THE ROLE OF AXOPLASMIC TRANSPORT

IN COLLATERAL SPROUTING

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By

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ABSTRACT

In the present research a comparison has been made of the effects of blockade of axoplasmic transport in a nerve to the hind limb of a salamander, with those of sectioning the nerve; the particular focus of attention was the peripheral fields of adjacent nerves to the same limb. The normal touch-receptive and motor fields of the spinal segmental nerves 15, 16 and 17, which innervate the hind limb, were found to be bilaterally symmetrical. Both after section of nerve 16, or after acute treatment of it with colchicine, a drug which blocks axoplasmic transport, the adjacent nerves 15 and 17 significantly increased (P <0.05) the size of their touch-receptive fields; increases in motor fields were statistically significant only after partial denervation. However, after colchicine, and in contrast to nerve section, the fields of the treated 16th nerve were normal. Electrophysiological and morphological studies showed that colchicine application did not provoke Wallerian degeneration in the treated nerve, and impulses conducted normally in it. However, colchicine treatment produced a blockade of the fast axoplasmic transport of catecholamines and cholinesterase, as shown by histochemical methods; presumably the axonal transport of other substances was also blocked. The colchicine also significantly reduced the number of microtubules in the treated axons. Taken as a whole, these findings are consistent with the concept that the size of peripheral nerve fields may be regulated by trophic factors which are continually supplied to the target tissues by fast axoplasmic transport. It is the reduction in the supply of these

factors along sectioned nerves which is responsible for the collateral sprouting of adjacent nerves.

ACKNOWLEDGEMENTS

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RESUME

Axoplasmic transport and the regulation of peripheral nerve fields

The present research was designed to study the mechanisms which
control collateral sprouting of nerve fibres. Collateral sprouting
designates the growth of new branches from parent nerve fibres. This
study, therefore, is also concerned with the mechanisms which regulate
the size of the peripheral fields of nerves, and relates particularly
to the spinal segmental nerves innervating the hind limbs of salamanders.
It has been demonstrated in a variety of experimental and clinical
situations (which include the salamander hind limb), that after partial
denervation, adjacent undamaged nerve axons can sprout, and take over,
wholly or in part, the denervated regions. From such studies it has
been suggested that the following factors may be responsible for collat-
eral sprouting: (1) unidentified products of nerve degeneration; (2)
a factor released by the denervated tissues; (3) a factor released by
proliferating Schwann cells, or (4) lack of function of the denervated
area. It has also been suggested that the nerve sprouts themselves may
"brake" the mechanism producing the stimulus for sprouting. Some of
these ideas are implicit in Cajal's (1919, 1928) hypothesis of neuro-
tropism; an appropriate density of epithelial innervation, he suggested,
may be achieved during embryological development by means of an equi-
librium between an "attractant" substance released by target (epithelial)
cells, and another substance released by nerve fibres which would be

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inhibitory to the further development of new branches, possibly by its interaction with the growth promoting factor.

If this kind of system survived throughout the life of the animal, then it is conceivable that it might be involved in the collateral sprouting after partial denervation. This reasoning provided the rationale for the present investigation. The hypothesis to be examined was that collateral sprouting after partial denervation is due to the elimination from the periphery of some neurotrophic factor, which normally offsets a sprouting stimulus. It was assumed that the neurotrophic factor would be synthesized in the neuronal soma, and be carried towards the periphery by axoplasmic transport. It would be predicted, therefore, that blockade of such transport, (by nerve section for example) would provoke collateral sprouting in the peripheral fields of nerves sharing a common territory with the nerve in which transport was eliminated. But could such transport be interfered with selectively, leaving the nerve otherwise intact and functioning?

The present investigation tested the hypothesis by allowing a comparison of the effects of nerve section with those of selective block of axoplasmic transport in the nerve achieved by other means. Colchicine is known to cause such a blockade. Therefore, an acute application of colchicine solution was made to one of the three spinal nerves which share a common territory in the salamander's hind limb. The salamander was selected as the experimental model because previous work by Stirling (1970a) showed (1) that the hind limb of the salamander is innervated by three spinal segmental nerves (15, 16 and 17) which have bilaterally symmetrical touch-receptive and motor fields, and (2)

section of the nerve with the largest field (16) provokes a significant increase in touch-receptive and motor fields of nerves 15 and 17 on the same side.

Since colchicine can kill nerves, the first step was to find a dose range which did not cause immediately detectable changes in nerve function. With that aim, nerve trunks were exposed _in vitro_ to colchicine solutions of various concentrations for varying times, and the effects of such treatment on compound action potentials were studied. It was found that application of 0.2M colchicine decreased the compound action potential, but lower doses of colchicine (0.025M to 0.1M) applied for 30 minutes were apparently ineffective in this regard. These doses, therefore, were used in the subsequent experiments, which were carried out in the following groups of animals: 1. normal or unoperated salamanders, 2. salamanders in which right nerve 16 was cut, 3. salamanders in which right nerve 16 was treated with colchicine solutions, 4. salamanders in which nerve 16 was treated with amphibian Ringer only, and 5. salamanders in which a combination was used of nerve 16 section and nerve 15 treated with colchicine. In all animals the following observations were performed: 1. visual inspection of the pattern of walking and reflex movements, 2. electrophysiological mapping of touch-receptive fields, and 3. electrophysiological mapping of motor fields of nerves 15, 16 and 17 on both sides. In addition, morphological studies, and histochemical ones of axoplasmic transport were carried out on selected nerves.

1. _Behavioural observations_: The principal aim of the behavioural observation was to assess any gross impairment in walking, i.e. limping,

which could be attributed to nerve damage, and to follow the time-course
of functional recovery. Limping was classified on the basis of the
degree of functional loss. It was found that section of nerve 16 caused
severe limping during the first post-operative week; but on the average
by the twenty-third post-operative day salamanders showed only minimal
limping, and by the thirty-fifth post-operative day they had recovered
normal walking. Colchicine-treated salamanders, however, showed minimal
or no limping, at any time. The limping observed in colchicine-treated
salamanders resembled that in saline-treated controls, and was attributed
to surgical trauma of hip muscles. Those salamanders in which nerve 16
was cut and nerve 15 treated with colchicine never recovered normal
walking.

Reflex withdrawal to skin stroking was impaired in some sala-
manders in the first few weeks after section of nerve 16, but not after
colchicine treatment.

2. Touch-receptive fields in hind limb: The skin was lightly stroked
with a bristle, and from some skin regions such procedure evoked a dis-
charge of impulses which were electrically recorded as "spikes" from the
whole nerve trunk. By exploring the hind limb skin under a dissecting
microscope a map of the area innervated by a given nerve trunk was con-
structed and measured in this way. In normal salamanders the area
innervated by a given pair of spinal nerves did not differ significantly
from side to side, i.e. the touch-receptive fields were bilaterally
symmetrical. After section of right nerve 16, the touch-receptive
fields of nerves 15 and 17 increased significantly on the operated side
within 8-10 days, the increase being first detected on the fifth post-

operative day. After treating right nerve 16 with colchicine solutions (0.03M to 0.1M) the total area innervated by right nerve 15 and 17 was also significantly larger than that inneryated by corresponding nerves on the untreated (left) side. There was no significant effect when right 16 nerve was treated with 0.025M colchicine or less. The amount or time-course of the increase (with 0.1M colchicine) resembled that after section of nerve 16. However, while section of right nerve 16 resulted in the complete elimination of its touch-receptive field, that of colchicine-treated nerve 16 did not differ significantly from that of the normal nerve 16 on the opposite side; this was true even though significant increases in the fields of nerve 15 and 17 occurred after colchicine treatment. When nerve 16 was sectioned and nerve 15 was treated with colchicine, the latter did not show any increase in its touch-receptive field, although nerve 17 did.

3. **Motor fields in hind limb:** After the mapping of touch-receptive fields was performed, the hind limbs were skinned, the nerve trunks mounted on a pair of platinum wire electrodes, and an electrical stimulus was applied to the nerve and gradually increased until the maximal electrical response was obtained from the muscle. Seven muscle groups were routinely sampled. The motor fields were assessed as the number of muscle groups which responded to the electrical stimulation of the nerve trunk. It was found that the motor fields of nerves 15, 16 and 17 were symmetrical in normal salamanders. In salamanders in which right nerve 16 was cut, nerves 15 and 17 increased significantly the size of their motor fields. In salamanders in which right nerve 16 was treated with colchicine (0.025M to 0.1M) the right nerve 15 was often found to

have taken over up to three muscle groups, as compared with left nerve 15 (untreated side); however, such changes were not significant for the whole group. In the group of salamanders in which nerve 16 was sectioned and nerve 15 was treated with colchicine, the motor fields of nerves 15 and 17 on the treated side did not differ significantly from those on the untreated (left) side.

The increase in the size of touch-receptive and motor fields has been attributed to collateral sprouting, since there is morphological and functional evidence of its occurrence after partial denervation in several amphibian and mammalian species. An alternative interpretation would be that non-functional but morphologically normal nerve endings were there from the beginning, and had become functional after partial denervation; this explanation lacks a direct demonstration, and for that reason I favour the interpretation that the increase in peripheral fields of innervation is due to collateral sprouting.

The fact that the peripheral fields of the colchicine-treated nerves did not differ significantly in size from untreated ones, and that the behaviour of such treated animals was normal, makes unlikely the possibility that lack of function or of impulse conduction may be the factor which triggered collateral sprouting. It is reasonable therefore to postulate that nerves are continually supplying the periphery with a presumed "trophic" factor which is concerned with the regulation of the size of peripheral fields of innervation. The complete elimination or a significant decrease in the supply of such a trophic factor (caused by nerve section or by block of axoplasmic transport) would then be responsible for the development of sprouting in the adjac-

ent nerves.

However, since it is known that colchicine treatment can lead
to Wallerian degeneration of nerves, the question arises of whether col-
chicine was effective simply because it caused significant nerve degen-
eration. This problem was approached in two ways: 1. electrophysio-
logical studies were performed 14 days after nerves were treated with
colchicine, and 2. morphological studies were performed on (a) normal
nerves, (b) nerves which were sectioned 3 or 14 days previous to fixa-
tion, and (c) nerves exposed to colchicine solutions 3 and 14 days
previous to fixation.

(i) The electrophysiological studies showed that the compound action
potential of colchicine-treated nerves did not differ significantly
from those of untreated nerves insofar as latency, time to peak amplitude,
and peak amplitude was concerned. (ii) Light microscopical and ultra-
structural studies showed that colchicine treated nerves did not differ
significantly from untreated nerves, except for a reduction in the
density of axoplasmic microtubules to about ¼ of the normal. Only two
out of 16 colchicine-treated nerves showed obvious degenerative changes
14 days after treatment with the highest dose of the drug. On the other
hand, 3 days after nerve section the axoplasm looked shrunken, and at 14
days all the nerves were in advanced stages of degeneration. Taken as a
whole, these data indicate that colchicine solutions (0.025M to 0.1M)
were not effective because they provoked Wallerian degeneration.

In order to assess whether or not colchicine doses used in the
present research did indeed interfere with axoplasmic transport of
materials, the histochemical methods for catecholamines (Falck-Hillarp

method), and for cholinesterase (acetyl-thio-choline method) were used to visualize the normal presence or reduction of axoplasmic transport. When a nerve was crushed there was an accumulation of a strongly fluorescent material, i.e. catecholamines, or an accumulation of a dark brown material, i.e. products of cholinesterase activity, proximal to the site of the crush. These findings are consistent with the hypothesis that such substances are transported along axons toward nerve endings, their accumulation being the result of the disturbance of axoplasmic transport caused by nerve crushing. When colchicine solutions (0.025M to 0.1M) were applied 1 hour previously to such nerve crushing, there was a decrease in the accumulation of both catecholamines and cholinesterase above a crush. With 0.025M colchicine such decrease was almost negligible, but with 0.1M colchicine, practically no accumulation of catecholamines was observed. The findings indicate that the doses of colchicine which were effective in causing sprouting of adjacent nerves produced a reduction in the axoplasmic transport of catecholamines and cholinesterase in a fairly dose-dependent manner; presumably, therefore, the axoplasmic transport of other substances carried by the fast phase of axoplasmic transport was also blocked.

Conclusion

It is concluded from these studies that the increase in touch-receptive fields of nerves 15 and 17 observed after both section of nerve 16 or after treating nerve 16 with colchicine solutions is due to the elimination or significant decrease in the supply to the periphery of a trophic factor which is concerned with the regulation of the size of peripheral nerve fields. This factor is carried there by fast axo-

plasmic transport in the peripheral nerves. It is possible that such a trophic factor normally inhibits sprouting in adjacent nerves or neutralizes the activity of stimulant substance(s) released by target tissues. This hypothesis, of course, rests on circumstantial evidence, and the chemical identity of the presumed trophic factor(s) is unknown; however, it offers a reasonable explanation of the known facts, is open to further investigation, and has the attraction of also explaining the establishment of nerve fields during primary development.

Part I   GENERAL INTRODUCTION

     This research is concerned with the mechanisms which provoke
collateral sprouting in nerve fibres, and therefore with the possible
existence of factor(s) which regulate the size of peripheral nerve
fields. It has been shown that after partial denervation of a struc-
ture, undamaged nerves adjacent to those which degenerate sprout into
the denervated area. This response of the normal fibres, in contrast
to regeneration of damaged ones, is known as collateral sprouting. Such
sprouting has been shown, for instance, in hind limb muscles of mammals
(Edds, 1950; Hoffman, 1950), for the cutaneous nerves in tail fin of
amphibian larvae (Speidel, 1933, 1941), in the spinal cord of cats (Liu
and Chambers, 1955, 1958), and in the septal nuclei of rats (Raisman,
1969; Raisman et al, 1973). These examples, which together with others
will be discussed in greater detail in subsequent sections, furnish
ample evidence of the phenomenon of collateral sprouting both in the
central, and peripheral nervous system.

     One hypothesis to explain collateral sprouting is that of Cajal
(1919, 1928), and proposes that the denervated tissues, or products of
nerve degeneration, or the proliferating Schwann cells, provide the
stimulus which triggers the nerve sprouting, and directs from a distance
the outgrowing nerve tips toward their target organs. Cajal's hypothesis
of neurotropism also suggested that an appropriate density of innervation
may be achieved by an interaction with this stimulus of a substance
released by nerve fibres themselves; this "neurotropic" agent would be

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inhibitory to their own further growth, as well as to the growth of other nerve fibres arriving later on into the new innervated territory.

Of course, denervation causes other changes than those defined in the above hypothesis; for example there could be important functional deficits - both behavioural and of the actual tissues deprived of their nerves. In addition, there will be a blockage of material movement along the cut nerve fibres, a point of great importance in the present study.

The observations that after nerve section the distal parts of neurons and often their effector organs underwent degenerative changes (for a review of the early literature see Cajal, 1928), led to the notion that the neuronal body certainly produced some "trophic" factors which were essential for the normal structure and function of axons and their target organs. That there is indeed a continuous supply of materials moving from the neuronal body toward the nerve endings was shown by Weiss and Hiscoe (1948), and it seems reasonable to assume that the proposed "trophic" factor(s) could be carried by such an axonal transport system.

The present research attempted to provide evidence that axoplasmically transported trophic factors may indeed have a role in determining the size and pattern of nerve fields in the skin and muscle of the salamander's hind/limb.

Part I-A STATEMENT OF THE PROBLEM

It has been shown previously by Stirling (1970a), that the salamander hind limb is usually innervated by 3 spinal segmental nerves (15, 16 and 17), and that section of the nerve with the largest touch-receptive and motor fields (nerve 16) results in an increase of both the touch-receptive and motor fields of the remaining nerves (15 and 17), presumably by their collateral sprouting. The following factors may be considered as the stimulus for this sprouting: 1. products of nerve degeneration, 2. the release of a sprouting promoting substance from the denervated skin and muscle, 3. idem by proliferating Schwann cells (this response is associated with Wallerian degeneration of the nerves), 4. loss of normal movement, reflexes and sensation from skin, or 5. the decrease in the supply of a trophic factor supplied to the periphery by the nerve which was sectioned.

Colchicine, a drug which blocks axoplasmic transport, could in theory, selectively result in phenomenon 5. without causing any of the other changes produced by nerve section, except a phenomenon perhaps analagous to that of 2. above. The question then was - can a selective impairment of axoplasmic transport cause collateral sprouting of un-treated adjacent nerves, like that seen after section of a nerve? If the experiment could be achieved, and if the answer were "yes", then the case for the existence of axoplasmically-transported neurotrophic factors concerned with peripheral field regulation, would be enormously strengthened.

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Part I-B HISTORICAL DEVELOPMENT OF CONCEPT THAT "TROPHIC" INFLUENCES
DETERMINE OUTGROWTH OF NERVES TO TARGET TISSUES

1. The neurotropism hypothesis

         We owe a great deal to Ramon y Cajal for much of our contemporary
thinking about "neurotropism", and indeed about the whole field of neuron
morphology and growth in general. When a nerve is sectioned, leaving
close apposition of the proximal and distal stump, the regenerating
fibres from the former grow into the distal cordons of proliferating
Schwann cells and degenerating nerve fibres. Furthermore, when the
distal stump is displaced so that both cut ends are side by side, some
of the regenerating nerve fibres make a 180° turn, and grow upwards
into the degenerating stump. Such observations were interpreted by
Cajal (1928) as evidence of the action of an "attractant" substance
released from the distal stump of the nerve; since the greatest neuro-
tropic influence occurs at a time when most of the myelin has been
resorbed, it seems likely that such an attractant substance may be
released by the proliferating Schwann cells. This suggestion has anal-
ogies with that contained in an earlier discussion by Cajal. Commenting
on the primary development of innervation of epithelial tissues such as
skin, Cajal (1919) pointed out that the pattern of cutaneous innervation
seemed appropriate, having neither vast aneurfttermes, nor redundant
nerve fibres; he suggested that such an appropriate density of inner-
vation may be achieved by an interaction between an attractant or stimu-
lant substance released by target tissues which would be in some sort

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of equilibrium with a substance released by nerve fibres themselves, the latter being inhibitory for their own growth as well as for the growth of other nerve fibres arriving later on into the now innervated territory. The hypothesis of "competitive" sprouting suggested by Edds (1953) for explaining collateral sprouting after partial denervation is based basically on the same assumptions. He was observing the re-innervation of hind limb muscles after sectioning one of the spinal segmental nerves which supply them. The terminal branches of undamaged nerves emitted sprouts which re-innervated the denervated endplates, and the number of new branches produced in a given region was in accord with local requirements. Edds suggested that when the earliest sprouts reached the cordons of proliferating Schwann cells, they began to "brake" the stimulus-producing mechanism, until at some critical level, sprouting ceased altogether, i.e. a local feed-back control developed.

In amphibian larvae, Hughes (1968) and Hughes and Tschumi (1958) observed that when an obstacle, i.e. a small mica plate was placed between the spinal cord and a hind limb before the limb had received any innervation, the nerves followed unusual pathways around the obstacle to find their way towards the limb. Such observations strongly suggested that the target tissues released substances which attracted and directed the growing nerve tips toward them.

When supernumary limbs were grafted on the thoracic region of salamanders, the fore limb nerves emitted new branches which were directed toward the added limbs, (Detwiler, 1936; Detwiler and van Dycke, 1934); this is another example in which nerve fibres apparently were attracted by some substance released from their target tissues. Such

stimuli may not be specific, since other grafts, i.e. a nasal placode, or eyes, also attracted nerve fibres toward them (Detwiler and van Dycke, 1934). The observation that nerve sprouts re-innervate the old site of denervated endplates seems difficult to interpret otherwise than in terms of a chemotactic phenomenon (see for example Cajal, 1928, volume I, chapter XI, or Miledi, 1960); of course the degenerating fibres may have provided some mechanical guidance (see below), but even so some extra factor(s) seem necessary.

The concept of neurotropism as originally proposed by Cajal (1892) and presented in a more elaborate form in his monograph on Degeneration and Regeneration of the Nervous System (Cajal, 1928), assumed that remote tissues by releasing specifical chemical substances could attract out-growing nerve fibres from a distance. Such an assumption implied: 1. that the supposed gradients were steady, durable, and undisturbed by any activity of the organism, and 2. that the nerve tips have means not only of perceiving the minute differentials of concentration, but also of translating them into corresponding steering actions. Neither of these premises, nor the basic assumption of distance attraction, has ever been critically demonstrated in the case of nerve fibres (Weiss, 1971b, pp 294). The original observations by Harrison (1907, 1910) that nerve fibres grow only on a substrate led to a series of experiments (Weiss, 1934, 1945) which suggested that nerve fibres are directed by the geometric characteristics of the medium on which they grow. Such experimental evidence will be presented in the following paragraphs.

2. Contact guidance as an alternative interpretation

Proliferating Schwann cells from explants of dorsal ganglia,

placed on a lamella of plasma clot provoked shrinkage of the colloidal substance, and the fibrin threads became oriented in a radial fashion (Weiss, 1934, 1941a). When two ganglia were explanted on such lamella, a bridge of orientated fibrin threads was formed between them, and this seemed to act as a substrate for the outgrowing nerve fibres which formed a fibre tract between both ganglia; this phenomenon was called the "two centre" effect by Weiss (1934, 1955), who suggested that nerve fibres were not directed by a presumed "neurotropic" influence, but by a "contact guidance" phenomenon. In this regard, it has been shown that the angle formed by the main body axis of frog embryos with the main axis of a hind limb bud experiences striking changes during the early limb bud stages, and this almost certainly produces mechanical stress in the "ground substance" of that region. The tension lines that would result from these mechanical forces, i.e. bending and shrinkage due to cell proliferation, etc., could give a plausible explanation for the direction taken by the outgrowing nerve fibres (Taylor, 1943).

In experiments of Weiss (1950, 1968), spinal cord explants and limb primordia were grafted in the loose connective tissue in the dorsal fin of amphibian larvae; when both grafts were placed in a common tunnel, axons from the spinal cord formed a clear nerve fibre tract across to the grafted limb, but when each graft was placed in a separate tunnel only a few nerve fibres reached the grafted limb, and did so by circuitous routes. Again, these observations were interpreted as suggesting that nerve fibres used mechanical guidance in reaching the target tissues, in this instance, by following the tracts created in the loose connective tissue by the tunneling procedure.

It should be noticed, however, that after partial denervation of hind limb muscles of rats (see Edds, 1953) adjacent undamaged nerve fibres sprouted and re-innervated endplates over a radius of 200 μ. Although it may be that nerve tips were directed toward denervated end-plates by tracks laid down by proliferating Schwann cells, the factor which triggered the sprouting must be of a chemical nature, since the ground substance organized or not, had always been there, while sprouting occurred only after partial denervation. It may be relevant that in slime molds (Dictyostelium sp.) cAMP has been shown to induce cell aggregation in a chemotactic manner (Bonner, 1947, Bonner et al. 1969, Konijn et al 1967, 1968; Barkley, 1969).

Perhaps the following remark by Hughes (1968) is a good summary of the situation in this field: "Two principles have been proposed as forces which direct the growing nerve fibres, the 'contact guidance' of Weiss (1941a, 1955), and the 'neurotropism' hypothesis of Cajal (1919, 1928); both rest on inference from the behaviour of growing nerve fibres under various circumstances, and at present on nothing more. Nobody would assert that one rather than the other was responsible for the direction of all growing fibres under every condition."

Even so, the stimulus for nerve growth, and for collateral nerve sprouting, has to be chemical in nature. This conclusion is fundamental to the rationale of the present investigation.

Part 1-C   THE PHENOMENON OF COLLATERAL SPROUTING AFTER PARTIAL DENER-
VATION

1.   Collateral sprouting in peripheral tissues

(a) Muscle nerves: Apparently this possibility was made explicit
for the first time by Exner (1884, 1885) who found that a muscle atro-
phies after complete denervation, but it does not after partial denerva-
tion. Regeneration of the cut nerve being excluded, he suggested that
either muscle fibres are innervated by more than one neuron or that
remaining undamaged nerve axons sprout and take over the denervated
muscle fibres.

The tibial nerve supplies the gastrocnemius muscle of rats, and
partial denervation of that muscle can be achieved by section of nerves
L4, L5 or both (Hines, 1942; Hines et al., 1945; Wehrmacher and Hines,
1945). These authors found that at the third post-operative day
electrical stimulation of the tibial nerve on the operated side produced
about 25% of the tension which the same muscle developed on the unoper-
ated side with similar stimulation. Between the ninth and the fiftieth
post-operative day, a gradual functional recovery occurred and stimu-
lation of the tibial nerve produced about 60% of the tension developed
by the gastrocnemius muscle on the control side. The wet weight of the
muscle had decreased to about 80% of the control value over the same
post-operative period. A careful dissection at the time of the final
experiment showed that regenerating nerve fibres had not grown into
the distal stump of the nerve. Hypertrophy of normally innervated muscle

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fibres could not be excluded, since no histological studies of muscle were performed, but the time-course of the functional recovery suggested to the authors that it may not be an important factor, and they postulated instead that the findings may represent a peripheral extension of the processes of viable axons to neighbouring denervated muscle fibres, or to an unmasking of functionless neuromuscular junctions (see below).

Van Harreveld (1945) studied the sartorius muscle of rabbits, which is innervated by a branch of the femoral nerve which has components from nerves L5 and L6. Electrical stimulation of the femoral nerve provoked a tension of about 150gr in the sartorius muscle, while stimulation of L5 alone produced one of about one third of this value; such values were not significantly different between both sides in unoperated animals. After section of L6, which was prevented from regenerating into the distal stump, he found that the tension developed in the sartorius muscle by stimulation of L5 on the operated side was significantly greater than that on the unoperated side. Measurements of a sample of about 100 muscle fibres suggested that there may be hypertrophy of some muscle fibres, but the changes were not enough to account for all the functional recovery. The wet weight of muscles were not presented nor counts of nerve fibres in the motor nerves, but the above findings were consistent with the idea that collateral sprouting had occurred in the remaining undamaged axons.

Similar but more complete studies were performed by Weiss and Edds (1946) who partially denervated the gastrocnemius and soleus muscles of rats by section of nerves L4 and L6 or L5 only. Observations were

performed on muscle weight, isometric tension after indirect (nerve) or direct (muscle) stimulation, and a thorough histological study was made on the cross sectional area of muscle fibres, and on the number of myelinated nerve fibres in the corresponding motor nerves. It was confirmed that partially denervated muscles showed a significant functional recovery as judged from the studies on muscle tension achieved by nerve stimulation. With respect to muscle weight it was clear from the second week on that muscles exhibited greater weight than was expected on the basis of atrophy of denervated portions. Indeed, the initial tendency to lose weight reverses, and the muscle regains part or all of its previous value. The histological studies revealed that there was a complete range from degenerating muscle fibres to those normally -innervated, and a significant group with intermediate characteristics; no regenerating motor nerve fibres had arrived at the distal nerve trunks, and the number of myelinated nerve fibres was less than that on the control side. These findings strongly suggested the possibility that intramuscular nerves had sprouted and taken over the denervated muscle fibres; this conclusion, however, rested on indirect evidence.

Similar studies, although not as complete as those of Weiss and Edds, indicated that collateral sprouting may also occur in other mammalian species, i.e. dogs (Frederick and Kosman, 1948), monkeys, (Black and Nulsen, 1951), and humans (Livingston, 1947).

Direct demonstration of collateral sprouting after partial denervation was achieved by histological methods in two different laboratories (Edds, 1949, 1950; Hoffman, 1950). Advantage was taken of a gold impregnation technique which allows the visualization in teased

preparations of a relatively large number of terminal nerve fibres and their associated muscle fibres. These authors found that after partial denervation undamaged axons within about 200μ of denervated endplates had sprouted, and these sprouts attached themselves to cordons of proliferating Schwann cells and reinnervated the denervated endplates. Some of the earliest sprouts were observed at stages in which they had not yet reached the denervated endplates. Initially nerve sprouts were significantly thinner than normal terminal fibres, but by the second post-operative month collateral sprouts had attained diameters within the range of normal fibres.

Edds (1950) pointed out that in normal serratus anterior muscle of rats the ratio of terminal motor fibres to the number of endplates innervated by them is 1:1:1, while after partial denervation the ratio becomes 4:7:1. This finding gave a quantitative estimate of the extent to which collateral sprouting had occurred.

It should be noted that the amount of collateral sprouting was in direct proportion to the number of endplates to be reinnervated, which suggests that the phenomenon of collateral sprouting may be under the control of some local factor, perhaps substances released by degenerating nerve fibres, proliferating Schwann cells, or from denervated target tissues (Edds, 1953). In this regard, Hoffman (1950); Hoffman and Springell (1951) showed that ether extracts of ox spinal cord, normal or denervated rabbit muscle, and egg yolk proved to be effective in inducing collateral sprouting in rats. Such extracts injected intramuscularly provoked in the normal anterior tibial muscle a significant amount of sprouting, seen particularly in the region of

terminal motor fibres.

The factor which provoked collateral sprouting was called "neurocletin", and an attempt at the chemical identification of it was performed. The crude ether extracts were treated with acetone, and the acetone soluble fraction contained the biologically active substance. From the latter fraction a mixture of glyceride fatty acids was saponified in the cold, subsequently acidified and extracted in an aqueous phase. Such a mixture of fatty acids was further purified either by low temperature fractionation or by urea fractionation. Spectroscopic data indicated that the active subfraction was still a mixture of fatty acids. In view of the loss of activity following even oxidation in air, it appeared that the fatty acids were unsaturated; further tests suggested that the biological activity was restricted to acids containing only one double bond (Hoffman and Springell, 1951). It seems that more recent techniques such as chromatography could elucidate the problem of the chemical identity of neurocletin, which the above authors solved only partially.

(b) Skin nerves: Collateral sprouting occurs also in the cutaneous nerves, as the following examples will illustrate. In the tail fin of amphibian larvae in vivo; it was shown by Speidel (1933, 1935, 1941) that after section of a cutaneous nerve branch which caused Wallerian degeneration of its distal part, adjacent nerve fibres sprouted into the denervated area. He often observed that the new sprouts joined cordons of degenerated nerve fibres and proliferating Schwann cells, and followed such tracks over considerable lengths, as if they were attracted and guided by some substance released from such structures.

However, as discussed in Part I-B, guidance of the growing fibres by mechanical means is another way of explaining the direction of growth of the collaterals-once they have formed.

After section of the sural nerve in rabbits, Weddell et al., (1941) found that the analgesic area produced over the lateral aspect of calf and foot gradually shrank over a period of about 12 weeks; the original anaesthetic area and its subsequent decrease was mapped with tattoo-marks. In the centre of the anaesthetic area, during the second post-operative week, only degenerating nerve fibres were found. However, between the second and the twelfth post-operative week, the areas which showed functional recovery also showed the presence of regenerating nerve fibres, which were orientated towards the denervated skin.

Livingston (1947) found that in a patient, two years after complete section of the median nerve, the motor function of the hand had recovered to a remarkably good extent, and there was no complete anaesthesia even at the tips of the index and middle fingers. The patient could distinguish between sharp and dull stimuli, could recognize light touch, and had a remarkably good ability to localize stimuli. A novocain block of the median nerve did not alter the motor and sensory status of the hand. However, a novocain block of the ulnar nerve rendered the thenar muscles completely functionless, the entire palmar surface of the hand anaesthetic. Even in the absence of confirmatory histological studies, these findings suggest that collateral sprouting occurs also in humans after partial denervation.

(c) An alternative hypothesis to collateral sprouting: It has been proposed that there may be morphologically normal nerve terminals which

are non-functional, but become functional after denervation of nearby
nerves (Pollock, 1920; Hines et al., 1945; Wehrmacher and Hines, 1945;
Cass et al., 1973). The last authors found that three days after
cutting one of the spinal segmental nerves (16) which innervates the
hind limb of A. mexicanum, the adjacent nerves (15, 17 and 18) had
invaded about 0.5mm into the margin of the denervated muscle area. This
small increase in the size of motor fields could be detected (they
claimed) because normally the latter are remarkably symmetrical (right
and left). Between one and two weeks later, these nerves (15, 17, 18)
had spread even further, and patches of innervated muscle fibres were
found in the centre of the denervated area. The sectioned fibres of
nerve 16 were allowed to regenerate into the distal stump, and about
30 days after nerve section, the increase in motor fields which the
remaining nerves (15, 17, 18) had acquired at the expense of the cut
nerve (16) began to disappear as the regenerating fibres of 16 grew
into the limb. Eventually, the normal symmetrical pattern of innerva-
tion was re-established. Cutting the 16 nerve a second time in animals
which had recovered completely from a previous denervation resulted as
before in an area of paralyzed muscle fibres in the expected part of the
limb. Now, however, the second spread of innervation was apparently
fairly sudden and complete three days after the second denervation. The
authors suggested that this unexpected rapid spread of innervation
occurring three days after cutting a nerve, either the first or the
second time, would be the result of the emergence of function in pre-
existing but functionless nerve terminals.

It should be noted, however, that unconnected nerve fibres can

certainly persist without resorption (Weiss and Traylor, 1944; Weiss and Edds, 1945; Weiss et al, 1945; Gutmann and Young, 1944), but if their numbers were so abundant as to account for all the phenomena observed by Cass et al., (1973) it seems doubtful that they would have gone unnoticed. It is possible that the interpretation of their findings may be correct, and that three days may be too short a time for sprouting of nerve terminals and formation of new endplates to occur, but this suggestion rests on indirect evidence. On the other hand, histological studies such as those by Edds (1950), Hoffman (1950), Speidel (1933, 1941) discussed above, gave a direct and unambiguous demonstration of the occurrence of collateral sprouting after partial denervation.

Mark et al., (1972) have also studied this problem in the superior oblique muscle (sup.obl.m.) of fishes which is normally innervated by the superior oblique nerve (sup.obl.n.). When the latter was cut and the inferior oblique nerve (inf.obl.n.) brought in the neighbourhood of the muscle, the inf.obl.n. reinnervated the muscle and by the third to fourth post-operative week the outcome of this abnormal innervation was that the reflex counterrotation of the eye reversed. When, however, the fibres of the original nerve were allowed to regenerate into the distal stump of the nerve, the normal reflex was recovered by the second post-operative month. This was interpreted as evidence that original fibres from the sup.obl.n. had regained control over the muscle and suppressed the nerve endings arising from the foreign nerve. In animals which had regained normal reflexes ultrastructural studies showed that all endplates looked morphologically normal; however, while section of

the sup.obl.n. five to seven days previous to fixation provoked de-
generative changes in a significant proportion of endplates, there were
also abundant morphologically normal ones. It was inferred that the
latter were still innervated by the inf.obl.n., whose endings, it was
suggested, were functionless. The behavioural observations are con-
sistent with such interpretation, but it seems that an electrophysio-
logical study of these endplates using intra- or extracellular recording
of muscle activity during appropriate nerve stimulation is much needed.
Otherwise Mark's experiments, although suggestive, are inconclusive.
It is conceivable that sprouts arising from foreign nerves could per-
sist as healthy nerve fibres if competition by regenerating "native"
nerves did indeed cause them to be "rejected" at the muscle fibres.
The latter explanation requires only one phenomenon, i.e. rejection in
a competitive situation, but available for growth and formation of
normal endings when required. The interpretation of Mark and his colla-
borators needs both this to be true, and the extra hypothesis that the
"rejected" endings persist in a way not detectably different from
normal ones morphologically, but lose their function. Obviously, more
experiments are needed in this controversial area.

2. <u>Collateral sprouting in sympathetic neurons</u>

        The superior cervical ganglion has been another experimental
model in which collateral sprouting has been shown to occur. Section
of rami communicantes (r.c.) T1 and T2 in one side, in cats, resulted
in degeneration of about 50% of the <u>pre</u>-ganglionic fibres arriving at
the ipsilateral superior cervical ganglion (Simeone <u>et al</u>., 1938).
However, three to six weeks after this partial denervation, stimulation

of the <u>other</u> r.c., T3-T4 gave contractions of the nictitating membrane on the operated side which were significantly greater than those on the control side. At that time (3-6 weeks) a comparison of the effects of intravenous injection of noradrenaline showed that supersensitivity in the partially decentralized nictitating membrane was no longer detectable. However, the effects of acetylcholine on the ganglionic neurons were not studied, and supersensitivity at that level was not excluded. Nevertheless, the findings were consistent with the view that the increased response in the nictitating membrane to stimulation of r.c. T3-T4 on the operated side was due either to an increase in the number of <u>pre</u>-ganglionic endings by sprouting, and take-over of the partially denervated ganglion cells, or to repetitive discharges (perhaps because of supersensitivity) from the post-ganglionic cells when they were normally activated by the T3-T4 pre-ganglionic volleys.

It was shown by Murray <u>et al</u>., (1958) that unilateral section of the r.c. T1-T3 caused Wallerian degeneration of about 90% of pre-ganglionic fibres to the ipsilateral superior cervical ganglion in the cat. Four to 8 weeks after such partial denervation the response of the nictitating membrane to preganglionic stimulation of r.c. T4-T7 was equal to that obtained by stimulation of the whole cervical trunk on the control side. It should be noticed that about 1 month after partial denervation supersensitivity to acetylcholine in the ganglion cells was no longer detectable and most of the experiments were performed at that time. Furthermore, stimulation of r.c. T4 to T7 provoked pupil dilation on the partially denervated side, but not on the control side. These findings suggested that the remaining pre-ganglionic fibres had sprouted

in the ipsilateral side and made connections with ganglion cells not previously innervated by them. This work was confirmed and extended by Guth et al., (1961) who also subsequently allowed the sectioned fibres from T1-T3 to regenerate into the distal stump of their pre-ganglionic trunks; 6 months after the original operation pupillary dilation was evoked by stimulation of the regenerated fibres from T1-T3, but no longer by stimulation of T4. Apparently, after fibres from T1-T3 had regenerated, and made appropriate connections with the pupillary ganglion cells, non-pupillary connections, i.e. the "foreign" collaterals from T4-T7, became functionally inactive. Some of these collaterals seem to persist, however, because simultaneous stimulation of T4-T7, unlike T4 alone, produced a significant pupillary dilatation on the operated side, but not on the control one. No morphological studies were performed, and it is unsettled whether or not the synapses which become non-functional also suffered degenerative changes.

