 3 M KCl electrode (KCL) in a microelectrode holder (H) containing a silver-silver chloride (Ag-AgCl) junction was used to impale muscle fibres (EDL). The signal from the microelectrode (KCL) was fed into an electrometer amplifier (i.e. a preamp, PA, WPI, W-P Instruments, U.S.A.), the signal from which was then displayed on a digital voltmeter (DVM, John Fluke, U.S.A.) and a storage oscilloscope (CRO, Tektronix, U.S.A.). The preparation was grounded by an Ag-AgCl electrode (GRD).

For iontophoretic application of ACh, the 3 M ACh electrode (ACh) was brought to about 100μ of the KCl electrode and positioned just extracellular to the muscle fibre. At this point, two sequential triggering signals (arrows 1 & 2 from TRG) were released from a triggering device (TRG; a Digitimer, Devices, England) which first (arrow 1) triggered the oscilloscope trace and then 200 msec later triggered (arrow 2) the stimulator (STIM, Devices, England) to produce a square wave, positive current pulse of variable intensity and duration (see text) across the ACh electrode. Included in the stimulator circuit was a "braking current" device (BUCK) which was used to prevent leakage from the elctrode (see text) and a 100MΩ resistance (R).

ACh receptor desensitization. The amount of braking current required for each electrode was determined by bringing the ACh pipette in close proximity to a denervated muscle fibre whose RMP was being monitored. If a depolarization of the muscle fibre was observed, then the braking current was increased until no leakage from the tip could thus be detected. ACh was expelled from the pipette by positive square wave current pulses of variable duration (2 - 75 msec) and intensity (0.5 to 3.0 x 10^{-7} Amps).

To test whether a muscle fibre exhibited extrajunctional sensitivity to ACh, it was impaled with a recording microelectrode usually first near the tendon region. The RMP was recorded and observed on a storage oscilloscope with a slow sweep speed (200 msec per cm). The iontophoretic pipette was brought to within 50 to 100 μ of the recording electrode and was positioned just extracellular to the muscle fibre. A positive current pulse was then applied to it. If the muscle fibre membrane was sensitive to ACh at that location, a slow, characteristic depolarization (an "ACh-potential", lasting 0.6 to 1.0 sec) of a few millivolts (0.5 to 9.0 mV) could be observed.

Membrane sensitivity to ACh was expressed in "Units" according to Miledi (1960); 1 unit of sensitivity being equal to 1 mV of membrane depolarization per 1 nanoCoulomb (1 x 10^{-9}C) of charge passed through the ACh pipette. The current being passed through the iontophoretic pipette was monitored on an oscilloscope. A 10 msec square wave pulse of 1 x 10^{-7} Amps current resulted in 1 nC of charge passing through the electrode.

c) DETERMINATION OF RESISTANCE TO TETRODOTOXIN (TTX)

Muscle fibres were excited directly by applying cathodal pulses through a 2 M K citrate, intracellular micropipette. The response of the stimulated muscle fibre was monitored by a recording microelectrode about 100 μ from the stimulating one. A DC hyperpolarizing current was passed through the stimulating electrode to obtain a steady membrane potential of about -90 to -100 mV as described by Redfern & Thesleff (1971a). The action of tetrodotoxin (TTX, Sankyo, Tokyo) on the spike generating mechanism was assessed after adding the toxin to the bathing fluid to obtain a concentration of 10^{-6} M.

6. AXOPLASMIC TRANSPORT MEASUREMENTS

Axonal transport was studied by observing the passage of tritium labelled material in the sciatic nerves of treated and untreated animals. This technique has been described by others (Ochs & Burger, 1958; Ochs, 1972) and consists of injecting ^{3}H-leucine into the spinal cord, in the vicinity of the motor neurons giving rise to the sciatic nerve. Measurements were made either on the ipsilateral side, contralateral side or both, 3 to 51 hours after colchicine injection into one sciatic nerve. Animals were anaesthetized with sodium pentobarbital and a laminectomy was performed over three vertebrae of the lumbar enlargement of the spinal cord. The precursor, L-leucine 4,5- ^{3}H(N), was obtained from New England Nuclear Co. (USA) and had a specific activity of

41.2 Ci/ m mole in a 0.01 N HCl solution at a concentration of 1 mCi / ml. One ml of this solution was dessicated to dryness and resuspended in 200 µl of isotonic saline to give a final concentration of 5 mCi / ml. Once the dura was opened, 5 injections were made in the selected half of the spinal cord of 4 µl each and 1.8 mm apart in a rostrocaudal direction at depths of 1.2 to 1.6 mm. Injections were made with Dow Corning glass micro-sampling pipettes (50 µl), graduated in 4 µl segments and pulled to have tips of about 30 µ in diameter. These were filled with the ^{3}H-leucine solution by aspiration. 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 to 37.5^{o}C. This was important because of the high dependence of the rate of axoplasmic transport on temperature (Ochs & Smith, 1971). Pentobarbital was administered as required to maintain a deep level of anaesthesia.

After the desired flow time of the labelled material had elapsed (3.25 to 6.25 hours) the animals were decapitated, the sciatic nerves and corresponding ventral roots (usually two roots) were isolated and the dorsal root ganglia dissected away. The nerves were partially dried at their in vivo length and were then divided into sequential 3 mm segments each of which was placed into a glass scintillation vial. Each sample was then rehydrated by the addition of 2 drops of distilled water and then solubilized by the addition of 0.5 ml of Protosal (New England Nuclear) and heating at 50^{o}C for 6 to 10 hours. After solubilization, each vial was analyzed for 10 minutes in a Beckman counter

following the addition of 10 ml of scintillation fluid. The composition of this was: 5 grams of PPO (2,5-diphenyloxazole) and 0.2 grams of POPOP (1,4-bis (2- (5-phenyloxazolyl)) in 1 litre of toluene. These chemicals were obtained from Fischer Chemicals (Toronto).

The quenching of radioactivity varied little from sample to sample. Since the number of counts per minute (CPM) measured by the scintillation counter is directly proportional to the number of disintegrations per minute taking place in the sample, the CPM was used as the indicator of sample radioactivity.

a) CALCULATIONS INVOLVING AXOPLASMIC TRANSPORT

The amount of radioactivity from each sample was plotted sequentially on semi-log graph paper to obtain a visualization of the profile of radioactivity in each nerve (see Fig. 3). The rate of axoplasmic transport was obtained by simply dividing the distance the radioactivity had reached peripherally in the nerve (arrow 2 in Fig. 3), by the time the process was allowed to continue. The distance that the radioactive "wave" had reached was determined as the point at which the slope of the advancing wave-front (dotted line at 1 in Fig. 3) of radioactivity intersected with the background level of radioactivity (BKG in Fig. 3). The "crest height" or amplitude (arrow 3 in Fig. 3) was taken as the maximum amount of radioactivity in the crest.

7. MEASUREMENT OF MUSCLE SENSITIVITY TO BATH APPLIED ACh.

EDL muscle were dissected out 20 to 110 hors after a systemic

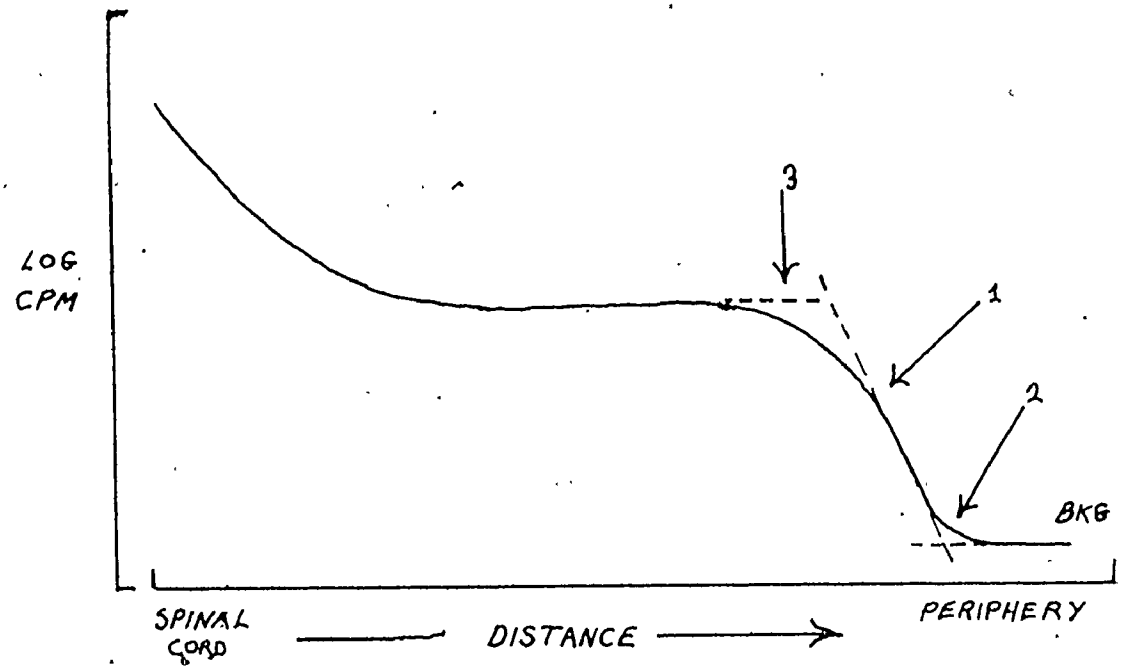

FIGURE 3. A diagrammatic representation of a typical profile of radioactivity observed in the sciatic nerve, 3-6 hrs following injections of ^{3}H-leucine into the spinal cord. No data points are represented for simplicity. The "crest height" or amplitude of the radioactive wave-front was taken as the maximum amount of radioactivity measurable as illustrated by the arrow at 3. The distance that the radioactivity had reached (intersecting dotted lines at arrow 2) was taken as the point at which the slope of the advancing wave-front (dotted line at arrow 1) intersected with the background level of radioactivity (BKG). Rate of axoplasmic transport was calculated by dividing the distance the radioactivity reached in the nerve by the time that this process was allowed to continue. The result was expressed in terms of millimetres (mm) per day.

injection of colchicine in 170 to 195 g rats. A ligature was placed on both distal and proximal tendons (see Fig. 1); one being attached to a strain guage, the other to a fixed point in a muscle bath. The whole preparation was positioned vertically in a small (volume about 10 ml) muscle bath. The muscle was then exposed to various concentrations of ACh (1×10^{-7} M to 1×10^{-3} M) dissolved in mammalian Krebs solution (composition previously given) which was radiply introduced into the chamber (~0.3 to 0.5 sec). The response of each muscle was recorded on a paper, strip-chart recorder (Grass Instruments, USA). A dose-response curve of the tension produced by each muscle in response to various concentrations of ACh was constructed.

8. MUSCLE CONTRACTION MEASUREMENTS

Isometric recordings of the EDL muscle contraction were performed in vivo, during pentobarbital anesthesia. The distal tendon was sectioned (see Fig. 1) 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 Hz) were applied to the sciatic nerve both proximal and distal to the site of colchicine application (injection or a nerve 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.

PART V

RESULTS

1. INJECTION OF COLCHICINE INTO THE SCIATIC NERVE: EFFECTS ON MUSCLE FIBRE MEMBRANE CHARACTERISTICS

The first question that was pursued in these investigations was, what effect would a block of axoplasmic transport, produced by an injection of colchicine into the sciatic nerve, have on the membrane characteristics of the muscle fibres in the EDL muscle of the rat?

a) SENSITIVITY TO ACh

Four to five days after the subepineural injection of colchicine (80-100 µg / 100 g b.w.) into one sciatic nerve of rats weighing 150-170 g , the ipsilateral EDL muscle fibres were examined for sensitivity to iontophoretically applied ACh. The sensitivity to ACh of normal EDL muscle fibres is confined solely to the end-plate region (Fig. 4). Fig. 5 illustrates the extrajunctional sensitivity of an EDL muscle fibre to ACh 108 hrs after an injection of colchicine into the ipsilateral sciatic nerve. Nine fibres in all were tested in this muscle, and everyone of them exhibited sensitivity to ACh at every tested position along their length. The mean sensitivity near the tendon region was 8.2 ± 2.5 units (mean ± S.E.M.). These results are in marked contrast to those from normally innervated EDL muscle fibres; in seven such fibres

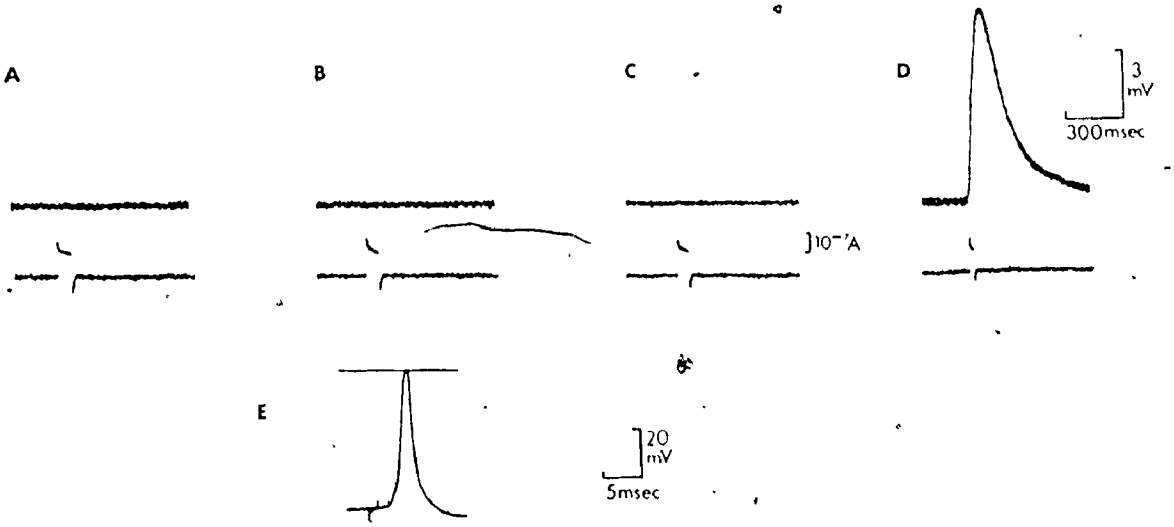

FIGURE 4. The responses recorded from a normally innervated EDL muscle fibre to iontophoretically applied pulses of ACh. A is at the tendon region, 4.6 mm from the end-plate region. B and C are 2.8 and 1.4 mm respectively from the end-plate region. The iontophoretic pulses in A, B and C are 75 msec in duration; at the end-plate region the pulse was 5 msec in duration. As can be seen, there was only a response to ACh at the end-plate region. E is an indirectly evoked action potential recorded at the tendon region A, indicating that this fibre was innervated. The RMP of this fibre was -61 mV by the time E was recorded, and this low level could account for the lack of overshoot of the action potential.

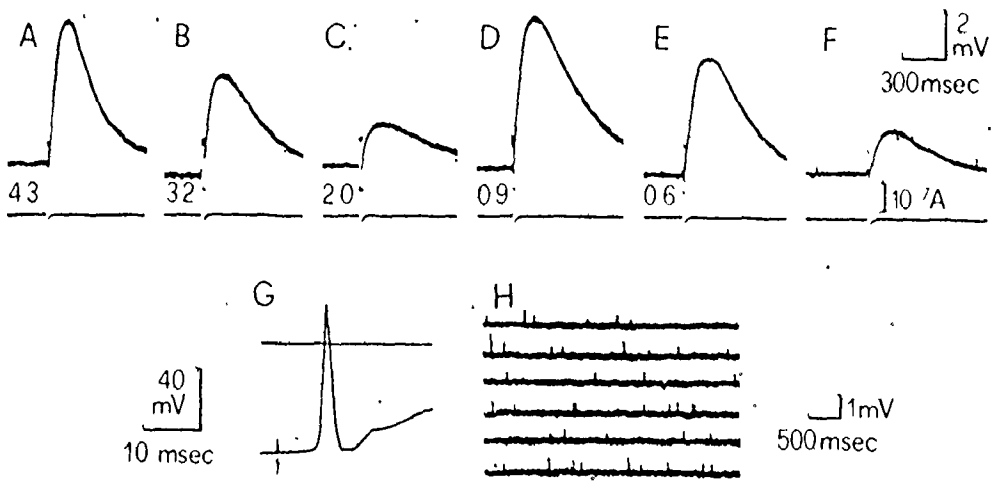

FIGURE 5. Responses recorded from an EDL muscle fibre ipsilateral to a sciatic nerve injected with 152 µg of colchicine 108 hours earlier (163 g rat) to iontophoretically applied ACh. A: tendon region ; F: end-plate region. Distances in mm from the end-plate are indicated below A to E. Iontophoretic 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 RMP consequent to the multiple penetrations, and to the non-optimal location of the ACh electrode. G: indirect spike recorded at the tendon region of the same muscle fibre, soon after the ACh potential shown in A was evoked; after the spike the muscle twitch dislodged the electrode. H: m.e.p.p.s recorded at position F. The average sensitivity at the tendon region was 8.2 ± 2.5 units (mean ± S.E.M., 9 fibres). Six out of the 9 fibres were excitable by nerve stimulation.

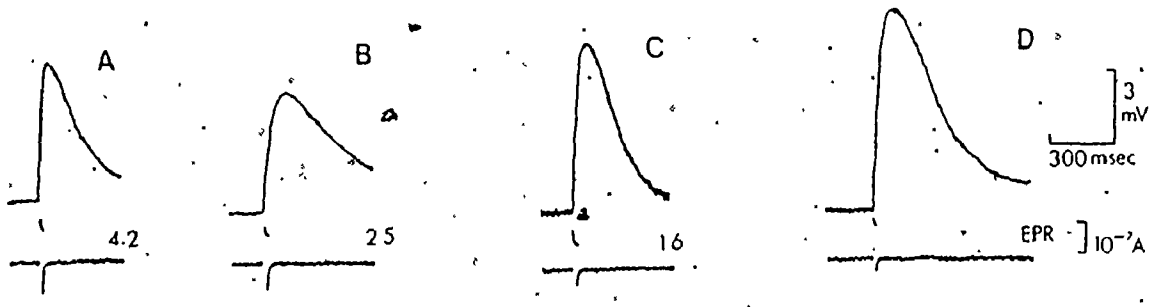

FIGURE 6. The responses recorded from a 5 day denervated EDL muscle fibre to iontophoretically applied pulses of ACh. The number below each response indicates the distance from the end-plate region that the recording was made. EPR indicates the response recorded near the end-plate region itself. Duration of the iontophoretic pulse was 5 msec in allcases except B and C where it was 10 msec. As can be seen, there was a response to ACh everywhere along the muscle fibre.

the sensitivity to ACh was observed always to be limited to the end-plate region (Fig. 4).