Murray and Thompson (1957) also showed by light microscopial studies, that collateral sprouts had arisen from remaining intact pre-ganglionic axons, and come into close apposition with the majority of ganglion cells denervated by the previous section of T1-T3. These findings were confirmed by Williams et al., (1973) who in addition performed ultra-structural studies. They found that after section of C8-T3 about 90% of pre-ganglionic fibres underwent Wallerian degeneration, and the large S1 axons of the pathway for pupillary dilation (Bishop et al., 1932; Eccles, 1935) were completely eliminated. Within 3 days after nerve section, numerous small groups of sheath cell "fingers" oriented parallel to surviving axons were found in the pre-ganglionic trunk. These sheath

cell assemblies were subsequently invaded by neurites, presumably collaterals from surviving pre-ganglionic fibres, which then ramified in parallel with the parent fibres. Groups of neurilemmal cell "fingers" accompanied by tiny neurites were observed very occasionally in normal tissue. It is possible that sprouting may be a slow and continuous process in normal animals, and that this phenomenon is greatly accelerated after partial denervation.

It might be relevant that nerve growth factor (NGF) which is obtained from mouse sarcoma, salivary gland of male rat, and from snake venom, provokes a remarkable sprouting of sympathetic neurons in vitro and in vivo, as well as cells from dorsal spinal ganglia; NGF is also present in small quantities in blood of many species, including humans (for a review see Levi-Montalcim and Angeletti, 1968). A further discussion on the effects of NGF is beyond the scope of this review however, and will not be dealt with further except to mention that the innervation of iris transplants placed in the rostral mesencephalon of rats, by cut fibres from the dorsal and ventral catecholaminergic bundles, and from dopaminergic fibres from the medial forebrain bundle, was greatly increased by intraventricular injections of NGF (20 to 2000 biological units) in a dose-dependent manner (Bjerre et al., 1973, Bjorklund et al., 1971a,b). These findings suggest the possibility that naturally occurring NGF may be involved in the collateral sprouting of adrenergic neurons, central and peripheral, but direct evidence of such an involvement is lacking. Furthermore, the almost complete elimination of sympathetic neurons in the adult animal seen after the administration of antiserum against NGF to newborn mice gives dramatic evidence suggesting the

importance of NGF during early development (Levi-Montalcini, and Cohen, 1960; Zaimis et al., 1972).

3. Collateral sprouting in the Central Nervous System

As with peripheral nerves, there is evidence that undamaged axons in the CNS in appropriate conditions can sprout. The evidence of this is mainly morphological, and functional studies have been less numerous, perhaps because the complex circuitry of the CNS imposes several technical limitations. Morphological studies will be presented first, followed by functional ones.

(a) Morphological studies on collateral sprouting in the CNS:

(i) The spinal cord of cats was partially denervated by cutting up to 10 dorsal roots above and below L7 on one side (Liu and Chambers, 1958). When the products of nerve degeneration had disappeared completely (up to 9 months) the remaining intact dorsal root on that side, L7, with the corresponding one on the untouched side, were cut. The central fields of the L7 roots were "mapped" using Nauta's silver impregnation for degenerating fibres. The degenerating fibres from L7 on the partially denervated side extended for a significant number of spinal segments more than on the control side, i.e. had increased their central fields. The Nauta method, however, does not show whether or not such nerve sprouts form synapses. Nevertheless this study of Liu and Chamber is a classical one, and in its day little recognized; it represents the first clear-cut investigation of collateral sprouting of undamaged axons in the CNS after partial denervation. That cut axons emit collateral branches in the CNS was shown earlier by Cajal (1928), but that is analagous to nerve regeneration after nerve section, as opposed to collateral

sprouting of undamaged axons after partial denervation.

(ii) Raisman and his collaborators (Raisman, 1969; Raisman et al., 1973) studied the inputs to the septal nuclei of the rat. The fimbria, one of the main afferent inputs was cut unilaterally, and this led to degeneration of the synaptic knobs on the dendritic spines of neurons of the medial septal nucleus. From the second post-operative week onwards, dendritic spines regained an innervation but the pattern of this showed a remarkable change, in that the number of synapses showing "multiple synaptic contacts" in the plane of section had increased significantly. These findings indicated that neighbouring undamaged axons had sprouted and taken over the denervated dendritic spines. Changes in the septal area after unilateral section of the fimbria have also been studied in the nucleus lateralis, and in the medial and posterior nuclei of that area, by the histochemical method specific for catecholamine containing fibres by Moore et al., (1971); they found that between 8 to 15 days after partial denervation an increase in the number of adrenergic nerve terminals had occurred in those nuclei. Section of the medial forebrain bundle produces a substantial loss in the adrenergic innervation of the septal nuclei on both sides. These findings, therefore, indicate that adrenergic fibres which normally innervate the septal nuclei will increase their field by collateral sprouting when a non-adrenergic input (the fimbria) to the septal nuclei is completely eliminated.

(iii) In normal rats most of the afferents to the outer molecular

layer of the hippocampus arise from the ipsilateral entorhinal cortex. Other important inputs, localized near to the body of the granule cells, are some cholinergic septo-hippocampal fibres, and fibres from the contralateral hippocampus which cross in the ventral hippocampal commissure. Unilateral extirpation of the entorhinal cortex in 11-day old rats resulted in a considerable extension of the fields of the remaining inputs as revealed by a histochemical method for cholinesterase, or by the Fink-Heimer method, respectively (Lynch et al., 1972, 1973; Cotman et al., 1973). In the second case, the ventral hippocampal commissure was sectioned 80 days after the original operation. Taken together, these findings would indicate that the precise pattern of innervation observed on the dendrites of granule cells does not depend on a kind of "matching" of chemical specificities, but rather on the interaction between a set of inputs sharing a given field of innervation; probably the timing of arrival of primary-growing inputs may be important in determining the size of the particular innervation fields. The basis, however, of the experimentally-observed phenomenon is the triggering of sprouting consequent to degeneration of nearby fibres. It is not known whether or not such sprouts form functional synapses, but the hippocampus seems to be particularly suitable for studying this problem (see for instance Eccles, 1964).

(iv) In the caudal thalamus-pretectal region there is a considerable overlap between (occipital) cortico-tectal and retinal fibres. Sixteen months after unilateral extirpation of the occipital cortex in adult rats, both eyes were removed (Goodman and Horel, 1966) and the fields of retinal fibres were morphologically mapped using one of the

Nauta methods for degenerating fibres. It was found that retinal fibres had sprouted; the phenomenon, however, was sharply localized to two loci: 1. the ventral lateral geniculate nucleus, and 2. the lateral nucleus of the optic tract. It is possible that other afferent inputs may have sprouted in preference to retinal fibres in other regions of the pre-tectal zone in which these inputs converge, or it may be that cortico-tectal and retinal fibres innervate different cells in the same nucleus, thus making more difficult the occurrence of collateral sprouting. It should be noted that important variables in the Nauta methods are the post-operative period before fixation and also the extent of suppression caused by slight modifications in the technique; such factors were not given attention in the above studies. Studies by Cunningham (1972) indicated that collateral sprouting of retinal axons is greater in neo-nate rats.

(v) Extirpation of one eye at birth followed by either extirpation of the remaining eye or the visual cortex 4 months later, in rats (Lund and Lund, 1971) resulted in an extension of the remaining uncrossed retino-tectal pathway across the entire colliculus instead of being confined to the small antero-medial region, and the cortico-tectal pathway extended to the surface of the colliculus instead of occupying only the deeper half of the stratum griseum superficiale; the Fink-Heimer method was used to visualize those changes. Ultrastructural studies showed that synapses in that region had the same proportion of small synaptic knobs with round vesicles relative to knobs with flat vesicles. It seems, therefore, that such sprouts formed synapses, but behavioural or electrophysiological studies were not performed.

Retrograde degeneration of tectal cells provoked by ablation of the visual cortex (Ralston and Chow, 1973) resulted in a significant decrease in axo-dendritic synapses together with an increase in axo-axonic ones. In this case there was a reorganization of the synaptic connections; collateral sprouting may have occurred, but could not be assessed with certainty.

(vi) There are, however, instances in which collateral sprouting did not seem to occur after partial denervation. In cats, the overlap in the distribution between cervical primary afferents and trigeminal ganglion fibres at the level of spinal segment C1 was suggested as presenting several favourable features with regard to the evaluation of potential sprouting by Kerr (1972). On the basis of Liu and Chambers' experiments (see above) it would be expected that partial denervation of spinal segment C1 by unilateral section of trigeminal roots might result in an extension of the central fields of dorsal spinal roots C1-C3. However, morphological studies using the Nauta and Fink-Heimer methods revealed no increase in such fields. It is possible that sprouting did take place in the region considered, but that such sprouts, if any, arose from interneurons in the nucleus. Alternatively, the actual overlap between trigeminal and C1-C3 dorsal spinal ganglia inputs may not be extensive on the same neurons, so that circumstances favour-able for collateral sprouting did not actually exist.

(vii) Unilateral destruction of the anterior ventral cochlear nucleus results in selective de-afferentation of ipsilateral lateral dendrites and contralateral medial dendrites of the medial superior olive (Liu and Liu, 1971). Golgi and Nauta's studies showed that by the

sixth post-operative week such dendrites had atrophied, and no evidence of collateral sprouting was found.

(viii) Unilateral extirpation of one eye in kittens (7-20 days old) results in the extension of un-crossed retinal fibres deriving from the remaining eye into layer A of the dorsal lateral geniculate nucleus, though not in adult cats,(Guillery, 1972) as judged from morphological studies using the Nauta and Fink-Heimer techniques. The author interpreted these findings as evidence of continued axonal growth in kittens, but not as actual sprouting. However, it can be argued that retinal axons can sprout in kittens, but not in adult cats.

(b) Functional studies on collateral sprouting in the CNS

(i) In cats and monkeys, from 3 weeks to 5 months after hemisection of the spinal cord, the electrical potential (N1a) attributed to spinal afferents, as recorded extracellularly at the entrance of dorsal spinal root L7 into the spinal cord, was significantly larger than that evoked in the control side (McCouch et al., 1958). These findings together with morphological evidence of collateral sprouting obtained in similar fashion to the Liu and Chambers' experiments, suggested that such sprouts conduct impulses, but detailed studies on the connections made by them would probably require intracellular recording from motoneurons and interneurons; synaptic function presumably did develop, however, since the animals showed increased knee reflexes and spasticity after spinal hemisection.

(ii) In the ventral posterior nucleus of thalamus in rats, the entire contralateral body surface is represented in an exact somatotypic map. After destruction of the nucleus gracilis, i.e. the hind

limb system, Wall and Eggers (1971) found that 3 to 17 weeks post-operatively there was an almost complete disappearance of responses in the leg-foot-toe projection in the thalamus, sites, where stimulation of such skin areas normally evoked activity in the thalamus, but there was an expansion of the arm area into the same regions. Furthermore, the thalamo-cortical projection of the arm area had also expanded into the leg area. The most likely explanation of such phenomenon is that sprouts have grown from terminal arborizations of intact axons from cuneate nucleus (the arm system) and have established successful contacts with deafferented neurons. Morphological studies were reported to be in progress in that laboratory.

(iii) In cats, the giant neurons in the red nucleus receive projections mainly from 1. nucleus interpositus (IP) in the cerebellum, particularly on the somatic membrane, and 2. sensory motor cortex of cerebrum, specially on the distal dendritic membrane. Normally, stimulation of cortico-rubral fibres evokes a small EPSP which has a slow time-course due to signal attenuation by the particular cable properties of dendrites. Two weeks after electrolytic destruction of the left IP nucleus (Tsukahara et al., 1974) stimulation of cortico-rubral fibres evoked EPSP which were larger and had, in addition, an earlier component with a significantly shorter time to peak amplitude. These findings are compatible with the assumption that dendritic cortico-rubral terminals sprout to form synaptic contacts with the denervated somatic membrane. Obviously these electrophysiological studies need a morphological correlation, and such studies are probably under way in that laboratory.

Part I-D GENERAL STRATEGY ADOPTED FOR THIS STUDY

The mechanism(s) which control the embryological development of
nerve fields may persist into adulthood, particularly in animals like
salamanders which show a high degree of plasticity in the nervous system.
It is conceivable then that they may operate during conditions when nerve
fields are experimentally interfered with, and when, as a consequence of
this, patterns of innervation reform, even in the adult. Suppose that a
given pattern of innervation is the resultant of an interaction between

1. a stimulus (presumably chemical) originating at the target tissues, and
2. a substance released by nerve fibres themselves which would be inhibi-
tory for any further nerve growth. (This essentially was Cajal's idea
of 1919.) The latter substance would be presumably synthesized in the
neuronal soma, transported along the axons to the nerve terminals, and
there be released. In adult animals the system would be in a steady state,
and the total number of nerve branches in the periphery would be constant,
The stimulant, and the "neutralizing" substance from the nerve, would
be in some sort of equilibrium.

It would then be expected that partial denervation, or a signifi-
cant blockade of axoplasmic transport by other means, would disturb this
equilibrium in the periphery, and the local stimulus to nerve sprouting
would be in the ascendency and provoke collateral nerve sprouting and a
change in the pattern of innervation of a given structure. In order to
test this possibility, the effects of partial denervation on the pattern
of innervation of salamander hind limb were compared with those provoked

by pharmacological blockade of axoplasmic transport. If the hypothesis
were correct, then the effects of such blockade - achieved without
causing degeneration of the nerves - should resemble those of partial
denervation, in that both would lead to peripheral sprouting of
neighbouring intact nerve fibres. Such a result would not prove the
hypothesis, but would be consistent with it, and would be inconsistent
with the hypothesis of "denervation sprouting" dependent for example on
chemical products of nerve degeneration, or functional loss due to
absence of nerve impulses. A positive result would also bring the
phenomenon of nerve sprouting after partial denervation into line with
plausible explanations of peripheral nerve sprouting de novo, during the
embryological establishment of peripheral nerve fields.

Part II   BEHAVIOURAL OBSERVATIONS

   The most important experiments in the present research involve
a comparison of the effects on the sizes of the peripheral fields of the
three hind limb nerves (15, 16 and 17) of section of nerve 16, with
those of treating nerve 16 with colchicine. In the present section the
appropriate operative procedures are described; and also the behavioural
observations made on animals which had received either nerve section or
colchicine treatment; these animals were subsequently used for the
mapping experiments described in Parts III and IV. The behavioural
observations were performed with the aim of assessing the extent of nerve
damage, when present, and to follow the time-course of functional
recovery.

Part II-A METHODS

1. Animal husbandry

   Adult male or female salamanders (Ambystoma tigrinum) of lengths (head to tail) 10 to 20 cm were used throughout. The salamanders were obtained from suppliers in North Carolina or Wisconsin, usually in lots of 50 to 200 animals. It was possible to buy salamanders during the period between April to September only, and therefore, a stock of salamanders obtained during the summer was maintained in the basement of a farm in Brantford. Groups of approximately 50 salamanders were housed in plastic tanks of 38 x 20 x 12 inches. The bottom of the tank had a layer of moss about 3 inches deep. In order to simulate the normal environment of salamanders, pieces of wood and bark (4 x 5 inches) were placed on top of the moss. Salamanders were apparently satisfied in these conditions, and either sheltered under the pieces of bark or buried themselves in the moss.

   A group of about 20 salamanders for "daily" use was kept in a small room adjacent to the laboratory. Each salamander was kept in an individual plastic cage of 12 x 6½ x 5 inches, the bottom of which was covered with a layer of moss about 2 inches deep; each cage was labelled with the code name for the animal. Initially, these animals were kept at room temperature; later on, it was found that the animals' health was better if kept at $16^{o}C$ and about 90% humidity.

2. Feeding of salamanders

   Salamanders do not readily feed in captivity, and so each animal

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was individually fed. A piece of liver or meal-worm was held in a forceps and waggled in front of the head of the animal; healthy salamanders will strike at such a target and eat voraciously 2 or 3 pieces, (more than that amount was not provided because they tended to become obese). If this maneouver failed, the salamander was force-fed in the following way: the salamander was held in the left hand and the right hand used to open gently the animal's mouth using a smooth metal spatula resembling the tongue depressor used by physicians. A second person then pushed a piece of food into the salamander's mouth; after removing the "tongue depressor" the salamander always swallowed the food.

3. Common diseases suffered by the salamanders and treatment provided

Diseases of probably fungal etiology seem to be the most common ailment of salamanders. As reported by Stirling (1970a), two varieties were frequently encountered:

(a) A "sore" type of disease which often appeared on the submaxillary and supra-sternal regions. The sore had a necrotic centre and the animal's health deteriorated very rapidly. It was treated with some success with topical applications of tetracycline powder, daily. This is a very contagious disease, and sick animals were isolated as soon as detected; the mortality rate was very high when this infection appeared in a colony.

(b) The "black spots"; this infection is also presumably of fungal etiology. The spots appeared particularly on the abdomen and the limbs. This disease caused a somewhat lower mortality than the "sore" type of disease; "Nystatin" ointment proved to be an effective treatment for this disease. As with the first condition, sick salamanders were isolated.

(c) A lot of salamanders brought from California showed a massive parasitosis by a mite, whose name and type I could not identify. These mites were found in the mesentery, muscles, and subcutaneous tissues. Eventually they migrated through the skin in a particularly offensive manner. I could find no reference on how to treat this parasitosis. Topical treatment with chemicals like potassium permanganate or antibiotic mixtures (Aurex rotenone drops, MTC Pharm. Ltd.) like those used in similar infections in dogs were ineffective. The most economical procedure was to discard these salamanders.

(d) Wound infections. Although the strict aseptic operative conditions appropriate for mammalian experiments were not necessary, wound infections were minimized by the following procedures: the surgical instruments were sterilized in Hibitane, Ayerst (1gr chlorhexidine acetate in 100 ml 70% ethyl alcohol) for 5 minutes, and after the operation, salamanders were kept for 3 or 4 days in individual plastic cages containing a pair of damp paper towels which were changed daily. After that period they were transferred to individual plastic cages with a layer of moss in the bottom. When these precautions were taken, wound infection was seen in only about 5% of operated animals.

Two salamanders that developed a severe wound infection had previously received antibiotic treatment (Tribiolean, MTC Pharm. Ltd.) topically. They were anesthetized, a "swab" sample was taken from the infected wound, and a blood sample from the heart. The samples were sent for bacteriological studies. Pseudomonas aeruginosa and Escherichia coli were isolated from one salamander, and E. coli from the other. Ps. aeruginosa was very likely a "super-infection" following the initial intro-

duction of antibiotics; E. coli was probably due to fecal contamination of the wound, and proved to be sensitive to a sulphonamide (Gantrisin).

4. Operative procedures

(a) Procedure to anesthetize salamanders

The operations were performed under anesthesia (0.1% MS-222, Sandoz); most of the animals recovered from anesthesia in approximately 60 to 75 minutes. A solution of 0.1% MS-222 was made in tap water; 1 litre for a group of 5 to 10 salamanders. It proved advisable to have an aeration device in the beaker containing the anesthetic, and for this a common fish-bowl bubbler was used. Five to 10 salamanders were placed in the anesthetic solution contained in a 2 litre beaker: usually it took 30 or 40 minutes for the salamanders to become completely anesthetized. It was considered that this stage was reached when the animals did not show any spontaneous motor activity, and when reflex responses (walking or writhing) to mechanical stimulation such as tail squeezing was abolished.

Exposure to MS-222 for approximately 1 hour seemed completely harmless to salamanders. However, when this period was exceeded by about 20 minutes, one or two animals out of 20 usually died. If the animals began to show signs of recovery from anesthesia before the surgical procedures were finished a cotton pad soaked in 0.5% MS-222 was applied over the head and thorax regions of the salamander, and proved adequate to re-induce a suitable depth of anesthesia.

After surgery was finished, salamanders were transferred to a jar containing aerated tap water. After total recovery, they were trans-ferred each one to its individual cage, and given a code name which was

written on the cage. A special form was filled out for each salamander (see next page). The whole procedure (anesthesia, operation and recovery) usually lasted less than 2 hours.

(b) Unilateral section of nerve 16

The position of the right ilium was determined by palpation, and a medio-lateral incision was made just in front of it; using watch-maker forceps a trough was made in the muscles until nerve 16 was exposed, then it was carefully freed of surrounding connective tissue with a fine glass rod. The nerve was cut at a distance of about 8mm from the midline, leaving a distal length of about 7 to 10mm before the plexus formation with nerves 15 and 17 began (see Text-fig. 2-1 and 2-2). The central stump of the nerve was tied off and displaced into the overlying muscle. The wound was closed with 2 or 3 sutures of 6-0 silk.

(c) Topical application of colchicine to nerve 16

A few mm length of nerve 16 was carefully exposed as in (b) above, and as near to the midline as possible. The trough made by the exposure route through the overlying muscle to the nerve trunk was filled with a solution of colchicine (BDH Ltd.) in amphibian Ringer solution (composition: NaCl 111 mM, KCl 1.9 mM, $CaCl_2.2H_2O$ 1.1 mM, Mg $SO_4.7H_2O$ 1.6 mM, $NaHCO_3$ 2.4 mM). A range of colchicine concentrations from 0.025 M to 0.1 M was normally used in this work. In control experiments amphibian Ringer only was used. After 30 minutes the trough was washed out with at least 2 ml Ringer solution, and the wound sewn up. (see Text fig. 2-2).

5. Determination of suitable colchicine doses for the present research

These experiments were performed to assess whether or not exposure

AGE...   WT.C./S.............................

HEIGHT.......

SIZE. AREOLA-GLANDS (cms.) ....

WEIGHT (g) ....

DISTRIBUTION VESSELS ....

....

DATE OF OPERATION ....

OPERATION DETAILS ....

....

....

POST-OPERATIVE EXTRACTION ....

....

SUBSEQUENT HISTORY

DATE (days)	REMARKS
RECOVERY O	

DATE OF EXAMINATION ....,   DISEASE SO-CON. (cms) ....

Vertebral column

Abdomen   15   16   17   Tail

15a
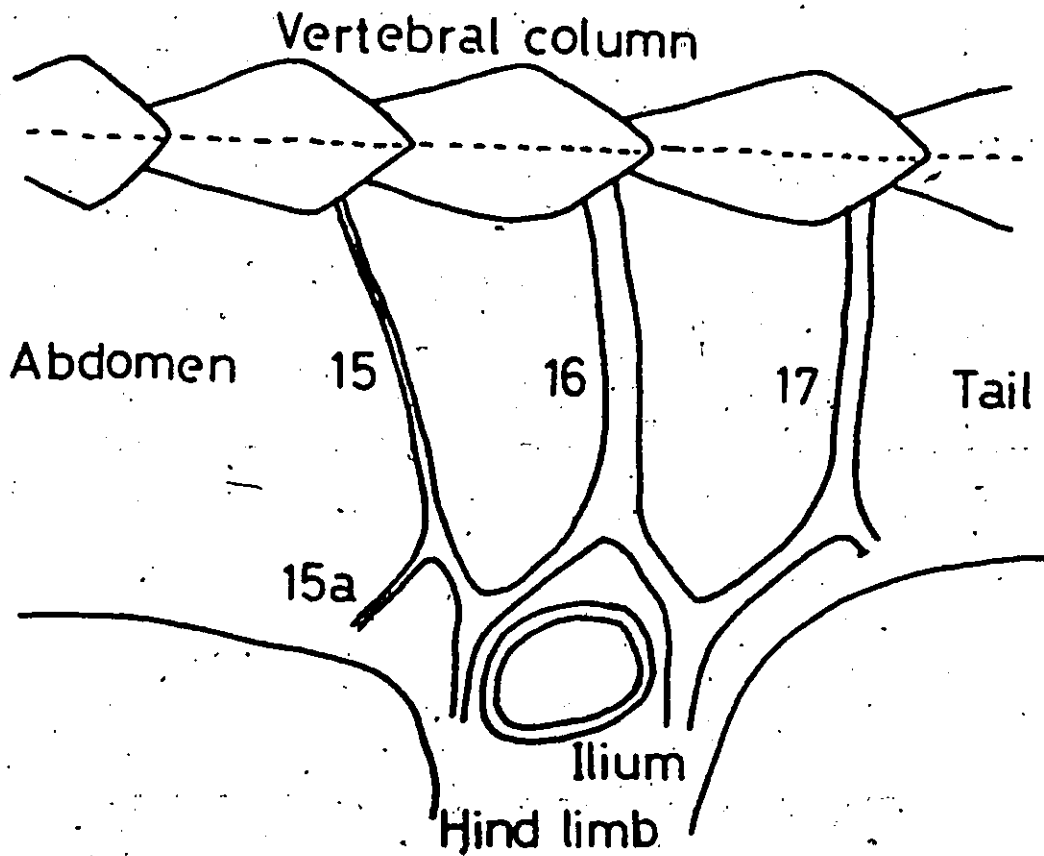
Ilium

Hind limb

FIGURE 2-1

Diagram of the hind limb plexus in salamanders. This represents the anatomical relationships of spinal segmental nerves which form the hind limb plexus in salamanders. The ilium and overlying muscles had been removed. The nerves are identified by their respective numbers (15, 16 and 17); (15a) indicates a branch of nerve 15 which innervates only skin and muscles of the body wall. Other anatomical landmarks are identified by their respective names.

FIGURE 2-2

Salamander in which right nerve 16 has been dissected. A trough was made just in front of the right ilium, and the right nerve 16 lies on the bottom of it; the nerve appears as a white and cylindrical object going from the the midline towards the hind limb, a piece of black plastic material was placed underneath the nerve for photographic purposes. Usually a smaller segment of the nerve was exposed, the dissection was larger in this salamander, in order to get a suitable illustration of this anatomical region. Each of the ruler divisions is 1 mm.

of the nerve 16 to colchicine solutions of various concentrations for
selected periods of time resulted in any immediate impairment of its
ability to conduct nerve impulses. This was assessed by observing the
compound action potential of the whole nerve caused by supramaximal
electrical stimulation in vitro.

Salamanders were anesthetized in 0.1% MS-222 and both nerves 16
were dissected from a point as near to the vertebral column down to the
level of the knee, removed and mounted on a chamber (see Text fig. 2-3
and 2-4) which included two pairs of platinum wire electrodes; one pair
was used for electrical stimulation of the proximal end of the nerve
trunk and the other for recording the compound action potential at the
distal end. Between the two compartments containing the electrode pairs
was a sealed chamber (see Text fig. 2-4) which could be filled with an
appropriate solution using a fine polyethylene tubing and a syringe. The
whole set up was covered with a glass slide smeared with vaseline to
prevent drying of the nerve. Usually the experiments lasted 2 or 3 hours.

A control record was taken at the beginning of the experiment,
then the nerve was exposed to the solution, i.e. amphibian Ringer or
colchicine (0.025M to 0.2M) for 30 minutes, and records were taken every
10 minutes. After that time, the central chamber was washed out with 1
or 2 ml amphibian Ringer, and another recording was taken 10 minutes
later. The right nerve 16 was exposed to colchicine solution and the left
one to amphibian Ringer only. Each pair of nerves was studied simultan-
eously using identical chambers, one for each nerve. The electronic set
up was the same as that described in Part III-B. Usually the gain was
set at 0.5 mV/cm on the cathode ray oscilloscope (CRO) screen, and the

FIGURE 2-3

Chamber used for electrical stimulation and recording of compound action potentials from nerve trunks. For clarity the glass slide covering the chambers as well as the leads for applying the electrical stimulus and for recording the compound action potential were removed, and only the pins connected to platinum wire electrodes are seen protruding from one of the walls of each chamber. The middle compartment and the tubings are filled with a coloured solution (0.01% methylene blue). A diagram of the chamber is shown in Figure 2-4. The whole set-up is placed on top of a paper which has squares of 0.5 x 0.5 cm.
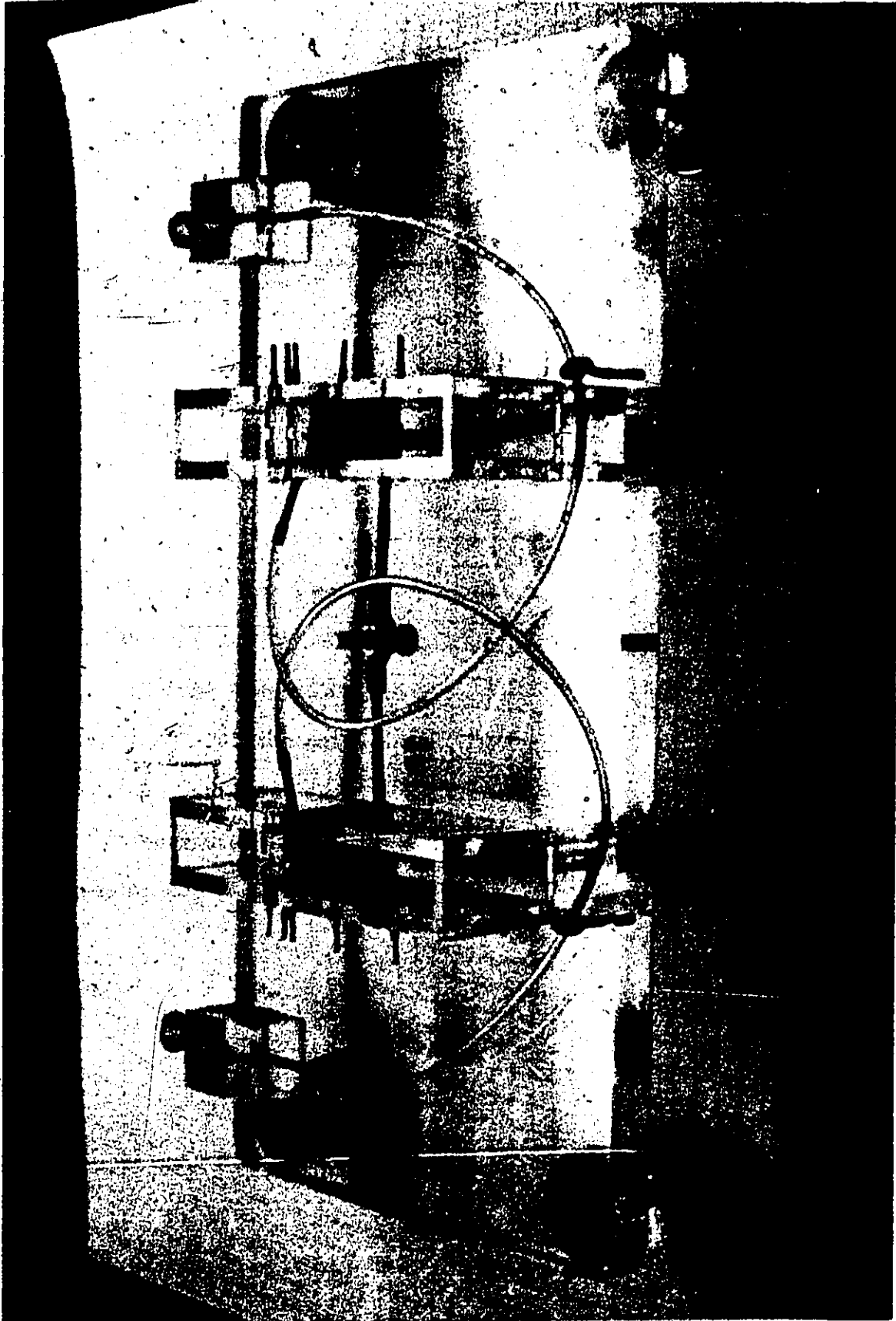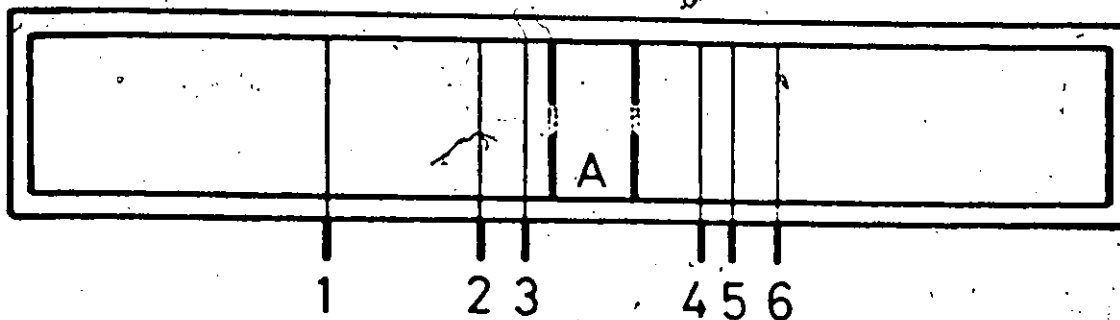
FIGURE 2-4

This is the top view of the upper of the two identical chambers shown in Figure 2-3. 'A' indicates the middle compartment, and the numbers 1 to 6 the pins connected to platinum wire electrodes. Depending on the length of the nerve trunk either '1' or '3', or '2' and '3' were used for recording from the distal end of the nerve trunk, while '4' and '5' were used for applying the electrical stimulus, '6' was used for grounding the preparation. The bar below the drawing represents 10 mm.

sweep speed was 0.5 msec/cm. A typical record is shown in Text fig. 2-5.
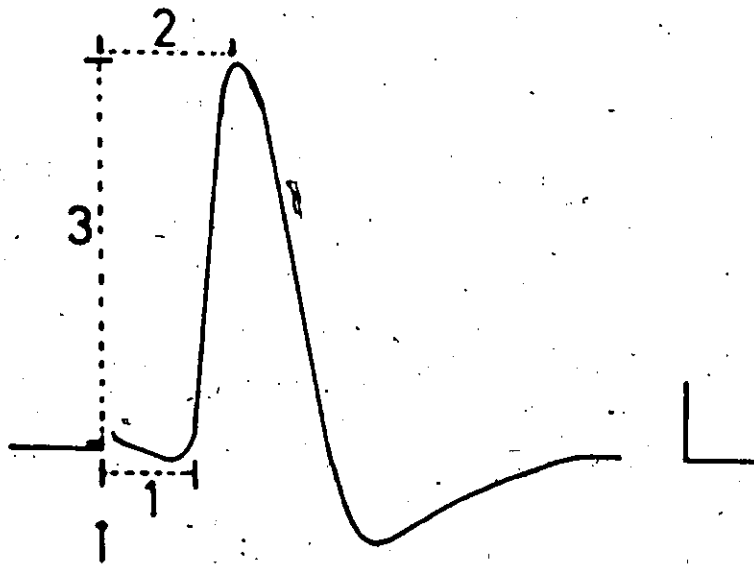
FIGURE 2-5

A typical recording of a compound action potential from a normal nerve. The electrical stimulus (2.1 volts, 50 μsec, 1 Hz) applied at the time indicated by the arrow gave a maximal response. 1 indicates latency (0.55 msec); 2, time to peak amplitude (0.85 msec), and 3, peak amplitude of compound action potential (2.7 mV); conduction velocity for the fastest fibres was 19.9 m/sec. Calibration marks indicate 0.5 mV (vertical), and 0.5 msec (horizontal).

Part II-B RESULTS

1. Normal motor behaviour

In this group of salamanders, as well as in the experimental

groups, i.e. colchicine-treated animals or those with sectioned nerves,

two types of behaviour were investigated. One was the response of the

animal to tactile stimuli, in particular the withdrawal of the limb when

it was lightly stroked with a bristle. More important, and studied in

more detail, was the pattern of movements during walking, since the

operative procedures caused variable degrees of limping which gave an

indication of the degree of nerve damage and subsequently the degree of

recovery after damage. Usually the animals were tested every 2 or 3 days.

- (a) The reflex withdrawal response

In unoperated salamanders, stimulation of the limb-skin with a

bristle resulted in a quick withdrawal of that limb, and it was not un-

common for the animal to start to move away from the stimulus if it was

applied a second time.

(b) Normal walking

This section will provide a brief description of the salamander's

normal walking; a more detailed analysis in the fashion of the myochrono-

grams such as those used by Weiss (1941b) for the analysis of the homolo-

gous response was not attempted in the present studies. The aim of these

observations was to provide a basis for the assessment of any impairment

of walking in the experimental groups of salamanders.

The order in which salamanders moved their legs was: left hind,

right fore, right hind, left fore limb, and so on (see Text fig. 2-6). In other words, the pattern was hind-fore on alternative sides successively. As the speed of walking increased, the interval between stepping by diagonal limbs decreased; in that case, the body was supported at any instant by only two limbs. Any functional loss, therefore, would become more obvious if the salamander would try to move quickly. It also became obvious that such losses were much more apparent when the animal became fatigued as after a period (about 5 minutes) of exercise.

For the present purposes, the following points are important in the movement of hind limb: 1. When the animal swings forward the leg in the phase of protraction, there is a considerable adduction of the limb, and in unoperated salamanders the foot ends up with the toes almost touching the abdomen; 2. Normally the foot is placed squarely on the ground at the start of retraction (see Text fig. 2-6).