Following denervation, skeletal muscle fibres become sensitive to ACh along their entire length (Axelsson & Thesleff, 1959). Fig. 6 illustrates the sensitivity of an EDL muscle fibre to iontophoretically applied ACh 5 days after the sciatic nerve was sectioned. Six fibres in all were examined in this muscle and everyone of them exhibited a high level of extrajunctional sensitivity to ACh. The mean sensitivity at the tendon region, about 5 mm from the end-plate region in these fibres, was 34 ± 2.5 units (mean ± S.E.M., 6 fibres).

Since the extrajunctional sensitivity to ACh in EDL muscle fibres following the injection of colchicine into the sciatic nerve so resembles that following denervation, it was important to test whether the drug was in fact causing a "chemical" denervation, i.e., producing neuromuscular transmission failure at the endings of the treated nerves. Two criteria were used to evaluate the condition of neuromuscular transmission: (1) could m.e.p.p.s be recorded at the end-plate region of muscle fibres? (2) could action potentials be recorded in the muscle fibres following stimulation of the sciatic nerve central to the site of colchicine injection?

Examination of EDL muscle fibres on the ipsilateral side following an injection of colchicine into the sciatic nerve 4 to 5 days earlier revealed that 94% of the tested fibres (192 fibres in 12 animals) exhibited m.e.p.p.s (Fig. 5H). These m.e.p.p.s had a frequency of 1.7 per sec ± 0.3 per sec (mean ± S.E.M.) and a mean amplitude of 0.70 ± 0.15 mV (mean ± S.E.M., measured in 5 fibres). This value was not

significantly different from that observed in normal fibres ($P > 0.20$). These EDL fibres, which displayed extrajunctional sensitivity to ACh and had m.e.p.p.s, also produced action potentials in response to stimulation of the sciatic nerve (Fig. 5G). The EDL muscle fibres that did not display m.e.p.p.s (6% of 192 tested fibres, 12 animals) also did not produce action potentials in response to stimulation of the sciatic nerve. Since the recording electrode impaled the muscle fibre at its tendon region, and the sciatic nerve was stimulated central to the site of colchicine injection, the presence of a muscle action potential indicated that conduction was not being impaired either in the nerve or muscle, and that transduction of the nerve impulse at the neuromuscular junction was taking place.

In summary then, as illustrated in Fig. 5, EDL muscle fibres examined 4 to 5 days after an injection of colchicine into the ipsilateral sciatic nerve, exhibited extrajunctional sensitivity to ACh. These fibres however, were not denervated since they displayed m.e.p.p.s (Fig. 5H) and responded to nerve stimulation with transmitted action potentials (Fig. 5G).

2. A SYSTEMIC EFFECT OF COLCHICINE

The EDL muscle on the contralateral side to the colchicine injection was examined as a routine control; unexpectedly, this muscle was also found to have fibres which exhibited extrajunctional sensitivity to iontophoretically applied ACh. Four to 5 days after the injection of 300-320 µg of colchicine into one sciatic nerve of rats weighing 320-440 g

(in 6 animals), 24 out of 29 (83%) ipsilateral EDL muscle fibres and 42 out of 55 (77%) contralateral EDL muscle fibres exhibited extrajunctional sensitivity to ACh. An example of the responses of EDL fibres contralateral to the colchicine injection is illustrated in Fig. 7. As found for the ipsilateral muscle fibres that exhibit extrajunctional sensitivity, the contralateral ACh-sensitive fibres all had m.e.p.p.s and transmitted action potentials.

A typical example of this possible systemic effect of a sub-epineural injection of colchicine (152 µg into a 166 g rat) into one sciatic nerve of a rat is illustrated in Fig. 7. Examined 75 hrs after the injection, every EDL muscle fibre tested in this animal, both ipsi- and contralateral, was found to exhibit extrajunctional sensitivity to ACh. All the examined contralateral fibres (14) had transmitted action potentials while 11 out of the 14 examined ipsilateral fibres responded with transmitted action potentials following stimulation of the sciatic nerve.

The EDL muscle fibres on the ipsilateral, treated side were compared to the fibres on the contralateral side with respect to the average amount of sensitivity to ACh measured at the tendon region. In 8 animals (weight range 145 - 170 g) in which one sciatic nerve had been injected 4-5 days earlier with 152 µg of colchicine, the values obtained were 6.7 ± 0.9 units on the treated side (mean ± S.E.M.; in 97 fibres examined) and 5.8 ± 0.6 units on the contralateral side (101 fibres examined). These values were not significantly different ($P > 0.1$). The range of the average values from animal to animal was 4.5 to 9.1 units.

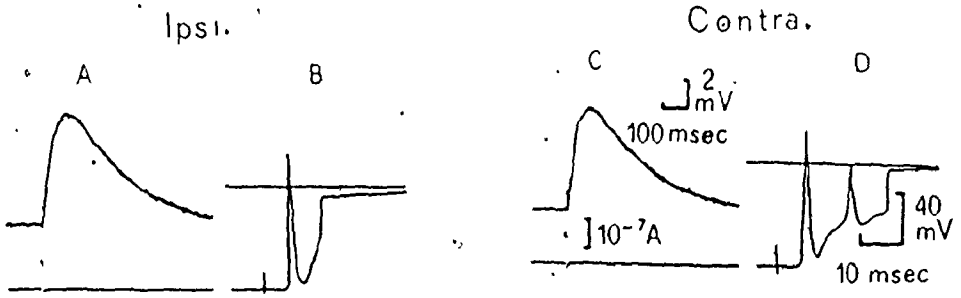

FIGURE 7. Extrajunctional sensitivity to iontophoretically applied ACh (examined at the tendon region) of two normally innervated EDL muscle fibres, one ipsilateral and the other contralateral to the sciatic nerve injected with colchicine (152 µg in a 166 g rat) 75 hours previously. A and C: ACh potentials elicited with a 5 msec iontophoretic pulse. B and D: spikes evoked in the same fibres with a single shock nerve stimulation. The average ACh sensitivity at the tendon region (14 fibres on each side) was 10.9 ± 2.9 units (mean ± S.E.M.) ipsilaterally and 6.8 ± 1.3 units contralaterally; the difference was not statistically significant ($P > 0.1$). Indirect spikes such as those shown in B and D were obtained in 11 ipsilateral and all 14 contralateral fibres.

When one-half of the usual dose (80-100 µg / 100 g b.w.) was injected into the sciatic nerve of 6 rats weighing 320 - 440 g , only 4 out of 35 ipsilateral fibres (12%) and 2 out of 50 contralateral fibres (4%) exhibited extrajunctional sensitivity to ACh. If this same amount of colchicine (i.e. 152-160 µg) was injected into proportionately smaller rats (140-170 g), extrajunctional sensitivity to ACh was observed in 82% of 97 tested ipsilateral fibres and in 88% of 101 contralateral fibres (8 animals).

These findings strongly suggest that colchicine is acting systemically even though injected locally into one sciatic nerve, and that the dose of the drug relative to the body weight (b.w.) of the animal is important in determining the bilateral appearance of extrajunctional sensitivity to ACh.

3. EFFECT OF COLCHICINE INJECTIONS ON THE IPSILATERAL SCIATIC NERVE

Since Kreutzberg (1969) had demonstrated that injections of colchicine into the sciatic nerve of rats produced large accumulations of acetylcholine esterase (AChE) proximal to the injection site, it was surprising that there was so little effect suggestive of a local action in the present experiments. A single subepineural injection of colchicine into one sciatic nerve produced bilateral extrajunctional sensitivity in EDL muscle fibres to an equal extent. The only side to side variation that could be attributed to the injection of colchicine

was the observation of a small number of fibres in the ipsilateral muscle exhibited extrajunctional sensitivity to ACh but did not have m.e.p.p.s or transmitted action potentials. In 7 animals (140-170 g.) examined 4-5 days following the injection of colchicine into one sciatic nerve, all of the contralateral EDL muscle fibres (142 fibres, 7 animals) exhibiting extrajunctional sensitivity to ACh were found to have m.e.p.p.s and transmitted action potentials. In contrast, 7% of the 137 examined ipsilateral EDL muscle fibres (7 animals) exhibiting extrajunctional sensitivity to ACh did not respond with transmitted action potentials following stimulation of the sciatic nerve. These fibres also did not display m.e.p.p.s. It was concluded that these muscle fibres were effectively denervated.

The number of ipsilateral EDL muscle fibres which could not be excited by stimulation of the sciatic nerve was increased when proportionately larger amounts of colchicine were injected into the sciatic nerve of large rats in order to maintain the usual dose of 80-100 µg / 100 g b.w. In 6 large rats (350-440 g), 4-5 days after an injection of colchicine into one sciatic nerve, 23 out of 104 (22%) ipsilateral EDL muscle fibres did not have transmitted action potentials. In contrast, all of the 89 contralateral EDL muscle fibres which were examined had transmitted action potentials. It thus appeared that the greater amounts of colchicine injected into the sciatic nerve of larger rats in order to maintain the whole animal dose of 80-100 µg / 100 g b.w. (that required to produce extrajunctional sensitivity to ACh) led to an increase in the number of ipsilateral EDL muscle fibres which appeared to have been denervated.

4. SYSTEMIC APPLICATION OF COLCHICINE

The finding that colchicine injected directly into one sciatic nerve produced extrajunctional sensitivity to ACh in EDL muscle fibres on both sides of the animal clearly indicated that the drug might be acting systemically. This idea was tested. A group of rats (5 animals, 140-170 g) were injected intraperitoneally (I.P.) or intramuscularly (I.M., into the lumbar muscles) with the same doses of colchicine used for nerve injections (i.e. 90-100 µg / 100 g b.w.). Four days later, the EDL muscle fibres in both hindlimbs were examined and found to exhibit extrajunctional sensitivity to ACh. The mean sensitivity of the EDL muscle fibres to ACh, measured at the tendon region was 6.9 ± 0.7 units (mean ± S.E.M., 93 fibres, 8 muscles). This sensitivity was similar to that of EDL muscle fibres to ACh following nerve injection of colchicine (see preceding section). Thus I.P. or I.M. injections of colchicine appeared to produce the same effects on muscle as injections of the drug into the nerve. In all the experiments, when the dosage was made optimal for the systemic action, there were essentially no differences in the changes of ACh-sensitivity between the right and left EDL muscles.

5. PROFILE OF EXTRAJUNCTIONAL SENSITIVITY TO ACh

Figure 8 illustrates the profile of the sensitivity to ACh observed in fibres of the EDL muscle contralateral to a nerve injected with colchicine. In this muscle, 15 adjacent fibres were examined for ACh sensitivity of the membrane between and including the end-plate and tendon regions. As can be seen in the figure (Fig. 8), the highest levels were recorded at the end-plate region (11.7 ± 1.4 units, mean ± S.E.M., 15 fibres). The tendon region exhibited the next highest values (6.3 ± 0.5 units, mean ± S.E.M., 15 fibres). The intermediate region (between end-plate and tendon) exhibited values between 1.8 and 3.1 units (45 tested sites in 15 fibres).

6. TIME-COURSE OF DEVELOPMENT OF SENSITIVITY TO ACh FOLLOWING COLCHICINE INJECTION

The time course of development of extrajunctional sensitivity to ACh in EDL muscle fibres was studied over a period of 2 to 7 days following a systemic injection of colchicine (I.P.) in 19 animals (309 fibres). The results are illustrated in Fig. 9. Maximal values of 7.1 ± 0.6 units (mean ± S.E.M., 5 animals, 83 fibres) were obtained at the tendon region, 5 days following the colchicine injection. After the 5th day following the injection, extrajunctional sensitivity to ACh decreased.

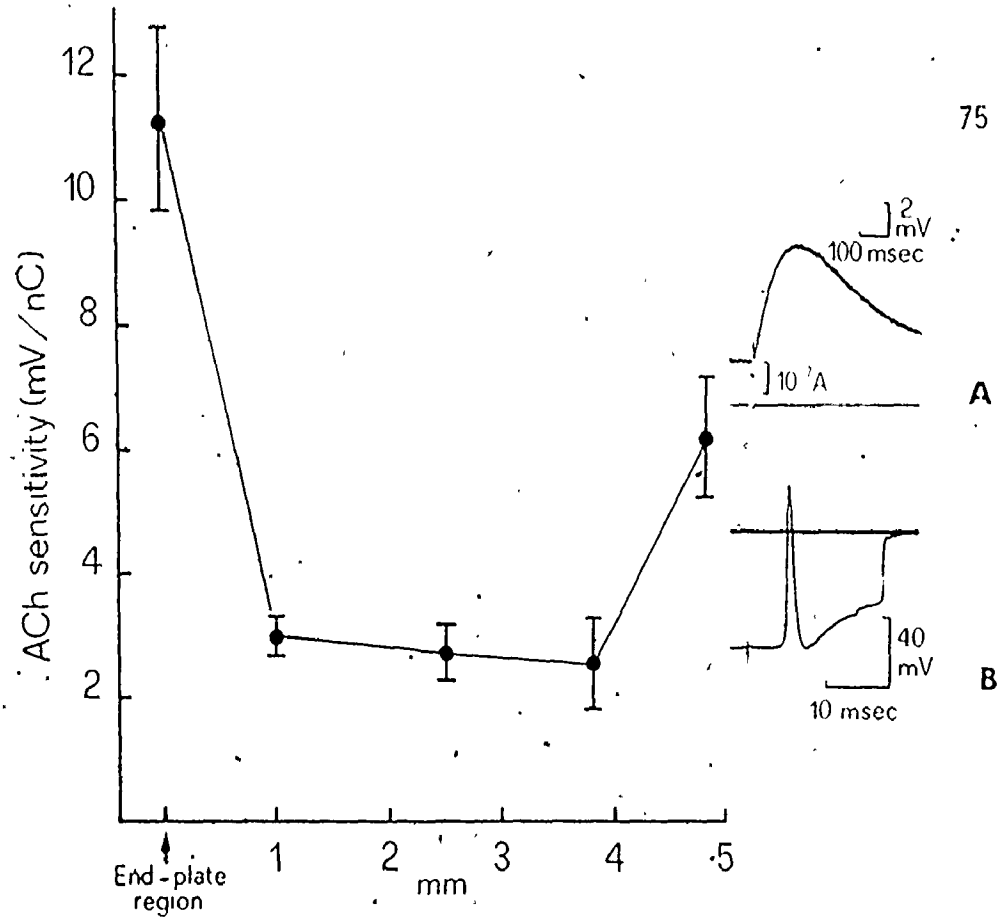

FIGURE 8. Profile of the extrajunctional sensitivity to ACh from the tendon to the end-plate region in 15 adjacent fibres of an EDL muscle, 100 hours after a direct injection of colchicine into the contralateral sciatic nerve (98 µg/100 g b.w.). All these fibres exhibited an action potential following nerve stimulation. Inset A illustrates the response of one fibre to a 5 msec iontophoretic pulse of ACh at the tendon region. Note that normal EDL muscle fibres have no detectable response to ACh outside the end-plate region, even at the tendon region. Inset B illustrates the typical indirect action potential that could be elicited in all of these fibres. In B, following the spike, the muscle twitched, dislodging the recording electrode.

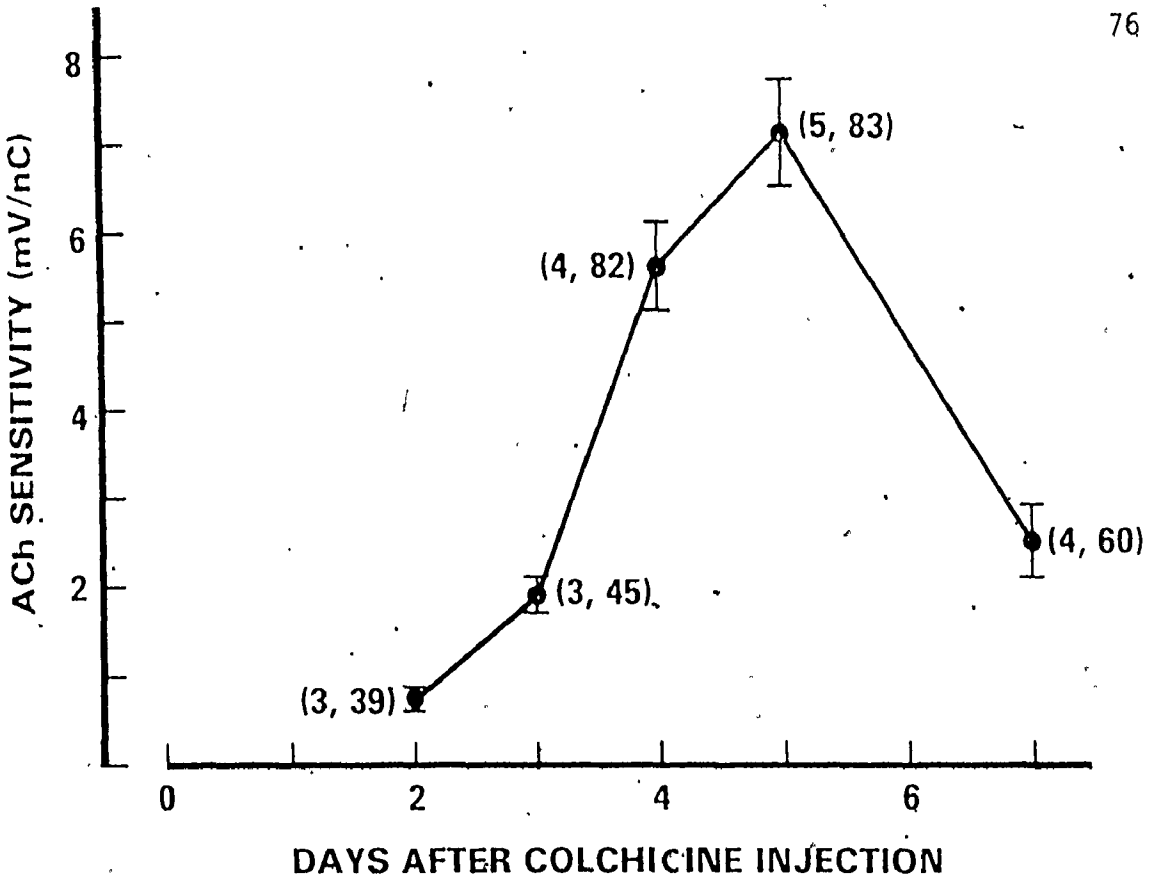

FIGURE 9. Time-course of acetylcholine sensitivity changes in EDL muscles at the tendon region due to systemic action of colchicine. The dose range was 90 - 100 µg / 100 g b.w.. The bars represent the standard error of the mean (S.E.M.) and the numbers beside each bar represent the number of animals and the number of muscle fibres examined respectively.

7. OTHER DENERVATION-LIKE PHENOMENA FOLLOWING COLCHICINE

After denervation, the spike generating mechanism of skeletal muscle fibres becomes partially resistant to concentrations of tetrodotoxin (TTX) that completely block action potentials in normal muscles (Redfern & Thesleff, 1971b). This was found to be true after colchicine injections as well. In 3 rats (180-190 g) examined 4 days after a systemic injection (I.M., 106 µg / 100 g b.w.) of colchicine, EDL muscle fibres on both sides were found to exhibit TTX-resistant action potentials. Figure 10 illustrates the results from one of these animals. Normally innervated muscle fibres did not produce regenerative action potentials when exposed to 10^{-6}M TTX (21 fibres, 2 animals). Four days after denervation however, 88% of 40 tested EDL muscle fibres (3 animals) exhibited TTX-resistant action potentials. Muscle fibres in the colchicine injected rats also exhibited TTX-resistant action potentials, and of 29 EDL fibres examined, 24 exhibited TTX-resistant spikes (83%).

In addition, the resting membrane potential (RMP) of EDL muscle fibres in colchicine treated animals was lower than that observed in the normal control animals. In 6 rats (140-190 g), 4-5 days after a systemic injection of colchicine (I.P.), the resting potential was observed to be -67.5 ± 0.9 mV (mean ± S.E.M., 156 fibres, 6 animals).

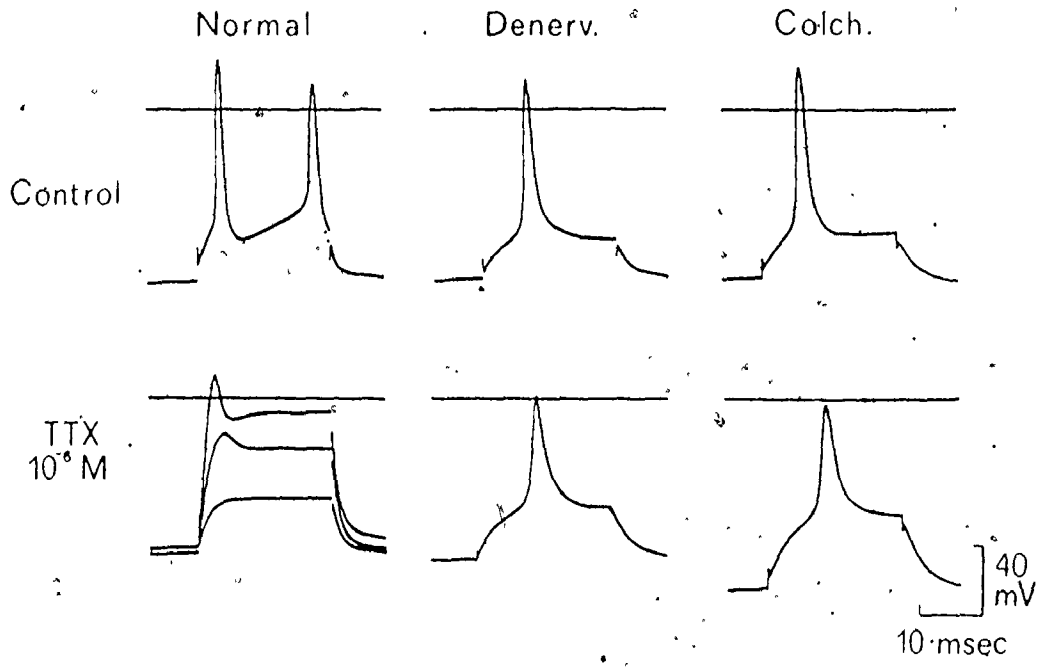

FIGURE 10.

FIGURE 10. Partial resistance to TTX of the directly evoked action potential in EDL muscle fibres 4 days after a systemic injection of colchicine (106 µg / 100 g b.w.). For comparison, examples from a normal and a 4 day denervated EDL muscle are shown. In the normal control fibre the depolarizing current pulse was strong enough to evoke two consecutive action potentials; in the normal fibre after TTX, 3 depolarizing pulses of increasing amplitude are superimposed. The pulse of the lowest amplitude (the bottom trace) displaced the membrane potential by 30 mV without producing any sign of a regenerative action potential. This is in marked contrast to denervated EDL muscle fibres and to muscle fibres from colchicine treated animals in which a membrane depolarization of only 2-5 mV produced the action potentials shown. Only with extremely large depolarizing pulses such as 60 and 80 mV (the middle and top traces for the normal-TTX fibre) was there any sign at all of spike activity, which was indicated by the initial humps associated with these depolarizations. The lack of overshoot observed in the other TTX treated muscle fibres is also due to the drug. In the normal muscle, the action potential was blocked by TTX in 11 tested fibres; whereas in the denervated muscle 7 out of 9 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.

This was significantly lower ($P < 0.001$) than the value observed in normally innervated EDL muscle fibres which gave values of -74.7 ± 0.7 mV (mean ± S.E.M., 42 fibres, 3 animals). EDL muscle fibres which had been denervated for 4-5 days exhibited the lowest values which were -54.6 ± 0.9 mV (mean ± S.E.M., 150 fibres, 5 animals).

8. DOSE-RESPONSE RELATIONSHIPS OF COLCHICINE

The action of colchicine in producing extrajunctional sensitivity of EDL muscle fibres to ACh apparently has a steep dose-response relationship, although this was not directly examined in detail. The threshold dose of the drug, injected either into the sciatic nerve or systemically, to produce a detectable amount of extrajunctional sensitivity to ACh was 40-50 µg / 100 g b.w. In 3 rats receiving this dose only 24% out of 46 fibres examined at the tendon region 5 days later exhibited extrajunctional sensitivity to ACh. These fibres had an average sensitivity of 1.2 ± 0.3 units (mean ± S.E.M., 11 fibres, 3 animals). Following the injection of higher doses of colchicine (80-100 µg / 100 g b.w.) systemically or into the contralateral sciatic nerve, virtually all the tested EDL muscle fibres were found to exhibit extrajunctional sensitivity to ACh. Typically, (as mentioned in preceding sections) animals injected with this dose exhibited 6 to 10 units of sensitivity to ACh at the tendon region, 4 to 5 days after the injection of colchicine. One group of rats (155-180 g, 4 animals, 19 fibres) for instance exhibited a mean sensitivity of 7.3 ± 0.9 units (mean ± S.E.M.) at the

tendon region 5 days after an injection of colchicine into the contralateral sciatic nerve.

9. TOXICITY SYMPTOMS FOLLOWING COLCHICINE INJECTIONS

Doses in excess of 105 µg / 100 g b.w. produced abnormal clinical signs in the treated rats. As observed in earlier studies (Ferguson, 1952), these consisted of diarrhea, a marked behavioural lethargy and an overall unhealthy appearance indicative of a generalized intoxication, marked by abnormal posture, movement and abnormal appearance of the fur. With doses of colchicine in the range of 140 to 150 µg / 100 g b.w., 72% of the animals died within 2 to 3 days (13 out of 18 animals in one experiment). This is in marked contrast to animals receiving doses of colchicine in the range of 90 to 100 µg / 100 g b.w. of which none exhibited any clinical signs of treatment whatsoever.

10. PARESIS FOLLOWING COLCHICINE INJECTIONS

In addition to the general symptoms of colchicine intoxication, an interesting feature of the toxic effects of high doses of the drug (120-150 µg / 100 g b.w.) injected systemically (I.P.) was the appearance of a marked bilateral paresis of the hindlimbs. Severly affected animals were unable to use their hindlimbs at all and dragged themselves around with their forelimbs. Following a "toxic" dose, the

paresis was most pronounced during the second day. It then regressed, and if the particular animals survived it was no longer detectable by the 4th to 5th day. The hindlimb paralysis was thus transient.

The observation of this phenomenon introduced the concern that this type of paresis might be produced in a milder form by the lower doses of colchicine which were usually used in the present experiments. If this was the case then the appearance of extrajunctional sensitivity to ACh might conceivable be secondary to "sub-clinical" levels of paralysis of otherwise normal EDL muscles (cf Lømo & Rosenthal, 1972, who demonstrated that muscle inactivity could lead to the appearance of extrajunctional sensitivity to ACh in rat EDL muscles).

The mechanism of this paresis was found to be explainable as a defect in neuromuscular transmission. A series of rats (24 animals, 175-185 g) were injected systemically with doses of colchicine ranging from 85 to 150 µg / 100 g b.w. and the tetanic tensions (see Methods) developed by the EDL muscles were measured 2 days later when the behavioural paresis described above was known to be at its maximum. The results of these experiments are shown in Fig. 11. With doses in excess of 105 µg / 100 g b.w. the average tetanic tension was significantly lower than the normal values obtained in control EDL muscles of 5 rats of the same size. With doses below this, no effect could be observed on the amount of tetanic tension developed by the EDL muscle (10 animals). The doses of colchicine used in these experiments to obtain extrajunctional sensitivity to ACh in EDL muscle fibres (85-95 µg / 100 g b.w.) was not observed to produce any measurable impairment in tetanic tensions. It was concluded that

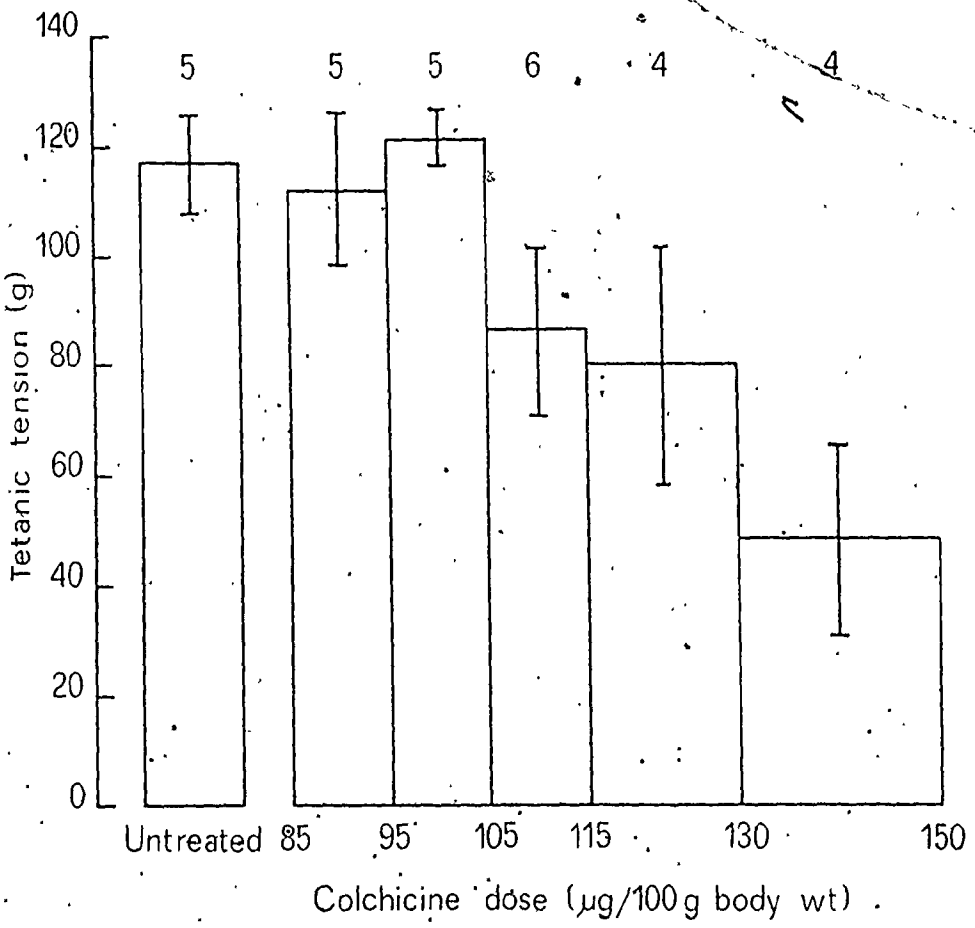

FIGURE 11. Tetanic tension evoked in the EDL muscle by nerve stimulation at 100 Hz, as a function of the dose of colchicine injected systemically 2 days earlier. Left-hand column shows results of control experiments. The figures above each column indicate the number of rats examined, and the bars represent the S.E.M.

the extrajunctional sensitivity to ACh of EDL muscle fibres examined on day 4 or 5 could not be attributed to muscle inactivity which might have occurred previously, e.g. on the second day following colchicine injection.

Preliminary observations indicated that the severe hindlimb paresis produced by a high dose of colchicine (130-150 µg / 100 g b.w.) injected systemically could be explained by a defect in the evoked release of ACh at the neuromuscular junction. Two rats (170-180 g) were examined on the second day after a toxic dose of colchicine (150 µg / 100 g b.w.). These animals exhibited an almost complete immobilization of the hindlimbs. Of the 38 EDL muscle fibres impaled at the end-plate region 31 (81%) failed to produce transmitted action potentials in response to nerve stimulation. These fibres were not denervated since they all had m.e.p.p.s.

11. THE EFFECT OF COLCHICINE INJECTIONS ON AXOPLASMIC TRANSPORT

The unexpected finding that colchicine was having a systemic action threw a different light on the original idea that the action of the drug might be explained by a primary effect on axoplasmic transport. This could however still be true. The hypothesis that was first tested therefore was whether colchicine, even though causing bilateral extrajunctional sensitivity to ACh, could be acting primarily on the motor nerves to impair the axoplasmic transport of neurotrophic

factors. If this were the case, then any changes observed in the muscle fibres would be secondary to the action of the durg on nerves. To test this possibility, measurements of the neuronal transport of ^{3}H-labelled material were made along the motor fibres of sciatic nerves in a series of rats (see Methods).

Following the injection of ^{3}H-leucine into the spinal cord in the region of the motor neurons giving rise to the sciatic nerve, a characteristic "wave" of radioactivity (see Fig.s 12 & 14) composed primarily of labelled proteins (Ochs & Johnson, 1969) can be observed to travel out from the spinal cord and peripherally, down the sciatic nerve (Fig. 12A). The wave of radioactivity has two readily measurable parameters: (1) its proximo-distal rate of movement and (2) its amplitude (i.e. the peak amount of radioactivity moving in the nerve). This latter parameter could give some insight into whether or not the amount of materials transported in the nerve was affected; it is of course influenced by the relatively ill-controlled amount of labelled material which is taken up by the nerve cells in the spinal cord, in this type of experiment.

a) AXOPLASMIC TRANSPORT IN NORMAL RATS

Fig. 12A illustrates two typical profiles of radioactivity measured from the sciatic nerves of two normal rats (230-250 g). One animal (open circles) was killed 3.25 hrs following the spinal cord injection of ^{3}H-leucine and the other (filled circles) was killed 6.25 hrs after the ^{3}H-leucine injections. As can be seen in the figure

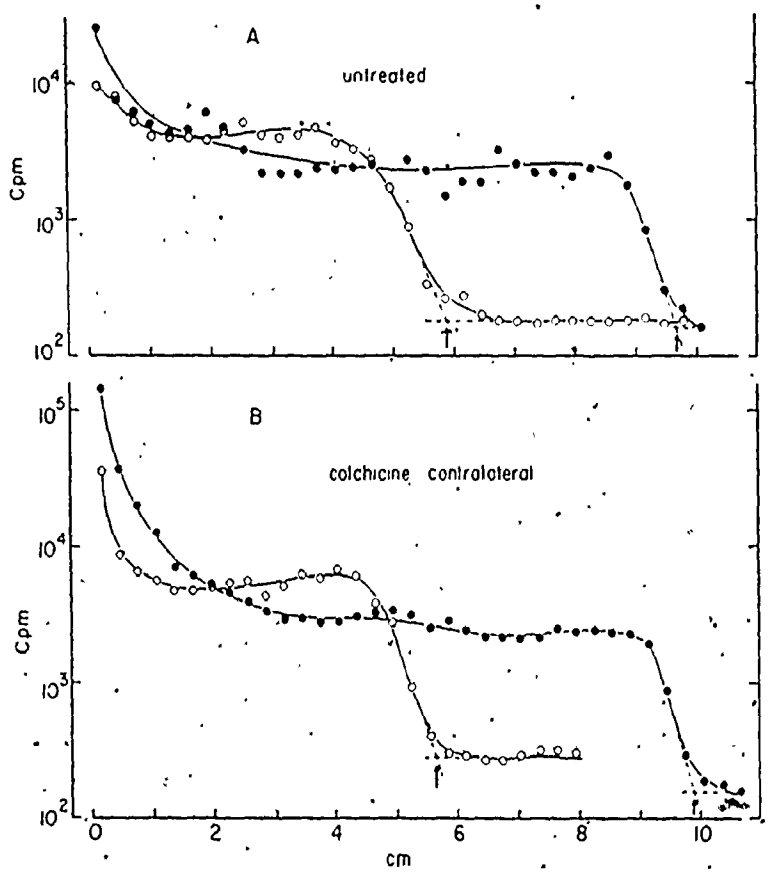

FIGURE 12. Profiles of ^{3}H-labelled material in the sciatic nerve 3.25 hrs (open circles) and 6.25 hrs (filled circles) after spinal cord injection of ^{3}H-leucine. A: two control rats. B: two rats injected with colchicine in the contralateral sciatic nerve about 20 hrs earlier, with a dose which routinely caused supersensitivity to ACh in muscles of both sides (100 µg / 100 g b.w.). Each point on the curve 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 being the portion of ventral roots emerging from the spinal cord segments injected with ^{3}H-leucine. The lines were drawn by eye to obtain the best fit of points. The arrows indicate the point that was taken as the distance that the wave-front had reached (see Methods, Fig. 3).

(12A), the wave-front of radioactivity was 39 mm further down the sciatic nerve in the latter rat than in the animal killed at 3.25 hrs.

Twenty rats (190-250 g) which were not treated with transport blocking drugs served as a control group. These animals were killed 5.5-6.25 hrs after receiving spinal cord injections of ^{3}H-leucine, and their sciatic nerves were dissected out and analyzed for radioactivity, using scintillation counting procedures. The rate of axoplasmic transport, calculated from the time taken for the wave-front of radioactivity to reach a measured distance down the nerve, as in Fig. 3, was found to be 399 ± 7.6 mm / day (mean ± S.E.M.). The amplitude of the radioactive wave-front observed in the control group was 3,582 ± 344 counts per minutes (CPM, mean ± S.E.M., 20 animals).

b). AXOPLASMIC TRANSPORT IN SYSTEMICALLY TREATED RATS

Axoplasmic transport was measured at various times between 3 and 51 hrs after colchicine treatment in a total of 45 rats. Twenty-eight (28) animals of this group were injected with the drug directly into the contralateral sciatic nerve and the remainder (17) received the drug systemically (I.P.). The dose was between 100 and 150 µg / 100 g b.w. which was equal to or greater than that previously found adequate to produce extrajunctional ACh sensitivity in EDL muscle fibres of both sides (see previous sections).