2. Behaviour after unilateral nerve section

   (a) The reflex withdrawal response

   After section of right nerve 16 there was sometimes a small region comprising the anterior border of the foot and the first two toes from which no withdrawal response could be provoked. This area, presumably anesthetic, would result from removing nerve 16 in salamanders which had fairly small touch-receptive fields of nerves 15 and 17 (see Part III-B-3); in these animals nerve 16 innervated exclusively the area now anesthetic. The latter, as tested in this way, eventually disappeared over a period of 2 or 3 weeks. The most reasonable interpretation of this phenomenon would be that collateral sprouting of neighbouring spinal nerves (15 and 17) had occurred, and these nerves had taken over the denervated area.
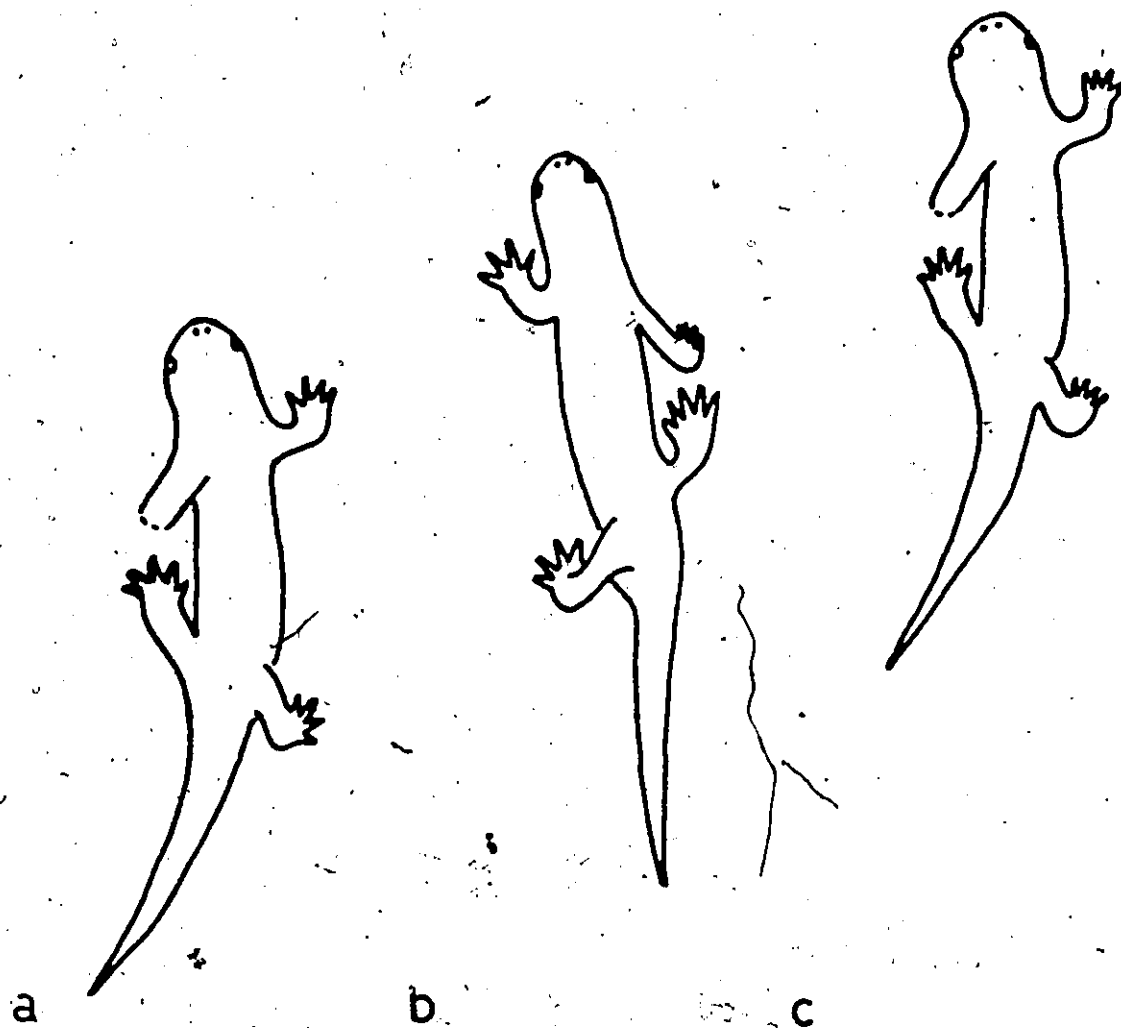
FIGURE 2-6

Pattern of walking movements in a healthy unoperated salamander. These drawings illustrate the sequence of limb movements in the following order: right forelimb and left hind limb (a), and left forelimb and right hind limb (b), and so on. Note that the knee is slightly flexed, that the foot rests squarely on the ground at the beginning of hind limb retraction (a), that the feet almost touch the abdominal wall at the end of retraction, right foot on (b), and left one on (c).

As will be mentioned (see Parts III and IV) in the group of unoperated salamanders the majority of animals had large motor and sensory fields of innervation of spinal nerve 15. That may explain why an anesthetic area after section of nerve 16 was only observed unambiguously in relatively few animals. Nevertheless, there are certain limitations of this test which deserve consideration. For instance, in smaller salamanders the determination of the minute anesthetic area in the limb was difficult and the results somewhat uncertain. Also, in salamanders which may be called hyperactive, this exploration was almost impossible to perform adequately. In that respect, salamanders apparently change in their overall reflex responsiveness from one day to the next.

(b) Walking after unilateral nerve section

Different degrees of limping were observed after section of right nerve 16. Animals were repeatedly stimulated to move by squeezing of the tail until fatigue was apparent, when limping became more obvious. Limping was classified in the following four categories: "Minimal" or (1) degree of limping was an abnormality which became apparent only after the animal had walked for a few minutes; "Slight" or (2) limping was apparent as soon as the animal moved, but the animal's progress was only slightly affected; "Moderate" or (3) limping was characterized by clear abnormalities in adduction, elevation and rotation of the limb during walking; and in particular by the onset of "limb dragging" when fatigue set in; "Severe" or (4) limping describes the situation when the limb was from the first almost totally incapable of being used to help propel the animal forwards. When charts showing the time-course of limping after surgery were plotted, the values (1) to (4), as defined above, were

used. Absence of limping, i.e. normal walking was represented by the value zero.

The most obvious effect of removing nerve 16 was a decrease in adduction of the limb, in most cases the animal could only swing their hind leg forwards through approximately 90° with respect to the main body axis. Usually, when normal animals move, the knee is in a moderate degree of flexion; in contrast, salamanders with 16 nerve cut had the knee in hyperextension. Also, the foot that normally rests squarely on the ground, in the present group tended to be flexed and pronated. It was conspicuous in these animals that they held the foot stiffly out to the side of the body or dragged it passively along (see Text fig. 2-7)

During the first two post-operative weeks all salamanders with a cut 16th nerve showed limping ranging from "slight" or (2) to "severe" or (4), the latter being the most frequent. The rate and degree of recovery was variable. From a set of 14 salamanders which were tested during a period up to 60 post-operative days all of them had improved to "minimal" or (1) degree of limping by the 21.6 $\pm$ 2.9 post-operative day (average $\pm$ SEM), with a range from the 8th to the 41st post-operative day for the amount of recovery; 12 of these salamanders recovered completely normal walking on the 35.6 $\pm$ 4.2 post-operative day (average $\pm$ SEM), with a range from the 15th to the 60th post-operative day. These results showed that the time-course of functional recovery after section of nerve 16 presented a considerable variation from animal to animal. However, by about the 5th post-operative week after section of nerve 16 most salamanders showed complete functional recovery insofar as walking pattern was concerned. Examples of the time-course of behavioural recovery are
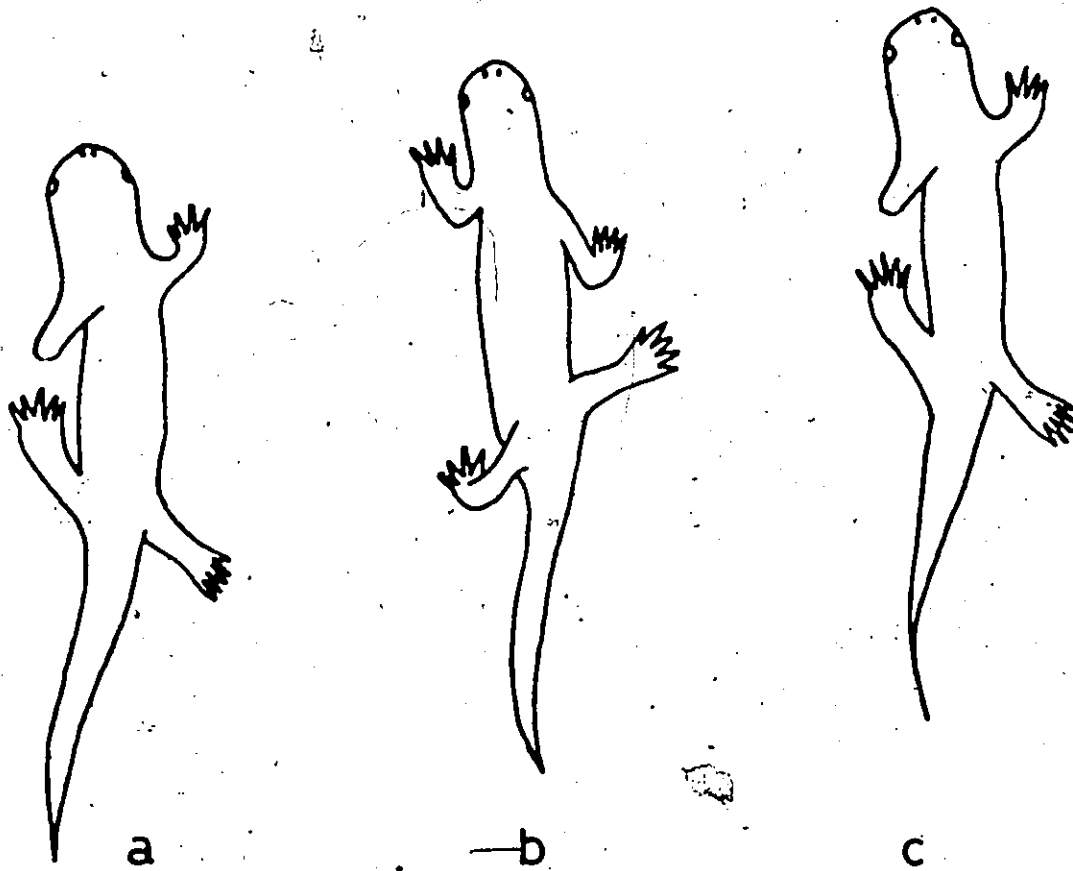
FIGURE 2-7

Pattern of walking movements in a salamander whose right nerve 16 was cut a week before. Note that the right hind limb is held out stiffly to the side of the body, and that the long axis of hind limb form an angle of about 90°, with the main body axis; although it is not apparent from the drawing, it was usually observed that the ankle was in extension and pronated. This drawing would illustrate a salamander with severe or (4) limping; compare such impairment of walking with the normal pattern of walking presented in Figure 2-6.

plotted on Text fig. 2-8 and 2-9.

3. Acute effects of colchicine on nerve conduction

Compound action potentials were recorded as described in Methods section, and the effects of various colchicine concentrations (0.025M to 0.2M) were measured with respect to the following parameters of compound action potential: latency, time to peak and peak amplitude. Text fig. 2-5 shows a typical recording of a compound action potential. The data for the control response before applying colchicine (0 minutes), those made at 10, 20 and 30 minutes after colchicine application, and that made 10 minutes after washing out the drug (40 minutes) will be presented.

(a) Latency

The histogram (Text fig. 2-10) summarizes the data for doses of colchicine ranging from 0.025M to 0.2M. The results show that none of these concentrations of colchicine acutely applied for 30 minutes (see Methods) caused any significant change in the latency of compound action potential.

(b) Time to peak amplitude

Histogram (see Text fig. 2-11) shows that colchicine solutions of 0.025M to 0.2M applied for 30 minutes caused no significant changes in the overall conduction velocity of compound action potentials. This velocity for the fastest conducting fibres was in the range of 19.9 m/sec.

(c) Peak amplitude of compound action potential

Histogram (see Text fig. 2-12) summarizes the data concerning the effects that colchicine solutions had on the peak amplitude of a compound action potential. For concentrations ranging from 0.025M to 0.1M there was no significant effect on the compound action potential of treated nerves

FIGURE 2-8

Time-course of the recovery of normal walking after section of nerve 16 (salamander 16-169). This salamander showed initially "severe" or (4) limping and it was not until the 39th post-operative day that normal walking was observed again. In this group of salamanders (section of nerve 16) statistically significant increases in the motor fields of innervation of nerves 15 and 17 were detected as early as the 5th post-operative day. This example illustrates that group which showed a late recovery of normal walking.

Limping

4
3
2
1
0

0 6 12 18 24 30 36 42 Days
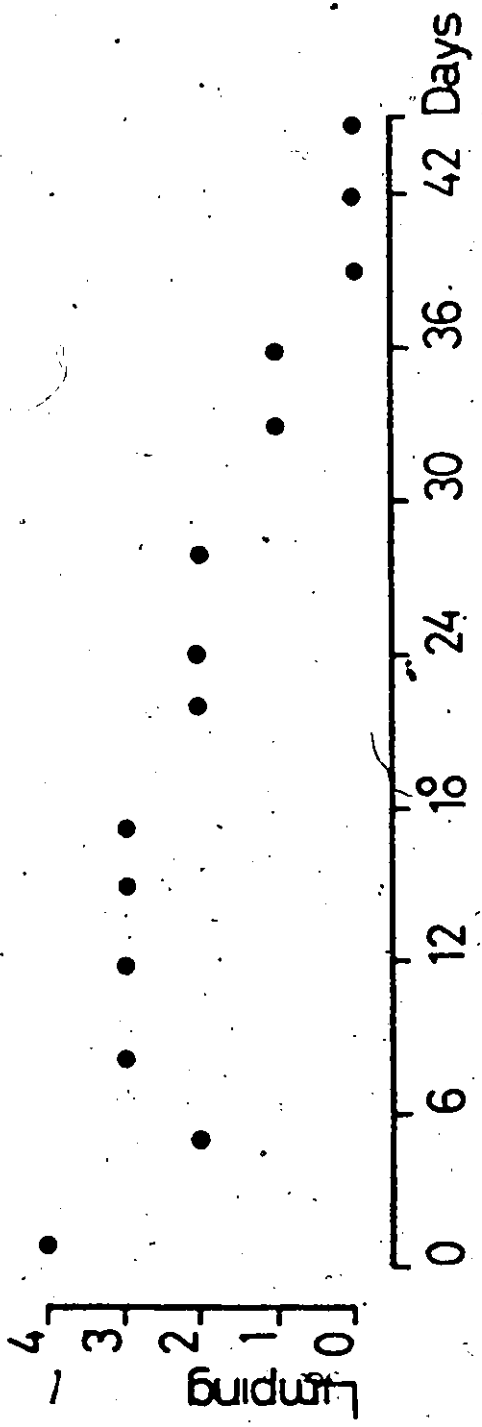
FIGURE 2-9

Time-course of recovery of normal walking after section of nerve 16 (Salamander 16-191). It is apparent that over a period of 15 days the degree of limping which initially was classified as "moderate" or (3) decreased progressively until normal walking was recovered at the 15th post-operative day. It is worth noting here that significant increases in the motor fields of innervation of nerves 15 and 17 were detected as early as the 5th post-operative day. This example is from that group of salamanders which showed a relatively quick recovery of normal walking.

A graph plotting Limping (y-axis, 0 to 4) versus Days (x-axis, 0 to 42).
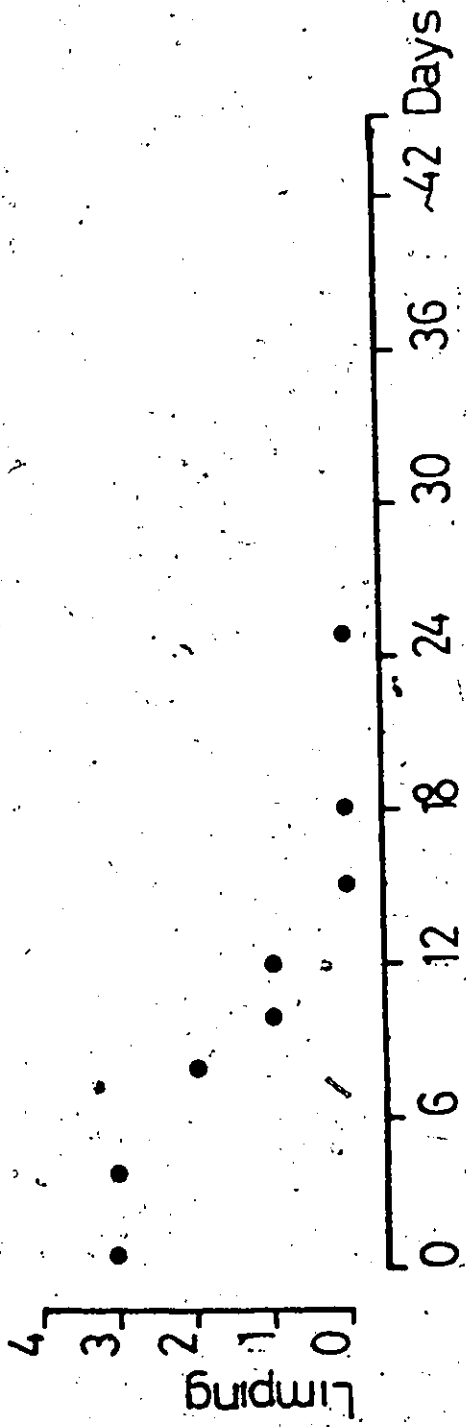
FIGURE 2-10

Effects of colchicine solutions on latency of compound action potentials. 0 minutes corresponds to observations made before colchicine was applied, 10, 20 and 30 minutes, to observations made during such exposure times to colchicine, and 40 minutes, to the period 10 minutes after washing out the drug. The left-hand column of each pair corresponds to the Ringer-treated control nerves, and the right to colchicine-treated ones. The doses are indicated below each row of histograms. Each column corresponds to the average ($\pm$ SEM). The number of experiments were for 0.025M colchicine (n = 5), 0.05M colchicine (n = 7), 0.1 M colchicine (n = 14), and 0.2M colchicine (n = 5). The difference between each pair of values was not statistically significant (level of significance P > 0.05).
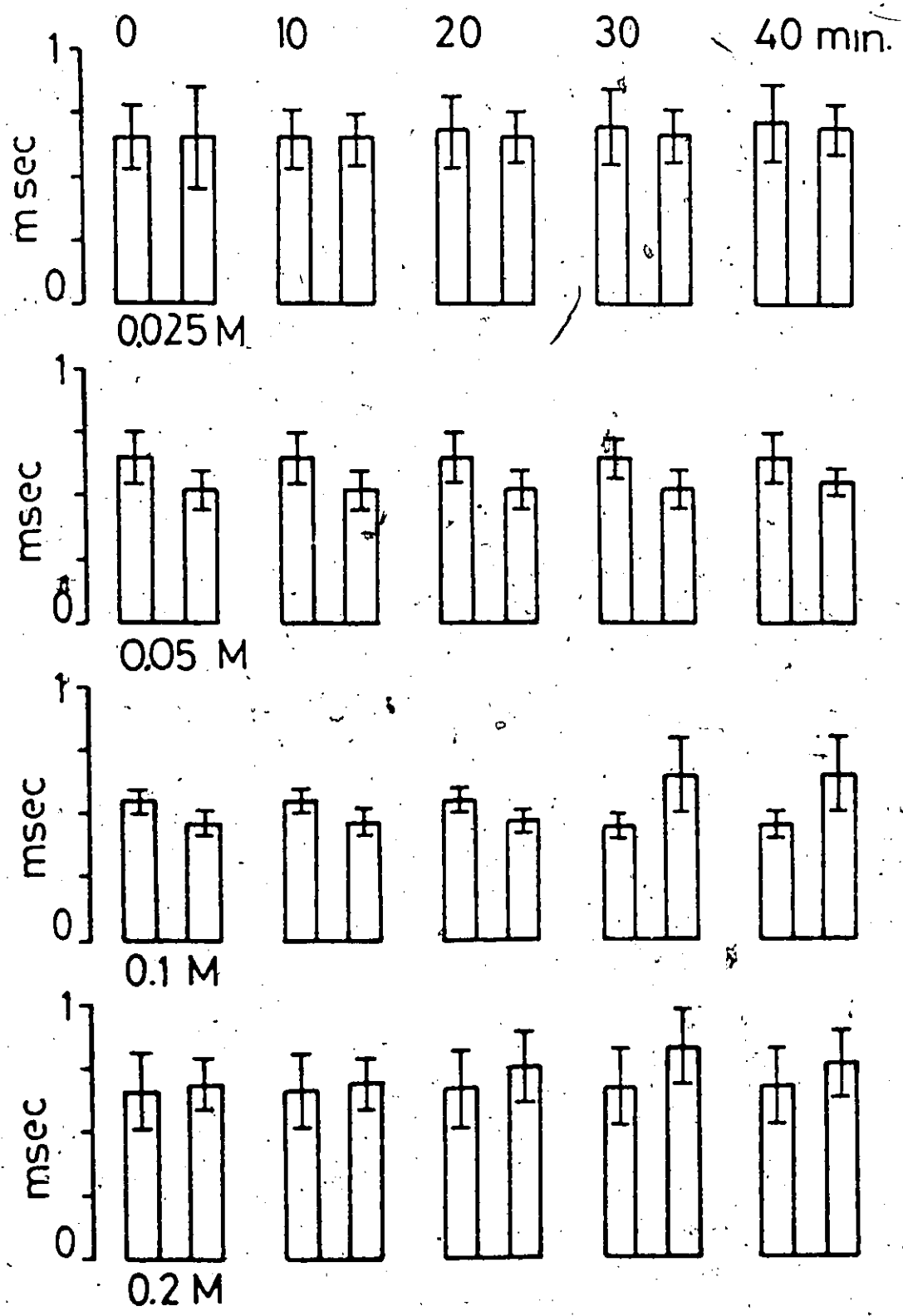
FIGURE 2-11

Effects of colchicine solution on time to peak of compound action potentials. 0 minutes corresponds to observations made before colchicine was applied, 10, 20, and 30 minutes, to observations made during such exposure times to colchicine, and 40 minutes, to 40 minutes after washing the drug. The left-hand column of each pair corresponds to the Ringer-treated control nerves, and the right to colchicine-treated ones. The doses are indicated below each row of histograms. Each value corresponds to the average ($\pm$ SEM). The number of experiments were for 0.025M col-chicine (N = 5), 0.05M colchicine (n = 7), 0.1M colchicine (n = 14), and for 0.2M colchicine (n = 5). With the higher concentrations the experi-mental values tended to be greater than the control ones, but such differences were not significant in any case (level of significance P = 0.05).
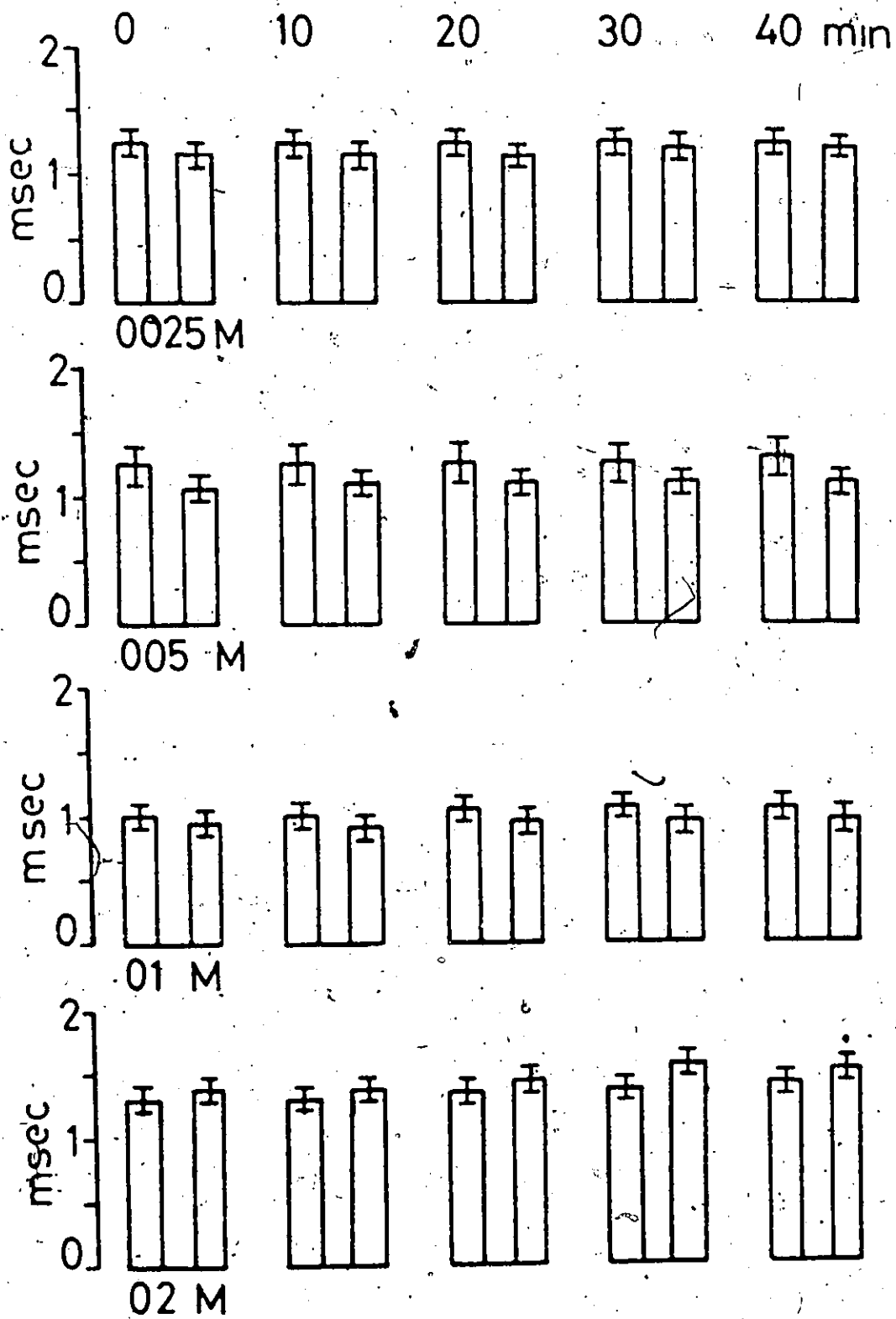
FIGURE 2-12

Effects of colchicine on peak amplitude of compound action potentials. 0 minutes indicates observations made before colchicine was applied, 10, 20 and 30 minutes, to observations made during such times of exposure to colchicine, and 40 minutes to ten minutes after washing out the drug. The left-hand column of each pair corresponds to Ringer-treated nerves, and the right to colchicine-treated ones. The doses are indicated below each group of histograms. Each column corresponds to the average ($\pm$ SEM). The number of experiments were for 0.025M colchicine (n = 5), 0.05M colchicine (n = 7), 0.1M colchicine (n = 15), and 0.2M colchicine (n = 5). Significance of difference is indicated by *P <0.05, and ** P <0.01.
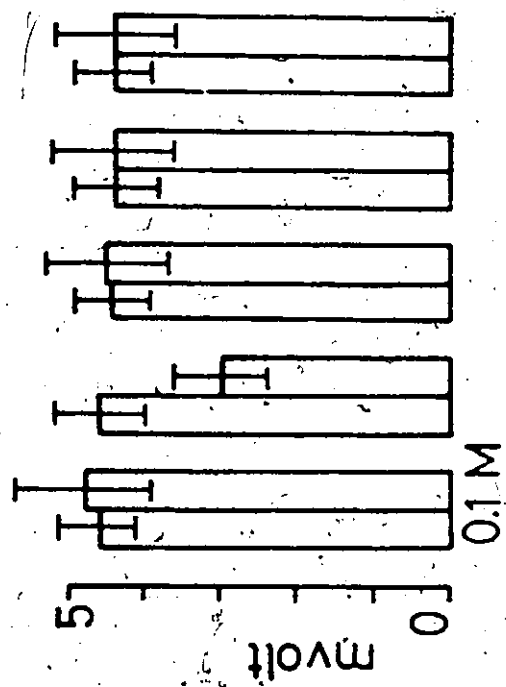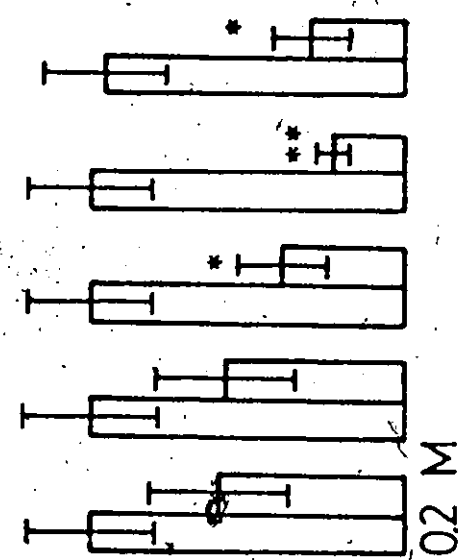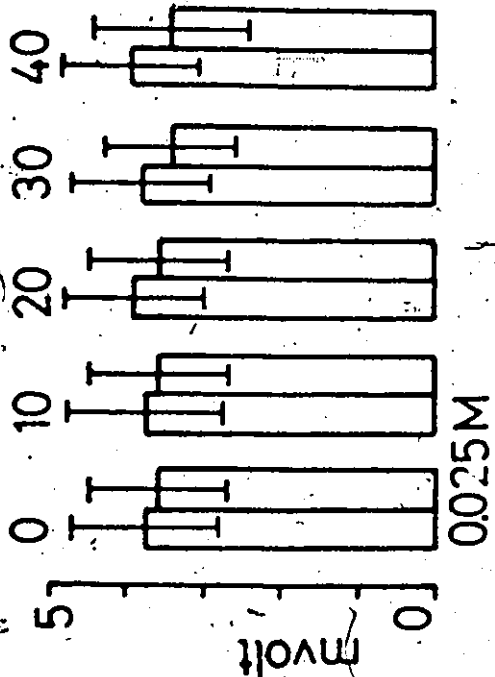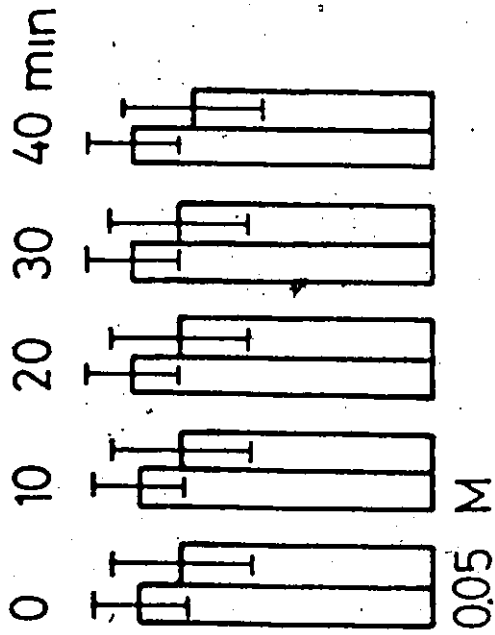
as compared to that of nerves which were exposed to amphibian Ringer only. Treating the nerve with 0.2M colchicine for 30 minutes, however, resulted in a significant decrease in the peak amplitude of the compound action potential. Such decrease was detectable as early as 20 minutes after exposure to 0.2M colchicine. Furthermore, in some of these nerves the compound action potential disappeared completely for about half an hour, and did not recover completely with continuous washing over a period of about 4 hours. Text fig. 2-13 and 2-14 illustrate typical examples from the present group of experiments. —

(d) Conclusion

The results indicate that colchicine doses ranging from 0.025M to 0.1M applied for 30 minutes to a nerve trunk did not cause any immediate effect on the compound action potential. However, a dose of 0.2M colchicine applied for 30 minutes caused a significant decrease in the amplitude of the compound action potential which was only partially reversible. These findings suggest that colchicine concentrations of 0.1M or less applied for 30 minutes may not have caused damage of nerve fibres, at least in the short term. Such doses were used in the following sets of experiments.

4. Motor behaviour after colchicine treatment

(a) The reflex withdrawal response

When the foot of the hind limb of colchicine treated salamanders was slightly stroked with a bristle the quick withdrawal of the foot was normal, and did not differ from that observed on unoperated salamanders, or from the "normal" side of unilaterally operated animals.

FIGURE 2-13

Effects of acute treatment of a nerve trunk with 0.2M colchicine for 30 minutes in vitro. (a) shows the control record taken at the beginning of the experiments; note that after 30 minutes of such treatment (b) there was a decrease in the amplitude of compound action potential and a lengthening of its latency and time to peak amplitude; (c) made 10 minutes after washing out the drug, shows that such changes were only partially reversible. The calibration marks indicate 0.5mV (vertical), and 0.5 msec (horizontal).
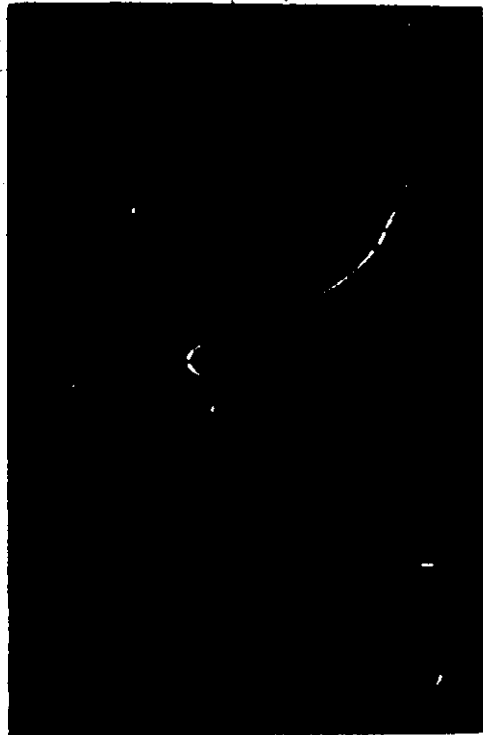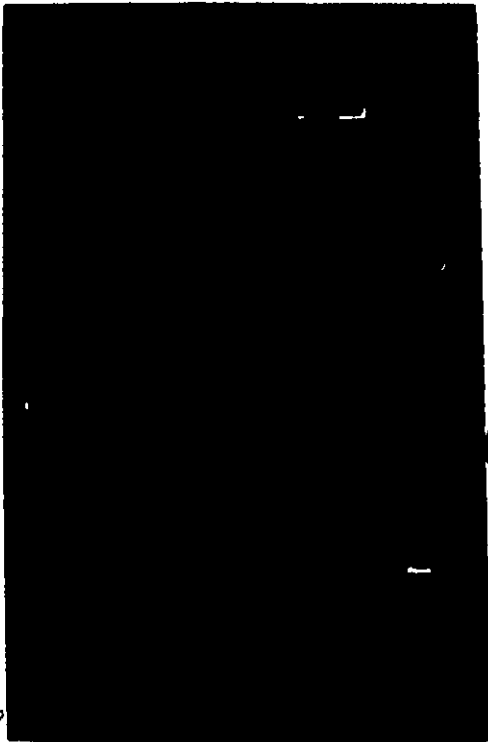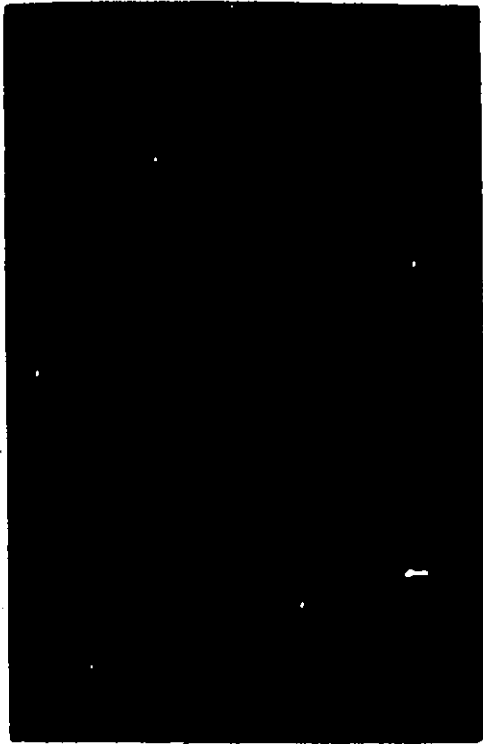
FIGURE 2-14

Effect of acuté application of 0.1M colchicine for 30 minutes to the "middle" region of a nerve trunk.. (a) shows the control record taken at the beginning of the experiment, (b) the record taken 30 minutes after the nerve trunk was exposed to 0.1M colchicine; there was a reduction of about 9% in the amplitude of compound action potential, as compared to the control record; (c) taken 10 minutes after washing out the colchicine solution shows that such small changes (when they occurred) were reversible, and moreover they were not significant (level of significance $P > 0.05$) for the whole group of experiments. The calibration marks indicate 0.5 mV (vertical) and 0.5 msec (horizontal).
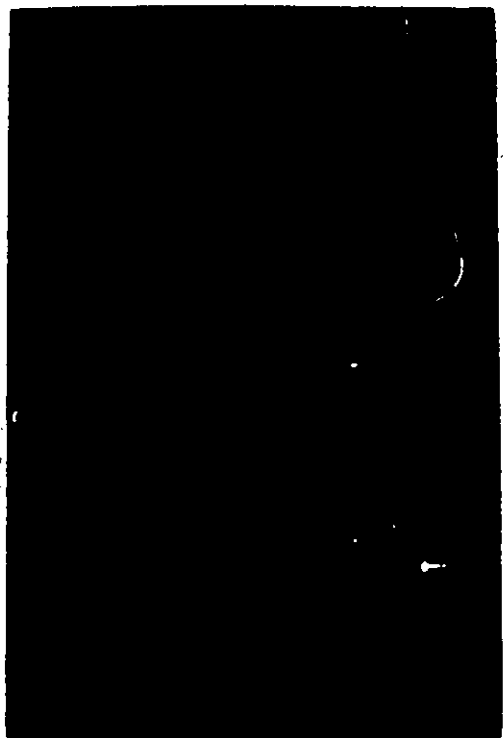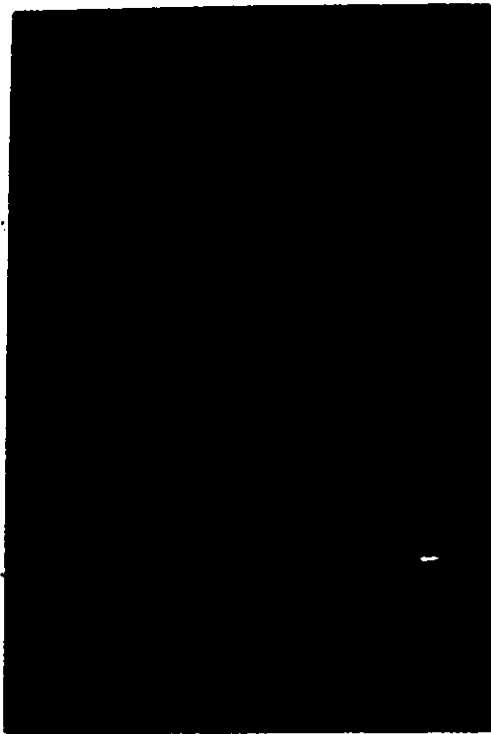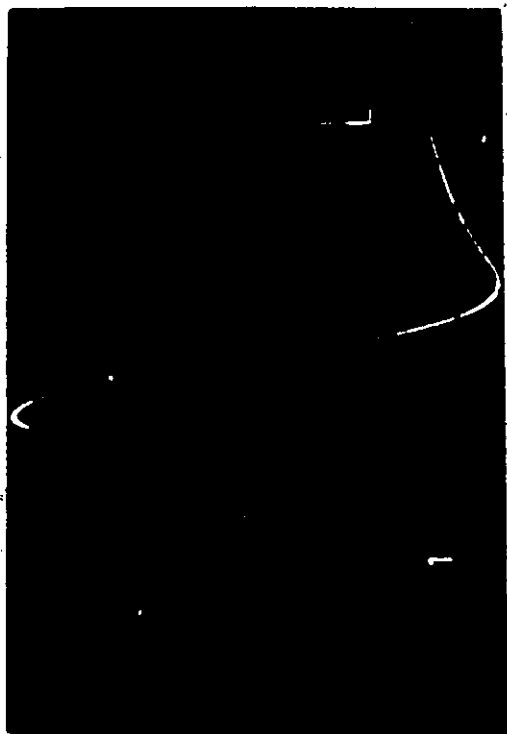
(b) Walking after colchicine treatment

Acute application of colchicine, as described in Methods, produced either no limping, or in a few animals a "minimal" or (1) impairment of walking up to the 12th post-operative day (see Text fig 2-15 and 2-16). The "minimal" limping observed was probably due to surgical trauma of the hip muscles, since it was seen up to the 7th post-operative day in some salamanders after simple exposure of nerve 16 in "sham" operations. In several of such animals "minimal" limping appeared only after they have walked for a couple of minutes, and after the 7th post-operative day even this degree of limping disappeared.

Practically all animals in this group placed the foot squarely on the ground and none of them presented the foot on the treated side in flexion and pronation. So far as behavioural observations are concerned, therefore, the various doses of colchicine (0.025M to 0.1M) which were used in these experiments caused no significant effects either on walking or on reflex withdrawal to mechanical stimulation of the foot. Although it was sometimes difficult to distinguish between normal walking and "minimal" limping, it should be noted that colchicine-treated salamanders never showed "slight" or (2) limping, which could always be recognized without any ambiguity.

(c) Conclusion

The time-course of recovery of colchicine and "sham" (Ringer)-treated salamanders was very similar. Most of them in fact showed no limping at all. These results are in sharp contrast to those from animals which had unilateral section of nerve 16. From the point of view of behaviour, therefore, the colchicine doses used in these experiments

FIGURE 2-15

Behavioural observations performed on salamander (C-76) whose right nerve 16 was treated with 0.1M colchicine, as described in Methods. This salamander is an example of those which did not show any limping after such a treatment. Compare this chart with the severe degrees of limping observed after section of nerve 16 (Figures 2-8 and 2-9).

Limping

4 3 2 1 0

0 6 12 18 24 30 36 42 Days
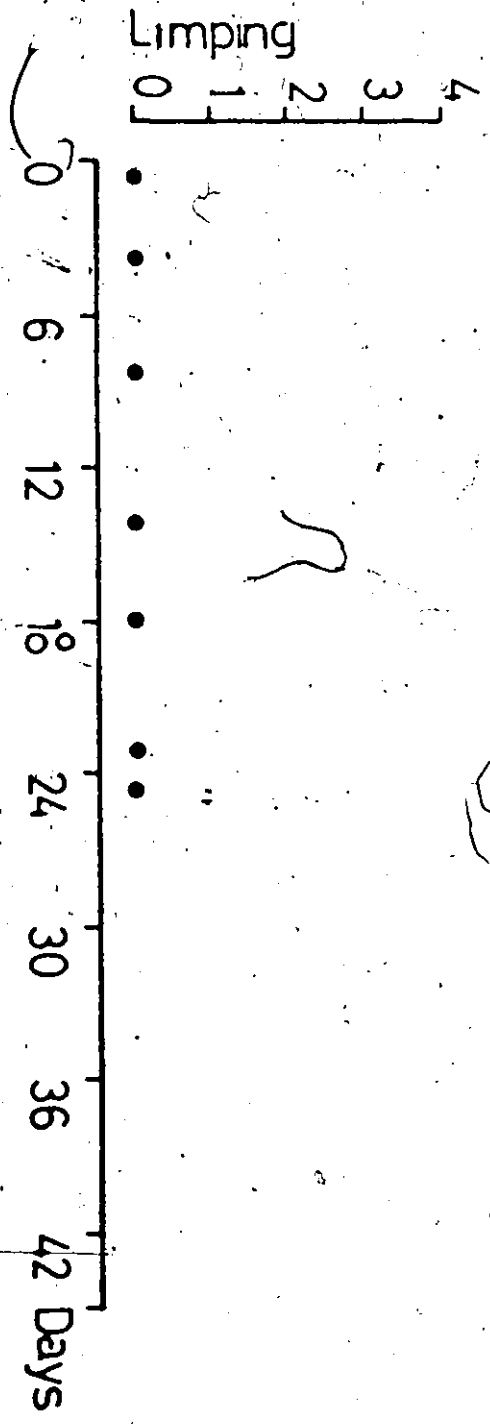
FIGURE 2-16

Time-course of the recovery of normal walking in a salamander (C-118) which was treated with 0.1M colchicine for 30 minutes on the right nerve 16, as described in Methods. This salamander showed only "minimal" or (1) degree of limping; the latter had disappeared by the 11th post-operative day. Colchicine-treated salamanders did not show higher degrees of limping, and recovery of normal walking occurred always before the 12th post-operative day when they presented "minimal" limping; often they did not limp at all. Compare this with the chart for an animal in which the 16th nerve was cut (Figures 2-8 and 2-9).
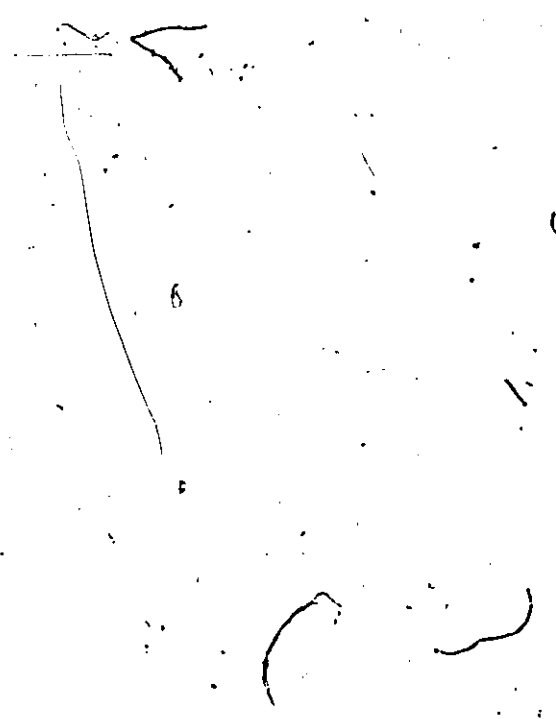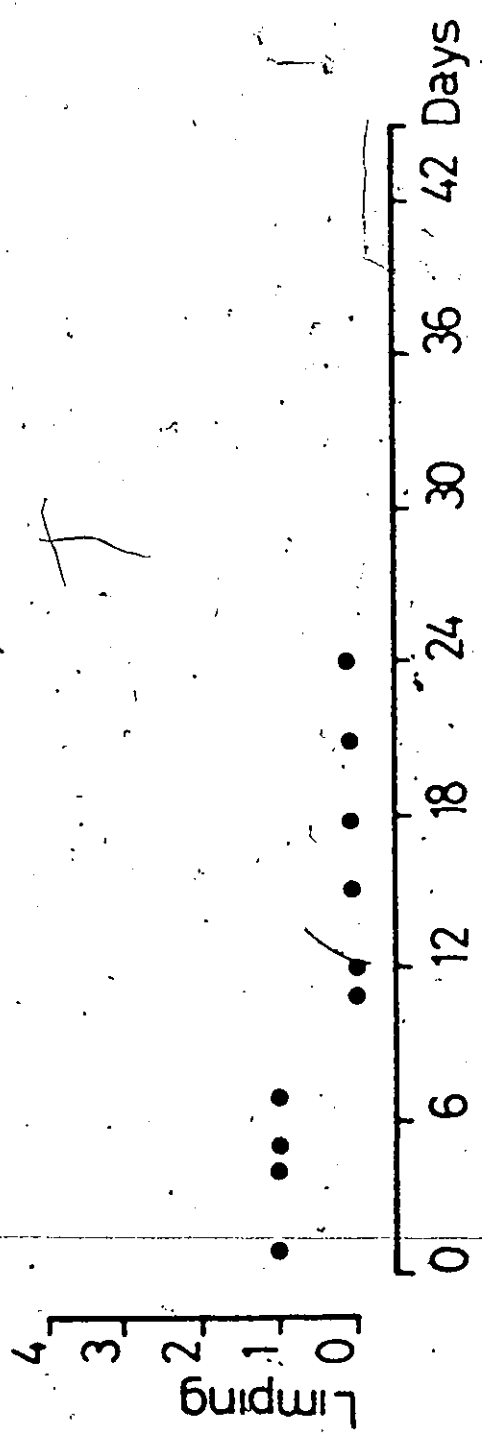
were totally ineffective, and this dramatizes somewhat the subsequent

findings which are based on other measures of colchicine actions.

Part II-C   DISCUSSION ON BEHAVIOURAL OBSERVATIONS

The most important finding has been that the severe limping
caused by cutting nerve 16 gradually reversed so that the normal walking
pattern was re-established after some 35 days or so, while after sham
operations or colchicine treatment no behavioural deficit was apparent
at any time in most animals. The recovery of the reflex withdrawal res-
ponse to touching of the denervated skin area may also have been gradual
but unlike the situation with larger animals, e.g. rabbits (Weddell et
al., 1941), and humans (Livingston, 1947) it was not possible to follow
the time-course of such recovery in salamanders because of the small size
of skin area involved.

As will be shown later in this thesis, at least part of the func-
tional recovery may be associated with the sprouting of nerves 15 and 17
which occurred both in the muscles and the skin, and prevention of this
sprouting in nerve 15 (see Part VII) also delayed or prevented this
recovery. However, sprouting was complete by the 10th post-operative day
at most, whereas full recovery of behaviour could take 3 times as long
(35 days on the average). There is normally a significant overlap of
innervation of muscles innervated by nerve 16 with both nerves 15 and 17,
and the sprouting of the latter nerves occurred into areas which were
usually synergistic, both sensory and motor. It seems surprising, however,
that peripheral sprouting without any central nervous system changes could
account for the recovery of normal behaviour, and the fact that the latter
took longer than did the completion of the peripheral sprouting supported

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this. Evidence that adaptive changes can occur in the spinal cord was found by Sterling (1970a, 1973) who showed that the reflex latency between nerves 15 and 17 became significantly shorter when nerve 15 had increased its touch-receptive field presumably by sprouting, after complete elimination of nerve 16. Moreover, it then became comparable to the reflex latency found in normal animals which already had a large touch-receptive field of nerve 15. Although she did not make a thorough study of the time-course of this phenomenon, it seems that the 23rd post-operative day was the earliest time at which such shortening of reflex latency was detectable. Exactly what adaptive changes would occur which could be appropriate for the functional requirements is a matter for speculation, which will not be explored here, except to mention that it could involve central sprouting and/or re-routing (respecification) of central connections; the latter has been implicated in analagous experiments of Miner (1956) and Jacobson et al., (1969).

With regard to the colchicine experiments, as will be shown later, peripheral sprouting of the adjacent untreated nerves also occurs; here we have the problem of how the central nervous system "handles" impulses coming along nerve fibres which have apparently sprouted into new skin as well as retaining connections with original skin; and at least in some salamanders how it deals with outgoing impulses in fibres which have probably taken over new muscles as well as retaining connections with the original muscles. It is of course possible that the resolution of the behavioural observations in the present experiments was not adequate to detect subtle malfunctioning in the motor system.

A key question which has not yet been answered is whether or not

sprouting after colchicine treatment results in changes in central reflex latencies between nerves 15 and 17 as Stirling found in the nerve-sectioning experiments; some preliminary experiments to investigate this possibility gave inconclusive results.

TOUCH-RECEPTIVE FIELDS OF HIND LIMB SKIN

INTRODUCTION

This section is concerned with the experiments in which the touch-receptive fields of hind limb nerves were mapped in unoperated salamanders and in salamanders in which either the 16th nerve had been sectioned or treated with colchicine. For each group of salamanders, individual experiments will be presented first, followed by a statistical analysis of the group.

Part III-A METHODS OF FIELD MAPPING

The methods of sectioning or colchicine-treating the 16th nerve with subsequent recovery of the animal have been described in Part II-A (d)

1. Preparation of nerves for recording

The salamanders were anesthetized with 0.1% MS-222 and then decerebrated as follows. An incision was made over the occipito-atlantoideal joint, the occipito-atlantoideal ligament was exposed and cut, then the upper part of the cranial vault was extirpated. A section was performed at the level of emergence of the 10th cranial nerve, the brain was removed with forceps and the cranial cavity filled with cotton.

After decerebration, the dissection of the hind limb plexus was performed in the following manner: A medial incision was made from vertebrae 14 to 18; at the end of this incision two lateral incisions were made, two skin flaps were then excised along a line parallel to the midline at a level with the groin. The muscles of the hip region were removed and the nerve trunks of nerves 15, 16 and 17 were exposed on both sides. Both ilia were cut as near to the vertebral column as possible, and close to the origin of the limb; the bones were removed. Great care was taken not to damage nerves 16 and 17 during this step.

Using a fine glass rod, the nerve trunks were dissected free of connective tissue to the region where they branch to form the limb plexus (Figure 2-1). In some animals, nerve 18 also participates in this plexus formation; such animals were not included in the present study. The

nerve trunks thus dissected were cut as near to the vertebral column as possible and a thread was tied round the cut end of the distal stump.

Usually, the amount of bleeding during the dissection was very slight, and a maintained blood flow through the small blood vessels of nerve trunks and the small skin capillaries could be seen without any ambiguity. In those few cases in which there was considerable hemorrhage, the blood flow was sluggish and sometimes difficult to observe; the latter group of animals was discarded.

2. Recording techniques

The recording electrodes were a pair of platinum wires across which the nerve was placed so that the cut end did not touch the body in any way, but was well positioned in the distal one of the platinum wires. The electrodes were connected to the amplifier imput via a filter device whose frequency limits were set at 150 HZ-10KHZ. The nerve signals were amplified and displayed on a Tektronix 5103 storage oscilloscope, and relayed through a loudspeaker which was used to monitor the nerve activity. Usually, the oscilloscope was set in the triggering mode "free running", at a gain of 20 or 50 μV/cm, and at a sweep speed of 5 msec/cm. The apparatus and the preparation were connected to a common ground (Figure 3-1).

3. Stimulation of touch-receptors

A holder containing a bristle was manipulated by hand so that light touch of the bristle was applied to the skin in single brief sweeps covering about 1 mm^2 of skin surface. With practice it was possible to define within 1 mm or less, the edge of the skin area which comprised the
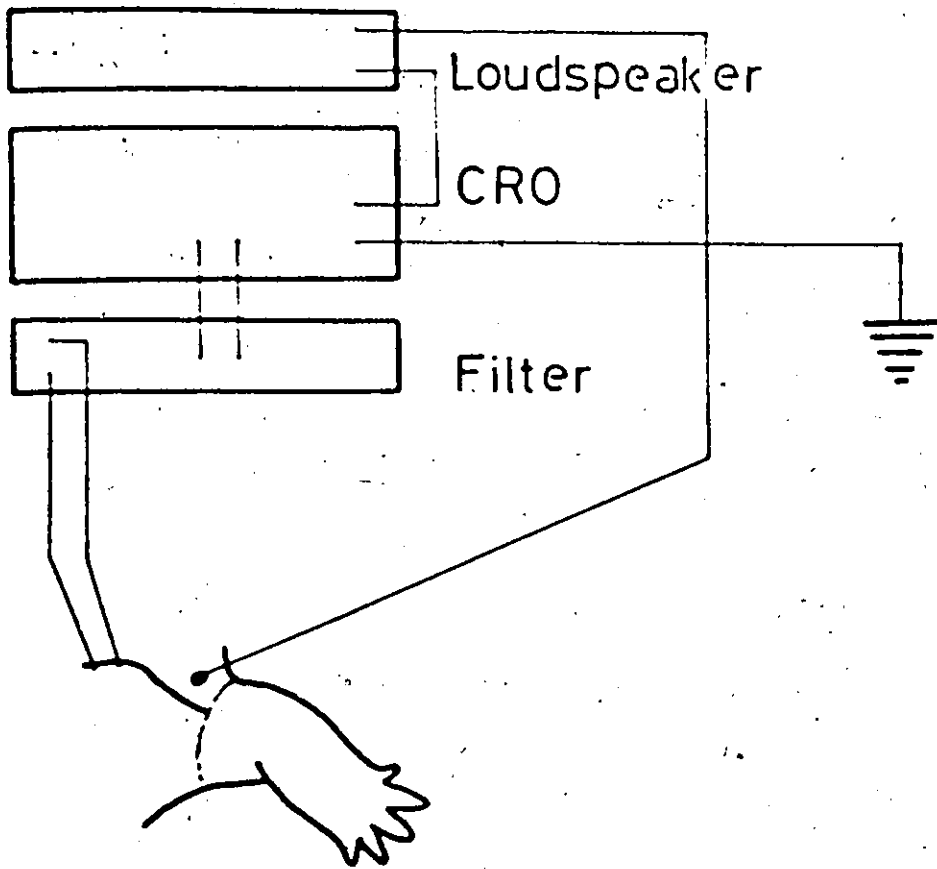
FIGURE 3-1

This diagram represents the connections between the several components of the set-up used for the mapping of touch-receptive fields. The hind limb skin was lightly stroked with a bristle and the action potentials thus evoked were recorded from a pair of platinum wire electrodes. The electrical signals passed through the filter before being amplified in the CRO; such amplified signals were relayed through a loudspeaker. The CRO was set on the triggering mode "free running". The apparatus and the preparations were grounded to a common point.

field of the nerve recorded from. The auditory monitoring of the impulses (over the loudspeaker) proved very sensitive in this regard. The field was that area which, when stimulated in this way, gave rise to impulses in the nerve. As it was defined during the experiment, it's outline was indicated in a "standard" map of the leg area in pencil. This "standard" map was a picture of the dorsal and ventral aspects of the hind limb amplified 20 times, the area enclosed within the outlines of the nerve fields were measured (in cm^2) using a planimeter, and subsequently converted into mm^2 of innervated skin. There is probably a small difference between the actual area of skin and the area circumscribed by the outline on the hind limb picture. but such differences seem negligible, since they were the same for both sides of the body. and leg size did not vary greatly from one animal to another.

Part III-B <u>RESULTS</u>

1. <u>Typical example of a mapping experiment</u>

From some regions of hind limb skin a barrage of spikes was
evoked in the selected nerve trunk when the skin was lightly stroked
with a bristle, while other regions were unresponsive (Figure a-2). The
spikes relayed from the CRO through the loudspeaker produced a
characteristic sound which was very useful in the mapping experiments
(see Methods). Figure 3-2 illustrates one such experiment in which
touching the skin lightly with a bristle in the three different points
marked on the shaded area of the hind limb diagram evoked a discharge of
spikes, the bars below the trace indicate the time during which the skin
was stroked. This procedure was performed while observing the hind limb
under a dissecting microscope. By carefully exploring the surface of the
skin, a map could be made of the innervated area (shaded area in Figure
3-2). When the rest of the skin was touched there was no discharge (trace
b, points 4 to 6). It is apparent from the record that there were some
occasional spontaneous spikes unrelated to the period of stimulus appli-
cation; a barrage of impulses such as that seen in record (a), however,
did not occur. The spontaneous low frequency firing of spikes was
observed often, and originated from the muscles and joints, since they
sometimes persisted after the limb was skinned; some spikes could also be
injury discharges from skin nerves.

When the hind limb was skinned and the muscle surface was lightly
stroked with the bristle, no equivalent bursts of impulses were evoked

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FIGURE 3-2

Mapping of touch-receptive fields. Action potentials were recorded
from the whole trunk of nerve 15. Record (a) shows the burst of spikes
evoked when the skin was lightly stroked during the periods indicated by
the bars below over the points indicated by 1, 2 and 3 in the drawing
beside the traces. Record (b) shows that touching the skin with a bristle
at points 4, 5 and 6 did not evoke bursts of spikes on nerve 15; only spon-
taneous spikes were observed. (The degree of spontaneous activity was
variable from one salamander to another). Record (c) which was taken from
another salamander shows an example of a quite large amount of spontaneous
activity in nerve 15. The calibration marks indicate 50 μvolt (vertical),
and 1.0 sec (horizontal); the bar below the hind limb drawing represents
10 mm.
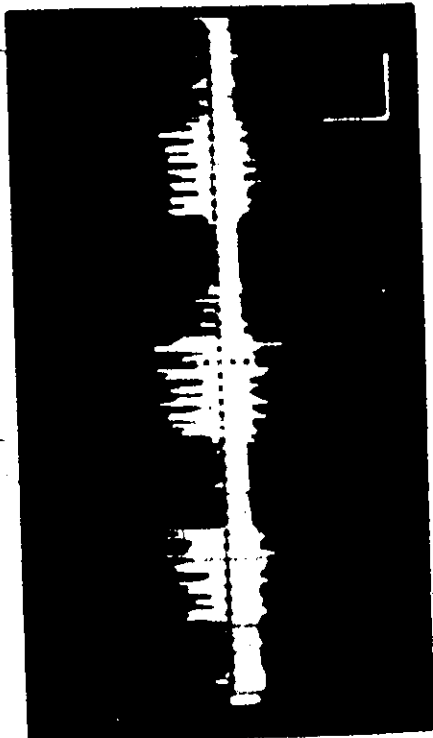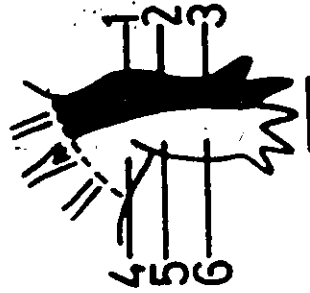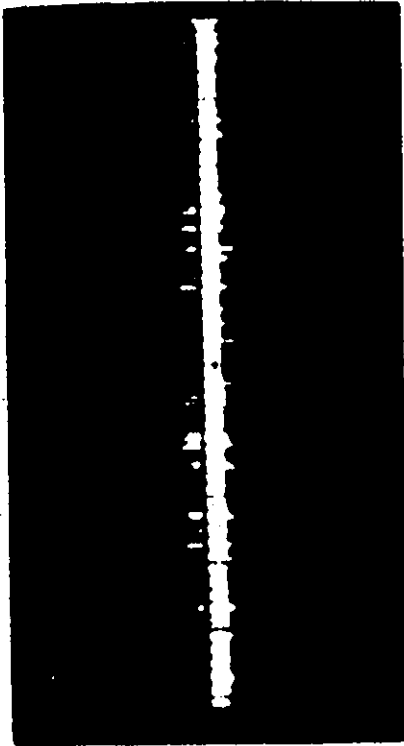
(Figure 3-3); however, prodding the muscle with a glass rod did evoke a discharge of spikes in the nerve trunk. These results show that the light stroking of the skin with the bristle stimulated selectively mechano-receptors in the skin; other kinds of "deep" mechano-receptors, particularly muscle spindle or tendon-organs, had a higher mechanical threshold for this kind of stimulus. The mappings performed as described above, therefore, represent the "touch-receptive" field of the nerve from which the action potentials were recorded. It is worth noting that the boundary between the innervated and non-innervated areas defined in this way, was quite sharply demarcated, and often a movement of the bristle of about 1 cm could distinguish the edge of the field.

The underside of the hind limb skin as well as its dorsal surface was explored in this way; however, for clarity only the results from the dorsal surface are presented in figures throughout this thesis. For the calculation of the areas innervated by a spinal nerve, both of the aspects of hind limb were taken into account.

2. Touch-receptive fields in un-operated salamanders.

(a) Individual examples: The results of the electrophysiological mapping of touch-receptive fields from two salamanders will be presented (salamanders N1 and N3).

Salamander N1:- The results from this experiment, (Figure 3-4), showed that the spinal nerve 15 on each side innervated approximately the anterior one third of the hind limb skin and the first and second toes; the area of skin innervated by each 15th nerve was 212 mm^2. The spinal nerve 16 on the right side innervated the skin of the whole hind limb which corres-

FIGURE 3-3

Mapping of touch-receptive fields. Record (a) shows the bursts of spikes which were evoked in nerve 15 when the points 1, 2 and 3 were lightly stroked with a bristle, during the time indicated by the bars below. Only from the shaded area of hind-limb could such bursts of spikes be evoked. Subsequently the hind limb was skinned; record (b) shows that prodding the muscles with a glass rod (upper trace), during the time indicated by the bars below, evoked bursts of spikes, while lightly stroking the muscle surface with a bristle (lower trace) was ineffective. Therefore, stroking of the skin with the bristle selectively stimulated mechano-receptors in the skin, and not muscle or other tissues, (joints, tendons). Calibrations are 50 µvolt (vertical) and 1.0 sec (horizontal), the bars below hind limb drawings represent 10 mm.
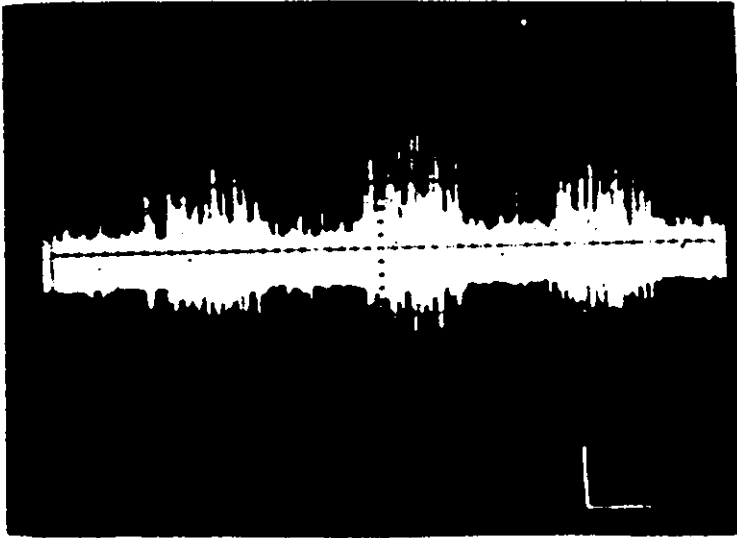
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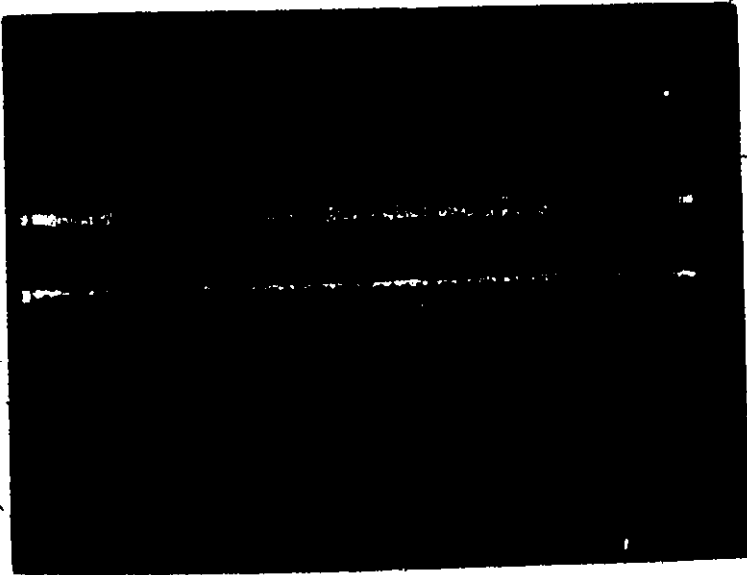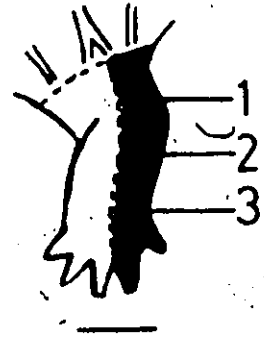
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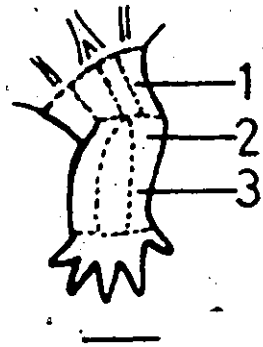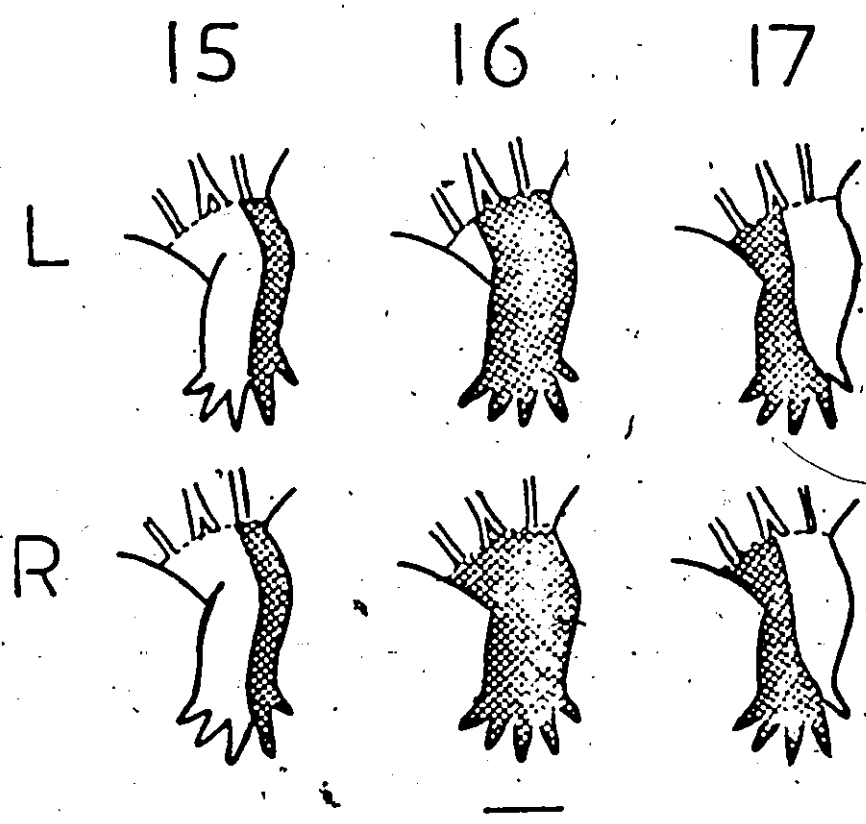
FIGURE 3-4

Touch-receptive fields of nerves 15, 16 and 17 of an unoperated salamander (N-1). In this example, and all subsequent ones, only the dorsal surface of hind limb will be presented, anterior (head on) is always to the right, and caudal (tail end) to the left of each drawing. The shaded area indicates in this salamander and all subsequent ones, the area of skin innervated by a given nerve. Note that nerve 15 innervates the most anterior aspect of hind limb skin and two toes, nerve 16 almost the whole hind limb skin, and nerve 17 the most caudal aspect of hind limb skin, and the toes from the second to the fif. The bilateral symmetry of the pattern of hind limb innervation is apparent from this example. The bar below the drawings indicate 10 mm.

ponded to an area of 572 mm^2, while the left nerve 16 differed from the right one in that it did not innervate the most caudal third of the thigh skin; its area of innervation was 544 mm^2. Both nerves 17 innervated approximately the caudal half of hind limb skin and the toes from the second to the fifth; the areas innervated by the right and left nerve 17 were 436 and 428 mm^2 respectively.

It is apparent that the areas innervated by any given pair of corresponding nerves (right and left) in this salamander were very similar, indicating a high degree of bilateral symmetry of their touch-receptive fields (see below).

Salamander N3:- The results from this experiment are shown on Figure 3-5. Both nerves 15 innervated the most anterior quarter of the hind limb skin up to the level of the ankle, and it should be noted that they did not innervate any toes at all; the total areas innervated by the right and left nerve 15 were 80 and 84 mm^2, respectively. Both nerves 16 innervated almost the whole skin of hind limb, except for the most caudal third of thigh skin; their areas of innervation being 552 mm^2 in both cases. The right and left nerve 17 innervated the most caudal third of hind limb skin plus the toes from the second to the fifth, and the areas of innervation were 340 mm^2 on both sides.

(b) Analysis of the bilateral symmetry of touch receptive fields: The main feature of the pattern of innervation in the examples presented above was the high degree of bilateral symmetry of the touch-receptive fields. The area innervated by a given pair of nerves did vary, however, from one animal to another, specially in the case of nerve 15. In this respect the
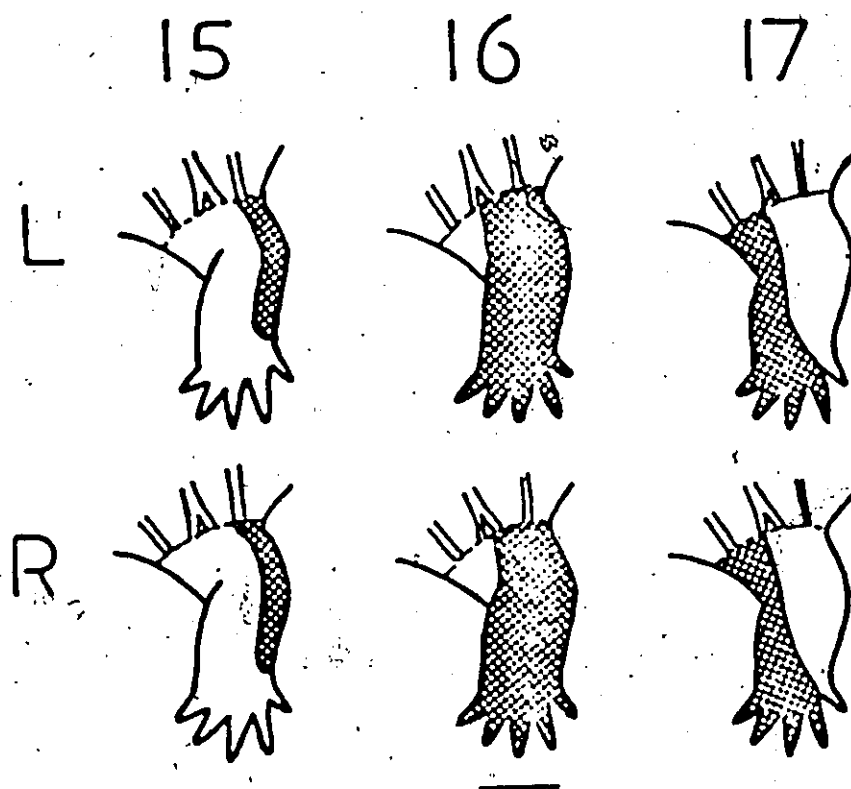
FIGURE 3-5

Touch-receptive fields of an unoperated salamander (N-3). Note that nerve 15 innervates the most anterior zone of hind limb skin, but no toes; nerve 16 innervates almost the whole hind limb skin except for a small corner on the caudal aspect of thigh; nerve 17 innervates the most caudal region of hind limb skin and the toes from the second to the fifth. Note the bilateral symmetry of the skin fields. The bar below the drawings indicate 10 mm.

two examples illustrated were the most extreme ones insofar as differences between individuals were concerned. A statistical analysis was performed on the results obtained from a group of such animals, in which the areas innervated by corresponding nerves on right and left sides were compared.

Statistical Methods: For each pair of nerves in a group of 12 healthy unoperated salamanders, the average ($\bar{x}$), standard deviation (SD), and standard error of the mean (SEM) were calculated for the difference between the areas of skin innervated by the right nerve minus the left. A "t" test for correlated measurements was then performed in order to find out whether or not the difference was statistically significant. A non-significant difference between the areas innervated by corresponding nerves (15, 16 and 17) on right and left sides, indicates bilateral symmetry in their fields of innervation.

Usually the number of experimental animals was about 12-20 in each month, and I was always aware of whether the animal was unoperated, or colchicine-treated and so on. It can be argued, therefore, that a subjective and unconscious bias might have existed when performing the mapping experiments. This may not be as serious as it may appear at first sight, since for instance, a common feature of sprouting (see below) was the take-over of extra toes, each of which represented a kind of "all or nothing" response (the presence or absence of bursts of spikes when the toe was touched). This response, although noted "subjectively" (since it was evoked by a manually applied stimulus) constituted in effect an "objective" observation as viewed on the CRO screen.

It was found that the average of the difference between both sides for nerve 15 was 3.00 ± 2.72 mm^2 (average $\pm$ SEM) and this difference was

not statistically significant (level of significance P > 0.05). For nerves 16 and 17 the figures were -3.32 ± 6.28 mm^2 and -0.32 ± 0.80 mm^2 respectively (average $\pm$ SEM). For both nerves 16 and 17, as with nerve, 15, the differences were not statistically significant (level of significance P > 0.05). These results, therefore, show that the touch-receptive. fields of all 3 nerves were bilaterally symmetrical. This is revealed in greater detail in the following sections.

(c) Nystatin-treated salamanders: It has been mentioned (Part II-B-1 Animal Husbandry) that some fungal diseases presented by salamanders were treated with Nystatin ointment (100,000 USP per gram). In order to test whether or not such a treatment may have an effect on the bilateral symetry of peripheral fields in hind limb (see above), 5 healthy unoperated salamanders were rubbed daily with Nystatin ointment on the right hind limb for a week, and then the mapping of touch-receptive fields in hind limb was performed. The data concerning the latter experiments is assembled on Table 3-1. This shows that the topical application of Nystatin. ointment did not induce any significant change in the bilateral symmetry of touch-receptive fields in hind limb; therefore this therapeutic agent was assumed not to interfere with other experimental procedures such as section of nerve 16 or treating nerve 16 with colchicine. The data from the group of Nystatin-treated healthy salamanders (n = 5) was therefore pooled with that from the previous group of 12 unoperated salamanders and is assembled on Table 3-1. Not only was bilateral symmetry unaffected, but the sizes of the fields (right and left) were within the range of untreated animals, indicating that the Nystatin treatment did not cause generalized (and symmetrical) changes in skin fields. From now on, the group of unoperated

TABLE 3-1

DIFFERENCE IN SIZE OF TOUCH-RECEPTIVE FIELDS IN SALAMANDER HIND LIMB

Group	Nerve 15	Nerve 16	Nerve 17
Unoperated (n = 12)	3.00 +2.72	-3.32 +6.28	-0.32 +0.80
Nystatin-treated (n = 5)	2.40 +1.80	-1.60 +7.00	0.80 +1.68
Pooled data (n = 17)	3.04 +2.44	2.84 +1.92	-2.84 +4.68

Each value represents the average (± SEM) of the difference between the right and left side touch-receptive field for each pair of nerves, in mm^2 of innervated skin. All salamanders included in this group were in good health. The differences were not statistically significant (level of significance $p > 0.05$).