Fig. 12B illustrates two typical profiles of radioactivity observed in the contralateral sciatic nerve of rats injected with colchicine into the ipsilateral sciatic nerve. One animal was killed

at 6.25 hrs after ^{3}H-leucine injections into the spinal cord (filled circles) and the other was killed at 3.25 hrs (open circles). Both animals had received an injection into one nerve, of colchicine (100 µg / 100 g b.w.) 20 hrs before the experiment. No significant difference can be seen between this result and the results obtained from normal, untreated animals (Fig. 12A). In this entire series of experiments no impairment of axoplasmic transport was ever observed in the sciatic nerve contralateral to a nerve injection of colchicine, or in either of the sciatic nerves of animals which received systemic injections of the drug. Fig. 13 shows that there was no measurable effect on either the amplitude of the radioactive wave-front (Fig. 13A) or on the rate of axoplasmic transport (Fig. 13B). These results apply to the periods both preceding and during the development of extra-junctional sensitivity to ACh (3-51 hrs) following colchicine treatment.

Even in 3 rats which were severly paralyzed by a systemic injection of a high dose of colchicine (150 µg / 100 g b.w.) there was no measurable impairment of rate (395 ± 9 mm / day, mean ± S.D.) or the amplitude of the radioactive wave-front (3,654 ± 452 CPM, mean ± S.D.).

It was concluded from all these observations that the extra-junctional sensitivity to ACh observed in EDL muscle fibres contralateral to a sciatic nerve injection of colchicine, was not preceded by or associated with a measurable impairment of axoplasmic transport in the sciatic nerves.

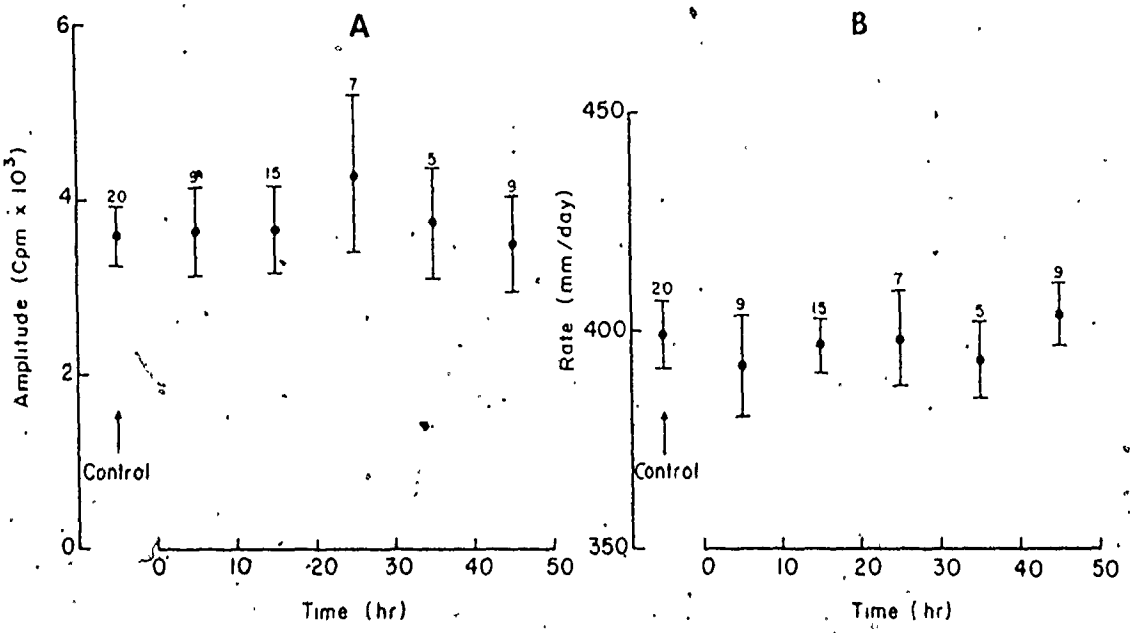

FIGURE 13. Peak amplitude (A) and rate of movement (B) of the wave-front of ^{3}H-labelled material in a series of rats, as a function of time after colchicine injection either in the contralateral sciatic nerve or systemically. Values for untreated rats (control) are shown to the left in both graphs. Bars represent the S.E.M.. The number of rats examined is shown on top of each bar. Note that extrajunctional sensitivity to ACh is already measurable at 48 hrs after such an injection.

12. EFFECT OF COLCHICINE ON THE INJECTED SCIATIC NERVE

While no detectable change in axoplasmic transport could be discerned in sciatic nerves contralateral to the colchicine injection, or in sciatic nerves of rats receiving systemic injections of colchicine, there was an effect on axoplasmic transport at the site of injection in the treated sciatic nerve. In 9 rats (140-170 g), axoplasmic transport was examined in the treated sciatic nerve at times varying from 54 to 108 hrs following a subepineural injection of 152 µg of colchicine. This dose was the usual one used to produce extrajunctional sensitivity to ACh on both sides of the animal. In all cases, there was an accumulation of radioactivity central to the site of colchicine injection. Fig. 14B is a typical result from this group. This animal was examined 49 hrs after a subepineural injection of 152 µg of colchicine and was killed 5.5 hrs after the ^{3}H-leucine injections into the spinal cord. As can be seen, there is an accumulation of radioactivity central to the injection site (at the arrow) of the drug. The wave-front of radioactivity can still however be seen 2 cm peripherally in the profile shown in Fig. 14B. The accumulation of radioactivity could be interpreted as being due to a partial block of axoplasmic transport of labelled materials. The rate of axoplasmic transport in these rats, estimated from the detectable edge of the wave-front (whose amplitude was reduced) was 394 ± 8 mm / day (mean ± S.E.M., 9 animals).

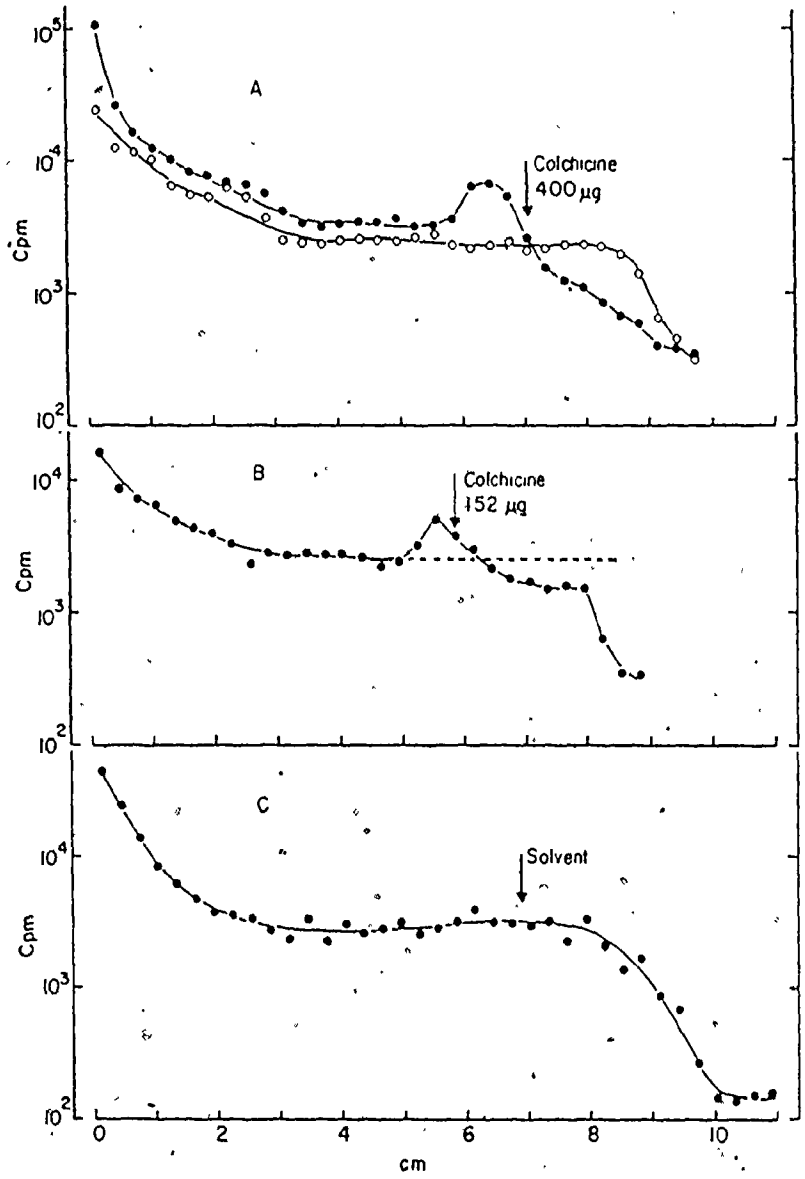
A
Colchicine
400 µg
B
Colchicine
152 µg
C
Solvent
Cpm
cm
0
2
4
6
8
10

FIGURE 14.

FIGURE 14. Block of axonal transport of ^{3}H-labelled material in sciatic nerves injected with colchicine. Flow time was 6.25 hrs in A, 5.5.hrs in B and 6.25 hrs in C. In A, there is a significant disruption of axoplasmic transport (distal to the injection site marked by the arrow) produced by a subepineural injection of 400 µg of colchicine, 49 hrs previously (filled circles). In the contralateral sciatic nerve of the same animal (open circles) no impairment of axoplasmic transport is apparent.

In B, the lower dose of colchicine (152 µg) which was found to produce extrajunctional sensitivity to ACh bilaterally and was the dose routinely used in this size of rat (140-170 g), can be observed to produce a "partial block" of axoplasmic transport; the wave-front can still be recognized distal to the site of drug injection (marked by arrow).

In C, no disruption of axoplasmic transport can be discerned following the injection (site marked by the arrow) of the solvent alone (see Methods).

When a large dose (400 µg) of colchicine was injected subepineurally into the sciatic nerves of 2 rats (380-390 g), there was, in addition to an accumulation of radioactivity central to the drug injection site (at the arrow in Fig. 14A), a total disruption of the wave-front. The results of one of these animals is illustrated in Fig. 14A, 49 hrs after a subepineural injection of 400 µg of colchicine. In the ipsilateral nerve (filled circles), there is no obvious production of the typical wave-front beyond the injection site as is observed with the lower doses of colchicine (compare to 14B) or in the contralateral sciatic nerve (open circles), which was normal. This animal exhibited a marked clinical paralysis of the injected hindlimb prior to the experiment.

To find out whether the procedure of injection into the nerve itself produced any detectable alterations in axoplasmic transport, axoplasmic transport was studied in 3 animals which received a subepineural injection of 5 µl of the solvent alone (see Methods). Fig. 14C is a typical observation of this group. No impairment of transport was observed as a result of the injection technique alone.

It was thus concluded that colchicine did in fact interefere with axoplasmic transport in a dose-dependent fashion, but only in the treated nerve itself. The impairment of axoplasmic transport was due to a specific action of colchicine since control injections alone had no effect. The contralateral nerve was unaffected. It should be recalled here that the contralateral muscle however showed extra-junctional sensitivity to ACh.

13. EFFECT OF NERVE INJECTIONS OF COLCHICINE ON NEUROMUSCULAR TRANSMISSION

To test whether the ipsilateral interference of axoplasmic transport described above was associated with any changes in neuromuscular transmission, the amount of tetanic contraction in the EDL muscles of colchicine injected rats was measured in vivo (see Methods). The results are presented in Fig. 15. Thirty-five (35) rats (145-180 g) were injected subepineurally with 152 µg of colchicine and the tetanic contractions, both ipsi- and contralaterally, were examined at times from 1 to 6 days following the injection. Nine (9) rats served as the control group (145-180 g). As can be observed in Fig. 15, the amount of tetanic tension that can be evoked in the ipsilateral EDL muscle (whose sciatic nerve was injected) decreases with respect to control EDL muscles. Muscles from the control group (9 animals) in this size of animal produced a mean tetanic tension of 112 ± 6 g (mean ± S.E.M.). By day 3, the tetanic tensions produced in the ipsilateral EDL muscle had decreased to 65 ± 7 g (mean ± S.E.M., 7 animals). The contralateral EDL muscles of these animals had a mean of 110 ± 6 g (mean ± S.E.M., 7 animals) which was not significantly different from normal.

From these reults, it was concluded that an impairment of neuromuscular transmission on the treated side could be attributed to a block of axoplasmic transport in the injected sciatic nerve.

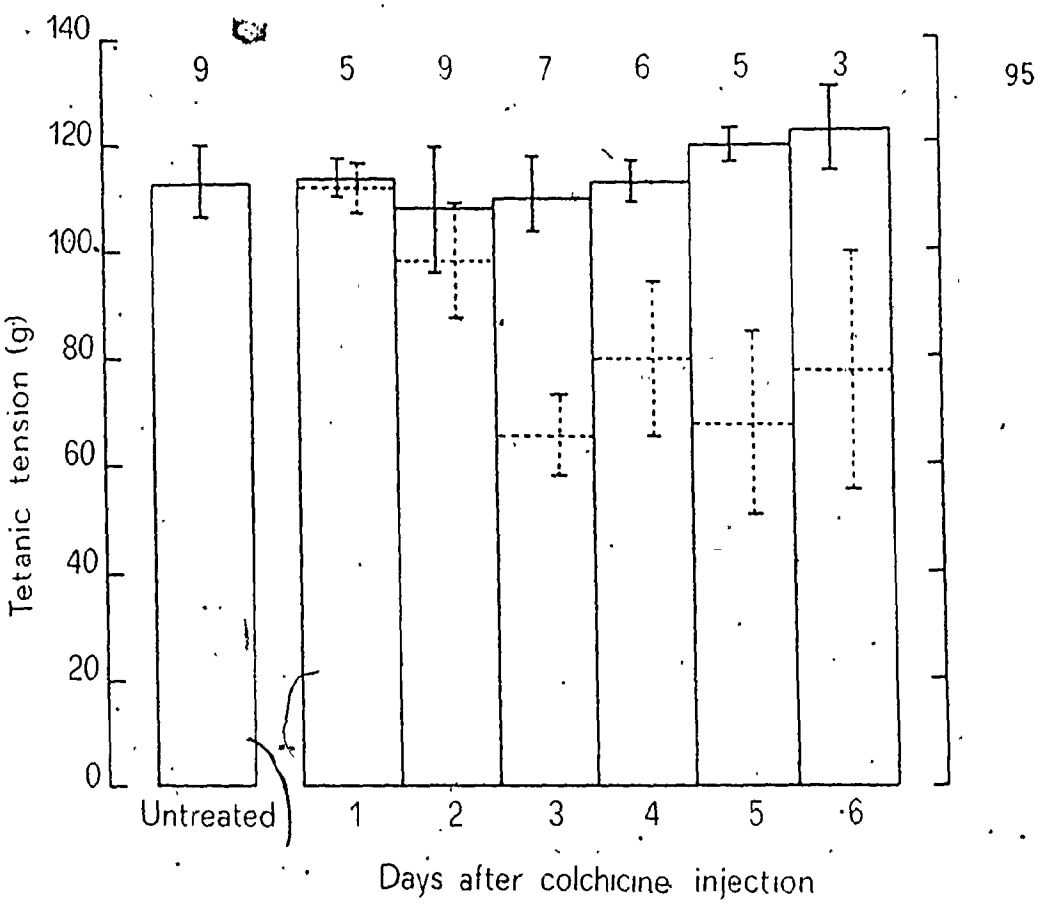

FIGURE 15. Indirect tetanic tension of ipsilateral (dashed columnns) and contralateral (closed columns) EDL muscles in a series of rats, as a function of time after an injection of colchicine (152 µg) into the ipsilateral sciatic nerve. The figure above the columns indicate the number of rats examined and the bars represent the S.E.M.. The left-hand column (untreated) indicates the tetanic tensions obtained from control rats of the same weight range.

14. SILICONE RUBBER NERVE-CUFFS

An attempt was made to avoid the systemic effect obtained following sciatic nerve injections of colchicine by placing drug-impregnated silicone rubber implants around the sciatic nerve (see Methods). It was hoped that this procedure would lead only to a local action of colchicine on the nerve. However, it was found that although changes in the EDL muscle were indeed confined to the treated side, the nerve-cuff produced effects of its own. In these experiments, a silicone cuff was applied as described in the Methods to one sciatic nerve in a total of 156 rats (270-350 g). The cuffs had an internal diameter (I.D.) of 1.6 mm to produce a snug but not overly tight fit around the sciatic nerve, which had a diameter of around 1.6 mm in these animals. Cuffs of 1.25 mm I.D. were also tried, but as can be seen in Table 1 (control cuffs), these proved to be too small and produced paresis in the majority of animals (13 out of 15, 87%). The silicone cuffs used in these experiments contained (1) 0.10% colchicine by weight (w/w), (2) 0.15% colchicine (w/w), (3) 0.10% vinblastine (w/w) or (4) no drug at all (control).

a) CONTROL NERVE-CUFFS

As can be seen in Table 1 (control cuffs), most (23 out of 30) of the rats which had control silicone nerve-cuffs (1.6 mm I.D.) applied to their sciatic nerves did not exhibit any clinically observable

signs of paresis at any time following this treatment (Animals were carefully examined twice a day as described in the Methods.). However, microelectrode investigation revealed that in the EDL muscles supplied by the cuffed nerves, some muscle fibres exhibited extrajunctional sensitivity to ACh. In 3 of these EDL muscles, 3 out 40 tested fibres were found to be sensitive to ACh at the tendon region (3.5 ± 1.2 units, mean ± S.D., 3 muscles) 5 to 8 days after the control nerve-cuff had been applied. These muscle fibres displayed m.e.p.p.s, indicating that they were not denervated. Of the 31 tested contralateral EDL muscle fibres examined in these 3 animals, not one of them was found to be sensitive to ACh at the tendon region.