TABLE 3-2

AVERAGE SIZE OF TOUCH-RECEPTIVE FIELDS OF HIND LIMB NERVES IN A.TRIGINUM

Side	Nerve 15	Nerve 16	Nerve 17
Right	176.2 ± 11.5	485.2 ± 17.8	351.3 ± 14.7
Left	174.4 ± 11.2	483.8 ± 16.8	354.0 ± 16.1

The values represent the average $\pm$ SEM of the skin innervated by each nerve trunk in mm^2. The group includes 17 healthy unoperated salamanders. The difference between each pair of values was not stastically significant (level of significance $P > 0.05$).

salamanders will comprise the pooled data from the 17 unoperated salamanders above.

(d) On the size of touch-receptive fields: The frequency distribution of the areas of skin innervated by nerves 15, 16 and 17 (right and left sides) is presented in Figures 3-6, 3-7 and 3-8, respectively. These show also that for any given pair of nerves the distribution of skin areas innervated is very similar for right and left sides, another finding which shows in more detail the bilateral symmetry of their peripheral fields of innervation.

In the case of nerve 15 the highest frequency is observed in the values of innervated skin areas ranging from 151 to 250 mm^2. In the case of nerve 16 almost all areas of cutaneous innervation are included in the values ranging from 401 to 600 mm^2 of innervated skin, and for nerve 17, the respective values are from 301 to 450 mm^2.

The average absolute values of the skin areas innervated by the nerves which supply the hind limb in salamanders were also calculated and are presented in Table 3-2 and Figure 3-9.

Conclusions: It is apparent from the above data that nerve 16 innervated the largest area of hind limb skin and nerve 15 the smallest one, while nerve 17 had an area with a value intermediate between the two. The right and left touch-receptive fields of innervation of given nerves were almost identical. Furthermore, the frequency distribution of those areas were very similar on both sides. In the subsequent experiments, therefore, differences between right and left fields could confidently be attributed to the experimental manoeuvers.
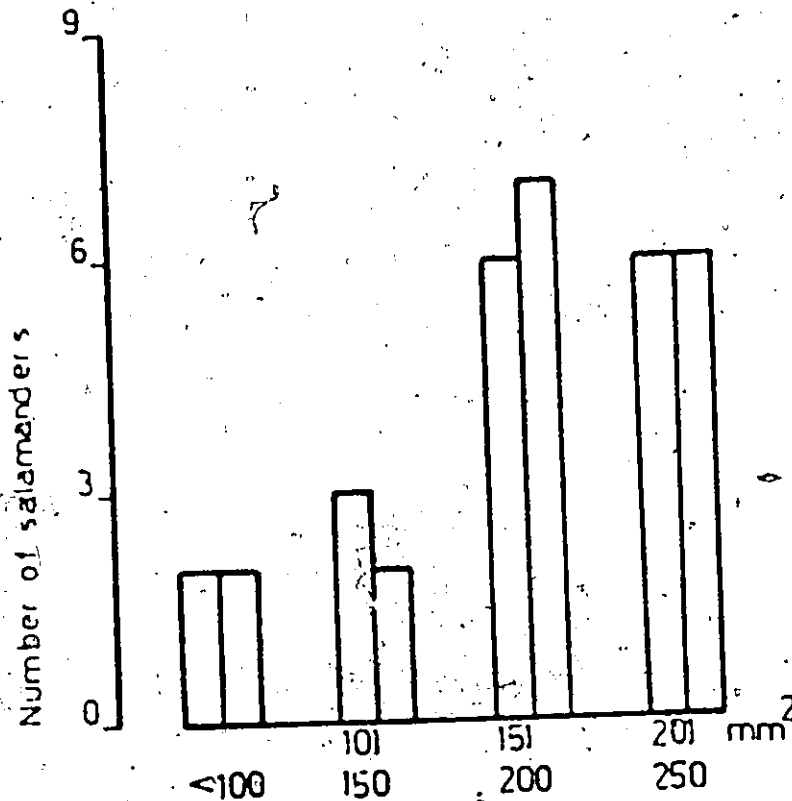
FIGURE 3-6

Frequency distribution of the size of touch-receptive fields for nerve 15 in unoperated salamanders (n = 17). The horizontal axis represents the area of skin innervated by nerve 15 in mm^2, in "bins" of 50 mm^2, and the vertical axis the number of salamander showing such value. The first column of each pair represents the right side, and the second the left side. It is apparent that the highest frequency occurred in the "bins" 151-200, and 201-250 mm^2, and that the frequency distribution for both sides was almost identical.
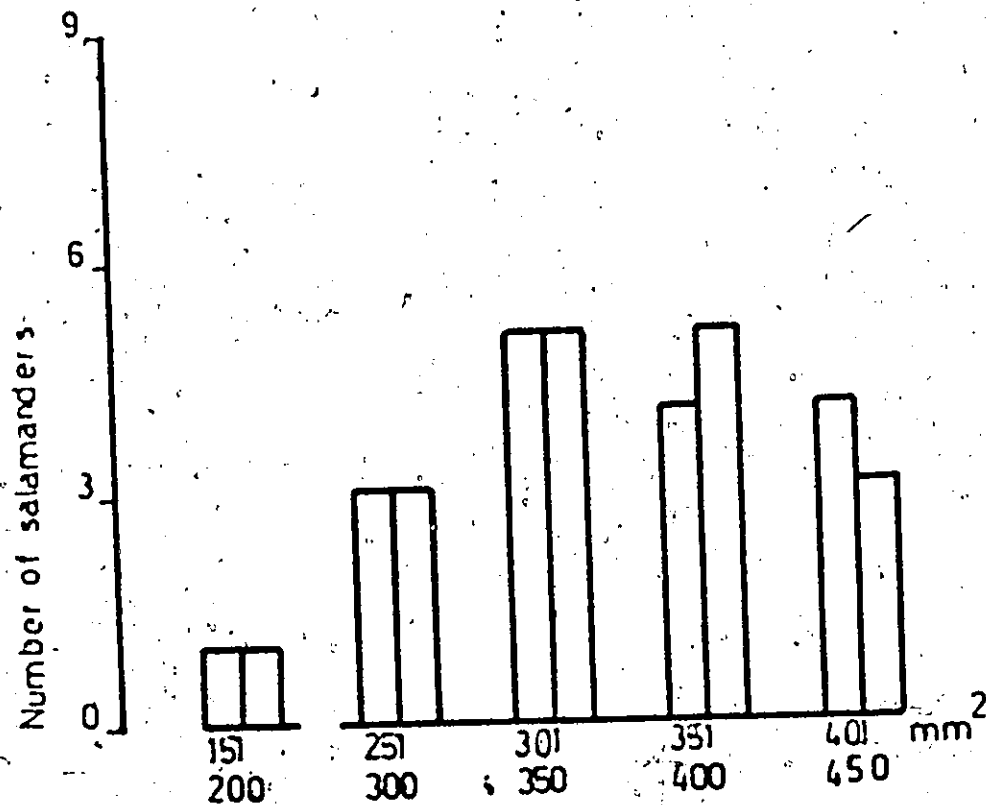
FIGURE 3-7

Frequency distribution of the size of touch-receptive fields for nerve 16 in unoperated salamanders (n = 17). The horizontal axis represents the area of skin innervated by nerve 16 in mm^2, in "bins" of 50 mm^2, and the vertical axis the number of salamanders showing such value. The first column of each pair represents the right side, and the second one, the left side. It is apparent that the highest frequency occurred in the "bins" 301-350, and 351-400 mm^2, and that frequency distribution for both sides was almost identical.
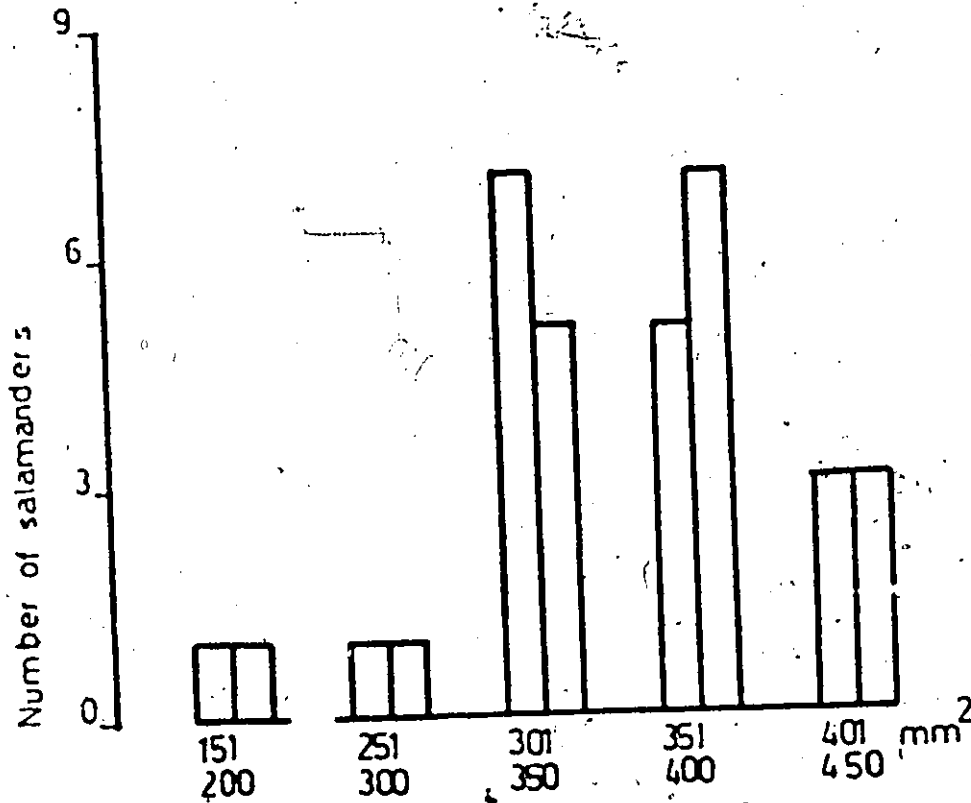
FIGURE 3-8

Frequency distribution of the size of touch-receptive fields
for nerve 17 in unoperated salamanders (n = 17). The horizontal axis
represents the area of skin innervated by nerve 17 in mm^2, in "bins" of
50 mm^2, and the vertical axis the number of salamanders showing such
value. The first column of each pair represents the right side, and
the second one, the left side. It is apparent that the highest fre-
quency occurred in the "bins" 301-350, and 351-400 mm^2, and that the
frequency distribution for both sides was almost identical.
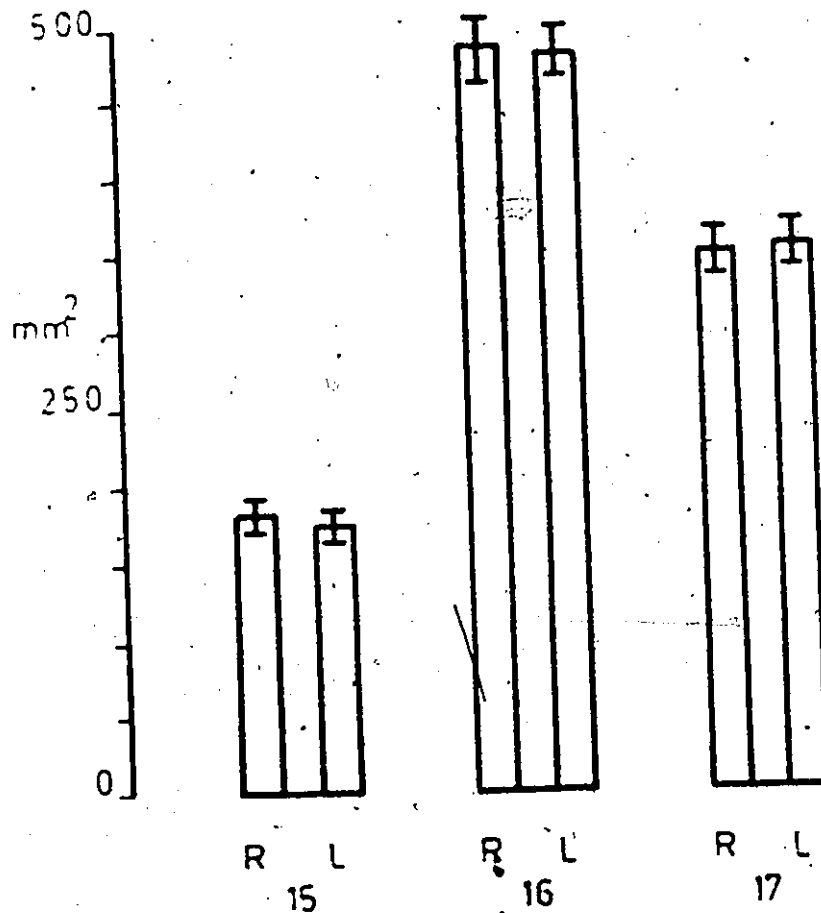
FIGURE 3-9

Size of the touch-receptive fields of right (R) and left (L) spinal segmental nerves 15, 16 and 17, in unoperated salamanders (n = 17). The height of the bars represents the average (± SEM) of the area of skin, in mm^2, innervated by each nerve. The difference between each pair of average values was not statistically significant (level of significance P > 0.05). It is apparent from this histogram that the smallest touch-receptive field was that of nerve 15, and the largest one that of nerve 16.

3. Touch-receptive fields in salamanders with cut hind limb nerves

One individual example will be presented initially, followed by a statistical analysis of the whole group.

(a) Individual example (Salamander 16-115): Nerve 16 was cut and a tie was applied to the proximal stump in order to prevent regeneration of the nerve into the distal portion, as described in Methods. This procedure caused severe limping which, had the animal been allowed to survive, would have gradually recovered such that by the third to the fifth post-operative week normal walking would have been almost totally restored (see Behavioural Observations). The mapping was performed 16 days after the nerve section. The results are presented in Figure 3-10.

Nerve 15 on the control (left) side innervated a narrow strip along the anterior border of the hind limb and it did not innervate any toes. The areas of skin innervated by the right and left nerves 15 were respectively 212 and 80 mm^2, i.e. the area innervated by the right nerve 15 was more than two and a half times larger than that of the left one.

Right nerve 16 did not innervate any skin, but left nerve 16 innervated the skin of the whole left hind limb with the exception of the fifth toe, i.e. an area of 564 mm^2. Right nerve 17 innervated the caudal half of the hind limb skin plus the five toes, while left nerve 17 innervated approximately the caudal third of hind limb skin and the toes from the third to the fifth. Their respective areas of innervated skin were 416 and 308 mm^2.

It is apparent from the above data that both nerves 15 and 17 seemed to have increased their areas of innervation in the right hind limb.
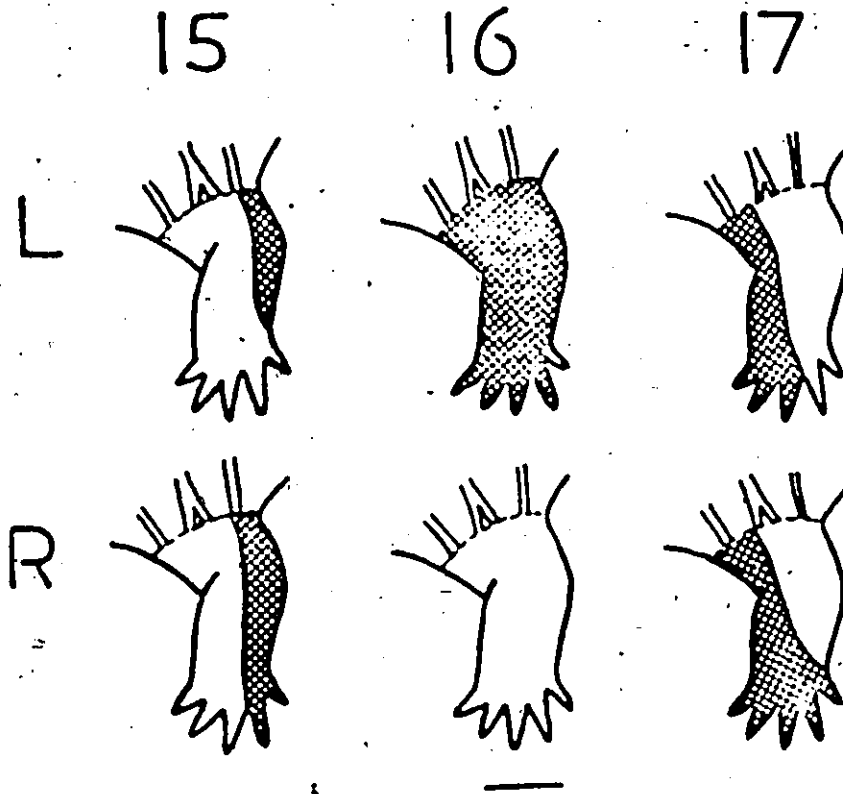
FIGURE 3-10

Touch-receptive fields of hind limb of a salamander (16-115) in which right nerve 16 was sectioned 14 days previously. Note that right nerve 15 innervated two toes whilst left nerve 15 innervated none. The touch-receptive field of right nerve 16 was completely eliminated by sectioning it. Right nerve 17 innervated a larger area of skin on the calf and two more toes than its left counterpart. It is apparent from this experiment that nerves 15 and 17 had increased the size of their touch-receptive fields after partial denervation of hind limb. The bar below the drawings represents 10 mm.

(b) <u>Analysis of the difference between right and left touch-receptive fields after section of right nerve 16:</u> The statistical procedure applied to the present group was identical to that described previously for the group of unoperated salamanders. The present group included 30 salamanders which were mapped individually at various periods after section of nerve 16, namely on the 7th to 28th post-operative day. In all of these sala-manders, the right nerve 16 had been successfully eliminated surgically, and did not innervate any skin of the hind limb. On the other hand, the left nerve 16 had a mean touch-receptive field of 495 ± 45.0 mm^2 (average ± SEM) which was not statistically different (level of significance P > 0.05) from the touch-receptive field of the left 16 nerve of the unoper-ated salamander group, which had corresponding values of 483.8 ± 17.1 mm^2.

The difference in the size of touch-receptive fields of the right and left 15th nerves was 48.4 ± 7.8 mm^2 (average ± SEM), the right being larger than the left. This difference was statistically significant (P < 0.01). Since normally (see Part III-B-2(b)) the touch receptive fields were bilaterally symmetrical, the findings showed that the right nerve 16 had increased the size of its touch-receptive field after total elimination of the right nerve 16.

The difference in the area of skin innervated by the 17 nerve, right minus left, was 35.2 ± 6.3 mm^2 (average ± SEM). This difference was statistically significant (P < 0.01), and as with the 15th nerve, the right nerve 17 had increased its size of touch-receptive field.

<u>Conclusion:</u> These results showed that total elimination of the right nerve 16 leads to a significant increase in the size of touch-recep-tive fields of the adjacent nerves 15 and 17. The results are summarized
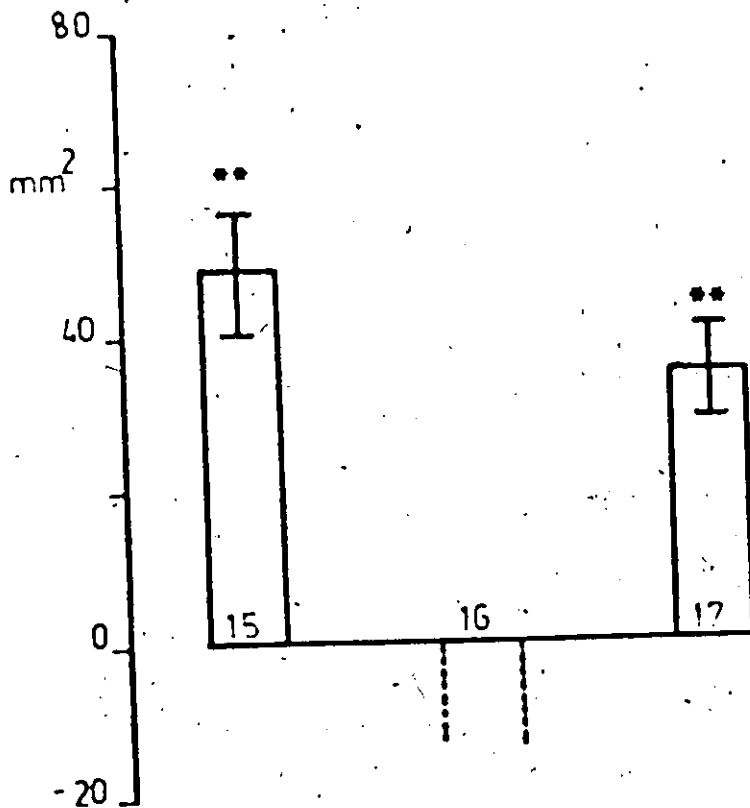
FIGURE 3-11

Changes in the touch-receptive fields of hind limb nerves produced by section of right nerve 16. Each column represents the average ($\pm$ SEM) of the difference in area of skin (mm^2) innervated by the right side minus the left side for each pair of nerves, indicated by the respective numbers 15, 16 and 17. Significance of the difference is indicated by ** $P < 0.01$. Positive values indicate that the right side had increased the size of its touch receptive field. The broken lines indicate that the touch-receptive field of right nerve 16 had disappeared completely and only that of left nerve 16 remained. The results are from 30 salamanders, the post-operative periods range from 7 to 28 days.

in Figure 3-11.

4. Touch-receptive fields in salamanders with colchicine-treated nerves

One individual experiment will be presented, followed by the statistical analysis of the whole group.

(a) Individual example: The right nerve 16 was exposed and colchicine solutions (0.025M to 0.1M) were applied to it for 30 minutes as described in Methods. The electrophysiological mappings were performed at various times up to the 30th post-operative day.

Salamander C-57:- Right nerve 16 was treated with 0.1M colchicine for 30 minutes. The field mapping was performed on the 14th post-operative day (Figure 3-12).

Right nerve 15 innervated a strip about one third the whole width of hind limb along the anterior border of the latter and the first three toes, while the left nerve 15 innervated a narrower zone and only two toes; the areas of innervated skin were respectively 316 and 176 mm^2, i.e. the touch-receptive field of right nerve 15 was more than one and a half times that of the left side.

Both right and left nerve 16 innervated the whole skin of hind limb. The innervated area being in both cases 572 mm^2.

Right and left nerve 17 innervated approximately the caudal half of hind limb skin; however, the right nerve 17 innervated 5 toes while the left one innervated only 4; the respective areas were 384 and 352 mm^2 (right and left). It was unusual for the variations of fields between right and left sides in normal animals to extend to the innervation of an extra toe on one side.
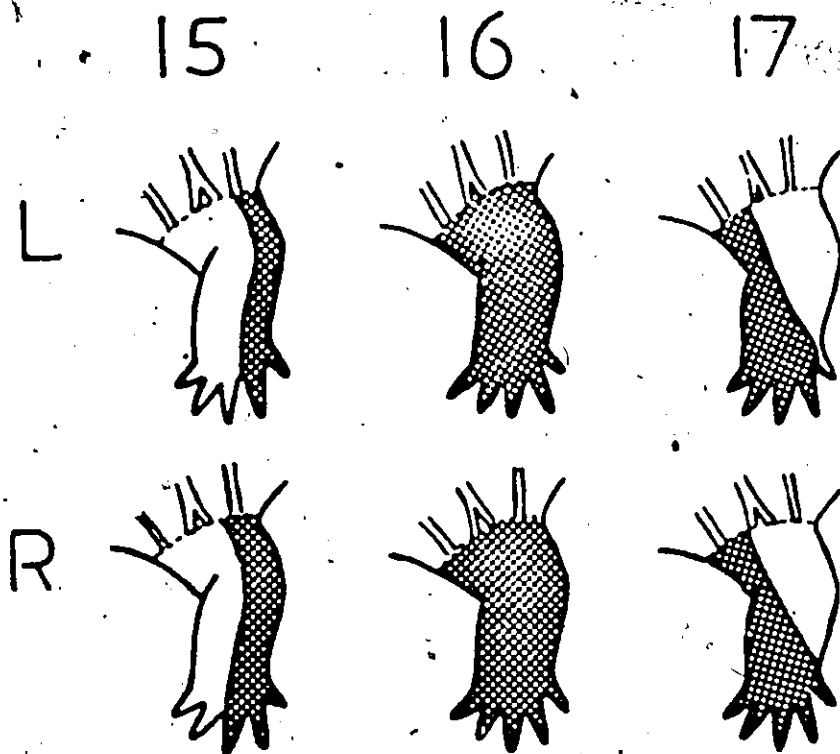
FIGURE 3-12

Touch-receptive fields of hind limb of a salamander (C-57) whose right nerve 16 was treated with 0.1M colchicine for 30 minutes, as described in Methods, 14 days before the mapping experiment. Note that right nerve 15 innervated a wider strip of skin along the anterior border of hind limb than the left nerve 15 and also one toe more; nerve 16 innervated the whole skin of hind limb on both sides, and right nerve 17 innervated one more toe than the left nerve 17. It is apparent from this example that treating right nerve 16 with 0.1M colchicine (see Methods) had resulted in an increase of the touch-receptive fields of the adjacent nerves (15 and 17); note that the area of skin innervated by nerve 16 was the same in the treated and untreated sides. The bar below the drawings represents 10 mm.

These results showed that nerves 15 and 17 had increased the size of their touch-receptive fields, while nerve 16 experienced no change, insofar as area of innervated skin was concerned after acute application of colchicine.

(b) Statistical analysis of the difference between right and left touch-receptive fields after treating right nerve 16 with colchicine: This set of experiments includes several groups of salamanders which were treated with different concentrations of colchicine solutions for 30 minutes, as described in Methods, and individual animals were mapped at times ranging from the 7th to the 28th post-operative day.

The data concerning the touch-receptive fields of nerves 15,16 and 17 are assembled in Table 3-3, and shown in Figures 3-13, 3-14 and 3-15.

It is apparent from the data presented in Table 3-3 that colchicine applied to right nerve 16 in the doses ranging from 0.1M to 0.05M provoked an increase of touch-receptive field of right nerve 15, and with 0.1M colchicine a significant increase of the touch-receptive field of right nerve 17 was also observed. That increase was dose-dependent (see section below). After treating right nerve 16 with 0.03M colchicine or with amphibian Ringer, no significant change in the bilateral symmetry of the touch-receptive fields of either nerve was observed.

The data concerning the touch-receptive fields of nerve 16 itself after treating the right nerve 16 with several doses of colchicine for 30 minutes, are assembled in Table 3-3. It is apparent that colchicine doses up to 0.1M did not cause any changes in the bilateral symmetry of the touch-receptive fields of nerves 16, and in this respect did not differ from salamanders which were treated with amphibian Ringer alone or from
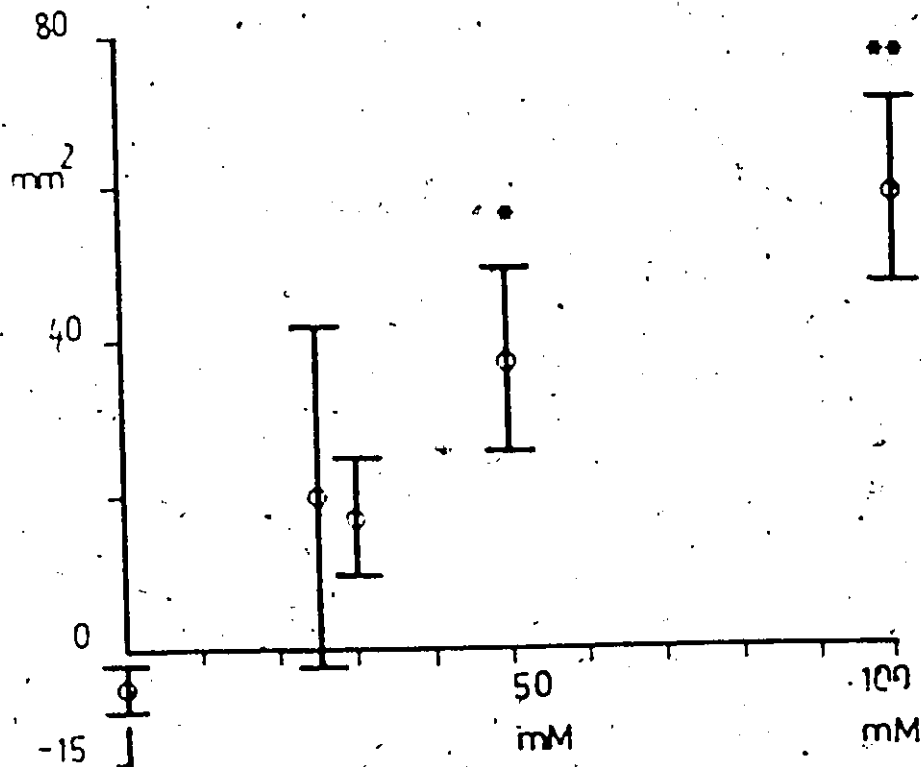
FIGURE 3-13

Changes in the touch-receptive fields of nerve 15 after treating right nerve 16 with colchicine solutions (0.025M to 0.1M) for 30 minutes, as described in Methods. Ordinate: increase in area of touch-receptive field of nerve 15 (right side minus left) in mm^2. Abscissa: concentration of colchicine to which right nerve 16 was exposed 6 to 28 days previously. Each point represents the average ($\pm$ SEM) of results from the following groups of salamanders: amphibian Ringer only or sham-treated salamanders (n = 6), 0.025M colchicine (N = 3), 0.03M colchicine (n = 11), 0.05M colchicine (n = 7), and 0.1M colchicine (n = 21). Significance of difference is indicated by * P <0.05, and ** P <0.01.
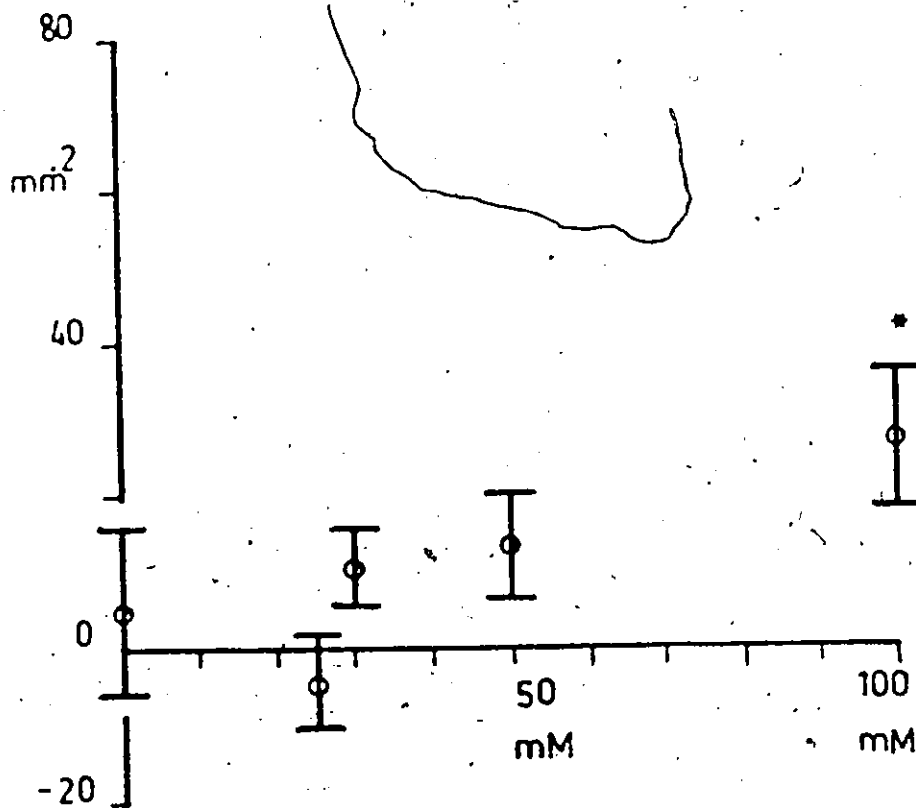
FIGURE 3-14

Changes in the touch-receptive fields of nerve 17 after treating right nerve 16 with colchicine solutions (0.025M to 0.1M) for 30 minutes as described in Methods. Ordinate: increase in area of touch receptive field of nerve 17 (right side minus left) in mm^2. Abscissa: concentration of colchicine to which right nerve 16 was exposed 7 to 28 days previously. Each point represents the average ($\pm$ SEM) of results from the following groups of salamanders: amphibian Ringer only or sham-treated salamanders (N = 6), 0.025M colchicine (N = 3), 0.03M colchicine (n = 11), 0.05M colchicine (n = 7), and 0.1M colchicine (n = 21). Significance of difference is indicated by * $P < 0.05$.
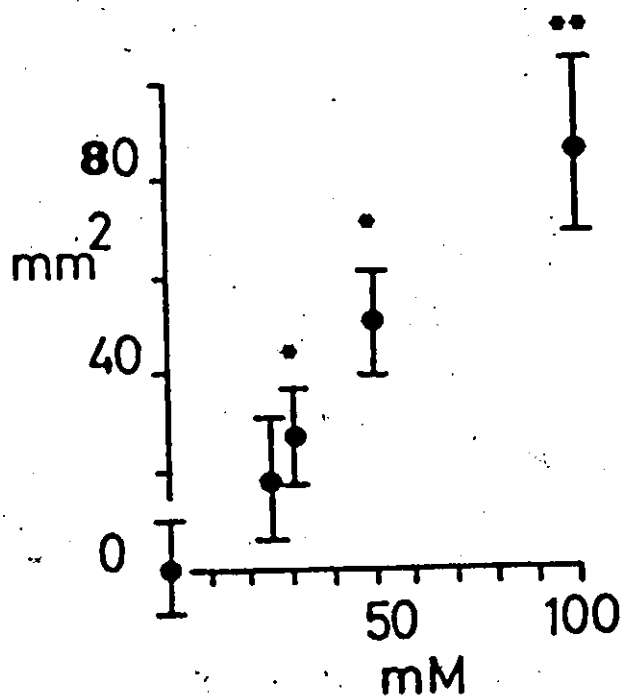
FIGURE 3-15

Relationship between dose of colchicine and resultant increase in touch-receptive fields of nerves 15 and 17. Ordinate: total increase in area of touch-receptive fields of nerves 15 and 17 (the difference between the field sizes on the right and left sides). Abscissa: concentrations of colchicine solution to which right nerve 16 was exposed 6-28 days previously, for 30 minutes. Each point represents the average (± SEM) of results from the following number of experiments: sham-treated salamanders (n = 6), 0.025M colchicine (n = 3), 0.03M colchicine (n = 11), 0.05M colchicine (n = 7), and 0.1M colchicine (n = 21). Significance of difference between right and left sides is indicated by * P <0.05, ** P <0.01.

TABLE 3-3

TOUCH-RECEPTIVE FIELDS OF NERVES 15, 16 AND 17 AFTER TREATING RIGHT NERVE 16 WITH COLCHICINE

Dose	Number experiment	Post-op p.	Nerve 15	Nerve 16	Nerve 17	Nerves (15 + 17)
0.1M	21	7-28	58.8 ± 12.4**	-2.5 ± 4.8	26.8 ± 9.6*	85.2 ± 14.3**
0.05M	7	7-28	37.2 ± 16.6*	2.7 ± 4.5	13.2 ± 6.8	50.2 ± 10.7*
0.03M	11	7-28	16.7 ± 7.5	0.36 ± 3.2	10.6 ± 5.0	27.3 ± 10.2*
0.025M	3	7-28	20.0 ± 27.3	0.0 ± 0.0	-4.0 ± 7.5	18.9 ± 12.9
Ringer-treated	6	8-14	-4.7 ± 2.1	-5.3 ± 3.9	6.4 ± 4.6	1.95 ± 9.8

The values are the average (± SEM) of the difference between the areas innervated by right and left nerves of a corresponding pair, in mm^2; the last column corresponds to the total (summed) area innervated by nerves 15 and 17. Significance of difference between right and left sides is shown by: * $P < 0.05$, ** $P < 0.01$.

unoperated salamanders.

The total (summed) increase in area of touch-receptive fields of nerves 15 and 17 (right minus left) was statistically significant for the following doses: 0.1M, 0.05M and 0.03M colchicine, but was not significant (level of significance P > 0.05) for 0.025M colchicine and for amphibian Ringer-treated salamanders.

(c) Time-course of the increase in touch-receptive fields: For ease of comparison, the time-courses of the increase in touch receptive fields after section of nerve 16 and after treating nerve 16 acutely with colchicine will be described together. The data concerning the changes in touch-receptive fields of nerves 15 and 17 (summed) have been plotted in Figure 3-16. It is apparent from this that the earliest detectable increase in touch-receptive fields after both section of right nerve 16 and after treating it acutely with colchicine solutions (0.05M and 0.1M) for 30 minutes occurred at about the fifth post-operative day; the increase seemed to be fully developed by about the eighth post-operative day.

In both types of experiments, nerve section and colchicine treatment, the data show a considerable scatter with regard to the magnitude of the increase in touch-receptive fields of nerves 15 and 17; also even at the longer periods at which the present observations were made, there were a few salamanders which did not show any increase at all.

In cases in which the left (control) nerve 15 innervated hind limb skin up to the level of the knee, while the right (operated side) nerve 15 had increased its innervation to take in the first toe, there were differences in length of the field along the main axis of the hind limb of the order of 10 to 20 mm, depending on the salamander. If the increase

FIGURE 3-16

Time-course of the summed increases in the size of touch-receptive fields of nerves 15 and 17 after section of right nerve 16 (•) and after treating right nerve 16 with 0.1M colchicine (▼) for 30 minutes. The ordinate represents the difference between the areas innervated by right nerves 15 and 17 minus the area innervated by left nerves 15 and 17. The abscissa represents the post-operative period in days; for clarity only the data up to the 15th post-operative day was included, although there were observations performed at longer post-operative periods. Although the data show a great deal of scatter it is apparent that the earliest detectable increases in the size of touch-receptive fields occurred in both cases at about the 5th post-operative day, and seemed to be fully developed over the next 2 or 3 days.
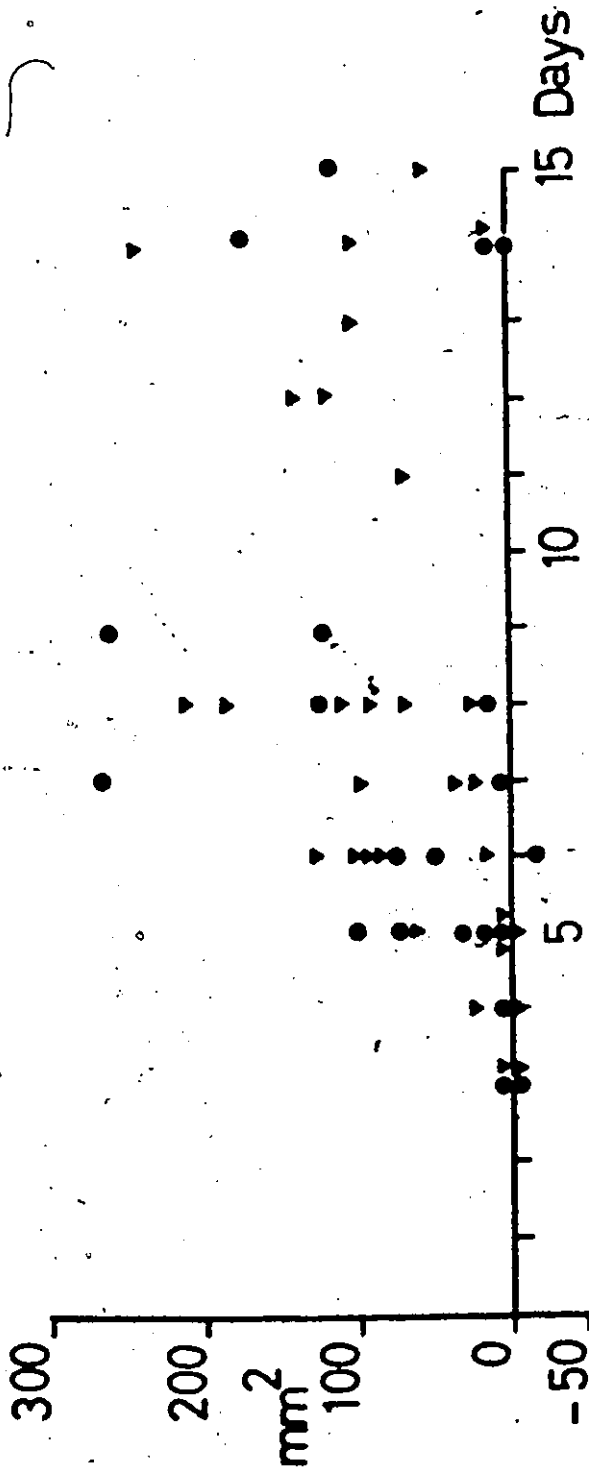
in touch-receptive fields is assumed to represent nerve sprouting, the rate of _linear_ nerve growth would be of the order of 2 to 4 mm/day; this assumes that the nerve fibres located at the margin of the touch-receptive fields were those which had sprouted into the toe.

Conclusion: The acute application of colchicine solutions (0.03M to 0.1M) for 30 minutes to right nerve 16 leads to increases in the touch-receptive fields of nerves 15 and 17. At doses below 0.03M the colchicine treatment was ineffective in this regard.

Part III - DISCUSSION ON TOUCH-RECEPTIVE FIELDS IN HIND LIMB

A most important finding from the experiments performed in un-operated salamanders was the bilateral symmetry of touch-receptive fields of corresponding pairs of nerves innervating the hind limb. This was apparent both from individual examples, and from a statistical analysis of the whole group. Therefore, any asymmetry in the touch-receptive fields detected in the experimental groups (cut or colchicine-treated nerves) could be confidently attributed to the conditions of the experiment.

Part of the present results confirm previous findings of Stirling (1970a) insofar as section of nerve 16 causes a significant increase in the size of the touch-receptive fields of the adjacent nerves (15 and 17); her analysis, however, was rather qualitative (sprouting or no sprouting), since her main concern was to establish a correlation between obvious changes in the peripheral nerve fields and adaptive changes in the spinal cord. The present work has included an investigation of the time-course of the field increases. Both after nerve section or after colchicine treatment the earliest detectable increase in size of touch-receptive fields of nerves 15 and 17 occurred by the fifth post-operative day, and seemed to be complete by the eighth to the tenth post-operative day. Since morphological changes of nerve degeneration occur several days later after colchicine treatment than after nerve section (Singer and Steinberg, 1972), it seems unlikely that the increase in touch-receptive fields observed after colchicine treatment may be due to Wallerian degeneration provoked by such

a treatment (see also Part V).

This increase can be attributed to collateral sprouting, rather than to the onset of function in endings which existed already, but were non-functional. (This has been discussed in Part I) Collateral sprouting is a phenomenon which has been observed in vivo in the tail fin of tadpoles; in this experimental model both the Wallerian degeneration of the distal stump of a sectioned branch, and the eventual re-innervation by sprouting of adjacent nerve fibres could be followed by daily microscopical examinations of the tail fin, (Speidel, 1933, 1941). Similar changes in the skin fields were observed in rabbits by Weddell et al, (1941) who found that after section of the sural nerve, the resultant area of anal-gesia gradually constricted as the fields of neighbouring nerves (tibial and peroneal) enlarged to fill the denervated area. Conversely, the sural nerve, this time left intact, increased its own field of innervation. After section of the sural nerve, histological studies showed only degen-erating nerve fibres in the anesthetic area; however, examination of those areas which showed subsequent functional recovery revealed the presence of new fibres which had indeed penetrated into the region. The difference between the functional and the neurohistological boundaries never differed by more than 1 mm.

In the present studies the results after colchicine treatment were very much the same as after nerve section, in that the adjacent spinal segmental nerves (15 and 17) increased significantly the size of their touch-receptive fields. Since the size of the touch-receptive field of nerve 16 on the colchicine-treated side did not differ significantly from that of the untreated side, or for that matter, from that of normal

untreated salamanders, it seems that hyper-innervation of the hind limb had occurred. Later in this thesis, more evidence will be presented that no degeneration occurred in nerve 16.

The results then, support the hypothesis being examined in the present work, namely that the pharmacological blockade of axoplasmic transport of materials can mimic the effects of nerve section, and that both treatments result in the release of a stimulus to adjacent nerves in the periphery to cause them to sprout.

In subsequent sections, the interference that colchicine treatment may produce on axoplasmic transport of materials will be discussed in greater detail. From the experiments just described it is concluded that insofar as touch-receptive fields are concerned, the effects of an acute treatment by colchicine solution closely mimic those of nerve section with regard to the increase in the size of skin fields of neighbouring nerves.

Part IV   MOTOR FIELDS IN HIND LIMB

This section will describe experiments on the mapping of motor
fields of spinal nerves 15, 16 and 17. The observations were made in the
following groups of salamanders: (1) normal (unoperated), (2) those with
previous section of right nerve 16, and (3) those in which right nerve 16
had been treated with colchicine solutions. For each group individual
experiments will be presented first, followed by a statistical analysis
of results from the whole group.

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Part IV-A METHODS OF MAPPING THE MOTOR FIELDS IN HIND LIMB

1. Operative procedures

The latter were identical to those described in Part III-A. After the touch receptive fields were mapped, the hind limbs were skinned, nerves 15, 16 and 17 were electrically stimulated, and electromyograms (EMG) were recorded (see below).

2. Procedure for mapping the motor fields

A concentric needle electrode was manipulated by hand, the hind limb was observed through a dissecting microscope, and the tip of the electrode was used to explore several points on the surface of each muscle group (see below) in order to assess whether or not electrical stimulation of the nerve trunk provoked an electrical response in it (see below). Such muscle response was monitored by means of a loudspeaker, or looking at the screen of the storage CRO; the exploration of the 7 muscles took about 5 minutes for each nerve trunk. Usually the response was obtained from the whole surface of the muscle group, and its morphology and magnitude did not vary significantly from one point to another in the same muscle group. In some cases the response could be detected over one third or half of the muscle, but not over the rest of it; this was more frequent in muscles like AT2 or AF2 which had two bellies. Changes in the morphology and magnitude of the EMG of different muscle groups were readily noticeable, the latter changes produced distinct sounds in the loudspeaker.

3. Muscle groups sampled in hind limb

Seven clearly distinguished muscle groups were routinely sampled, all easily accessible from the dorsal surface. Six of these muscles were dorsal and one ventral; the nomenclature proposed by Stirling (1970a) was used, and the muscle groups are illustrated in Figure 4-1.

Each individual muscle group was counted as one unit of muscle innervation field; density of innervation of individual muscle groups or of individual muscle fibres could not be measured with the extra-cellular recording techniques used.

4. Stimulating and recording techniques

For electrical stimulation the nerve was mounted on a pair of platinum wire electrodes which were connected to the output of an isolated stimulator (Devices, Mark IV). The EMG from the muscles were recorded with a concentric needle electrode whose tip abutted neatly on the muscle surface. This electrode was connected to a filter device whose frequency limits were set at 150 Hz-10 KHz. The signals were amplified and displayed on a Tektronix 5103 storage oscilloscope (CRO) and relayed through a loudspeaker to monitor those signals. Usually a gain of 50 μV/cm on the CRO screen, and a sweep rate of 5 msec/cm were used. Pulses derived from a crystal controlled pulse generator (Digitimer) were used to trigger the CRO; such a pulse occurred at the beginning of each cycle of stimulation (1Hz) and originated from the first unit of the pulse generator, whose second unit was used to drive the isolated stimulator, usually with a delay of 5 msec with respect to the beginning of the cycle (see Figure 4-2). Rectangular monophasic stimulating pulses (50 usec, up to 3.0

volts, 1 hz) were normally applied to the nerve trunk by the stimulator.
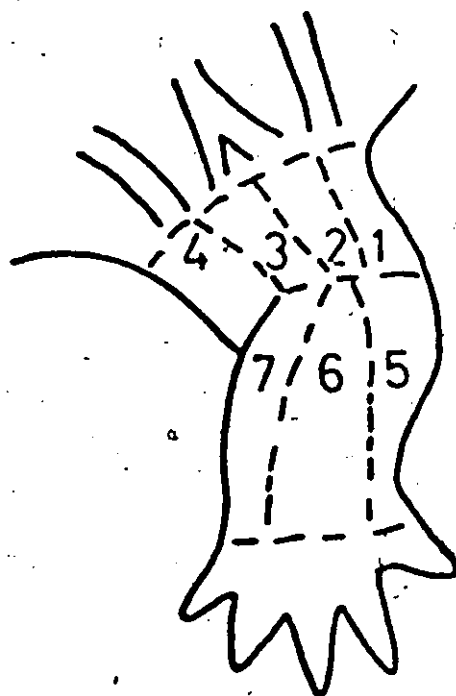
FIGURE 4-1

Muscle groups which were sampled in salamander hind limb. Salamander's head would be on the right-hand side of the drawing. The numbers from 1 to 7 identify the following muscle groups: 1 = AT1, anterior thigh 1 - adducts and elevates the limb; 2 - AT2, anterior thigh 2 - elevates the limb; 3 - AT3, anterior thigh 3 - flexes knee and elevates limb; 4 - VF, ventral flexor - flexes limb at hip, flexes knee and ankle; 5 - AF1, ankle flexor 1 - ankle dorsi-flexion and promotion; 6 - AF2, ankle flexor 2 - ankle dorsi-flexion, toe extensor; 7 - AF3, ankle flexor 3 - ankle dorsi-flexion, toe extensor.
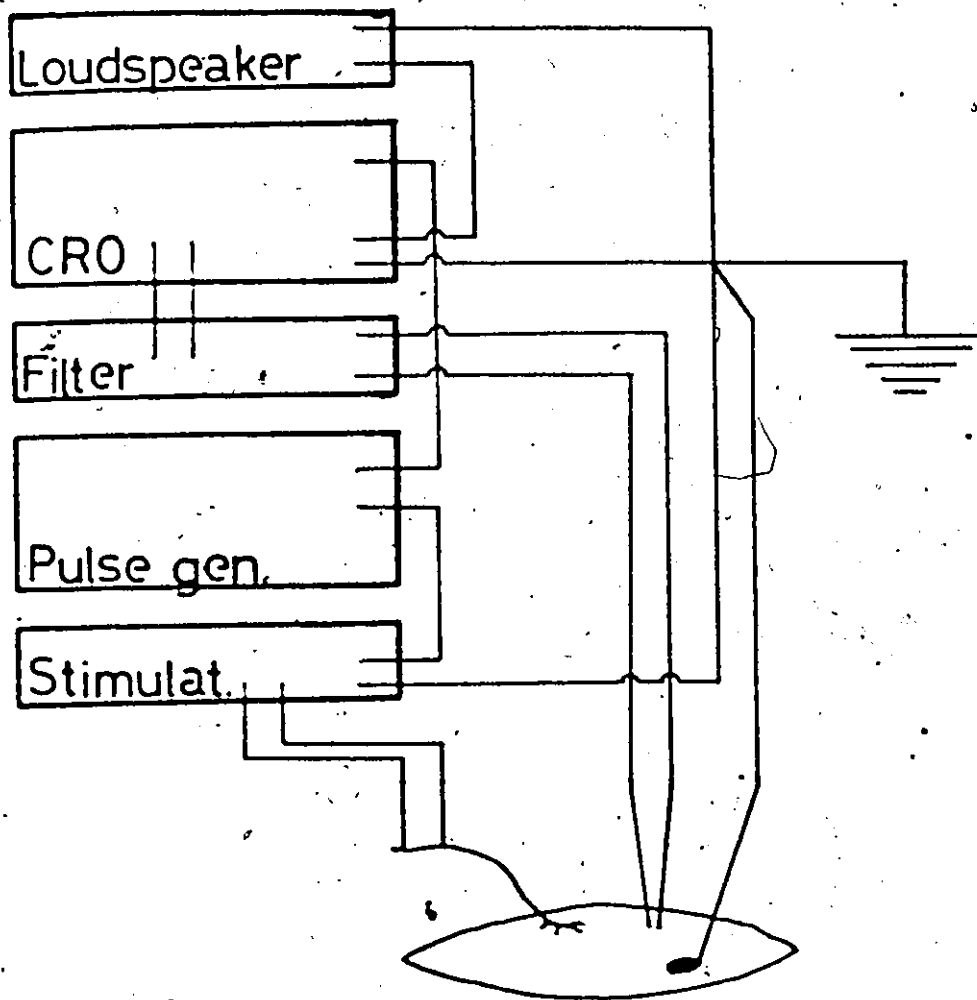
FIGURE 4-2

This diagram represents the connections between the several components of the set-up used for the motor mapping experiments. The crystal controlled pulse generator was used to trigger the oscilloscope (CRO), and the isolated electrical stimulator (Stimulat.). All apparatus were grounded to a common point.

Part IV-B <u>RESULTS</u>

1. <u>Typical example of a motor mapping experiment</u>

       The following example is taken from an unoperated salamander.
Figure 4-3A shows that a sub-threshold electrical stimulus (0.9 volt)
applied to nerve 17 did not evoke any response at all (muscle AT3 trace
is shown in Figure 4-3A), but a slightly stronger pulse (1.05 volt) was
just above threshold and evoked a small response on AT3; the response
was maximal with a pulse of 2.5 volts. The EMG had a latency of 3.3 msec
and an amplitude of 75 μvolts when the stimulus was near threshold, and
of 1.6 msec and 300 μvolts, respectively when the stimulus was supra-
threshold.

       Figure 4-3B illustrates the observations made when nerve 15 was
stimulated with a pulse of 50 μsec and 3.0 volts, delivered at 1 Hz. It
is apparent from the above figure that muscle groups AT1 (trace B-i),
AT2 (trace B-ii), and AF1 (trace B-iii) gave a clear response, which did
not increase with further stimulus strengths, i.e. these were the maximal
responses. From VF (trace B-iv) no response was evoked, nor from the
other muscle groups (AT3, AF2, AF3). Nerve 15 thus innervated only muscle
groups AT1, AT2 and AF1. In the drawings, which will be presented subse-
quently, the muscle groups which responded to the electrical stimulation
of the nerve are represented by a shaded area, and muscles which remained
silent are unshaded.

2. <u>Motor fields in hind limb of unoperated salamanders</u>

    (a) <u>Individual examples</u>: The results of the electrophysiological

<center>133</center>

FIGURE 4-3

A: Electromyograms recorded from muscle group AT3 when nerve 17 was stimulated with electrical pulses of different strengths. Record A(i) shows the trace the electrical stimulus was subthreshold; A(ii) shows the response to a stimulus of 1.05 volt, 50 μsec, delivered at Hz, which was slightly above threshold; A(iii) shows the maximal response of AT3 obtained with a supra-threshold electrical stimulus to the nerve of 2.5 volt and 50 μsec.

B: Electromyograms recorded from several muscle groups when nerve 15 was stimulated with a supra-threshold electrical stimulus, (3.0 volt, 50 μsec, 1 Hz), which evoked a maximal response from all those muscle groups innervated by that nerve. Three muscle groups (AT1, AT2 and AF1) showed an unambiguous electrical response with a delay of about 4 msec after nerve 15 stimulation, but VF did not; i.e. the first 3 muscle groups were taken as being innervated by nerve 15, but not VF. The remaining muscle groups (AT3, AF2 and AF3) were not innervated by nerve 15 in this particular example. Calibration marks apply to all traces, and indicate 50 μvolt (vertical) and 5 msec (horizontal). Arrows show the moment of application of stimulus.

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B

iii)AF1

iv)VF

i)AT1

ii)AT2

period
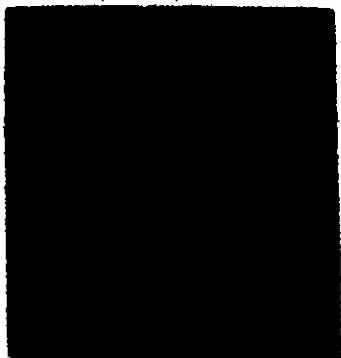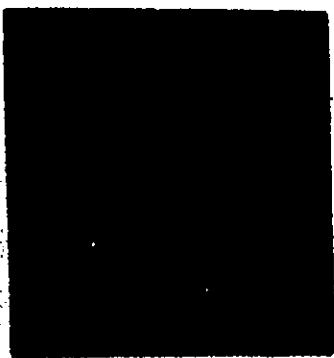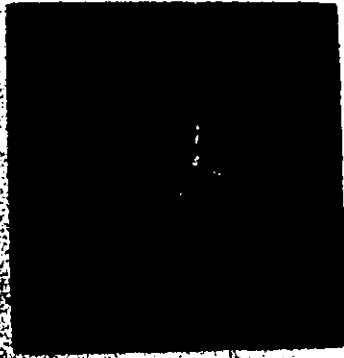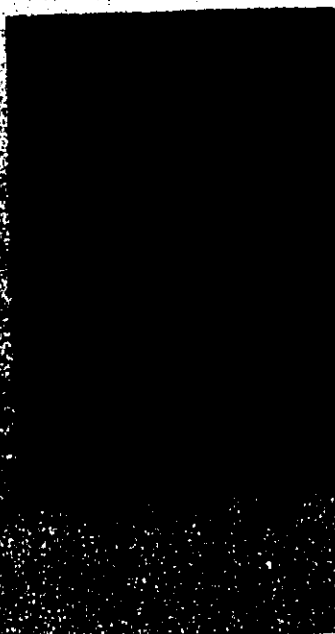
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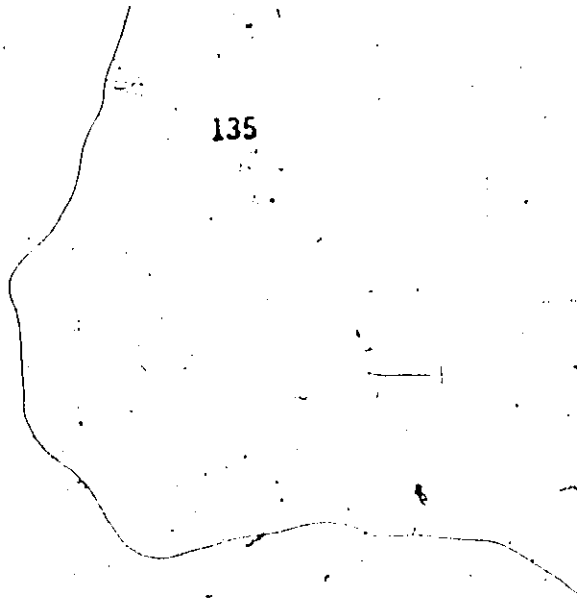
mapping of motor fields from two salamanders will be presented (salamanders N1 and N3).

Salamander N1:- The results from this experiment (Figure 4-4i) showed that the right nerve 15 innervated all 7 muscle groups that were sampled, and left nerve 15 innervated 6 of them. Nerve 16 innervated all 7 muscles on each side, as did nerve 17.

It is apparent from this example that the number of muscle groups that are innervated on each side by a given nerve is almost identical, in other words the results showed a high degree of bilateral symmetry of motor fields of the whole nerves.

Salamander N3:- The results from this experiment are given in Figure 4-4ii, and show that nerve 15 did not innervate any of the 7 muscles sampled. In cases like this it was usual to observe that muscles of the body wall and of the underside of the hind limb twitched when the nerve was stimulated. Nerve 16 innervated all 7 sampled muscles on each side, as did nerve 17.

It may be noted that this salamander (N3) had a touch-receptive field of nerve 15 that finished at the level of the wrist and did not innervate any toe, which suggests that the size of the touch-receptive field may "match" that of its motor field; similar observations were made in S. salamandra by Stirling (1970a). This possibility has not been specially investigated in these experiments, though on an approximate basis it seemed to be supported. The principal feature of these results from normal animals is the high degree of bilateral symmetry of the motor fields of innervation of a given pair of nerves.

FIGURE 4-4

Motor fields of innervation in salamander hind limb. (i) Unoper-
ated salamander (N-1): in this example each muscle group was innervated
by all three spinal segmental nerves (15, 16 and 17) except for muscle
groups AF3 which was not innervated by nerve 15 on the right side. It
is apparent from this example that there is a great amount of overlap in
the motor fields of innervation of the above nerves, and more important,
such motor fields of innervation are basically bilaterally symmetrical.
This was confirmed by the statistical analysis of the whole group (see
text). (ii) Unoperated salamander (N-3): in this example nerve 15 did
not innervate any of the 7 muscles which were sampled; however, muscles
from the underside of hind limb and from the abdominal wall were observed
to twitch when the nerve 15 was stimulated. Nerves 16 and 17 innervated
all 7 muscles on both sides. As with the previous example (N-1), the main
feature of the pattern of motor innervation of hind limb is its bilateral
symmetry. (iii) Salamander (16-115) whose right nerve 16 was cut and tied
16 days before the mapping experiment was performed. Note that the control
or left nerve 15 did not innervate any of the 7 muscle groups which were
sampled, while right nerve 15 innervated AT1; the motor field of right
nerve 16 was completely eliminated, and right nerve 17 appeared to have
taken over one extra muscle group (AT1). These findings suggest that nerves
15 and 17 may have increased the size of their motor fields of innervation
after partial denervation of hind limb. (iv) Salamander (C-57) whose right
nerve 16 was treated with 0.1M colchicine for 30 minutes, 14 days before

the mapping experiment was performed. Note that right nerve 15 inner-
vated 3 muscle groups more than the left nerve 15; such a difference in
the number of muscle groups innervated by a given pair of nerves was
never observed in unoperated salamanders. Nerves 16 and 17 showed a
difference of 1 muscle group when compared with the respective nerves on
the left side; such differences were occasionally observed in normal
unoperated salamanders. The above findings strongly suggest that nerve
15 had certainly increased its motor field of innervation, while the
other changes were within normal range of variations observed in the
pattern of muscle innervation in the hind limb of salamanders. In all
drawings head or anterior end of salamander is at right side of the
picture; hatching indicates muscles innervated by corresponding nerves;
L and R indicate left and right hind limbs respectively; the bar below
the drawings indicates 10 mm.

i) N-1

ii) N-3

iii) G-115
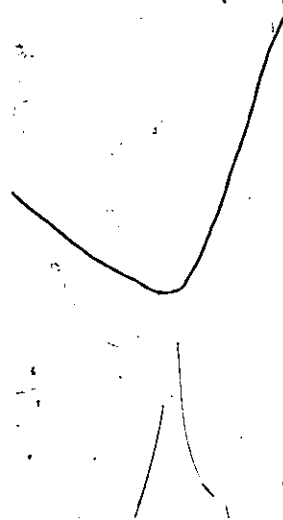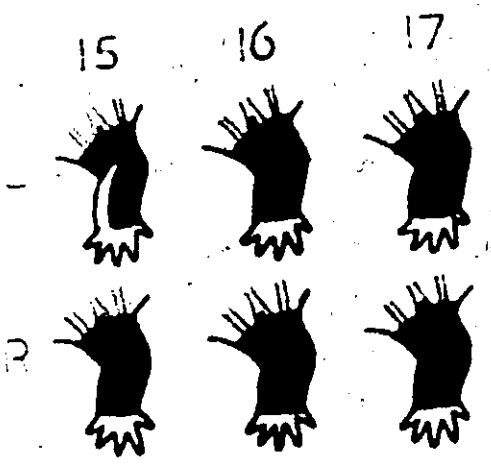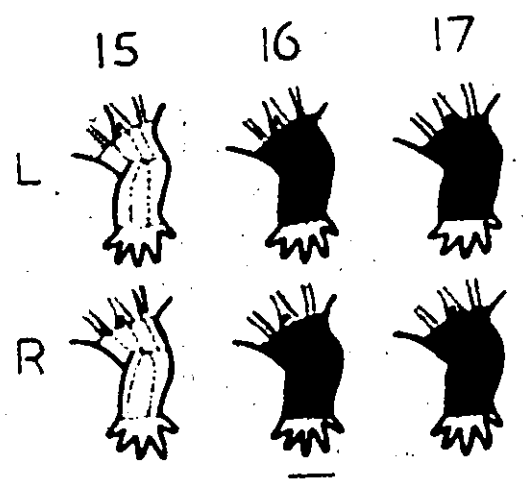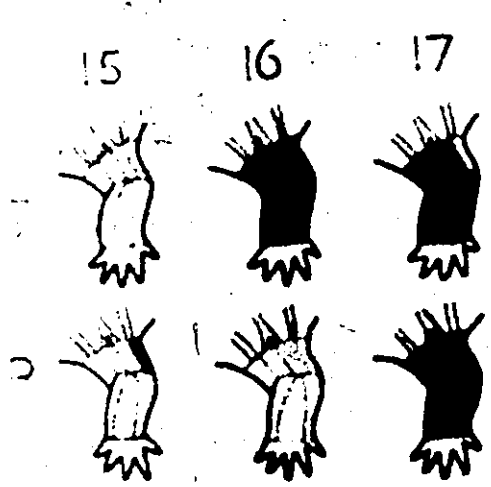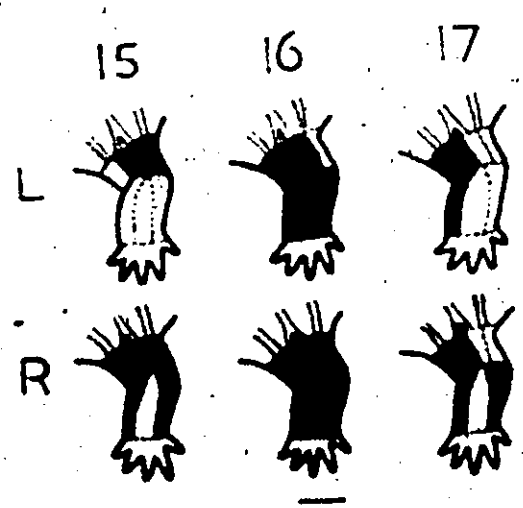
iv) C-57

(b) <u>Analysis of the normal motor fields</u>: The statistical methods used were similar to those described in the previous section on touch-receptive fields. The group analyzed includes the same 12 healthy unoperated salamanders.

The results showed: (1) the difference in the number of muscle groups innervated by right and left nerve 15 was -0.58 ± 0.16 (average ± SEM); this difference was not statistically significant (level of significance P > 0.05) which indicates that the motor fields of nerve 15 were bilaterally symmetrical; (2) in every case nerve 16 innervated all 7 muscles on each side, and therefore the right and left motor fields were identical and obviously bilaterally symmetrical; (3) the corresponding results here were that the difference between the right and left motor fields of nerve 17 was -0.08 ± 0.20 (average ± SEM); this difference was not significant (level of significance P > 0.05), and as with nerves 15 and 16 the motor fields were bilaterally symmetrical.

Conclusion: The conclusion then is that any significant asymmetry of muscle fields found in subsequent groups of experimental animals can be confidently attributed to the conditions of the experiment. In this regard the situation resembles that of the sensory fields.

(c) <u>Experiments performed in healthy unoperated salamanders which were treated with Nystatin</u>: It has been mentioned previously (see section on Animal Husbandry) that some salamanders presented a fungal disease which was treated with Nystatin ointment. In order to test whether or not this therapeutic procedure provoked a change in the peripheral fields of inner-vation of the hind limb, a group of 5 healthy salamanders was treated daily by rubbing the right hind limb with Nystatin ointment during one week, and

subsequently a mapping of the peripheral fields of innervation was performed in a similar way as described previously. The data from this group is shown in Table 4-1. It shows that the treatment did not significantly affect the peripheral fields of innervation, which were bilaterally symmetrical, and within the normal range. In order to increase the size of the control sample, for comparison with results from other experiments, the data from healthy unoperated salamanders and the data from Nystatin-treated, healthy unoperated salamanders were pooled (Table 4-1), and henceforth the results from "unoperated" salamanders refers to this pooled data.

(d) On the size of the motor field of innervation: The experiments presented above have shown that although a given pair of the spinal nerves 15, 16 and 17 innervate almost the same muscle groups on both sides, the number of muscle groups innervated varies from one animal to another. With the aim of measuring this variation, the average (± SEM) of the number of muscle groups innervated by each nerve trunk was calculated. That data is presented in Figure 4-5.. These data show that nerve 16 had the largest motor field of innervation, and approximately its field comprised all 7 muscles, nerve 15 had the smallest motor field of innervation (about 3 muscles on each side). It is important to note that the average of the number of muscle groups innervated by a given pair of nerves was very similar, which indicates a bilateral symmetry in the pattern of hind limb innervation. The differences between such averages were not statistically significant (level of significance $P > 0.05$).

The frequency with which a given spinal nerve trunk innervated each of the 7 muscle groups was also investigated. These date are presented in
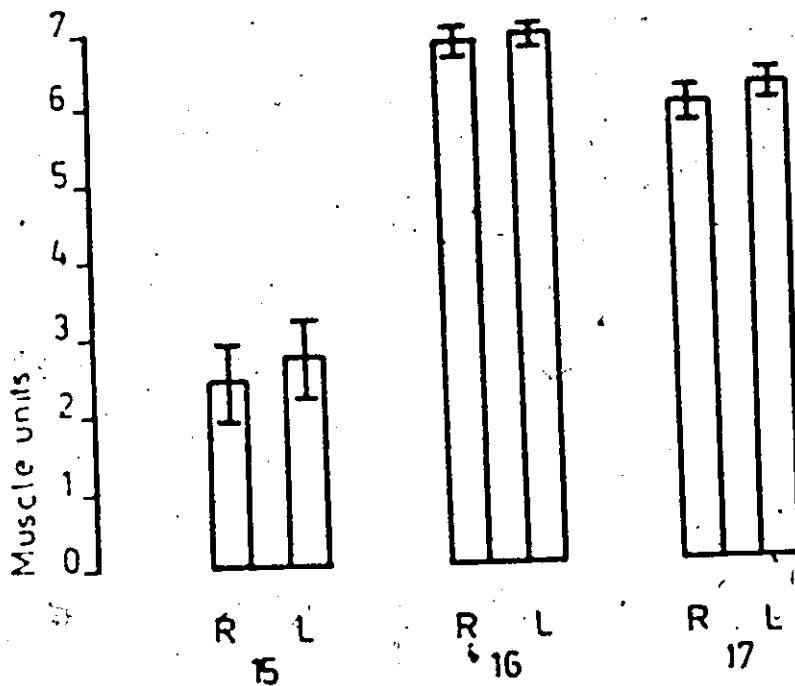
FIGURE 4-5

Number of muscle groups innervated by right (R) and left (L) spinal segmental nerves 15, 16 and 17 in unoperated salamanders (n = 17). The height of the bar represents the average (± SEM) of the number of muscle groups innervated by each nerve. The difference between each pair of average values was not statistically significant (level of significance P > 0.05). It is apparent from this histogram that the smallest motor field was that of nerve 15, and the largest that of nerve 16, and that for a given pair of nerves the motor fields of innervation were bilaterally symmetrical.

TABLE 4-1

DIFFERENCE IN THE NUMBER OF MUSCLE GROUPS INNERVATED BY A GIVEN PAIR OF NERVES (RIGHT MINUS LEFT)

Group	Nerve 15	Nerve 16	Nerve 17
Unoperated (n = 12)	-0.58 +0.16	0.0 +0.0	-0.08 +0.20
Nystatin-treated (n = 5)	0.20 +0.42	-0.20 +0.22	0.00 +0.35
Pooled data (n = 17)	-0.35 +0.18	-0.06 +0.24	-0.06 +0.16

The values given are the average ($\pm$ SEM) of the above difference. The group comprises 12 healthy unoperated salamanders and 5 healthy unoperated salamanders treated daily with Nystatin ointment (100,000 USP/ per gr) for 7 days before the mapping experiments. The differences were not statistically significant (level of significance, $P > 0.05$).

Figure 4-6. These data showed that the muscles more frequently inner- vated by nerve 15 were the more anterior muscle groups (AT1, AT2 and AF1). Nerve 16 innervated approximately the 7 muscles in every case. Nerve 17 innervated more frequently the more caudal muscle groups (AT2, AT3, AF2, AF3 and VF). It is important to note that the frequency distribution for the muscle groups innervated by a given pair of nerves was very similar which indicates a bilateral symmetry in the pattern of innervation of hind limb.

.Conclusion: The difference between the number of muscle groups innervated by a corresponding pair of nerves was not statistically signifi- cant, neither the difference between the average number of muscle groups innervated by right and left corresponding nerves in normal salamanders. Also, the frequency distribution of muscle groups innervated by such nerves was very similar on both sides. These findings are evidence of bilateral symmetry in the motor fields of innervation in unoperated salamanders.

3. Salamanders with 16 nerve cut

(a) Individual example: The data from the same salamander which was presented in Part III-B-3 will be presented here, i.e. salamander 16-115. The mapping was performed in the 16th post-operative day; this salamander showed some behavioural recovery at the time of the experiment. The results of the motor mapping are presented in Figure 4-4iii, and show that after complete elimination of motor fields of right nerve 16, the adjacent nerves (15 and 17) had apparently increased the size of their motor fields by one muscle group.

(b) Analysis of the difference between right and left motor fields

FIGURE 4-6

Frequency with which each of the muscle groups sampled in the hind limb of salamander was innervated by (i) nerve 15, (ii) nerve 16, and (iii) nerve 17, in a group of 17 unoperated salamanders. 100% means that in all 17 salamanders a given muscle group was innervated by one of those nerves. It is apparent from these histograms that nerve 15 innervated the more anterior muscle groups (AT1, AT2 and AF1) in a higher percentage of cases. Nerve 16 innervated all 7 muscles in almost every instance. Nerve 17 innervated always the more caudal muscle groups (AT2, AT2, AF2, AF3, and VF). Note that the frequency distribution for both sides was almost identical. The first column of each pair represents the right side, and the second one the left.
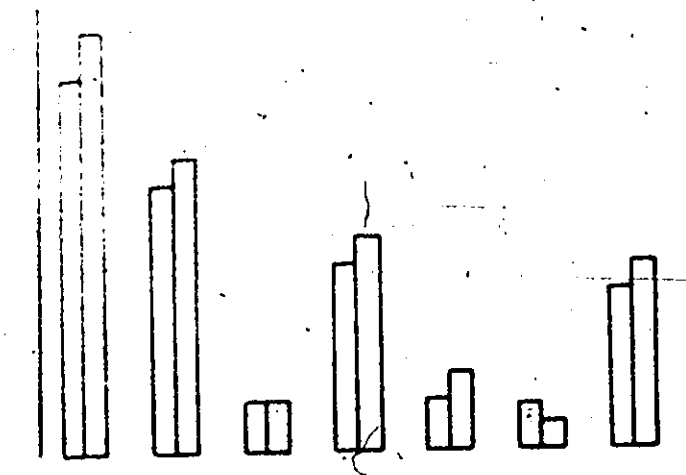
i) Nerve 15
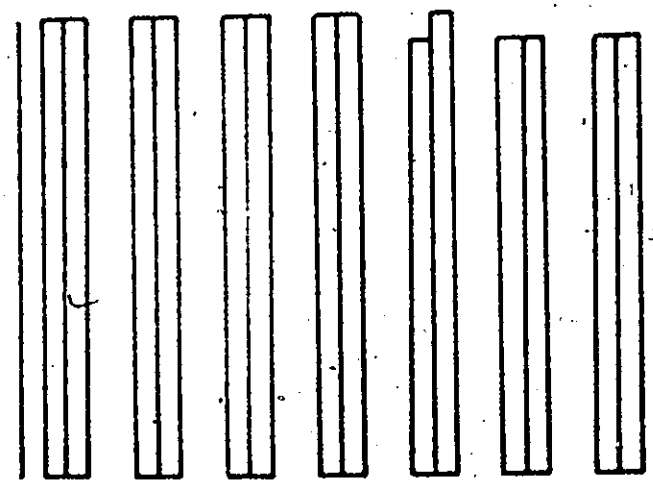
ii) Nerve 16
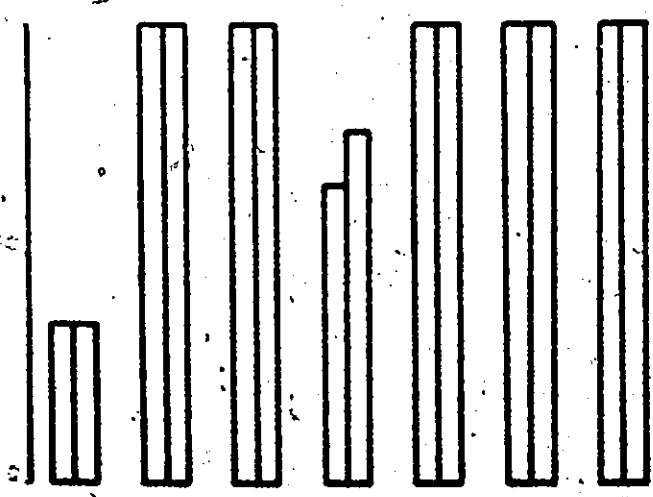
iii) Nerve 17

AT1 AT2 AT3 AF1 AF2 AF3 VF

after section of right nerve 16: The present group included 30 salamanders which were mapped during a period ranging from the 7th to the 28th post-operative day. In all of these salamanders the right nerve 16 did not innervate any muscle group at the time of mapping, while the left one innervated 6.53 ± 0.02 (average $\pm$ SEM) muscle groups; the latter value was not statistically different (level of significance $P > 0.05$), from the average of muscle groups innervated by the left 16 nerve in unoperated salamanders. Thus the surgical procedure had succeeded in totally eliminating the 16th nerve innervation of all muscles sampled in these experiments.