It was concluded from these observations that a small number (perhaps around 7-8%) of EDL muscle fibres could be affected by the mechanical presence of the control cuff alone on the sciatic nerve.

b) COLCHICINE-IMPREGNATED NERVE-CUFFS

Even though a few ipsilateral EDL muscle fibres exhibited an extrajunctional sensitivity to ACh that could be attributed to the mechanical effects of the control nerve-cuffs, it was still possible that drug-impregnated cuffs would produce a significant ipsilateral effect by pharmacological block of axoplasmic transport. Silicone nerve-cuffs containing either 0.10% or 0.15% colchicine (w/w) were placed on one sciatic nerve of 47 rats (270-350 g.). Seventy per cent (70%) of these animals (see Table 1) developed clinically observable signs of paresis (the rats were observed for up to 8 days). The

TABLE 1 - INCIDENCE OF LEG PARESIS WITH NERVE-CUFFS

Type of cuff	1.6 mm I.D.		1.25 mm I.D.	
	Total No. of rats	No. and % with paresis	Total No. of rats	No. and % with paresis
Control	30	7(23%)	15	13(87%)
Colch. 0.10%	23	13(57%)	9	9(100%)
Colch. 0.15%	24	20(83%)	9	9(100%)
Vinbl. 0.10%	24	16(67%)	-	-

TABLE 1. Incidence of paresis of the rat hindlimb following the application of silicone-rubber nerve-cuffs to the sciatic nerve. Cuffs of two different diameters (1.6 and 1.25 mm I.D.) were tried in order to provide the best fit around the nerve while producing the least amount of trauma. Under the column "paresis" are included all the rats which were affected, irrespective of the degree of paresis which ranged from mild to complete. The weight range of the animals used in this series of experiments was 270-350 g. The observations from these experiments are discussed in the text.

ipsilateral EDL muscle fibres of 11 of the apparently non-paretic 30% of the animals were examined with microelectrodes, 5 to 8 days after the application of a drug-impregnated nerve-cuff. In these muscles, 65 out of 191 (34%) ipsilateral fibres exhibited sensitivity to ACh at the tendon region (13.8 ± 1.8 units, mean ± S.E.M.). Most of these fibres (88% of 65 fibres) displayed m.e.p.p.s, indicating that they were not denervated. However, only a few of these fibres (8 out of 57, 14%) had transmitted action potentials following stimulation of the sciatic nerve. The rest responded only with end-plate potentials (e.p.p.s). This may have been due in part to the fact that these fibres had a low RMP (-58.6 ± 1.8 mV, mean ± S.E.M., 49 fibres).

One feature of the ipsilateral EDL muscle fibres that exhibited extrajunctional sensitivity to ACh following the application of a colchicine-impregnated nerve-cuff was that relatively few (only about 34%) were found compared to the number observed after systemic colchicine injections (all) ; furthermore, these affected fibres were found to be scattered among the rest of the muscle fibre population, which were normal. Of the EDL muscle fibres examined contralateral to nerve-cuffs containing colchicine (0.10% and 0.15%, w/w.) none could be found to exhibit extrajunctional sensitivity to ACh (71 fibres, 8 animals). It was thus concluded that changes in the muscle fibres were confined to the treated side with this procedure; however, they could not be unequivocably attributed to the blocking of trophic factors in the nerve.

c) VINBLASTINE-IMPREGNATED NERVE-CUFFS

Vinblastine (0.10% w/w), another potent anti-mitotic drug, was also mixed into a few cuffs (1.6 mm I.D., see Methods) and applied in 24 animals (see Table 1). Of these animals, 67% (16 out of 24) developed a paresis of the treated hindlimb by the second day following the application of the nerve-cuff. Since a greater number of animals appeared to develop an ipsilateral hindlimb paresis with vinblastine impregnated nerve-cuffs than with colchicine (67% vs 57% for equivalent concentrations, see Table 1) the use of vinblastine was discontinued.

15. DEVELOPMENT OF IPSILATERAL HINDLIMB PARESIS FOLLOWING NERVE-CUFF APPLICATION

One point of concern throughout this investigation, as mentioned earlier, was the possibility that the cuff itself could produce a mechanical disturbance of the nerve. As can be seen in Table 1, a large number of animals treated with drug-impregnated nerve-cuffs developed a clinically detectable paresis (56 out of 101, 55% of all treated animals). The paresis was of variable severity and started about one day after the application of a drug-impregnated nerve-cuff. Interestingly, the paresis was frequently transient. A rat which may have exhibited almost complete paralysis of the treated leg one day after surgery, might be judged clinically normal by the 5th to 8th day.

Because of this feature of the paresis, animals were examined twice a day (see Methods) for any clinical signs of impairment. In one group of 8 rats that developed a moderate to severe paresis of the treated leg on the second day following the application of colchicine-impregnated (0.15% w/w) nerve-cuffs, only 1 had any sign of muscle weakness by the 7th day. It was also interesting to note that there was a higher frequency of occurrence of paresis (see Table 1) with higher levels of colchicine (i.e. 0.15% w/w) in the cuffs than with 0.10% colchicine in the cuffs (57% vs 83%).

The nature of the ipsilateral paresis of the hindlimb following the application of a drug-impregnated cuff was investigated in 6 rats exhibiting moderate to severe paresis on day 2 following surgery. In these animals, the sciatic nerve was stimulated in vivo, both above and below the level of the cuff, and the isometric tetanic contractions of the EDL muscles were recorded. Fig. 16 illustrates the results from one animal of this group. As can be observed, stimulation distal to the cuff evoked much greater responses than stimulation proximally. This was interpreted as being due to a conduction block produced by the drug-impregnated cuff at the region of the cuff itself. Drug-impregnated cuffs however produced a greater occurrence of paresis than control cuffs of the same diameter (see Table 1). It thus appeared that there was a synergistic effect between the drug and the cuff in producing conduction block.

From these experiments it was concluded that the observations obtained with colchicine-impregnated cuffs could not be used to support

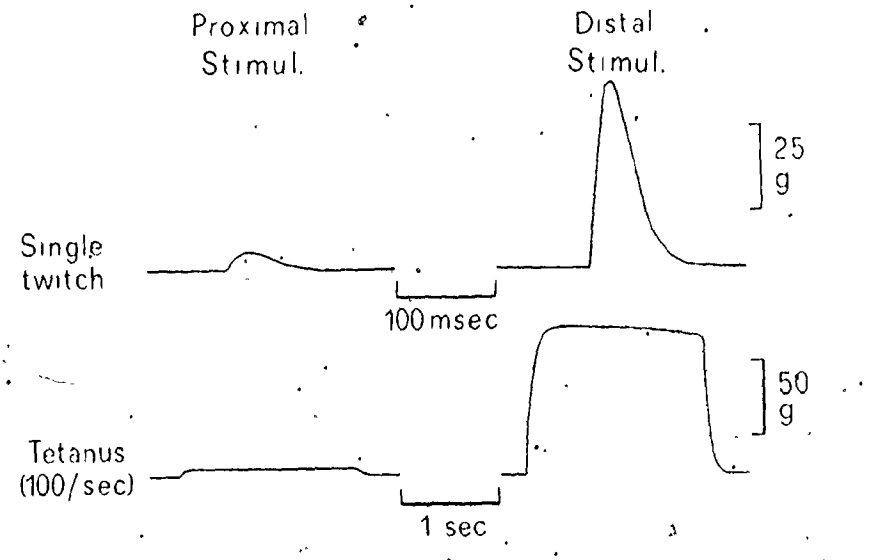

FIGURE 16. Conduction block in the sciatic nerve at the site of application of a colchicine impregnated cuff (1.6 mm I.D., 0.15% colchicine w/w), implanted 4 days earlier. A sub-total paresis of the cuffed leg had appeared about 20 hrs after cuff application. Isometric contractions of EDL muscles are shown, caused by supramaximal stimulation proximal (Proximal Stimul.) and distal (Distal Stimul.) to the cuff in.vivo. Tetanic tension of the contralateral normal EDL muscle was 192 g.

unequivocably the hypothesis that trophic factors were being blocked in the nerve. These considerations are discussed later.

16. A POSSIBLE DIRECT ACTION OF COLCHICINE ON EDL MUSCLE FIBRES

Since colchicine, injected either systemically or subepineurally, did not appear to produce denervation-like changes in the EDL muscle by an action on the sciatic nerve, the possibility was considered that the drug was acting directly on the muscle fibre itself. In a few preliminary experiments the action of the drug on the denervated EDL muscle was investigated. If the effect of colchicine on the EDL muscle were nerve-mediated, then no action of the drug on denervated muscle could be expected. However, it was hoped that, if the effects of colchicine were exerted directly on the muscle fibres, they might sum in some way with the effects of denervation, rather than be masked by them. In the preliminary experiments reported here however, a direct action of colchicine on the EDL muscle could be observed.

Rats were denervated and simultaneously injected with a systemic dose of colchicine (90-100 µg / 100 g b.w., I.M.). The sensitivity of EDL muscle fibres to ACh was studied by measuring the tensions produced by EDL muscles in vitro following exposure to various concentrations of ACh (10^{-7} to 10^{-3}M, see Methods). Fig. 17 illustrates the change in response to bath-applied ACh undergone by EDL muscles following denervation. A concentration of ACh (5×10^{-4}M) that barely evoked a

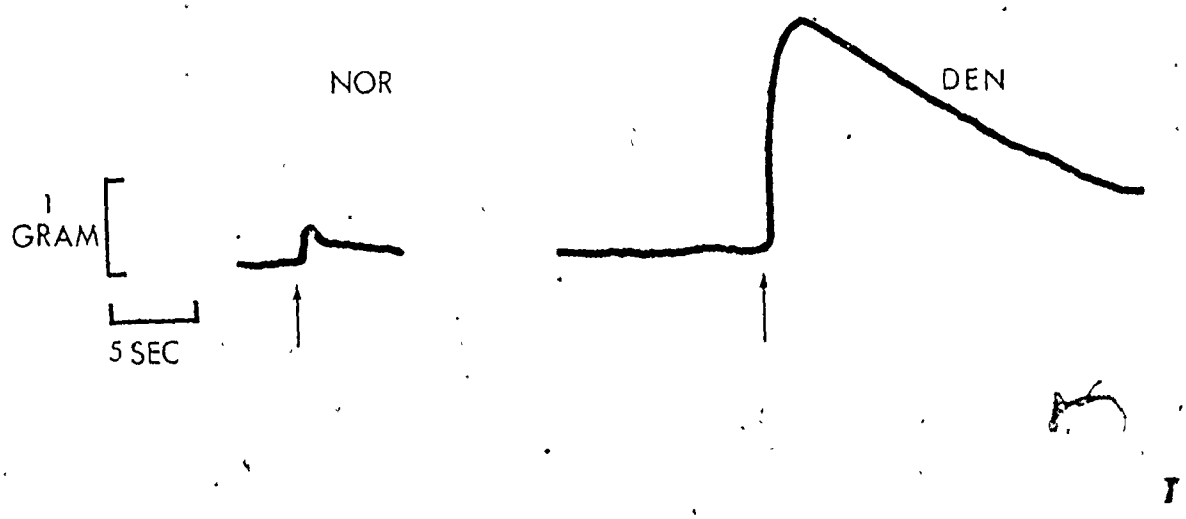

FIGURE 17. Comparison of the response of normally innervated (NOR) and a 54 hour denervated (DEN) EDL muscle to the bath application of 5×10^{-4} M ACh. Both muscles were taken from the same animal. The time of introduction of the ACh is marked by the arrows. The curves are direct tracings from the original record. As can be seen, the denervated muscle produces a large contraction in response to this concentration of ACh, which for the normal muscle is just above threshold.

response in the normal muscle stimulated the denervated one to produce a significant amount of tension. The principle objective was to compare the dose-response characteristics of normal, denervated and denervated plus colchicine treated EDL muscles.

Fig. 18 illustrates the markedly altered dose-response characteristics of denervated EDL muscles (DEN, 100 hrs following denervation) and of EDL muscles from colchicine treated animals (COLCH, 90-100 µg / 100 g b.w., I.M., injected 100 hrs previously). Not only are the thresholds lowered but also the amount of tension produced with respect to normal muscles (NOR) is significantly increased. The denervated EDL muscles (DEN) were 100 times more sensitive to ACh than normal muscles, exhibiting a threshold at around 1×10^{-6}M ACh whereas the normal muscles (NOR) responded only at 1×10^{-4}M ACh. The EDL muscles from colchicine treated rats (COLCH) also exhibited a lowered threshold (about 1×10^{-5}M ACh), although not as great as for denervated muscles. At a concentration of 5×10^{-5}M ACh, which was one-half of the threshold dose of normal muscles (1×10^{-4}M), the denervated muscles (DEN) produced 3.7 ± 0.4 g (mean ± S.E.M., 6 muscles) of tension while the muscles from colchicine treated animals (COLCH) produced 0.30 ± 0.08 g (mean ± S.E.M., 5 muscles). As can be seen in the figure (18), the normal muscles did not respond at all at this concentration.

To investigate whether or not some interaction may have occurred, the EDL muscles from a series of rats (5 animals, 175-190 g) were examined 30-44 hrs following a colchicine injection (systemically, I.M., 100 µg / 100 g b.w.) and simultaneous denervation. A dose-response

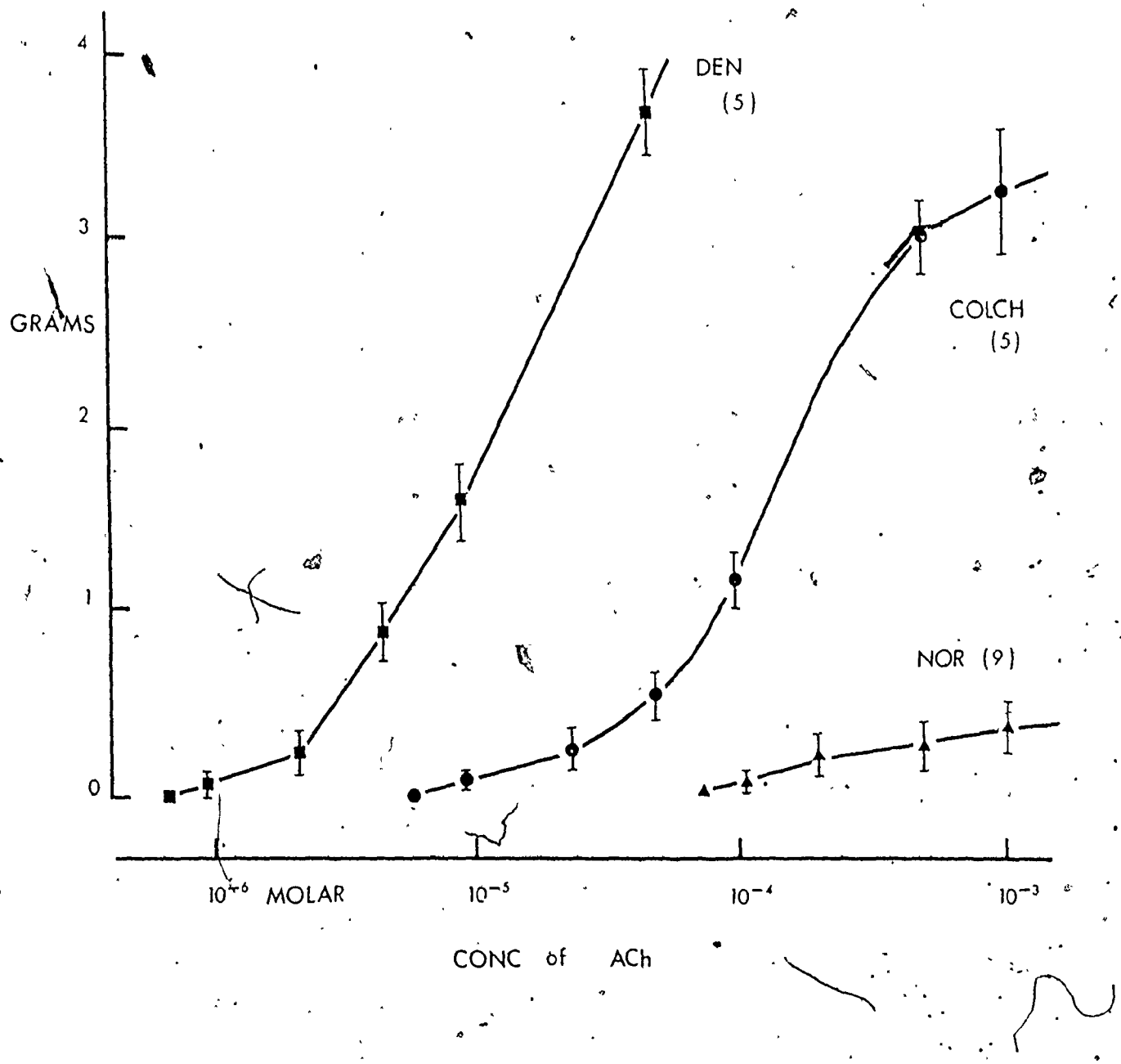
4
3
2
1
0
GRAMS
DEN
(5)
COLCH
(5)
NOR (9)
10^{-6} MOLAR
10^{-5}
10^{-4}
10^{-3}
CONC of ACh

FIGURE 18.

FIGURE 18. Dose-response curves of EDL muscles to bath-applied ACh following various treatments. Normally innervated (NOR) EDL muscles exhibited a threshold response at about 1 x 10^{-4} M.ACh. Normally innervated EDL muscles from animals which had received a systemic injection of colchicine (COLCH) 100 hrs previously, exhibited a threshold which was one-tenth that of untreated animals (NOR) and in addition produced much greater tensions in response to various concentrations of ACh. EDL muscles which had been denervated (DEN) 100 hrs previously displayed the greatest amount of sensitivity to ACh both in terms of a greater production of tension and in terms of a lowered threshold (100 times lower than normally innervated muscles). The numbers in brackets associated with each curve represent the number of muscles tested. Each point represents the mean value of tension obtained in response to the corresponding concentration of ACh and the error bars represent the S.E.M. The curves were drawn by eye to obtain the best fit of the points.

relationship was obtained and compared to the dose-response characteristics of EDL muscles which had been denervated alone for 30-45 hrs. The results are illustrated in Fig. 19. The denervated EDL muscles from colchicine injected animals responded to significantly lower concentrations of ACh than did denervated EDL muscles alone. At an ACh concentration of 5×10^{-4} M the colchicine plus denervated muscles produced 0.65 ± 0.08 g of tension (mean ± S.E.M., 5 muscles) compared to 0.20 ± 0.08 g of tension (mean ± S.E.M., 6 muscles) for denervated muscles alone.

It was concluded from these preliminary results that colchicine has some direct effect on EDL muscle fibres.

a) MICROELECTRODE INVESTIGATION

A few preliminary experiments were also carried out with microelectrodes to examine whether a direct effect of colchicine could similarly be detected at the single fibre level. Four (4) rats (180-195 g) were systemically injected with colchicine (100 µg / 100 g b.w.) and simultaneously denervated. The EDL muscles were then removed 40-46 hrs later and the muscle fibres were tested for extrajunctional sensitivity to iontophoretically applied ACh at the tendon region. Of the 49 tested fibres, all exhibited extrajuntional sensitivity to ACh with a mean value of 23.8 ± 1.7 units (mean ± S.E.M., 49 fibres, 4 animals). These values were compared to the values measured in EDL muscle fibres from 3 rats (175-190 g) which had been denervated 41-46 hrs earlier. The extrajunctional sensitivity observed in 37 fibres from

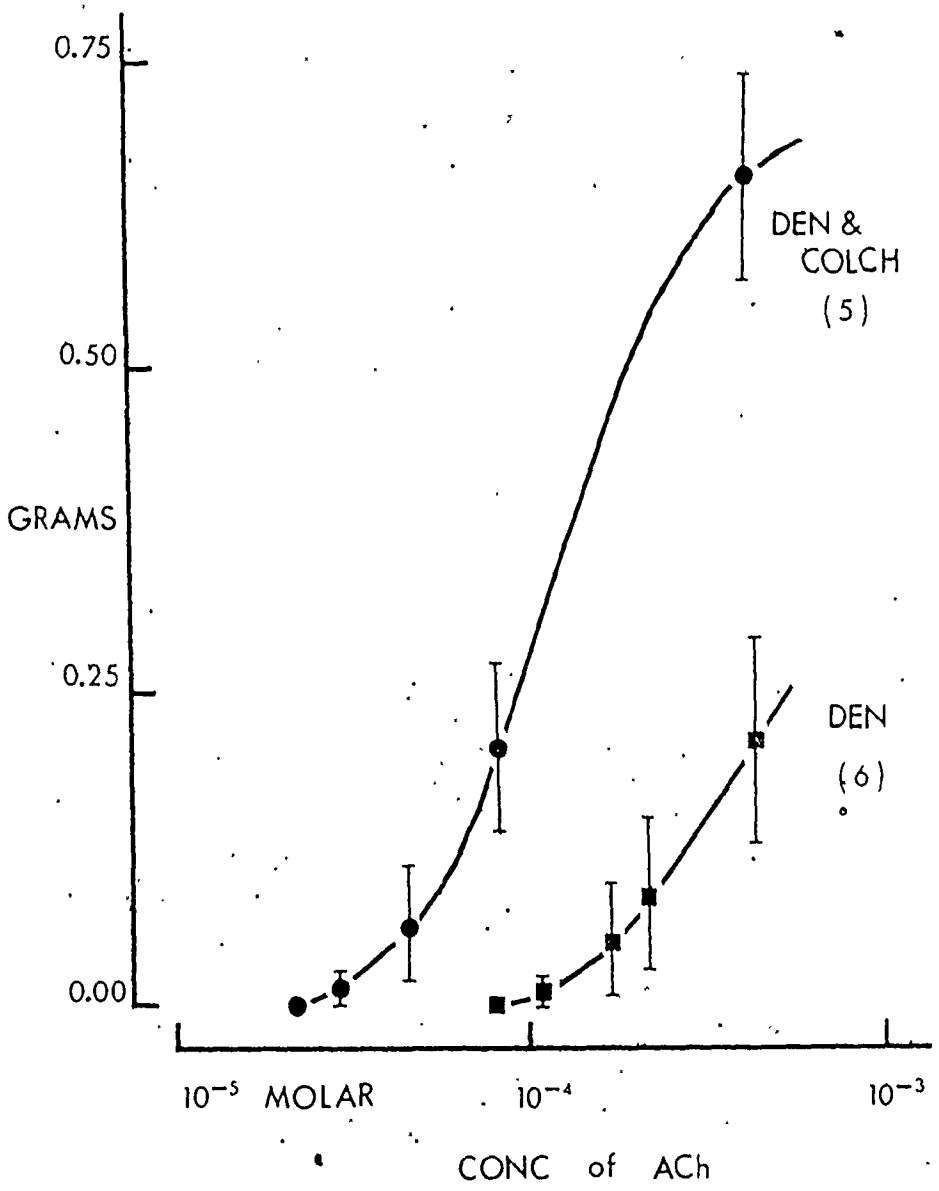

FIGURE 19. Comparison of dose-response curves of denervated EDL muscles (DEN) to EDL muscles from animals which in addition to denervation, simultaneously received a systemic injection of colchicine (DEN & COLCH). All muscles were tested 30-44 hrs following treatment. The number associated with each curve represents the number of muscles tested. Each point represents the mean value of tension obtained in response to the corresponding concentration of ACh. The error bars represent the S.E.M.. The curves were drawn by eye to obtain the best fit of the points.

these animals at the tendon region was 13.8 ± 2.0 units (mean ± S.E.M., 37 fibres, 3 animals). These results, which show a significant ($P < 0.001$) difference between the ACh-sensitivity of muscle fibres from the two groups of animals also suggest that colchicine has a direct effect on the EDL muscle fibres.

17. RESTING MEMBRANE POTENTIAL (RMP) OF EDL MUSCLE FIBRES FOLLOWING DENERVATION

Following denervation, the RMP of skeletal muscle fibres decreases (see Introduction). This is also the case following systemic injections of colchicine (see previous sections) although the decrease in RMP is not as great. It was hoped that some preliminary experiments could be carried out to observe whether there might also be some interaction between the effects of colchicine and denervation in regard to this phenomenon. Although time did not allow this series of experiments to be completed, some interesting points emerged from this study.

Following a few hours of denervation, the end-plate region of EDL muscle fibres was observed to depolarize significantly ($P < 0.01$) by 6 to 8 mV. At this time, no alteration was yet evident in the RMP measured at the tendon region. In the two muscles that were examined, the mean RMP was -71.1 ± 1.1 mV (mean ± S.E.M., 65 fibres) at the end-plate region, 10-12 hrs following surgery. At the tendon region, these fibres displayed a mean RMP of -77.4 ± 1.2 mV (mean ± S.E.M., 58 fibres). The motor nerve terminals at this time still appeared to

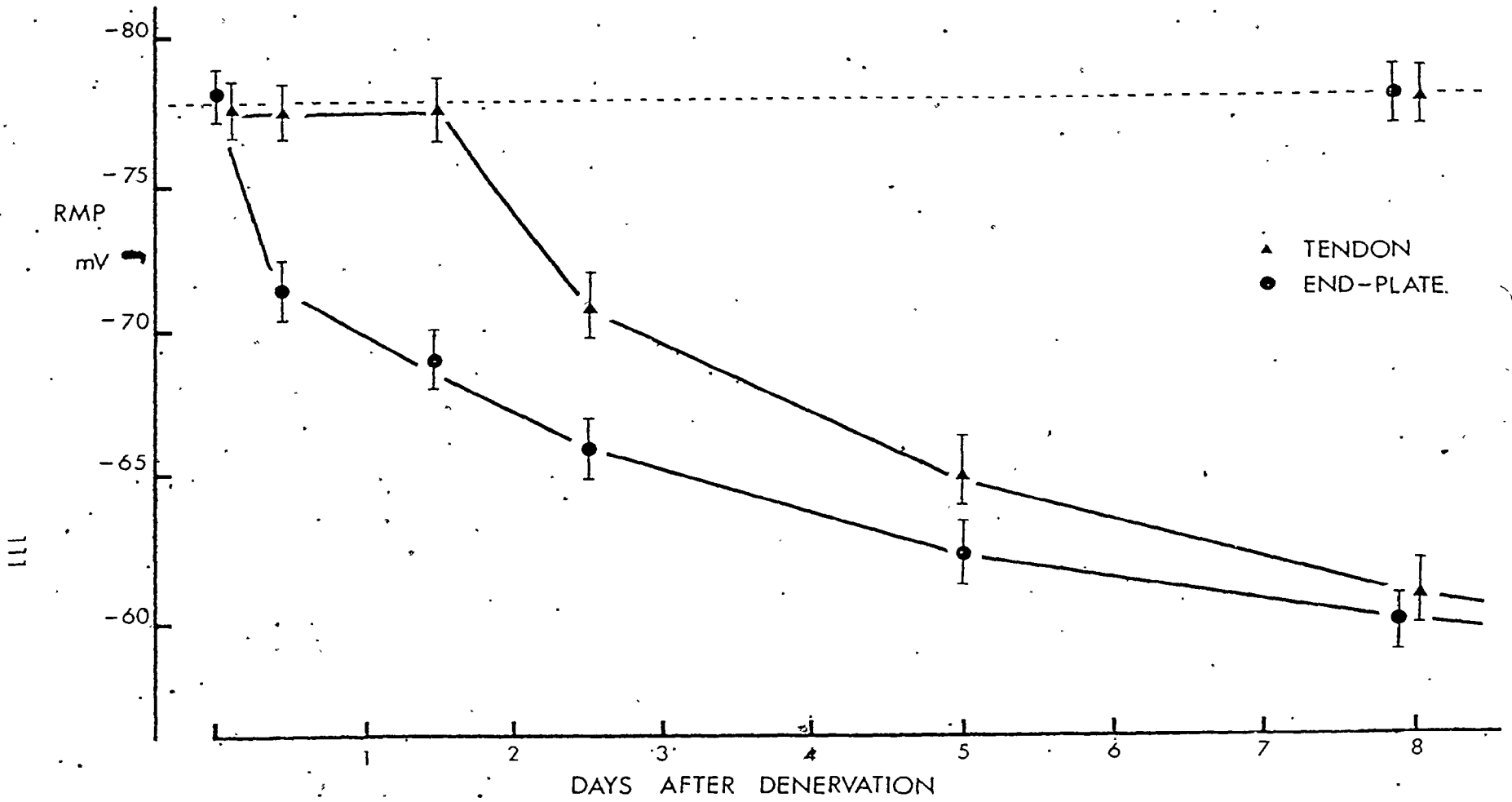
-80
-75
RMP
mV
-70
-65
-60
TENDON
END-PLATE
1
2
3
4
5
6
7
8
DAYS AFTER DENERVATION

FIGURE 20.

FIGURE 20. Comparison of the time-course of the fall in the RMP of denervated EDL muscle fibres measured at the end-plate region to the RMP measured at the tendon region. Each point represents the mean value of at least 30 muscle fibres and the error bars represent the S.E.M.. The triangles and circles represent observations at the tendon region and at the end-plate region, respectively.

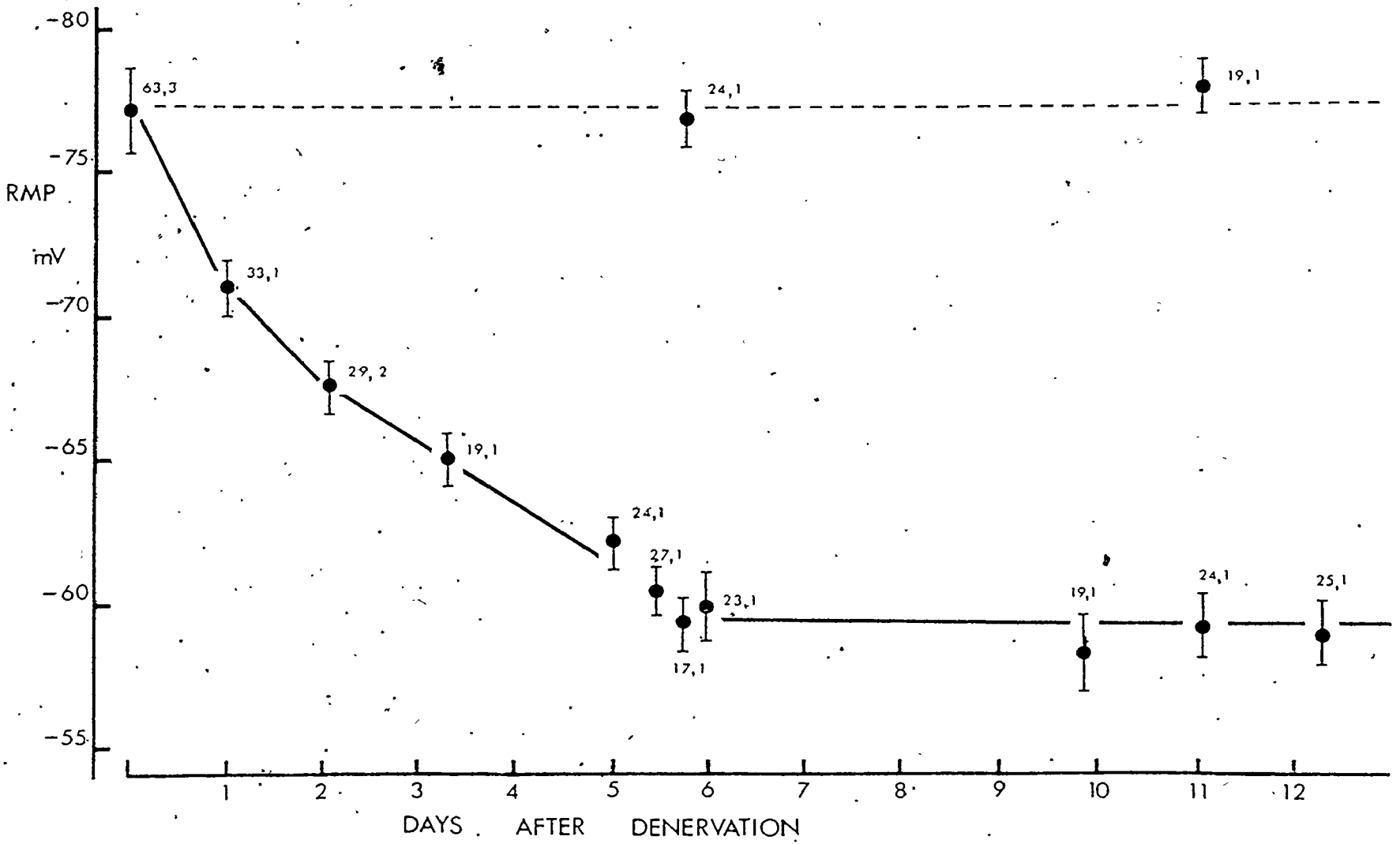
-80
-75
RMP
mV
-70
-65
-60
-55
63,3
33,1
29,2
19,1
24,1
27,1
17,1
23,1
24,1
19,1
24,1
25,1
19,1
1
2
3
4
5
6
7
8
9
10
11
12
DAYS AFTER DENERVATION

FIGURE 21.

FIGURE 21. The time-course of drop in the resting membrane potential (RMP) of EDL muscle fibres following denervation. Muscle fibres were impaled with KCl microelectrodes at the end-plate region and the RMP noted. Each point represents the mean value of RMP and the error bars associated with eadh point, the S.E.M.. The numbers associated with each data point represents the number of muscle fibres impaled and the number of muscles examined, respectively. The dotted line represents the RMP of normally innervated fibres.

be physiologically normal since m.e.p.p.s could be recorded at virtually every end-plate (62 out of the 65 fibres examined) and virtually every fibre tested exhibited transmitted action potentials (14 out of 15 tested fibres). Only after 36 hrs of denervation, as illustrated in Fig. 20, was a drop in RMP observed at the tendon region. These results generally confirm the observations of Albuquerque, Schuh & Kauffman, (1971) who reported similar findings.

Fig. 21 illustrates the general time-course of the drop in RMP of denervated EDL muscle fibres as measured at the end-plate region. After 5 days of progressive decline, the RMP in denervated EDL muscle fibres reached a minimum value around -60 mV. No difference could be observed in the RMP measured at the end-plate or tendon regions.

The observation of an "early" membrane depolarization (i.e. a membrane depolarization at a time when the motor nerve still appears, at least functionally, to be physiologically normal) raises some interesting ideas which are discussed later. These results will be followed up in subsequent studies to see whether or not colchicine influences them, as it does the increased ACh-sensitivity which follows denervation.

PART VI

DISCUSSION OF THE RESULTS

WITH CONCLUSIONS AND SPECULATIONS

1. A SYSTEMIC ACTION OF COLCHICINE

Following a single subepineural injection of colchicine (dose range 90-100 µg / 100 g b.w.) into one sciatic nerve of rats, extrajunctional sensitivity to inotophoretically applied ACh could be detected in the majority of ipsilateral EDL muscle fibres, when examined 4 to 5 days later with microelectrodes. This was not due to denervation of these fibres by the experimental procedure, for the majority (94%) of the ipsilateral EDL fibres displaying extrajunctional sensitivity to ACh also had m.e.p.p.s and transmitted action potentials. (Those ipsilateral fibres (about 6%) that did not have m.e.p.p.s and transmitted action potentials but displayed extrajunctional sensitivity to ACh were considered to have been denervated). Unexpectedly, contralateral EDL mucle fibres also exhibited extrajunctional sensitivity to ACh. These fibres were all apparently normally innervated, since they had m.e.p.p.s and transmitted action potentials.

The bilateral effect of colchicine following a sciatic nerve injection is interpreted as being due to an effect of the drug diffusing from the injection site and acting systemically. When a low dose of colchicine was injected into the sciatic nerve, only a few EDL muscle fibres on either side could be found to exhibit extrajunctional sensitivity to ACh. With a higher dose, the majority of tested EDL muscle fibres on both sides of the animal were found to exhibit extrajunctional sensitivity

to ACh. Thus the factor that was important in the appearance of bilateral extrajunctional sensitivity to ACh appeared to be simply the amount of colchicine injected per body weight of animal. When deliberate systemic injections were made (I.P. or I.M.) of the same doses of colchicine used for the nerve injections (i.e. 90-100 µg / 100 g b.w.), a similar bilaterally-expressed extrajunctional sensitivity to ACh occurred. It thus seems clear that even though colchicine is injected directly into one sciatic nerve, it is essentially acting systemically.

2. A LOCAL ACTION OF COLCHICINE

In the initial experiments, the only effect that could be ascribed to a local action of colchicine on the treated side was the appearance of a few muscle fibres of the ipsilateral EDL, referred to above, which displayed neither m.e.p.p.s nor transmitted action potentials, and were considered to have been denervated. This was not the result of the procedure alone, but was due to the action of the drug, since animals injected only with the solvent did not give such results.

The "local" versus the "systemic" effect of the drug could be clearly identified when the size of the rats receiving nerve injections of colchicine was altered, while maintaining a constant whole-animal dose of the drug (i.e. 90-100 µg / 100 g b.w.). In very large rats, the nerve injection of colchicine produced a greater number of ipsilateral non-responding (i.e. denervated) fibres than that observed in

the smaller animals. In contrast, contralateral EDL muscle fibres displaying extrajunctional sensitivity to ACh all exhibited transmitted action potentials, regardless of animal size. It can be concluded therefore that colchicine, injected locally into the sciatic nerve, caused a loss of function in the endings of a small percentage of nerve fibres, depending on the dose used. In rats of larger size, a greater amount of the drug was injected into the sciatic nerve to maintain a whole-animal dose of 90-100 µg / 100 g b.w. and thus a greater amount of the drug was available to act locally. The systemic action of the drug was unaffected since the whole-animal dose remained the same.