The data concerning changes in the size of motor fields of nerves 15 and 17 after section of nerve 16 is assembled in Table 4-2 and Figure 4-7. It is apparent that both nerve 15 and 17 had significantly increased their motor fields of innervation; the increase is attributed to collateral sprouting for the reason which will be discussed later.

4. Salamanders with colchicine treated nerves

(a) Individual experiments: The same salamanders whose touch-receptive fields were presented in Part III-B-4 will be presented here.

Salamander C-57:- Right nerve 16 was treated with 0.1M colchicine 14 days previously to the mapping experiment. Right nerve 15 innervated 3 muscle groups more than the left one (Figure 4-4iv); such a very large differ-ence was never observed in unoperated salamanders. Right nerve 16 inner-vated one muscle group more than left nerve 16, and right nerve 17 inner-vated also one muscle group more than the left one. These results strongly suggest that right nerve 15 had increased the size of its motor field, but
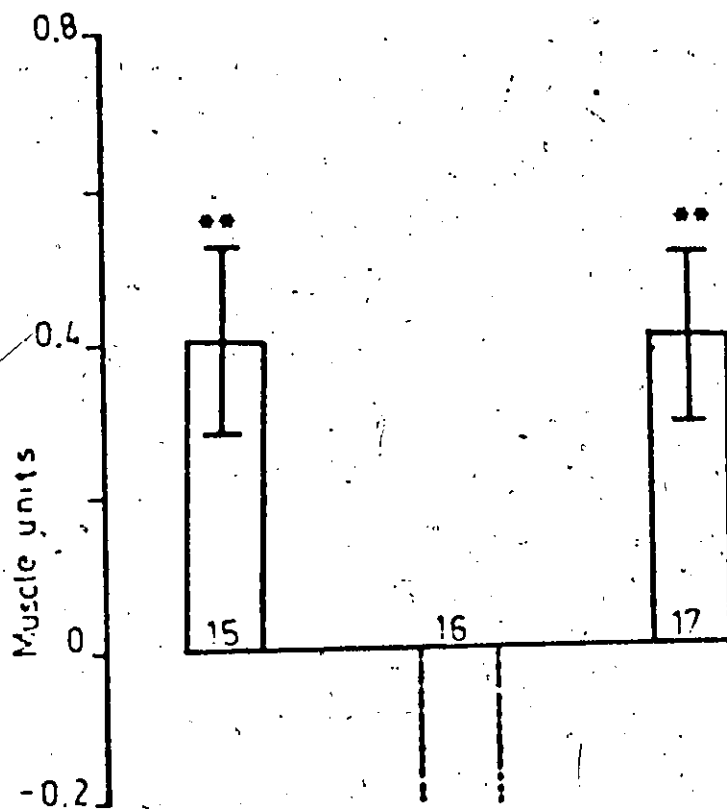
FIGURE 4-7

Changes in the motor fields of hind limb nerves produced by section of right nerve 16. Each column represents the average (+ SEM) of the difference of number of muscle groups innervated by the right side minus those of the left side for each pair of nerves, indicated by the respective number 15, 16 and 17. Significance of the difference is indicated by ** P <0.01. Positive values indicate that the right side had increased its motor field of innervation. The broken line indicates that the motor field of right nerve 16 had disappeared completely and only that of left nerve 16 was present. The results are from 30 salamanders which underwent section of right nerve 16 seven to 28 days previously.

TABLE 4-2

CHANGES IN THE SIZE OF MOTOR FIELDS OF NERVES 15 and 17 AFTER COMPLETE

ELIMINATION OF MOTOR FIELDS OF RIGHT NERVE 16

Nerve	Difference (R-L)
15	0.40 ± 0.13**
16	-
17	0.40 ± 0.11**

The values given are the average ($\pm$ SEM) of the difference in the number of muscle groups innervated by a given pair of nerves (right side minus left). Number of experiments was 30. The P values indicate that the right 15th and 17th nerves had increased their motor fields of innervation significantly as a consequence of the elimination of nerve 16,7 to 28 days previously. Significance of difference is indicated by ** $P < 0.01$.

the changes observed in nerve 16 and 17 are within the range of normal variability of motor innervation of hind limb.

(b) Analysis of the difference between right and left motor fields after treating right nerve 16 with colchicine: The results of the experiments to be presented include the same groups of salamanders presented in the section on touch-receptive field changes after treating right nerve 16 with colchicine (Part III-B-4); the details concerning dose, number of experiments, and duration of the post-operative period were exactly as described there.

The data concerning the changes in size of motor fields of innervation after such colchicine treatment are assembled in Figure 4-8. It is apparent that the number of muscle groups innervated by a given pair of nerves after treating right nerve 16 with colchicine solutions (0.025M to 0.1M) for 30 minutes was not statistically significant (level of significance P = 0.05; right minus left), i.e. such treatment did not provoke any change in the bilateral symmetry of the motor fields of innervation, insofar as number of muscles innervated by a given pair of nerves is concerned. It should be noted, however, that the smallest difference that could be detected with the technique used here was one whole muscle, which is a rather gross unit of measurement; more subtle changes such as a change in density of innervation of a given muscle, even if it were massive, and detected, were not included in the present data.

Conclusion: Although the results in some individual cases strongly suggested that after treating right nerve 16 with colchicine solutions there was an increase in the number of muscles innervated by nerves 15 and 17 on the treated side, such changes were not statistically

significant for the whole group (level of significance P $>$ 0.05).

FIGURE 4-8

Changes in the motor fields of hind limb nerves after treating
right nerve 16 with colchicine solutions (0.025M to 0.1M) for 30 minutes,
as described in Methods. Each column represents the average ($\pm$ SEM) of
the difference of the number of muscle groups innervated by the right
side minus the left side for each pair of nerves indicated by the res-
pective numbers 15, 16 and 17. Group (a) corresponds to the control or
amphibian Ringer-treated salamanders (n = 6), (b) to salamanders treated
with 0.025M colchicine (n = 3), (c) 0.03M colchicine (n = 11), (d) 0.05M
colchicine (n = 7), and (e) 0.1M colchicine (n - 21). None of the changes
were statistically significant (level of significance $P > 0.05$); the
broken lines indicate that the average and the SEM had a value of zero.
The post-operative periods range from 7 to 28 days.
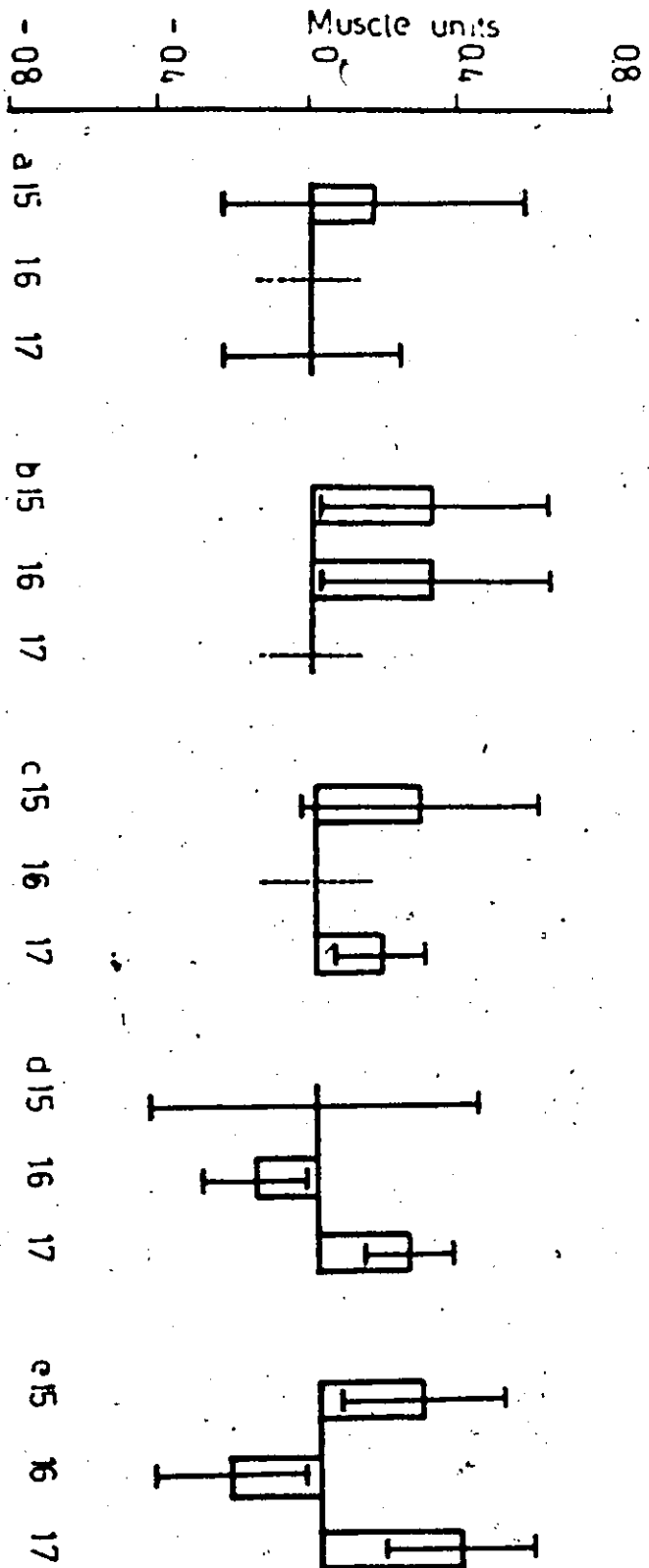
Part IV-C DISCUSSION ON MOTOR FIELDS

As in the case of the skin field (see Part III-C), a most impor-
tant finding in these studies was the bilateral symmetry of the motor
fields of corresponding nerves (15, 16 and 17) in unoperated salamanders.
Therefore, any change in the bilateral symmetry of motor fields could be
attributed to the experimental procedure performed on the treated side,
either nerve section or acute application of colchicine.

After complete elimination of the motor field of nerve 16, the
adjacent nerves (15 and 17) significantly increased the size of their motor
fields of innervation. As was discussed in Part I, the latter increase
could be explained either by (1) collateral sprouting of undamaged axons,
or (2) the emergence of function in pre-existing terminals at endplates,
which were morphologically normal but otherwise functionless. The reasons
for accepting the first interpretation have been mentioned and it seems
necessary here only to refer to direct histological demonstrations of its
occurrence in partially denervated hind limb muscles of rats (Edds, 1950,
1953; Hoffman, 1950), in the rat septal nuclei (Raisman, 1969; Moore et
al., 1971), and in the central processes of the dorsal spinal ganglia L7
in cats (Liu and Chambers, 1958).

After colchicine treatment of nerve 16 some individual experiments
strongly suggested that a genuine increase in motor field of nerve 15 had
occurred. However, the change for the whole group was not statistically
significant (level of significance $P > 0.05$). In that respect, there are
certain points in the pattern of innervation of salamander muscle cells

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which deserve consideration. The type of innervation is intermediate between that of slow and fast type of muscle, and each muscle cell receives 6 to 8 nerve endings which display a variety of morphological features; it seems probable that salamander muscle cells may be poly-neuronally as well as multiply-innervated (Tiegs, 1953; Marks et al., 1966).

The problem then is that an increase in motor fields could occur which would not be detected in the present experiments; these required that a whole muscle which was previously not innervated would become innervated to be scored as an increase. Assume that a single identified muscle had 100 muscle cells, that it was innervated by 2 spinal segmental nerves, and that each nerve innervated exclusively 10% of the muscle cells, while the remaining 80% were innervated by both nerves. After section of one of the nerves 10% of muscle cells would be totally denervated, while 80% would be partially denervated. If the remaining nerve took over the denervated territory a considerable increase in its density of innervation would have occurred, but the size of its motor field would have increased by a mere 10%; in any event, this would not be detected in the present experiments. Further experiments on density of innervation after either nerve section or colchicine treatment of nerve 16 are in progress in this laboratory.

Part V WAS COLCHICINE EFFECTIVE BECAUSE IT CAUSED NERVE DEGENERATION?

In order to assess whether or not the acute application of colchicine solutions used in these experiments caused nerve degeneration, nerves exposed in the standard way described in Methods were studied both electrophysiologically and morphologically 14 days after treatment, and compared with untreated nerves, usually from the same animal.

Part V-A **METHODS**

1. Electrophysiological experiments

A group of 3 salamanders were taken, in each of which nerve 16 on the right side was treated with 0.05M colchicine for 30 minutes in the usual way; 14 days later both nerves 16 were dissected from a point near the vertebral column up to the level of the knee, removed and set up in a chamber which has been described in Part II-B-3. The proximal end of the nerve trunk was stimulated with an electrical pulse (50 μsec, up to 3:0 volts, 1 Hz) and the compound action potential recorded from the distal end. Another pair of nerves, usually nerve 15, were dissected as above, and studied in a similar way. Latency, time to peak amplitude, and peak amplitude were measured from each compound action potential.

For comparison, 3 salamanders in which right nerve 16 was cut 14 days previously were studied in a similar fashion as above.

2. Morphological studies

(a) Operative procedures: These were similar to those described previously (Part II-B-3). This group included 16 salamanders in which the right nerve 16 was treated with colchicine solutions (0.025M to 0.1M), and 4 in which right nerve 16 was cut and tied. The salamanders were allowed to recover, and at either the 3rd or 14th post-operative day, they were re-anesthetized, and segments of both nerves 16 which included the treated region and a few mm distal to it were removed and processed either for light-microscopical or ultrastructural studies as detailed

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below.

(b) <u>Light microscopy studies. Histological techniques</u>: The nerve segment (see above) was placed in a small flask containing about 5 ml of 3% glutaraldehyde in 0.2M cacodylate buffer (pH 7.3) for 2 hours, and then post-fixed in Dalton's chrome-osmium fixative (2% OsO_4, pH 7.2). The specimen was embedded in Spurr epoxy resin and 1 to 2 u thick cross sections were cut. The sections thus obtained were stained with 1% toluidene blue in 1% borax, mounted, and covered with a coverslide.

(c) <u>Electronmicroscopical studies</u>: Nerves were fixed and embedded as above. Then ultrathin "silver" sections were cut using a Reichter microtome with a diamond knife, and subsequently stained in 25% methanol uranyl acetate (5 minutes) followed by Reynold's lead citrate (for further details see Kay, 1965), and examined with a Phillips 300 electronmicroscope.

Part V-8 <u>RESULTS</u>

1. <u>Electrophysiological studies</u>

Table 5-1 summarizes the data concerning the effects that treating

nerve 16 with 0.05M colchicine may have on the compound action potential,

as studied on the 14th post-operative day. Such data indicate that

colchicine treatment did not result in any deleterious effect on conduc-

tion velocity of compound action potentials nor on the peak amplitude at

the 14th post-operative day. Figure 5-1 illustrates the results from

one of the experiments presented above.

For comparison, a group of 3 salamanders which had undergone

section of right nerve 16, 14 days previously, were studied in an

identical way as the previous group which was treated with 0.05M colchi-

cine. It was found that stimulating electrically right nerve 16

(sectioned) did not evoke any compound action potential at all, while

the contralateral nerve 16 did.

Two more salamanders were treated with 0.1M colchicine and

studied as the group presented above; the nerves conducted apparently

normally impulses, but the number in the group was too small and the

scatter of the data too wide to justify a statistical analysis. The im-

portant point, however, is that such nerve trunks propagated compound

action potentials which certainly fell within the normal limits of vari-

ability.

Conclusions

The chronic experiments showed that 0.05M colchicine did not pro-

FIGURE 5-1

Effect of treating right nerve 16 with 0.5M colchicine for 30 min-
utes (see Methods) on the compound action potential recorded on the 14th
post-operative day. Record (a) shows the superimposed traces of the com-
pound action potentials from colchicine-treated (right nerve 16) and
untreated nerves (left 16). Record (b) shows the superimposed traces from
another pair of nerves, both untreated, taken from the same salamander.
These findings indicate that colchicine treatment did not produce a
detectable decrease in the peak amplitude of the compound action potential
of right nerve 16, which makes unlikely the possibility that such a
treatment may have caused Wallerian degeneration in the colchicine-treated
nerve fibres. The arrows indicate the time when the electrical stimulus was
applied. Calibration marks indicate 0.5 mvolt (vertical) and 0.5 msec
(horizontal).
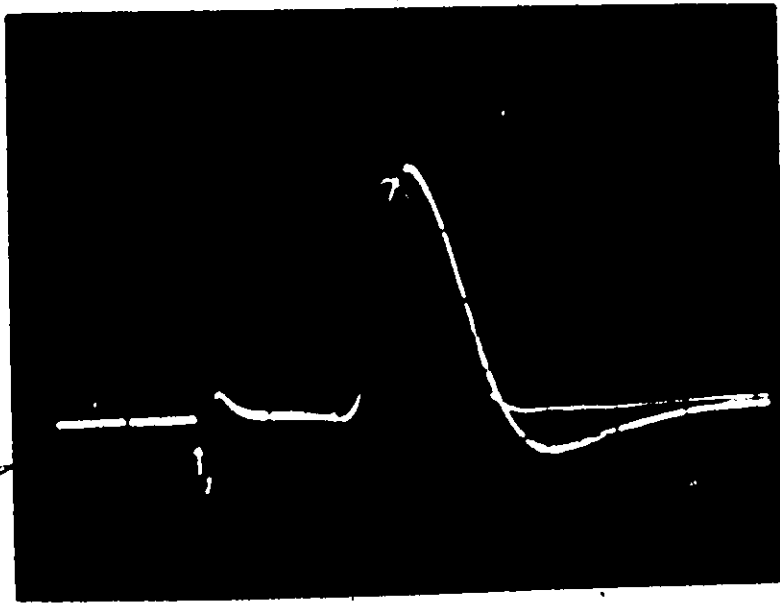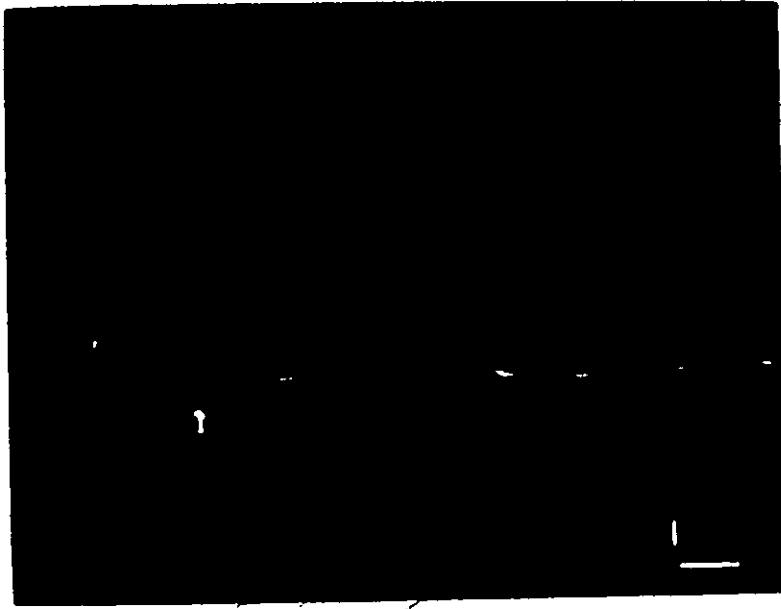
TABLE 5-1

EFFECT OF ACUTE APPLICATION OF COLCHICINE

ON COMPOUND ACTION POTENTIAL

Nerve	Latency (msec)	Time to peak (msec)	Peak amplitude (mV)
Right 16 (treated)	0.17 $\pm$ 0.02	0.45 $\pm$ 0.04	2.21 + 0.45
Left 16 (untreated)	0.17 $\pm$ 0.01	0.42 $\pm$ 0.02	2.38 $\pm$ 0.22
Right 15 (untreated)	0.18 $\pm$ 0.01	0.49 $\pm$ 0.07	3.66 $\pm$ 0.73
Left 15 (untreated)	0.16 $\pm$ 0.02	0.46 $\pm$ 0.04	4.36 $\pm$ 0.69

Each value represents the average ($\pm$ SEM) of observations performed on nerves taken from 3 salamanders which had received 0.05M colchicine on right nerve 16 14 days previously. The difference between each pair of values (right vs. left) of the latency, time to peak amplitude, and peak amplitude of compound action potential of the nerves, was not statistically significant (level of significance $P > 0.05$).

voke any deleterious effect on the conduction of compound action potentials, as studied on the 14th post-operative day; a decrease of the amplitude of compound action potential on the treated nerves would have indicated a severe loss of nerve fibres, probably due to Wallerian degeneration. The extreme case would be analogous to nerve section, in which no action potentials at all were recorded from such degenerated nerves.

2. Light microscopical studies

(a) Control or untreated nerves (Figure 5-2): Myelin sheaths were observed as structures of a deep blue colour (black in the photographs) which had rather unusual shapes, specially the small myelinated fibres; this contrasts sharply with the rather uniformly circular cross-sections of myelinated fibres which are usually pictured for mammalian myelinated fibres; see for example Figures 29 and 30 from Peters et al., (1970), or Figures 9 to 14 from Maximow and Bloom (1961). Schwann cells could be easily identified and had a large, irregularly shaped nucleus with some chromatin clumping which stained more deeply than the nucleoplasm; the cytoplasm of Schwann cells was scant and had a faint blue colour. The axoplasm appeared as a material with a faint blue colour, but cell membranes could not be resolved in these specimens.

(b) Three days after nerve section: There were a few fibres in which the axons looked somewhat shrunken, specially in larger fibres; it appeared to be a slight increase in the number of Schwann cells, but counts of Schwann cells were not performed; otherwise the microscopic appearances did not seem to differ from those observed in control nerves.

(c) <u>Fourteen days after nerve section</u> (Figure 5-3): Advanced de-
generative changes were obvious. Myelin sheaths in most cases had
collapsed; in those axons in which myelin sheaths resembled normal ones,
the axoplasm was either shrunken or filled with vacuoles; these changes
departed considerably from the histological picture of the control nerve
(compare Figures 5-2 and 5-3).

(d) <u>Nerve 3 days after colchicine treatment</u>: These nerves did not
differ considerably from untreated ones.

(e) <u>Nerve 14 days after colchicine treatment</u> (Figure 5-4): The histo-
logical picture of a colchicine-treated nerve (14 days) looked very much
the same as that of an untreated nerve. Schwann cells appeared to be
more frequent in the colchicine-treated nerve than in the control one
(compare Figures 5-2 and 5-4), but counts of Schwann cells were not per-
formed. It is obvious that colchicine treatment did not cause any of the
degenerative changes which were found in nerves cut 14 days previously
(compare Figures 5-3 and 5-4). Only in two cases out of 16 did colchicine
treatment (0.1M) provoke signs of degeneration such as breakdown of
myelin sheaths and hypertrophy of Schwann cells.

3. <u>Ultrastructural studies</u>

The ultrastructural studies that will be presented here were per-
formed 14 days after either nerve section or colchicine-treatment, since
the light microscopical studies showed that at that time Wallerian
degeneration was well established in sectioned nerves.

(a) <u>Control nerves</u>: Axons with cross sectional shapes like those ob-
served with light microscopy were also seen in low power (.5700X)

electron micrographs. The axonal membrane and myelin lamellae which had a regular spacing could be resolved. Inside the axon mitochondria, microtubules, neurofilaments, and profiles of smooth endoplasmic reticulum were observed. The Schwann cell cytoplasm appeared to be more electron dense than the axoplasm; a few unmyelinated fibres could be seen in the low power electron micrograph (see Figures 5-5 and 5-6).

(b) Nerves 14 days after nerve section: Myelin sheaths were folded, some had collapsed, and the orderly array of myelin lamellae was lost in several fibres. Some nerve fibres were loaded with electron-dense bodies, with dilated tubules (smooth endoplasmic reticulum?), and swollen mitochondria; it is not possible to tell whether these organelles were of axonal or of Schwann cell origin. To summarize, the above changes are indicative of severe Wallerian degeneration (Figures 5-7 and 5-8).

(c) Nerves 14 days after colchicine treatment (Figures 5-9 and 5-10): The results after treating the nerve with 0.1M colchicine will be described in some detail, but such description applies also to other colchicine doses (0.025M and 0.05M).

The axonal cross sections were similar to those observed in untreated nerves; myelin lamellae presented an orderly array, axonal membrane could be identified at the periphery of all axons; mitochondria, microtubules, neruofilaments, and profiles of smooth endoplasmic reticulum all showed a normal morphology. There were some electron-dense bodies which may correspond to microtubules altered by colchicine treatment, or to glycogen granules; such structures were not observed in untreated nerves, at least not so often. It is apparent from these electron micrographs that colchicine treatment did not provoke the dramatic

degenerative changes observed 14 days after nerve section (see Figures
5-7 and 5-9). Some unmyelinated nerve fibres could also be seen and did
not differ from those seen in untreated nerves.

(d) Microtubule density after colchicine treatment: A preliminary
survey showed that colchicine treatment caused a reduction in the number
of microtubules in the axons; the data concerning microtubule counts are
assembled in Table 5-2. For ease of comparison an electronmicrograph of
a control nerve and a nerve treated with 0.1M colchicine 14 days prev-
iously to fixation is shown in Figure 5-11.

TABLE 5-2

EFFECT OF COLCHICINE SOLUTIONS (0.1M and 0.05M) ON MICROTUBULE DENSITY

Treatment	Microtubule density
Untreated	13.3 ± 2.07
0.1M colchicine	3.4 + 0.68**
0.05M colchicine	4.1 ± 0.43**

Each value represents the average (± SEM) of the number of microtubules per um^2 of axoplasm. The number of observations were for untreated nerves (n = 7), 0.1M colchicine (n = 5), and for 0.05M colchicine (n = 6). Significance of difference between untreated and colchicine-treated groups is indicated by ** $P < 0.01$.

FIGURE 5-2

This picture was taken from a normal nerve. The myelin sheaths appeared as dark structures of rather irregular shapes, filled with axoplasm. Schwann cells with a darker cytoplasm and irregularly shaped nuclei are also to be seen. This and all subsequent light microscopy pictures were taken with a Neofluar objective 45X/NA 0.75. The bar at the bottom of the picture indicates 10 μ.
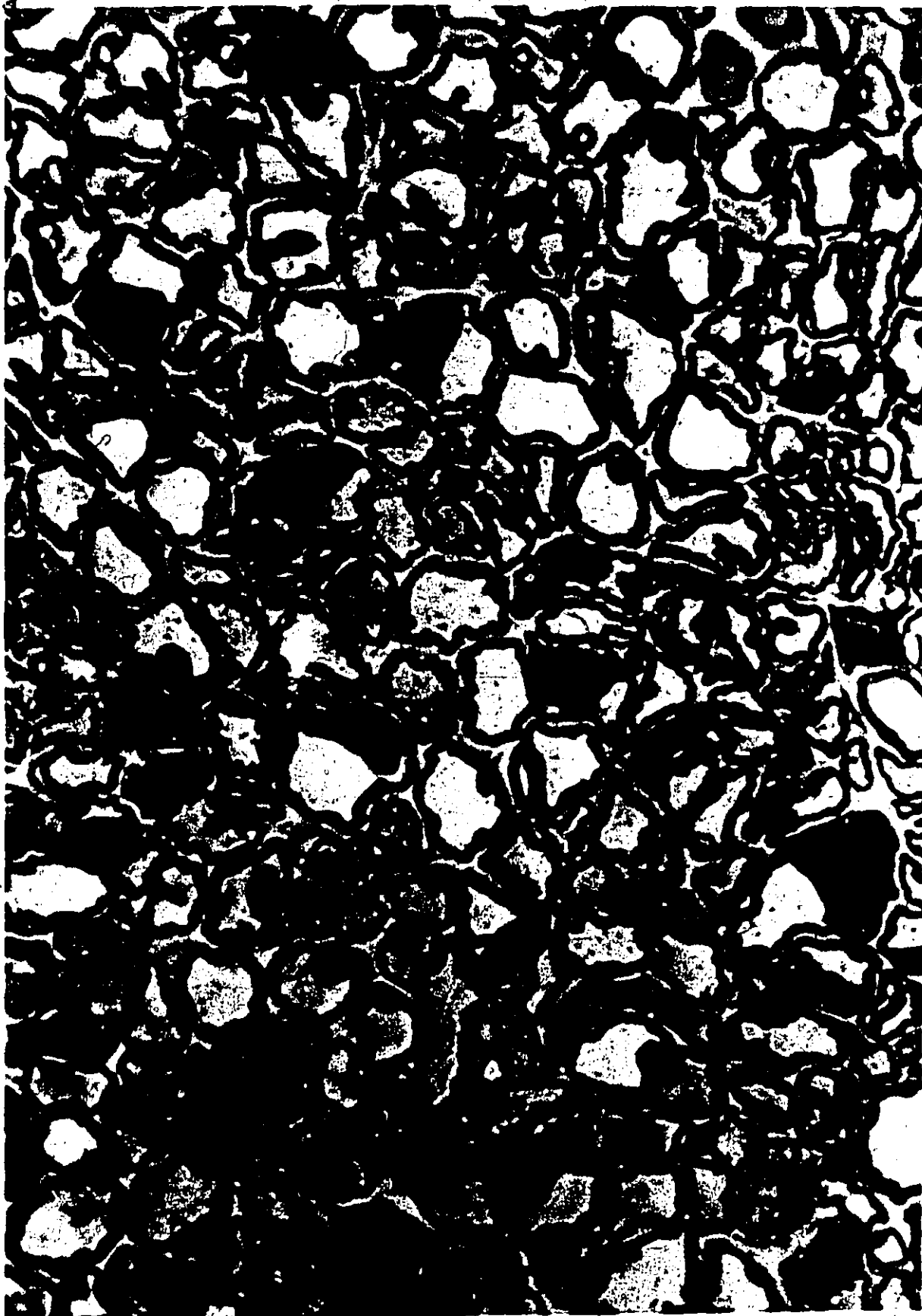
FIGURE 5-3

This picture was taken from a nerve which had been sectioned 14 days before. In most axons in which the myelin sheath still resembles the morphology of normal myelin sheaths, the axoplasm looked shrunken and filled with vacuoles. Compare with control nerve (Figure 5-2), and with a nerve 14 days after colchicine treatment (Figure 5-4). It is obvious that nerve section resulted in severe degenerative changes. The bar at the bottom of the picture indicates 10 μ.
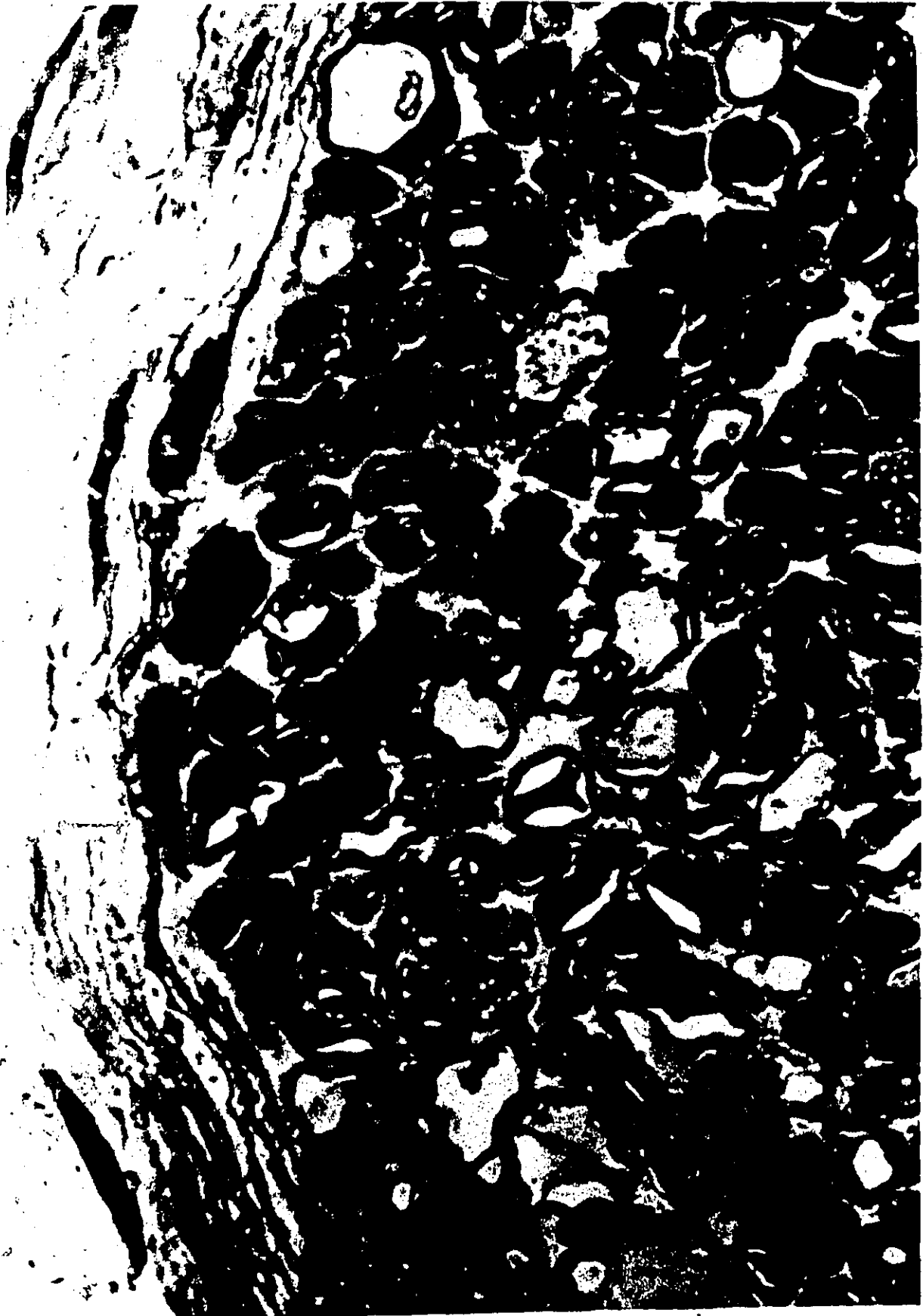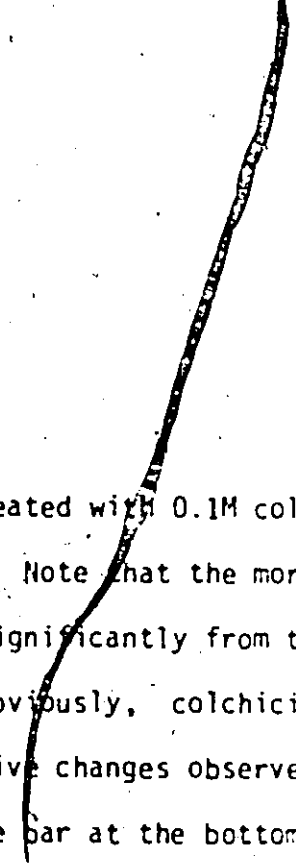
FIGURE 5-4

This picture was taken from a nerve treated with 0.1M colchicine for 30 minutes 14 days previously to fixation. Note that the morphology of myelin sheath and axoplasm did not differ significantly from that of untreated nerves (compare with Figure 5-2). Obviously, colchicine treatment did not provoke the severe degenerative changes observed 14 days after nerve section (see Figure 5-3). The bar at the bottom of the picture indicates 10 μ.
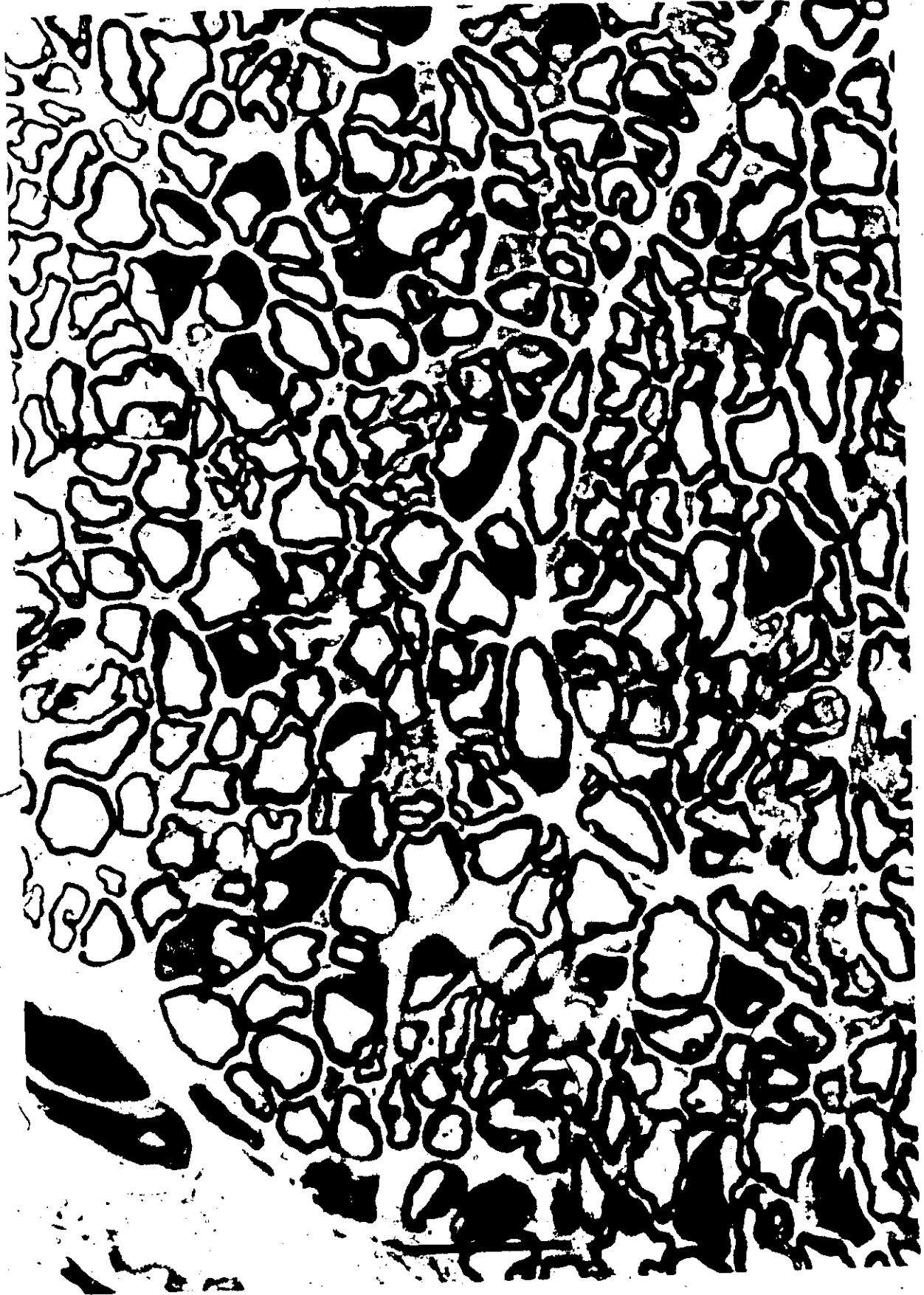
FIGURE 5-5

Low magnification (5700) electronmicrograph from a control (untreated) nerve. Myelinated axons of characteristic (for salamanders, cross sections were observed; some unmyelinated fibres were also seen. Some mitochondria and neurofilaments could be observed in the axoplasm; the axonal membrane could be resolved in this specimen. The nucleus of a Schwann cell could be observed at the left hand corner of the picture. The bar at the bottom of the picture indicates 2 μ.