3. THE NATURE OF THE EXTRAJUNCTIONAL SENSITIVITY TO ACh FOLLOWING COLCHICINE INJECTIONS

The extrajunctional sensitivity ot ACh of EDL muscle fibres (at the tendon region) was found to be relatively low (about 0.7 units) when examined with microelectrodes two days after a systemic (I.P.) injection of colchicine. At later times after the injection, the sensitivity increased, and reached maximal levels (around 8 units) at about day 5. After this time however, the sensitivity diminished until it was no longer detectable.

The profile of extrajunctional sensitivity to ACh along EDL muscle fibres in colchicine treated rats was similar to that in denervated muscle fibres, in that the highest levels were recorded at the tendon region (about 6 units). The intermediate region (between the end-plate and tendon region) exhibited lower levels (1:8 - 3.1 units).

The end-plate region had the highest sensitivity (about 12 units).

In all aspects therefore except magnitude, it appeared that the extrajunctional sensitivity of EDL fibres to ACh following a colchicine injection was similar to that reported for denervated muscles (Redfern & Thesleff, 1971). Typically, denervated EDL muscle fibres exhibit 50 - 80 units of sensitivity to iontophoretically applied ACh, while the maximal values obtained after colchicine were in the range of 6 - 10 units.

4. OTHER DENERVATION-LIKE CHANGES FOLLOWING COLCHICINE

In addition to the extrajunctional sensitivity to ACh developed by EDL muscle fibres contralateral to a sciatic nerve injected with colchicine, or following a systemic injection of the drug, other characteristics of denervated muscle fibres appeared, namely action potentials which were resistant to the effects of the neurotoxin TTX, and a lowering of the resting membrane potential (RMP). This drop in RMP, -67.5 ± 0.9 mV, as compared with the normal value of -74.7 ± 0.7 mV, was not however as great as that observed for denervated EDL fibres (-54.6 ± 0.9, mean ± S.E.M.).

5. "TOXIC" EFFECTS OF COLCHICINE

Colchicine injections in higher doses (greater than 105 µg / 100 g b.w., injected either systemically or into one sciatic nerve) produced generalized symptoms of drug intoxication, a striking feature

of which was a marked bilateral paresis of the hindlimbs, which was consistent with an impairment of neuromuscular transmission; interestingly, this paresis was transient. Following a toxic dose of 120-150 µg / 100 g b.w., the paresis was most pronounced during the second day and then regressed over the next few days until it completely disappeared in surviving animals. Because of this observation, the possibility that colchicine treatment could cause a transient muscle inactivity had to be taken into consideration as a factor which could cause denervation-like changes in the EDL muscle fibres.

6. WHAT IS THE MECHANISM OF ACTION OF COLCHICINE?

It seemed paradoxical that apparently normally innervated skeletal muscle fibres should display characteristics of denervated muscle fibres, particularly since none of the treated animals could be clinically distinguished in any way from their untreated litter mates. Three main arguments could be put forward to explain these findings: (1) Colchicine was impairing neuromuscular transmission and thus reducing the level of muscle activity, which causes "denervation-like" changes per se (Lømo & Rosenthal, 1972; Lømo & Westgaard, 1975); (2) Colchicine, even though acting systemically, was primarily affecting the nerve and was blocking a neurotrophic factor from reaching the EDL muscles; or (3) It was possible that the drug was acting directly on the EDL muscle fibres themselves to bring about the expression of denervation-like changes in them, without affecting their responses to the normal motor nerve drive.

a) IMPAIRMENT OF NEUROMUSCULAR TRANSMISSION?

A systemic (I.M. or I.P.) injection of colchicine in doses greater than that required to produce the bilateral appearance of extra-junctional sensitivity to ACh, i.e. greater than 105 µg / 100 g b.w., produced a measurable impairment of neuromuscular transmission (see Fig. 11). The possibility was therefore considered that the denervation-like changes occurring following administration of even small (but effective) doses of colchicine might be attributable to a barely detectable but nevertheless significantly decreased level of activity of EDL muscles. This however, appeared unlikely for several reasons. Tetanic tension measurements (Fig. 11 and contralateral EDL muscles (closed columns) in Fig. 15) failed to reveal any degree of neuromuscular transmission impairment at any time, with the usual doses of colchicine used (i.e. 80-100 µg / 100 g b.w.); moreover, the treated animals were clinically indistinguishable from their control litter mates. It may be possible that the amount of ACh released from the motor nerves is somewhat decreased in the treated rats (cf. Katz, 1972). However, because of the large safety factor associated with neuromuscular transmission, a major impairment would be required before neuromuscular transmission was affected to the extent that consequent changes in the activity of the muscle would occur. Certainly, such an impairment would have been clinically apparent and would have been detected with the tetanic tension measurements.

Finally, one must consider the possibility that the firing frequency of the motor neurons may have been somewhat decreased, perhaps by some direct action of colchicine on the cell bodies themselves. However, such a condition need not necessarily lead to any denervation-like changes in skeletal muscle, since even low levels of neuromuscular activity may apprently be adequate to maintain normal skeletal muscle fibre characteristics (Lømo & Westgaard, 1975). In fact, a large decrease (85-90%) of the normal level of activity of motor neurons is required before a minor increase in extrajunctional sensitivity to ACh in rat soleus muscles can be observed (Fischbach & Robbins, 1971). From such considerations, it appeared unlikely that the denervation-like changes observed in the EDL muscle fibres following colchicine treatment (systemic injection or injection into the contralateral sciatic nerve) are due to a decrease in the normal activity of these muscles.

b) BLOCK OF NEUROTROPHIC FACTORS?

Even though acting systemically, it was quite possible that colchicine was primarily affecting nerves and blocking trophic factors from reaching the EDL muscles on both sides of the animal. Nerves contain the most abundant source of microtubules. Colchicine has been demonstrated to block axoplasmic transport when applied to nerves (Dahlstrom, 1969; Kreutzberg, 1969) apparently by its ability to bind to microtubules (Taylor, 1965). Indeed, it is this blocking action, taken together with affinity of colchicine for microtubulin, which has led to the belief that microtubules are causally associated

with axoplasmic transport (for reviews see Jeffry & Austin, 1974; McClure, 1972). It was conceivable therefore that axoplasmic transport was being impaired, even when the drug was used systemically. Perhaps the drug could be acting systemically along the entire length of the sciatic nerve (which, including the ventral roots, can be as long as 11 cm in 300-400 g rats) producing small local impairments of axoplasmic transport which in summation, could result in a major reduction of trophic factors arriving at the muscle.

To check this possibility, the transport of ^{3}H-labelled material was studied in treated and untreated rats. With this technique, no impairment whatsoever could be detected in either the rate of axoplasmic transport or the amount of materials transported at any time, either in the contralateral sciatic nerve after an impsilateral sciatic nerve injection, or in either sciatic nerve after systemic injections of colchicine. This applied even in rats which were severly paralyzed by toxic doses of the drug. It can be concluded from these results that the denervation-like changes observed in the EDL muscles following colchicine treatment by systemic injection, or injection into the <u>contra-lateral</u> sciatic nerve, was not preceded by or associated with any measurable impairment of axoplasmic transport. (The situation in the <u>ipsilateral</u> sciatic nerve after it was injected is dealt with later. The results from those experiments in no way affect the conclusion being drawn from the experiments being considered at this point.)

c) A POSSIBLE DIRECT ACTION OF COLCHICINE ON EDL MUSCLE FIBRES

The simplest interpretation for the appearance of denervation-like changes in the EDL muscle fibres following the colchicine injections in these experiments is that the drug is acting directly on the muscle fibres themselves. Direct evidence for this was obtained from the preliminary experiments in which an interaction of the effects of colchicine with the effects of denervation was studied.

If the action of the drug were nerve-mediated, it was reasoned, no effect should be observed on denervated muscle. On the other hand, if the drug was acting directly on the muscle fibres then an effect might be detected if it interacted in some way with the reaction of the muscle fibres to denervation. In the preliminary experiments reported here, there was a significant effect on the ACh dose-response curve (see Fig. 19) of EDL muscles from animals that were denervated and simultaneously injected with colchicine, compared with the curve for the denervation-only experiments. This was interpreted as evidence of a direct action of colchicine on the EDL muscle. It appeared that there might in fact be a summation of the effects of colchicine with those of denervation.

Preliminary observations with microelectrodes also disclosed that EDL muscle fibres from animals which had been denervated and simultaneously treated with a systemic injection of colchicine exhibited greater levels of sensitivity to iontophoretically applied ACh at the tendon region then muscle fibres which had been denervated alone (23.8 units versus 13.8 units respectively).

These results are supported by the recent findings of Lømo (1975) who also obtained results indicating a direct action of colchicine on denervated rat soleus muscles. Direct electrical stimulation *in vivo* of denervated rat soleus muscles can markedly reduce their extra-junctional sensitivity to ACh, usually close to the normal value. Following an injection of colchicine however, Lømo (1975) observed that stimulation of denervated muscles could not reduce their extra-junctional sensitivity to ACh back to normal. This was interpreted as being due to a direct action of the drug on the muscle fibres.

d) CONCLUSION

The evidence from the experiments described in this thesis suggests that colchicine acts in some way directly on the EDL muscle fibres, causing them to exhibit denervation-like changes. From the results it seems unlikely that the drug produces these changes by causing either an impairment of neuromuscular transmission or an impairment of axoplasmic transport.

7. BLOCK OF AXOPLASMIC TRANSPORT BY COLCHICINE

While no detectable change in axoplasmic transport could be discerned in the sciatic nerves of animals receiving colchicine injections systemically or into the *contralateral* sciatic nerve, there was however a significant effect on the injected sciatic nerve. Proximal to the site of colchicine injection, there was an accumulation of radio-

activity which was interpreted as being due to an interference with axoplasmic transport. Since the block of transport was not complete (the wave-front continued past the injection site) when for example 152 µg was injected, it is referred to as a "partial" block. With larger amounts of colchicine injected into the sciatic nerve (400 µg), almost complete disruption of the radioactive wave-front was observed. These impairments of axoplasmic transport were solely the result of the action of the drug since control injections of the solvent alone had no effect.

When a "partial" block of axoplasmic transport was produced in the ipsilateral sciatic nerve, there was invariably a detectable impairment in neuromuscular transmission on the treated side. Tetanic tension measurements demonstrated that on the third day following a colchicine injection into one sciatic nerve, there was a significant decrease in the amount of tension the muscle could produce. The contralateral muscles in these animals produced normal tensions. This finding, combined with the observations made with microelectrodes, that in some fibres no transmitted action potentials could be recorded, is interpreted as indicating either the presence of nerve fibres killed as a result of the action of colchicine, or at least a failure at the terminals of a number of axons. It can be concluded from these observations that in the rat a demonstrable block of axoplasmic transport probably always results in some degree of neuromuscular failure in some nerve fibres.

8. CONCERNS AND SPECULATIONS ABOUT EXPERIMENTS INVOLVING THE BLOCKING OF AXOPLASMIC TRANSPORT

One point of concern that may be raised in these experiments is the degree of resolution of the present technique (using ^{3}H-leucine) for the measurement of axoplasmic transport. Could the block of a small but significant amount of material be detected? From the present experiments, an overall reduction of at least 15 to 20% of the mean amplitude of the radioactive wave-front would have to occur before it could be stated unequivocably that there was indeed a significant decrease in the amount of materials transported in the nerve. This then defines to some extent, the resolution of this technique as used in the present experiments. Consider the situation in which, say, there was a measured 30% decrease in the amount of material carried by axoplasmic transport. This could either mean that axoplasmic transport in 30% of the nerve fibres was being blocked completely, leaving 70% of the fibres totally unaffected, or that axoplasmic transport was reduced by 30% in each individual axon in the nerve. These considerations indicate the uncertainties in this type of study and limit the confidence with which the presence or absence of "trophic" changes in target organs can be attributed to changes in neuronal transport.

It is also not quite clear what the primary consequences of a block of axoplasmic transport really are on the nerves themselves.

Certainly, when a motor nerve is sectioned or ligated in mammals (thereby producing an immediate and total block of axoplasmic transport at the site of treatment) degenerative changes are observed within a matter of hours in the nerve terminals (Miledi & Slater, 1970). Presumably then, motor nerve terminals in mammals are highly dependent for their viability on the integrity of axoplasmic transport. It seems likely then, that if axoplasmic transport was in any way (mechanically or pharmacologically) interrupted, the viability of the nerve terminals would be affected first (cf Perisic & Cuenod, 1972; Cooper, Diamond & Turner, 1977); this then raises the question of detection of the early stages of denervation in contrast to changes which occur most dramatically when degeneration is well advanced.

Finally, even if it could be demonstrated that for example, 30% of axoplasmic transport was being blocked in each individual nerve fibre, would such a reduction be adequate to account for denervation-like changes, on the grounds of an inadequate delivery of trophic factors to the post-synaptic structure? Are trophic factors transported in a special separate "channel", or are they intermingled with other transported axonal materials? If they are intermingled, then a 30% block of axoplasmic transport in each individual nerve fibre would mean that 70% of the trophic factors would still be getting through. At this stage, the question of "safety factor" would have to be considered. By what percentage would trophic factors have to be reduced to cause detectable effects on a muscle fibre? Answers to these questions do not yet exist. If trophic factors were transported in a separate "channel" then an

argument could be put forward that colchicine might preferentially and significantly interfere with it, but the associated reduction in the total measured axoplasmic transport might go undetected. There is no evidence for or against such a view at the moment, and in this case there seems no compelling reason to argue that poor resolution of the axoplasmic transport measurement has caused a wrong interpretation of the present findings.

From the results presented in this thesis, it is obvious that great care must be exercised when interpreting experiments which purport to block axoplasmic transport. The amount of block produced in each nerve fibre should be known in addition to what effect such treatment has on the nerve terminal itself, before any inferences can be made concerning the block of postulated trophic factors.

9. THE USE OF DRUG-IMPREGNATED NERVE-CUFFS

The chronic application of colchicine to the sciatic nerve with a silicone-rubber implant did indeed restrict EDL muscle fibre changes to the ipsilateral side. However, the technique itself introduced an insidious complication. A large number of animals developed a transitory paresis of the treated hindlimb (57% of those treated with 0.10% colchicine-cuffs and 83% of those treated with 0.15% colchicine-cuffs, see Table 1). The paresis was not due simply to the mechanical presence of the rubber implant since much fewer animals (23%) were affected with the control cuffs alone. It appeared that some form of synergism was

taking place. Perhaps the paresis was due to the swelling of the nerve inside the rubber cuff as a result of trauma, and because of this, a block due to compression was occurring in the nerve. The irritation and consequent swelling of the nerve may have been greater with the colchicine-impregnated cuffs, causing a higher frequency of paresis in these animals. The swelling of the nerve can also account nicely for the transient nature of the paresis, since when the swelling subsides, impulse conduction in the sciatic nerve could be expected to return to normal.

Since the paresis was transient, it was very important in this type of experiment to check that an animal had at no previous time demonstrated clinically detectable impairment of its hindlimb function. In the relatively few rats that passed this stringent criterion (less than 40% overall of animals treated with 0.10-0.15% colchicine-cuffs) the interpretation of the ipsilateral changes in the EDL muscle still remained highly equivocable. Only about 34% of the examined fibres were found to exhibit extrajunctional sensitivity to ACh, and these were scattered among therest of the muscle fibre population which were found to be normal. There was no certain way of knowing whether these fibres had actually been inactive for a few days prior to the experiment or not. In fact, there can be at least three different possible interpretations for the presence of these fibres exhibiting extrajunctional sensitivity to ACh. (1) The appearance of denervation-like changes in these fibres is due to the block of trophic factors in the nerve. (2) The changes in the muscle fibres are due to their inactivity, produced by a conduction block by the nerve-cuff (interruption of muscle activity for as little

as two days is sufficient to cause a generalized supersensitivity to ACh of some days' duration, Lømo & Slater, personal communication). Finally, (3) it is possible that these muscle fibres may have been denervated by the killing of their nerves by the pressure of the nerve-cuff, and that they may have just newly been reinnervated by axon collaterals from neighbouring muscle fibres.

Because of the many reasonable doubts and possible interpretations for the appearance of denervation-like changes in the ipsilateral EDL muscle fibres following the application of a colchicine-impregnated nerve-cuff to one sciatic nerve, it is concluded that the results of such cuff experiments cannot be used as unequivocable evidence to support the existence of neurotrophic factors.

10. MEMBRANE DEPOLARIZATION AND NERVE-STUMP LENGTH

Although no experiments were carried out to specifically test the effect of altering the length of the distal nerve stump on the onset of denervation changes, several interesting points emerged from the preliminary experiments involving the measurement of resting membrane potential (RMP) of EDL muscle fibres following denervation. First, the observation of Albuquerque, Schuh & Kauffman (1971) was confirmed, that the end-plate region of denervated EDL muscle fibres is depolarized by 6 to 8 mV as early as 2 hrs following denervation. In the present experiments, animals were denervated at mid-thigh level, leaving a nerve stump length of about 30 to 35 mm. In the EDL muscles of two such animals

examined at 10 to 12 hrs following sciatic nerve section, the RMP at the end-plate region was indeed 6 mV lower than at the tendon region (approximately). At this time, allof the examined fibres had m.e.p.p.s and 14 out of the 16 fibres examined also had transmitted action potentials, indicating that the nerve terminals were still physiologically normal. Albuquerque et al (1971) interpreted their findings in this regard as evidence in favour of trophic factors from the nerve regulating this property of muscle fibres. There is no strong evidence to corroborate this view at the moment.

Interestingly, when the results of the present work are compared to the results of Albuquerque et al (1971) and to the results of Redfern & Thesleff (1971), both groups studying the effects of denervation on the rat EDL muscle, a relationship between the rate of fall of RMP and the length of the distal nerve stump can be observed. Albuquerque et al (1971) crushed the nerve at its point of entry into the EDL muscle, leaving essentially a "zero" length of nerve stump. The consequently denervated EDL muscle fibres depolarized to about -60 mV in about 2 days. Redfern & Thesleff (1971) cut the nerve to the EDL muscle leaving about 5 to 10 mm of distal nerve stump. In their experiments, the RMP of the muscle fibres reached their denervated levels in about 3 days. The denervated EDL muscles in the experiments reported here, had distal nerve stumps of about 30 to 35 mm. As can be seen in Fig. 21, the RMP drops for about 5 days before reaching the final denervated levels of about -60 mV. Taken together, these experiments and results confirm the observations of Locke & Solomon (1967) who

observed that the rate of decline in the RMP following denervation of the cat gastrocnemius muscle was more rapid with distal than with proximal nerve section. The correct interpretation of this phenomenon is not yet possible with the presently available information. It might involve neurotrophic factors released from the motor nerve, the consequences of degenerating nerve terminals, inactivity of the denervated muscle fibres or some combination of these possibilities.

11. CONCURRENT STUDIES BY OTHERS

During this investigation, a number of concurrent reports appeared concerning physiological changes in mammalian skeletal muscles as a consequence of blocking axoplasmic transport. In these studies, all of the investigators interpreted their findings as being due to a block of neurotrophic factors in the nerve. However, because of the concerns raised in this present work, their findings must be more critically evaluated.

Hofmann & Thesleff (1972) injected colchicine into the sciatic nerve of rats, in a similar manner to that described in this thesis, and observed the appearance of extrajunctional sensitivity to ACh and TTX-resistant action potentials in the EDL muscle. They failed however to examine the contralateral EDL muscle with microelectrodes and thus had no way of assessing whether in their experiments the drug was producing a systemic effect. Because of this important omission, their evidence can not be used to support the existence of neurotrophic factors.

Fernandez & Ramirez (1974) injected colchicine into the hypoglossal nerve in the cat and observed only an ipsilateral increase in sensitivity to intra-arterially injected ACh of the geniohyoid muscle in the neck. They interpreted their findings as being due to a block of trophic factors in the treated nerve. While this may be true in the cat, it is still necessary to exclude allother reasonable possibilities first, especially in view of the fact that many different situations (i.e. denervation, inactivity, direct effect of the drug etc.) can all produce changes in skeletal muscles. The level of neuromuscular transmission for instance, in the geniohyoid muscle is difficult to assess clinically and even a complete but transient paralysis following treatment may go entirely unnoticed. Thus a period of inactivity prior to the experiment could be the reason why this muscle becomes supersensitive to intra-arterially injected ACh. In addition, in their report, Fernandez & Ramirez (1974) did not assess in any way the extent to which they were blocking axoplasmic transport. Thus we have to be assured that they were indeed blocking axonal transport in the manner they assumed, and we must have strong assurances that muscle inactivity (particularly transient inactivity) did not occur at any time in their experiments, before their results may be accepted as "proof" of a trophic influence of the motor neuron on skeletal muscle fibre properties.

Albuquerque and coworkers (Albuquerque, Warnick, Tasse & Sansone, 1972) applied silicone-rubber nerve-cuffs impregnated with colchicine and vinblastine to the sciatic nerve of rats, and reported that they observed innervated EDL muscle fibres which exhibited denervation-like

changes (i.e. extrajunctional sensitivity to iontophoretically applied ACh). They interpreted their findings as being due to a block of trophic factors in the nerve. In view of the complications inherent in this technique, which have been mentioned previously, great care must be exercised before such an interpretation can be accepted however. They do not mention for instance, that any of their treated animals had any impairment of the treated hindlimb at any time, even though they were using cuffs with a surprisingly small I.D. of 0.8 mm. In the present experiments, the use of cuffs with an I.D. of 1 mm or less in the same size of rat that Albuquerque and coworkers used had to be abandoned because of the extensive paresis and conduction block which it produced. It is still possible to select animals with no detectable paresis, but it is then very important to know what percentage of muscle fibres exhibit extrajunctional sensitivity to ACh. This information was not given by Albuquerque and coworkers and if, in fact the percentage of these fibres was small, then it becomes very difficult to rule out that their results were not the result of muscle inactivity due to a conduction block in the nerve (see discussion on the use of drug-impregnated nerve-cuffs.).

In summary, unequivocable direct evidence has not yet been obtained from experiments in which colchicine has been injected into the nerve or applied chronically with silicone-rubber nerve-cuffs that trophic factors participate in the control of skeletal muscle fibre properties.

12. CONCLUSIONS AND SPECULATIONS

The major finding in these experiments, if the interpretations are correct, is that colchicine has a direct action on skeletal muscle fibres, causing them, paradoxically, to exhibit "denervation-like" changes. The function of the muscles however, seems otherwise unimpaired. The mechanism by which this colchicine effect occurs is unknown. Although the participation of possible neurotrophic factors in the regulation of skeletal muscle fibre properties remains unresolved, this finding may still provide some insights on the nature of nerve-muscle relationships. What, for instance constitutes the signal which triggers denervation changes in skeletal muscles? Following systemic injections of colchicine, the appearance of denervation-like changes certainly seems unlikely to be due to either muscle inactivity or to the loss of trophic factors. Still, some alteration similar to that caused by interruption of the normal nerve-muscle relationship occurs. The denervation-like changes that appear following colchcicine injections appear to differ from the changes following denervation in two ways; the magnitude of the changes following colchicine is much smaller than following denervation, and they persist during apparently normal neuromuscular activity.

Following the subepineural injection of colchicine into the sciatic nerve, an impairment of the transport of ^{3}H-labelled material

can be observed. If this block also interrupts the transport of trophic substances controlling the sensitivity of EDL muscle fibres to ACh, one would expect this effect to sum with the moderate systemic effect of colchicine to produce a more pronounced extrajunctional sensitivity to ACh on the injected side. The extrajunctional sensitivity to ACh however, was the same in the innervated EDL muscle fibres of both legs. The only observation that can be correlated with the observed impairment of axoplasmic transport in these experiments, was an impairment of neuromuscular transmission on the treated side, suggesting that a block of axoplasmic transport leads to an impairment of nerve function, and perhaps even to nerve death.

In summary, colchicine, following subepineural injections, may affect nerves and muscles by various mechanisms depending on the dose used. First, it appears to act systemically directly on the EDL muscle inducing denervation-like changes in innervated fibres. Second, with higher doses it also appears to act systemically in producing a reversible, bilateral impairment of neuromuscular transmission. Finally, it also blocks axoplasmic transport at the site of application on the nerve, producing an ipsilateral impairment of neuromuscular transmission and possibly nerve fibre death as well.

With the use of drug-impregnated silicone-rubber nerve cuffs to confine the action of colchicine primarily to the sciatic nerve, the problem of conduction block of nerve impulses was encountered. Microelectrode investigation revealed that even with control nerve cuffs in animals displaying absolutely no clinically detectable signs of paresis

at any time, a few scattered muscle fibres could be found which displayed extrajunctional sensitivity to iontophoretically applied ACh. This complication might be discounted if following the application of a colchicine-impregnated nerve cuff, the majority of examined EDL muscle fibres would exhibit denervation-like changes without previous signs of paresis. This however, is not the case. Relatively few of these fibres can be seen following colchicine-cuff application, and there is no certain way of knowing whether these fibres had been at any previous time inactive.

The conclusions of this report are that colchicine can exert a direct effect on skeletal muscle fibres producing, paradoxically, "denervation-like" changes in them. In higher doses, colchicine injected systemically, can also reversibly impair neuromuscular transmission, perhaps by directly affecting the release of ACh at the nerve terminals. Colchicine injected into the sciatic nerve produced a dose-dependent block of axoplasmic transport which could be correlated with an ipsilateral impairment of neuromuscular transmission thought to be due to killed nerve fibres.

Finally, this report brings into focus the stringent criteria that must be adhered to in order to obtain clear evidence concerning the nature of nerve-muscle "trophic" relationships. One must be certain beyond any reasonable doubt that nerve impulse conduction is not impaired and that the pharmacological agents used do not have direct effects of their own on the muscle fibres.

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APPENDIX

STATISTICAL CONSIDERATIONS

Full statistical analyses of the data obtained in these experiments were not presented in the main body of the thesis for the sake of simplifying the presentation of the findings. This seemed justified on the grounds that no crucial argument pertaining to the thesis was based merely on the manipulation of a set of numerical data. Since the omission of some of the statistical considerations from the main body of the thesis could justifiably produce some misunderstanding of the nature of data acquisition and treatment, this appendix has been added to allay some of those concerns.

POOLING OF DATA FOR COMPARISONS

One question that may arise in the treatment of the data in this thesis concerns the justification for pooling certain data obtained from individual muscle fibres from a number of different animals. Pooling of data would imply that each value obtained was an independent measure. Although this in reality appears to be the case, statistical justification for this was not presented in the main body of the thesis. As can be seen from the following examples of raw data similar to that obtained throughout this work, pooling of data for the specific comparisons made in this thesis appears to be justified. The main basis for pooling data was that an analysis of variance of the raw data

demonstrated that the EDL muscle fibres from any similarly treated group of animals appeared to belong to the same population of muscle fibres even though they were obtained from different animals. The variance of data obtained within animals in any similar treatment group was much greater than the variations observed between the means of different animals. Because of this, a sample population of EDL muscle fibres from one muscle could not be distinguished as being different from sample populations from other muscles within the same treatment group. Since there appeared to be no difference in this regard, the data was pooled for ease of manipulation and comparison.

The animals presented in this appendix are different from those which were described in the text of the Results section and exhibit variances which are somewhat lower. Because of this, the possibility that some slight side to side differences in extrajunctional sensitivity to ACh or in RMP may have occurred following sciatic nerve injections of colchicine can not be entirely ruled out.

TABLE A1. Example of raw data obtained demonstrating extrajunctional sensitivity of ipsilateral EDL muscle fibres to iontophoretically applied ACh near their tendon regions, following an injection of colchicine into the sciatic nerve four to five days earlier. The values represent units of extrajunctional sensitivity to ACh (see Methods).

MUSCLE	1	2	3	4
	4.8	8.4	6.8	6.1
	7.9	7.4	7.5	7.8
	8.0	3.3	4.9	4.9
	6.7	5.8	8.8	5.5
	3.2	5.9	6.2	8.1
	7.1	4.7	5.9	5.7
	6.3	7.4	7.7	6.4
	5.9	7.6	5.8	6.3
	8.6	3.7	6.9	6.0
		4.3	4.1	5.8
				6.7
				5.1
				7.9
				6.0
SUM	58.5	58.5	64.6	88.3
MEAN	6.50	5.85	6.46	6.31
n	9	10	10	14

An analysis of variance (simple randomized design) was carried out on the results measured in the ipsilateral EDL muscle fibres. This analysis indicated that statistically, one could not reject the idea that the populations of the sampled muscle fibres had equal means and that they were actually obtained from a single population of muscle fibres. A sample population of ipsilateral EDL muscle fibres from one muscle appeared to behave like the sample populations from the other ipsilateral EDL muscles examined. Thus, since the probability was high that all the measurements obtained were actually taken from a single population, it appeared that the results could justifiably be pooled.

ANALYSIS OF VARIANCE OF THE DATA PRESENTED IN TABLE A1

The analysis of variance that was carried out was of the simple randomized design.

The total sum of squares was 85.36.

SOURCE	DEG. FREEDOM	SUM SQ.	MEAN SQ.
Among groups	3	2.62	0.87
Within groups	39	82.74	2.12

Calculated F value = 0.87 / 2.12
= 0.41

This F value indicates that there is a high probability that the sample means are equal. It is thus concluded from this test that the variances observed in the data could have occurred by chance alone.

TABLE A2. Example of raw data obtained demonstrating extrajunctional sensitivity of contralateral EDL muscle fibres to iontophoretically applied ACh near their tendon regions, following an injection of colchicine into the sciatic nerve four to five days earlier. The values in the table represent units of extrajunctional sensitivity to ACh.

MUSCLE	1	2	3	4
	6.8	6.3	6.1	4.4
	4.3	3.8	6.3	6.2
	5.4	7.4	7.2	7.3
	6.7	4.9	5.9	7.1
	6.4	4.2	6.5	5.0
	6.9	5.9	6.2	5.3
	6.8	4.6	5.4	5.5
	7.5	5.4	6.6	6.1
	6.6	5.7	5.8	6.0
	5.3	5.8		5.6
	5.8	5.1		6.2
SUM	68.5	59.1	56.0	64.7
MEAN	6.23	5.37	6.22	5.88
n	11	11	9	11

An analysis of variance (simple randomized design) was carried out on the data presented in table A2. This analysis indicated that for the contralateral EDL muscle fibres as well, the hypothesis that each muscle fibre was obtained from the same population could not be rejected and as can be seen from the data, the variances were much greater within animals than between them. Since it was highly probable, using this treatment, that each muscle fibre obtained was from the same population of muscle fibres, the data was pooled for later comparisons.

ANALYSIS OF VARIANCE OF THE DATA PRESENTED IN TABLE A2

The analysis of variance that was carried out was of the simple randomized design.

The total sum of squares was 33. 46 .

SOURCE	DEG. FREEDOM	SUM SQ.	MEAN SQ.
Among groups	3	5.17	1.72
Within groups	38	28.29	0.74

Calculated F value = 1.72 / 0.74
= 2.32

This F value indicates that there is a high probability that the sample means are equal and that the variances observed in the data could have easily occurred by chance alone.

COMPARISON OF POOLED DATA

Since each measurement appeared to be an independent observation, data with respect to extrajunctional sensitivity of EDL muscle fibres was pooled. The pooled ipsilateral measurements were compared to the pooled contralateral ones using a standard t-test (one tailed for related measures).

	IPSILATERAL	CONTRALATERAL
n	39	39
MEAN	6.33	5.96
STD. DEV.	1.42	0.93
S.E.M.	0.23	0.15

Using a one tailed t-test for related measures, t was found to be 1.19 in this case. The degrees of freedom was taken as 38. With the above t value, the P value was found to be greater than 0.1, which would indicate that there is no significant difference between the two populations of muscle fibres.

To be certain that no obvious side to side variations were missed in these animals, analysis was carried out to observe whether significant differences could be detected between muscle pairs.

ANALYSIS OF MEASUREMENTS BETWEEN MUSCLE PAIRS

The data presented in tables A1 and A2 were obtained from 4 rats. Since the ipsilateral muscles correspond to the contralateral ones (i.e. ipsilateral muscle 1 and contralateral muscle 1 were obtained from the same animal), a paired analysis could be done. The standard t-test (one tailed for related measures) was used to examine whether any significant side to side variation could be observed.

MUSCLE		n	MEAN	DEG.FREE.	t
1	IPSI	9	6.50	8	0.16
	CONTRA	9	6.38		
2	IPSI	10	5.85	9	0.57
	CONTRA	10	5.40		
3	IPSI	9	6.72	8	0.93
	CONTRA	9	6.22		
4	IPSI	11	6.30	10	0.92
	CONTRA	11	5.88		

In all of the above cases, P was greater than 0.1, indicating that the observed differences from side to side in these animals could have been due to chance alone.

In the foregoing treatment, one will note that the number of observations used differs slightly from the data tabled in A1 and A2. For the t-test for related measures, it is necessary to compare groups of observations of equal size. Thus for this treatment the last few observations were dropped in those columns which exceeded the required number. This procedure can be justified on the basis that the observations were obtained and tabled randomly and that dropping the last one or two measures should still leave a proper sample of the examined muscle fibre population.

TABLE A3. Example of raw data of resting membrane potentials (RMP) measured in normal EDL muscle fibres. The values as measured are in units of negative (-) millivolts (mV) but for statistical purposes they can be considered as positive values.

MUSCLE	1	2	3
	79	78	76
	81	79	72
	68	67	74
	69	63	73
	67	76	75
	62	75	77
	71	76	74
	73	77	68
	76	70	78
	74	73	77
	75	71	79
	73		
SUM	868	805	823
MEAN	72.3	73.2	74.8
n	12	11	11

ANALYSIS OF VARIANCE OF THE ABOVE DATA

The analysis of variance that was carried out was of the simple randomized design.

The total sum of squares was 702.2 .

ANALYSIS OF VARIANCE

SOURCE	DEG. FREEDOM	SUM SQ.	MEAN SQ.
Among groups	2	36.3	18.15
Within groups	31	665.9	21.50

Calculated F value = 18.15 / 21.50
= 0.84

This F value indicates that there is a very high probability that the sample means are equal and that the variances observed in the data could have easily occurred by chance alone.

TABLE A4. Example of raw data of resting membrane potentials (RMP) in denervated EDL muscle fibres.

MUSCLE	1	2	3
	59	62	54
	63	55	60
	57	56	62
	64	59	53
	58	61	59
	60	60	61
	61	58	58
	62	62	57
	61	61	62
		57	61
			58
			56
SUM	545	591	701
MEAN	60.5	59.1	58.4
n	9	10	12

ANALYSIS OF VARIANCE OF THE DATA IN TABLE A4

The analysis of variance that was carried out was of the simple randomized design.

The total sum of squares was 221.9 .

SOURCE	DEG. FREEDOM	SUM SQ.	MEAN SQ.
Among groups	2	23.9	11.95
Within groups	28	198.0	7.07

Calculated F value = 11.95 / 7.07
= 1.70

This F value indicates that the sample means are probably equal and that the variances observed are due to chance alone.

COMPARISONS OF RMPS OF NORMAL AND DENERVATED EDL MUSCLE FIBRES

As can be seen from table A3, the average RMP of normal EDL muscle fibres is -73 mV, in this particular instance. Furthermore, by the tests applied, it appears that the individual values measured behave independently, thus justifying the pooling of the results. The denervated EDL muscle fibres in table A4 exhibited an average RMP of -59.3 mV. In this case also, there appeared to be valid justification for pooling data since by the tests applied, each value could be considered to be an independent measure.

ANALYSIS OF THE POOLED RESULTS

The standard t-test (one tailed for related measures) was carried out on the pooled data to examine whether any differences existed.

MUSCLE	n	MEAN	STD.DEV.	S.E.M.
NORMAL	30	73.4	4.85	0.89
DENERVATED	30	59.4	2.70	0.49

From the t-test, a value of 14.7 was obtained for t (degrees of freedom = 29), indicating that the two values were indeed significantly different. P was very much less than 0.01. This indicated that such differences as observed above in the mean RMPs could not have been due to chance alone.

ANALYSIS OF RMP MEASUREMENTS OBTAINED BETWEEN MUSCLE PAIRS

The data presented in tables A3 and A4 were obtained from 3 rats in which one hindlimb was denervated while the other one remained as the control. In this way, a paired analysis could be done (i.e. the normal EDL muscle 1 and the denervated EDL muscle 1 were both obtained from the same animal). The standard t-test (one tailed for related measures) was used to examine whether any significant side to side variation in RMP could be observed in these muscles.

ANALYSIS OF RMP MEASUREMENTS

MUSCLE		n	MEAN	DEG.FREE.	t
1	NORMAL	9	71.8	8	5.50
	DENERVATED	9	60.6		
2	NORMAL	10	73.4	9	7.99
	DENERVATED	10	59.1		
3	NORMAL	11	74.8	10	13.95
	DENERVATED	11	58.6		

In all the above cases, P. was very much smaller than 0.01, indicating that the above differences in the mean RMPs could not have been due to chance alone.

In the above treatment, one will note that the number of observations is slightly different from the data tabled in A3 and A4. For the t-test for related measures, it is necessary to compare groups of equal size. Thus for this specific treatment, the last few observations were dropped to produce equal numbers of observations. This can be justified on the basis that the observations were obtained and tabled randomly and that dropping the last one or two measures should still leave a proper sample of the examined muscle fibre population.