FIGURE 5-6

High magnification (57,000) electronmicrograph from a control (untreated) nerve. The axonal membrane and the innermost myelin lamellae can be observed on top of the figure. Mitochondria, microtubules, neuro-filaments, and profiles of smooth endoplasmic reticulum are to be seen. The bar at the bottom of the picture indicates 0.2 μ.
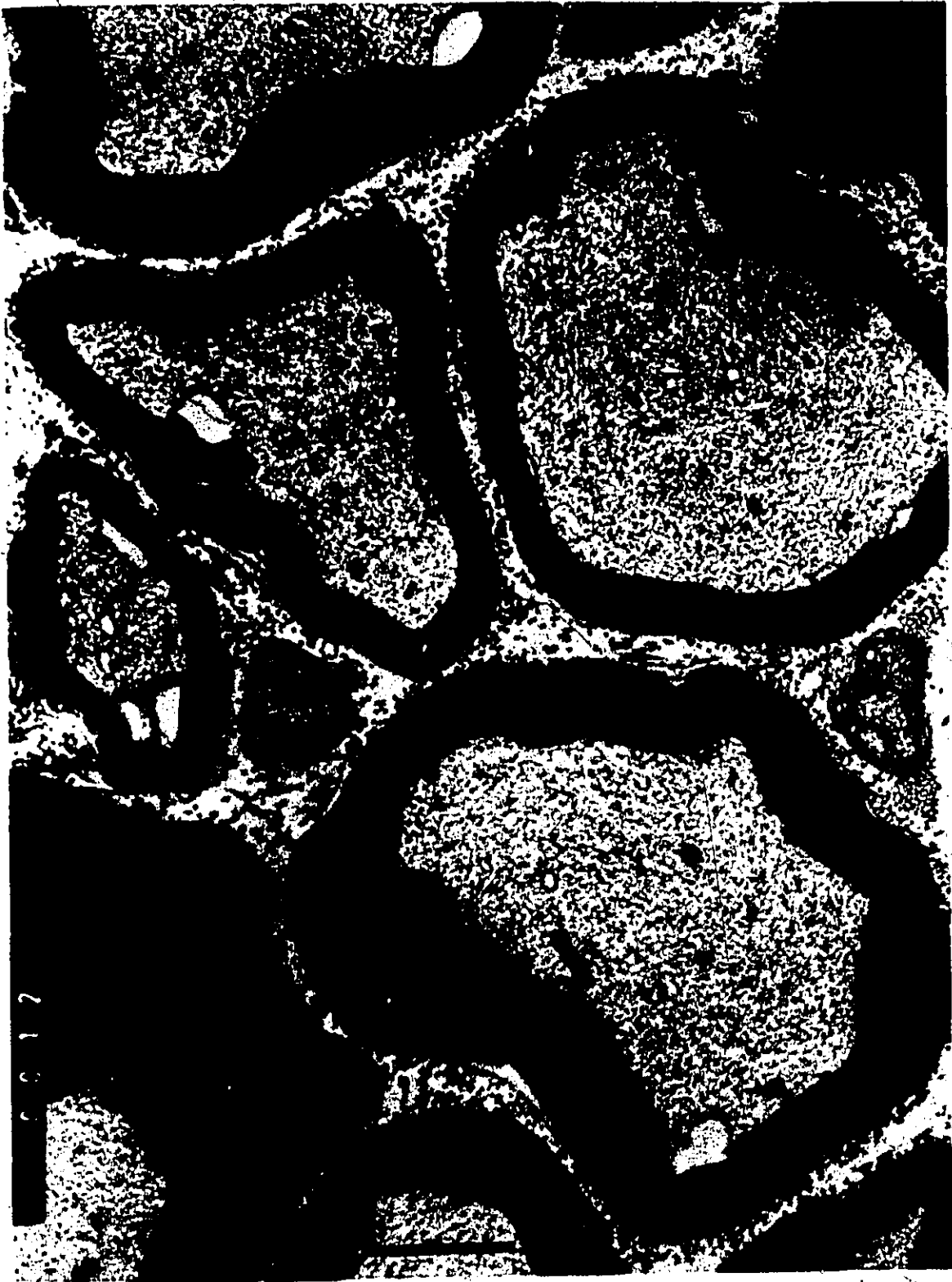
FIGURE 5-7

Low magnification (5,700) electronmicrograph from a nerve sectioned 14 days previous to fixation. In some axons the myelin sheath has broken down or collapsed, and lost its regular spacing. Other axons are loaded with swollen mitochondria, and electron-dense bodies (lipo-lytic bodies). The bar at the bottom of the figure indicates 2 μ.

FIGURE 5-8

High magnification (57,000) electron micrograph from a nerve which was sectioned 14 days previous to fixation. Swollen mitochondria could be observed, as well as other structures in advanced stages of degeneration which made their identification unreliable. The bar at the bottom of the picture indicates 0.2 μ.
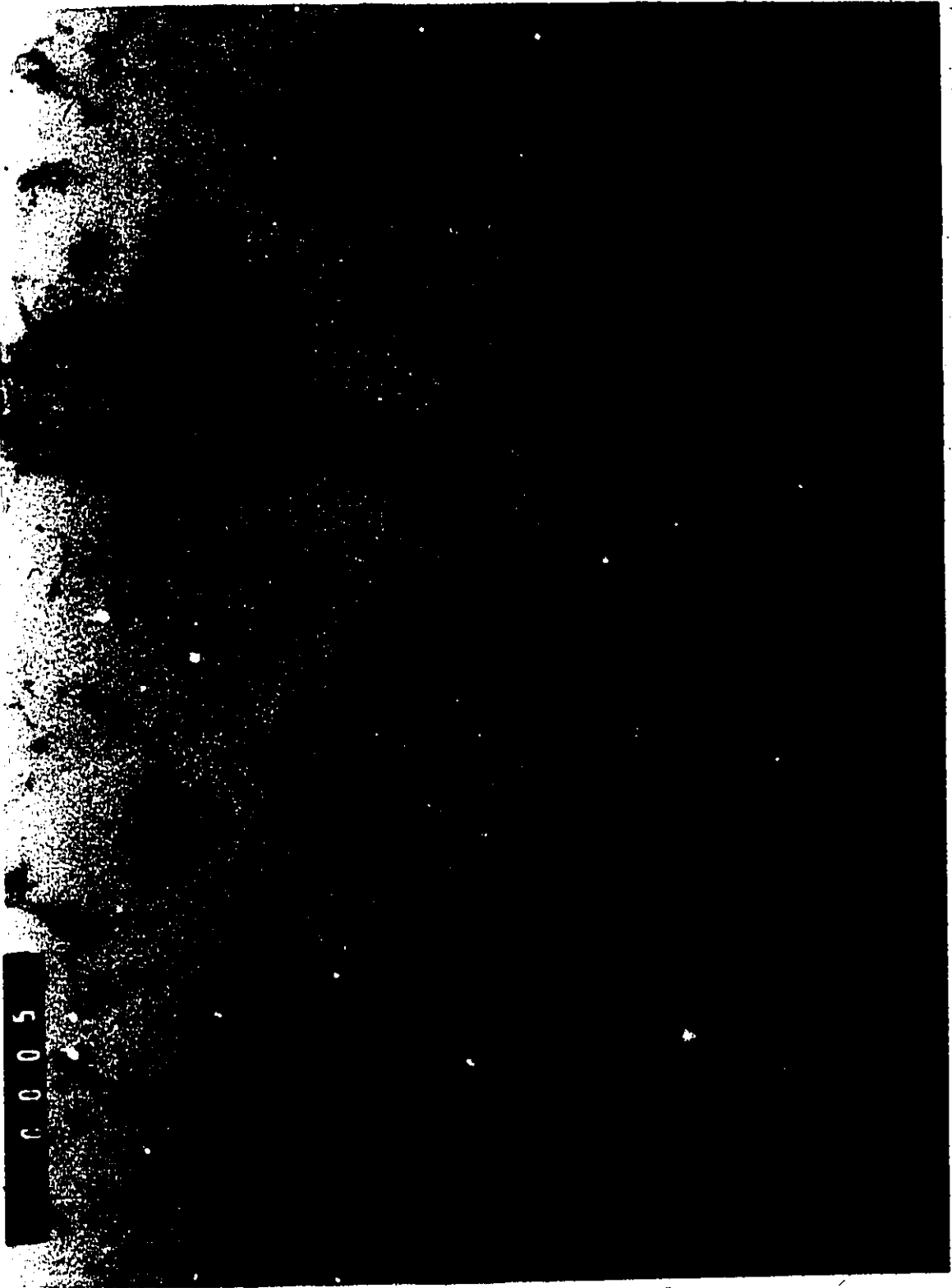
COO5

FIGURE 5-9

Low magnification (5,700) electronmicrograph from a nerve
treated with 0.1M colchicine, for 30 minutes, 14 days previous to fixa-
tion. Myelin sheath did not differ significantly from those observed
in untreated nerves. Mitochondria, neurofilaments, and profiles of
smooth endoplasmic reticulum could be seen, and their morphology seem
identical to that observed in control, nerves at similar magnification.
The axonal membrane looks intact. There are also some unmyelinated
fibres similar to those observed in untreated nerves. Compare with the
latter (Figure 5-5) and with the nerve 14 days after section (Figure 5-7)
The bar at the bottom of the picture indicates 2 µ.

FIGURE 5-10

High magnification (57,000) electronmicrograph from a nerve
treated with 0.1M colchicine, for 30 minutes, 14 days previous to fixa-
tion. The axonal membrane and myelin lamellae are observed at the lower
right hand corner. A few microtubules are present, as well as neuro-
filaments, and profiles of smooth endoplasmic reticulum, all had normal
morphology. There were some electron dense bodies which were not ob-
served, at least not so often in untreated nerves. Compare with the
normal nerve (Figure 5-6), and with the nerve cut 14 days previously to
fixation (Figure 5-8) at the same magnification. The bar at the bottom
of the picture indicates 0.2 µ.

FIGURE 6-11

For ease of comparison an electronmicrograph from an untreated nerve (lower picture), and that of a nerve treated with 0.1M colchicine 14 days previous to fixation are shown side by side. Note that the main difference between both axons is the absence of microtubules in the colchicine-treated one. The bar indicates 0.2 μ.
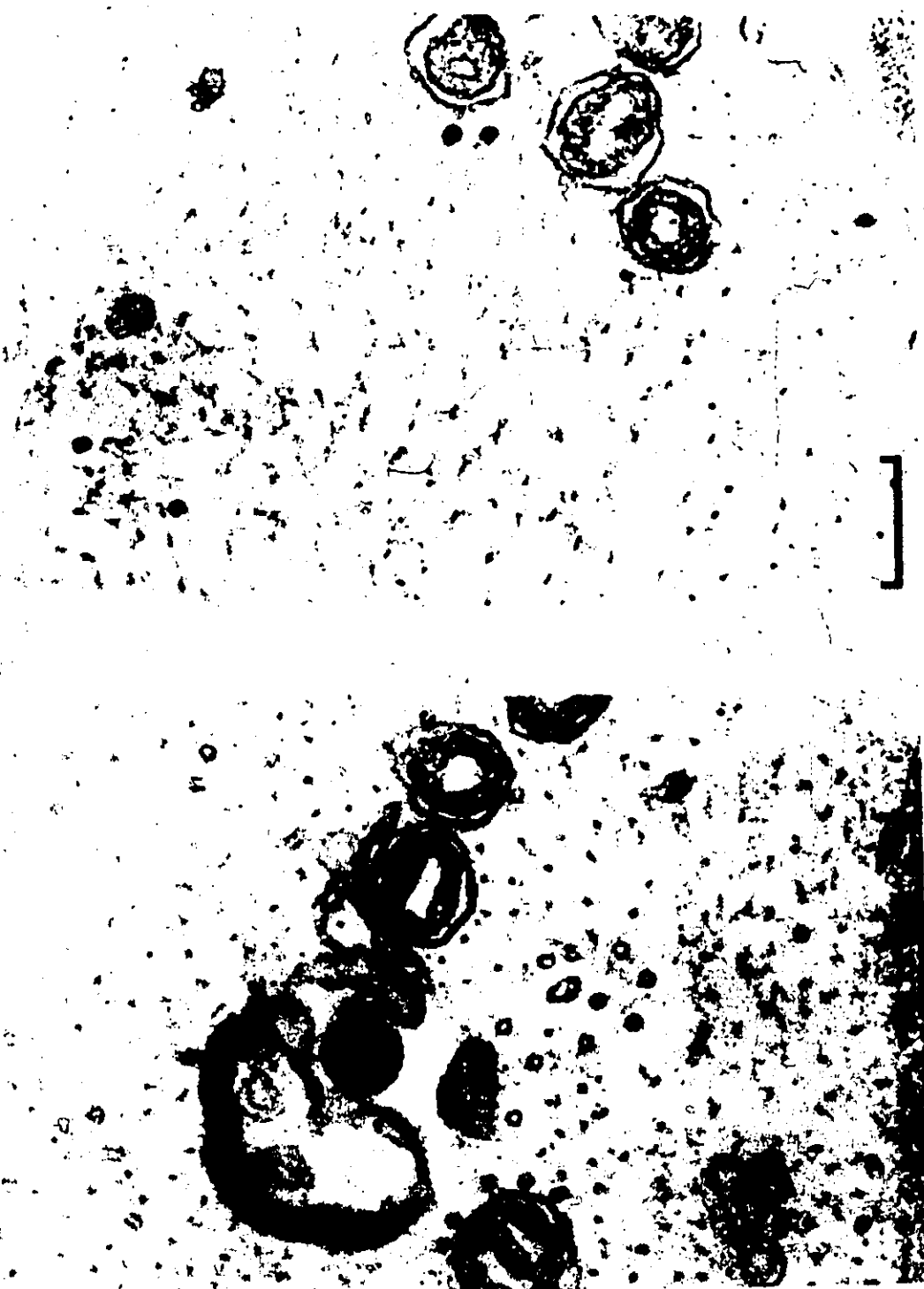
0.2μ

Part V-C DISCUSSION ON NEUROTOXICITY OF COLCHICINE

The data presented above showed that acute application of colchicine solutions (0.025M to 0.1M) for 30 minutes did not result (in nerves studied up to the 14th day after treatment) in any impairment of ability to conduct nerve impulses, nor (except in 2 cases out of 16) in morphological changes which could be described as Wallerian degeneration.

The rather unusual morphology of untreated nerve fibres may give rise to suspicions as to, e.g. fixation. However, untreated nerves in Triturus showed the morphology found in the present research. See for instance Singer et al., (1972) or Hay (1960). It is possible that such morphology may be peculiar to urodele nerve fibres.

The earliest evidence of Wallerian degeneration in Triturus (Singer et al., 1972) was the breakdown of the axonal membrane where it comes into close contact with the adaxonal membrane of Schwann cell, and the subsequent commingling of the cytoplasmic content of axon and Schwann cell. It was clearly shown in the above electronmicrographs that there was a normal integrity of the axonal membrane, and this was observed in all cases, except in two salamanders which were treated with 0.1M colchicine, which showed evidence of Wallerian degeneration. It is reasonable, therefore, to conclude that in most cases colchicine treatment, as described in Methods, did not cause Wallerian degeneration.

One finding of particular interest was the apparent reduction in the density of microtubules caused by colchicine treatment, since such a finding is considered as the best evidence, though circumstantial, that

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microtubules may be involved in the mechanism of fast axoplasmic transport (Banks et al., 1971b; Hokfelt and Dahlstrom, 1970, 1971).

We have not yet studied whether or not colchicine treatment provokes early degenerative changes confined to the nerve terminals. That is a possibility which deserves further investigation, since in the pigeon visual system, intraocular injection of colchicine (10 to 100 ug) did not provoke any impairment of conduction of nerve impulses in the optic tract, but caused a reversible depression of synaptic transmission in the tectum (Perisic et al., 1972), and early degenerative changes in the optic nerve terminals similar to those observed 12 to 24 hours after optic nerve section (Cuenod et al., 1972). Since the latter changes appeared at a time when fast axoplasmic transport was maximally depressed, this suggested that a material transported by the fast phase of axoplasmic transport was essential for the normal morphology and function of optic nerve endings.

The conclusion from the present studies is that the colchicine doses used in the present experiments did not cause loss of impulse conduction or significant degeneration of fibres in the treated nerves, except in a very few cases, for the highest doses of colchicine (0.1M). The reduction in microtubule density in treated axons is consistent with the accepted story from other worker's findings, namely that mechanism of axoplasmic transport (see Part VI- A) is related to microtubules since colchicine affects both of them.

Part VI   DOES COLCHICINE AFFECT AXOPLASMIC TRANSPORT?

It has been shown in previous sections that both partial dener-
vation of the salamander hind limb, and treating nerve 16 with colchi-
cine solutions result in an increase of the touch-receptive fields of
nerves 15 and 17. This finding lends support to the hypothesis that
the size of peripheral fields, at least in the salamander skin, is
regulated by a "neurotrophic" factor(s) carried along the axons to the
periphery. The movement of substances along nerve axons is implicit
in the terms "axoplasmic flow" and "axoplasmic transport".

Part VI-A <u>HISTORICAL REVIEW OF "AXOPLASMIC TRANSPORT"</u>

Due to the special morphology of neurons, substances synthe-
sized in the neuronal soma, for instance proteins, may have to be
exported over large distances to relatively remote regions such as the
nerve endings of motoneurones. The concept of transport mechanisms in
axons has changed our understanding of the neuron from that of a rather
static structure suggested by histological appearances to that of a
continuously modifying and possibly continually growing dynamic system.
There are now new possibilities for explaining how nerves can influence
their target cells by means other than the immediately effective action
of neurotransmitter substances on post-synaptic structures.

This section will cover some of the classic papers which estab-
lished the existence of the phenomenon of axoplasmic transport, par-
ticuarly of those substances (cholinesterase and catecholamines) which
were used as "markers" of such transport in the present research. Of
course, there is evidence that other substances are transported along
nerves, such as glycoproteins (Bondy and Madsen, 1971), RNA (Bondy, 1971),
and mitochondrial enzymes (Kreutzberg, 1969), but a thorough account of
that material will not be attempted in this discussion; comprehensive
reviews have been presented by e.g. Jeffrey and Austin (1974), Weiss
(1969, 1970, & 1971b), Dahlstrom (1971a), Barondes (1969), Davison (1970a,
b), and Grafstein (1969). Included here, however, will be the evidence
indicating that colchicine can block axoplasmic transport, probably by
interfering with the proteins which form certain structural entities
within the axoplasm.

1. The axon as a stream of axoplasm

The first clear demonstration that indeed there was an axonal transport of materials was given by Weiss and Hiscoe (1948). The experiments consisted of cutting the sciatic nerves of rats and allowing them to regenerate for periods of 4 to 35 weeks into regions of partial constriction produced by arterial cuffs round the nerves. The regenerating fibres which succeeded in growing into the distal region remained permanently undersized, while the proximal segments near the entrance of the constricted zone enlarged markedly. The impression given was that of a column of axoplasm pressing distally, which had become dammed up where the channel narrowed (Weiss 1969, 1972). It was found that the removal of the arterial cuff resulted in a sort of axoplasmic flood wave whose advancing front could be recognized microscopically during the first 2 days after releasing the constriction. The rate of advance of the axoplasmic column was about 1-2 mm/day. More recent experiments (see below) have shown that there are also higher rates of axoplasmic transport, which cannot be accounted for by this "slow" flow described by Weiss and his colleagues.

2. Axoplasmic transport of proteins

Proteins are an example of substances which are usually synthesized in the perikaryal region of neurons (Spirin et al., 1969; Utakoji and Hsu, 1965), and then transported toward the nerve endings. However, it should be kept in mind that there exists the possibility that proteins may be synthesized in axons (Eddstrom 1966; Koening, 1967), synaptosomes (Morgan and Austin, 1968; Austin et al., 1970), or may be transferred

from Schwann cells to axons (Singer et al., 1966).

The first clear demonstration that proteins synthesized in the neuronal body were subsequently transported along axons was given by Droz and Leblond (1963). These authors administered tritium-labelled amino acids (^{3}H-a.a.) intraperitoneally and later on visualized the localization of labelled material using an autoradiographic technique (see below). Specimens of cerebral cortex, cerebellum, and spinal cord were fixed within minutes after the injection of ^{3}H-a.a., and were subsequently processed histologically. An autoradiographic reaction (silver granules) was detectable over neuronal bodies, but not over the axon hillock, nor over either central or peripheral axons. By repeating the experiments at various times after the initial injection, the radio-activity which was responsible for the positive autoradiographic res-ponse was shown eventually to diminish in the neuronal soma, but to move down the axons, both in the CNS and in the periphery (sciatic nerve) at a rate of about 0.8 mm/day in full grown rats, and 1.5 mm/day in young ones. It is conceivable that the difference may reflect the need for extra materials required for growth in the young animals, but it is interesting that this would affect rate of movement of labelled material (presumably protein in this case), and not just quantity. The authors did not make any reference to higher rates of transport; the failure to detect such rates may be due to the fact that "fast" axoplasmic transport (rates higher than 2 mm/day) carries a smaller amount of proteins than "slow" flow (Karlsson and Sjosstrand, 1971a; Sjosstrand et al., 1969; McEwen and Grafstein, 1968), and also because the background radioactivity was rather high (3 grains/10 μ^2) in the above studies, and disallowed therefore the resolution of fast rates which may have been present.

3. Techniques used to study axoplasmic transport of ^{3}H-proteins

The basic principles of two of the more often used techniques will be briefly considered here, but a detailed account will not be attempted; for further details see Wang et al., (1965).

(a) Autoradiographic techniques: The rationale of these experiments is that when ^{3}H-a.a. are administered to a preparation either in vivo or in vitro, those cells which are in the process of protein biosynthesis will incorporate ^{3}H-a.a. into proteins, thus tagging the proteins themselves. ^{3}H-a.a. which becomes incorporated into proteins would remain (it is presumed) in the specimens during the fixation procedure, but free ^{3}H-a.a. would be washed out. In sections prepared for autoradiography, and subsequently processed histologically, an autoradiographic reaction, i.e. silver granules visible in the coating emulsion after development, would be detected over those places where enough of the labelled protein existed at the time of fixation. Such labelled sites, if they were known not to be there where protein synthesis occurs, indicate the existence of some mechanism, such as axoplasmic transport, capable of conveying the synthesized protein. Other experiments in which scintillation counting techniques have been used, showed that ^{3}H-proteins moved as a sharp peak along 20 mm of nerve trunk, but the peak took up about 1 mm, and moved with no decrement in time or space (Fernandez et al., 1970); these findings would exclude laminar flow and simple diffusion as mechanisms responsible for the observed shift in the labelled proteins.

(b) Liquid scintillation counting techniques: This technique is based on the emission of low energy beta particles from samples containing isotopes like ^{3}H or ^{14}C. The energy of the nuclear radiation is

...ultimately, released as photons which are detected and measured, usually,
being expressed as disintegrations per minute per mg of protein.

As with the autoradiographic experiments, ^{3}H-a.a. incorporated
into proteins will tag the latter, which can be precipitated from tissue
homogenates and so provide an estimate of the incorporation of ^{3}H-a.a.
into such proteins. In some experiments (McEwen and Grafstein, 1968)
further demonstrations of this incorporation was obtained by breaking
down the proteins into amino-acids by acid hydrolysis subsequently
separating the a.a. by chromatography, and visualizing them by ninhydrin
staining of the spots which were subsequently processed for scintilla-
tion counting as described above.

4. Investigations of axoplasmic transport in selected groups of neurons

Systemic administration of ^{3}H-a.a. may result in the labelling
of all cells (neurons and non-neurons) which happened to be in the pro-
cess of protein biosynthesis at that time. Experiments were designed
therefore to permit the labelling of selected groups of neurons. This
has been accomplished in the following examples: (a) application of
[3]H-a.a. to the nasal mucous membrane of frogs, which resulted in the
labelling of the olfactory nerve (Weiss and Holland, 1967), (b) the dorsal
spinal ganglion of mice can be placed in a chamber with a partition which
allows one to expose the ganglion to radioactive tracers without directly
affecting the axons of the neurons (Weiss, 1967) and the injection of
[3]H-a.a. into the dorsal ganglion of cats in vivo. (Lasek, 1966, 1968a),
(c) the intraocular injection of ^{3}H-a.a. which resulted in the labelling
of the visual pathway in birds (Bondy et al., 1971a,b), goldfish

(Grafstein, 1967, Grafstein and Murray, 1969), rabbit (Karlsson and Sjostrand, 1971) and rat (Sjosstrand and Hansson, 1971). In the above examples either autoradiographic studies or scintillation counting techniques (see above) showed that a wave of radioactive proteins moved away from the neuronal bodies, and was largely confined to the axons of the neurons concerned. The amount of labelling carried by the proximo-distal convection of fluid in the endoneurial space which Weiss et al., (1945) estimated to occur at a rate of 24-72 mm/day appeared to be small, since the silver grains visible over connective tissue in nerves were negligible at a region 7 mm from the site of injection of ^{3}H-a.a. (dorsal spinal ganglion of cats) while the more peripheral axon profiles were overlaid with a studding of silver grains (Lasek, 1968a).

5. Rates of axoplasmic transport

The experiments of Weiss and Hiscoe (1948), and Droz and Leblond (1963) discussed above, showed that there was an axoplasmic transport of materials at a rate of 1-2 mm/day. Apparently, clear evidence of higher rates of axoplasmic transport was given for the first time by Grafstein (1967) in the goldfish visual pathway, and by Lasek (1966) in the cat dorsal spinal ganglion. (These experiments were mentioned above, see also Table 6-2). A considerable number of experimental models have been investigated and a list of relevant examples is given in Tables 6-1 and 6-2. In most of these experiments, the rate of axoplasmic transport has been calculated from the rate at which the front of a wave of radio-active protein moved along axons after the administration of ^{3}H-a.a., using either autoradiographic or scintillation counting techniques.

TABLES 6-1 and 6-2

Legend

The numbers in brackets refer to the techniques used, as follows:

(1) scintillation counting.

(2) scintillation counting and parallel studies using light and/or EM autoradiography to show axonal localization of radioactive protein.

(3) light microscope autoradiography; silver grain counts.

(4) proteins purified using ion exchange columns followed by poly-acryl-amide gel electrophoresis or isoelectric focusing; the protein thus obtained (bound to ^{3}H-colchicine) was then processed for scintillation counting.

(5) Light microscopic observation of a visible axoplasmic "flood wave".

TABLE 6-1

RATES OF "SLOW" AXOPLASMIC TRANSPORT OF PROTEINS

References	Preparation	Label	Rate mm/day
Austin et al., (1966); Bray and Austin (1968, 1969)	Sciatic nerve of chicken (1)	^{14}C-a.a.	2-3
Carlsson et al., (1971)	Sciatic nerve (motor), cat (1)	^{3}H-a.a.	5
Chou (1970)	Optic pathway of monkey (1)	^{3}H-a.a.	1
Cooper and Diamond (1974, unpublished)	Nerve 16 of salamander (1)	^{3}H-colchicine	1-2
Cuenod and Schombach (1971), Schombach and Cuenod (1971)	Optic pathway of pigeon (2)	^{3}H-a.a.	1
Droz and Leblond (1963)	Sciatic nerve, young rats (3)	^{3}H-a.a.	1.5
	Sciatic nerve, adult rats (3)	^{3}H-a.a.	0.8
Fernandez (1969) Fernandez et al., (1970, 1973; Samson (1971)	Abdominal ganglia of crayfish (1)	^{3}H-a.a.	1.1
Grafstein (1967), Grafstein et al., (1969, 1972), McEwen & Grafstein (1968)	Visual pathway of goldfish, 20°C (1)	^{3}H-a.a.	0.4
Hendrickson and Cowan (1971)	Visual pathway of rabbit, 6-day old (2)	^{3}H-a.a.	5
	Visual pathway of rabbit, 1-month-old(2)	^{3}H-a.a.	2
Jakoubek et al., (1969)	Isolated Mauthner axon, fish (3)	^{14}C-a.a.	3.5
James et al., (1970a)	Sciatic nerve of chickens (1)	^{3}H-a.a.	2

TABLE 6-1 continued

References	Preparation	Label	Rate mm/day
James et al., (1070b)	Sciatic nerve of chicken (4)	^{3}H-colchicine	2
Karlsson and Sjostrand (1970, 1971c)	Optic nerve of rabbit (4)	^{3}H-colchicine	1.5-2
Karlsson & Sjostrand (1971a,b)	Optic nerve of rabbit (1)	^{3}H-a.a.	1.5
Lasek (1966, 1967, 1968a,b, 1970)	Sciatic nerve (sens. & motor), cats	^{3}H-a.a.	1
	Dorsal root ganglion cells, 2-4 month old kittens (1,2,3)	^{3}H-a.a.	2.9
McEwen et al., (1971)	Optic nerve of goldfish (4)	^{3}H-colchicine	0.5
Sjostrand & Hansson (1971)	Visual pathway of rat (1)	^{3}H-a.a.	2
Smith (1971)	Abdominal ganglia of cockroach (1)	^{14}C-a.a.	1-2
Taylor & Weiss (1965)	Visual pathway of mice (1)	^{3}H-a.a.	1-2
Weiss (1967)	Dorsal spinal ganglion cells, in vitro, mice (1,3)	^{3}H-a.a.	1
Weiss & Hiscoe (1948)	Sciatic nerve of rat (5)	-	1-2
Weiss & Holland (1957)	Olfactorynerve of frogs (3)	^{3}H-a.a.	1

See page 194 for legend

TABLE 6-2

RATES OF "FAST" AXOPLASMIC TRANSPORT OF PROTEINS

References	Preparation	Label	Rate mm/day
Bray and Austin (1969)	Sciatic nerve of chicken (1)	^{14}C-a.a.	250-30
Cangiano and Fried (1974)	Sciatic nerve of rat (1)	^{3}H-2.2	400
Carlsson et al., (1971)	Sciatic nerve (motor), cat (1)	^{3}H-a.a.	139
Chou (1970)	Visual pathway, monkey (1)	^{3}H-a.a.	100
Cuenod & Schombach (1971), Schombach & Cuenod (1971)	Optic pathway, pigeon (2 & 3)	^{3}H-a.a.	20-500
Diamond & Fried (personal commun.)	16 nerve of salamander (1) at 20-23°C	^{3}H-a.a.	40
Elam & Agranoff (1971)	Optic pathway, goldfish (1) at 23°C	^{3}H-a.a.	70-100
Eddstrom & Mattson (1972)	Sciatic nerve, frog, in vitro (1) at 17°C	^{3}H-a.a.	60-90
Fernandez et al., (1970, 1971, 1973), Samson (1971)	Abdominal ganglia, crayfish (1)	^{3}H-a.a.	10
Grafstein (1967, Grafstein et al., (1969, 1972), McEwen & Grafstein (1968)	Visual pathway, goldfish, at 20°C (a)	^{3}H-a.a.	60
Hendrickson & Cowan (1971)	Visual pathway, rabbit 6-days old (2)	^{3}H-a.a.	120
	Visual pathway, rabbit 1-month old (2)	^{3}H-a.a.	240
Jakoubek et al., (1969)	Isolated Mauthner axon, fish (3)	^{14}C-a.a.	30-40

TABLE 8-2 continued

References	Preparation	Label	Rate mm/day
James et al., (1970a)	Sciatic nerve, chicken (1)	^{3}H-a.a.	300
Karlsson & Sjostrand (1971a,b)	Optic nerve, rabbit (1)	^{3}H-a.a.	110-150
Lasek (1966, 1967, 1968a,b)	Sciatic nerve (sensory) cat (2,3)	^{3}H-a.a.	500
	Sciatic nerve (motor) rat (2,3)	^{3}H-a.a.	100
Livett et al., (1968a,b)	Splenic nerve, cat (1)	^{14}C-a.a.	96-120
Lux et al., (1970)	Motoneurons, cat (3)	^{3}H-a.a.*	12-240
Marchisio et al., (1971)	Optic nerve, chick embryo (18 days) (2)	^{3}H-a.a.	10-100
Ochs & Smith (1971), Ochs & Hollingworth (1971)	Sciatic nerve (sensory) cat (1)	^{3}H-a.a.	400
Ochs & Johnson (1969)	Sciatic nerve (motor) (1), cat	^{3}H-a.a.	930
Schonatolla et al., (1972)	Optic pathway, zebra fish embryo (3)	^{3}H-a.a.	11.5
Sjostrand et al., (1969)	Optic nerve, rabbit (1)	^{3}H-a.a.	110-150
Sjostrand (1969, 1970a, b)	Vagus nerve, rabbit (1)	^{3}H-a.a.	26-400
	Hypoglossal nerve, rabbit (1)	^{3}H-a.a.	5-300
Sjostrand & Hansson (1971)	Visual pathway, rat (1)	^{3}H-a.a.	120-140
Utakoji & Hsu (1965)	Dorsal spinal ganglion cells, chick embryo, in vitro (3)	^{3}H-a.a.	10

See page 194 for Legend

* Iontophoretic application

Usually the rate of 1-2 mm/day is referred to as "slow" axoplasmic transport (Samson, 1971; Dahlstrom, 1971a). A rate as high as 26 mm/day has been called "slow" (Sjosstrand, 1969, 1970) because in the same experiments a clear separation was achieved (by scintillation counting techniques) between that rate and another rate up to 400 mm/day. However I myself concur with the view that the term "slow" axoplasmic transport should be restricted to rates of 1-2 mm/day; there is a possibility that this may correspond to the bulk movement of axoplasm as originally conceived by Weiss and Hiscoe (1948); higher rates than this probably relate to different components of the "fast" axoplasmic transport. It should be noticed, however, that the axoplasmic transport in general is a rather selective system, including even the slow one; for instance mitochondria are transported at a rate of 1 mm/day along axons (Weiss, 1971a; Kreutzberg, 1969), but ribosomes which are abundant in the neuronal body, are conspicuously absent from axons (Peters et al., 1970). In addition, there is good evidence that axoplasmic transport occurs in the reverse (i.e. distal-proximal) direction also (Kristensson et al., 1970, 1971). In particular, evidence exists that cholinesterase, a substance of interest in the present studies, moves both distally and proximally, presumably in the same axons (see below).

6. Axoplasmic transport of cholinesterase

Cholinesterase is the enzyme used in the present research as an indicator of normal or reduced axoplasmic transport in nerves. By using either histochemical methods or determinations of enzymatic activity the transport of particular proteins (enzymes in this instance) have been

measured, in contrast to studies involving the administration of radio-active precursors (^{3}H-a.a.) which results in the labelling of several kinds of proteins, which have not been separately characterized.

Swayer (1946) found that after cutting the sciatic nerve of rats the regenerating fibres of the proximal stump showed an increase of about 300% of the control value (for an equivalent length of nerve trunk) in cholinesterase activity. Since histochemical methods showed that a considerable amount of products of cholinesterase activity was present on axonal surfaces, he attributed this phenomenon to the increase in cell surface of the numerous regenerating axons.

Using several modifications of the acetyl-thio-choline method for cholinesterase (Fukuda and Koelle ,1959) showed in the cat ciliary ganglion that the pattern of cholinesterase staining corresponded very closely with that of the Nissl substance. Following total inactivation of ganglionic cholinesterase with diisopropylfluorphosphate, the enzymatic activity appeared first in a region around the nucleus, then at more peripheral regions of the neuronal body, and later on at cell membranes; presumably it occurred also in axons and axonal endings, although the latter point was not assessed. These findings were inter- preted as indicating that cholinesterase was synthesized in the endo-plasmic reticulum, and then transported via the endoplasmic reticulum canaliculi to the cell surface and processes. In isolated nerve segments (peroneal nerve of dogs) of several cm length there was an accumulation of cholinesterase at both ends of the nerve segment, although it was higher at the distal than at the proximal end; there was a corresponding decrease of the enzymatic activity in the middle region of the nerve seg-

ment, so that the total enzymatic activity did not change (Lubinska, 1964; Lubinska et al., 1964; Lubinska and Nimierko, 1971). These findings were interpreted as evidence of a bidirectional transport of the enzyme; from the rate of accumulation it was calculated that such transport occurred at a rate of 260 mm/day in the proximo-distal direction, and at a rate of 130 mm/day in the retrograde one. The existence of a bidirectional transport of cholinesterase has been confirmed in other laboratories, and some of the values reported are given in Table 6-3. Although the values are not identical for different experimental models, it is apparent that the rate of transport in the proximo-distal direction is greater than in the opposite one. The wide variation in the values of the transport rate of cholinesterase, e.g. from 5 up to 430 mm/day, may reflect species differences, although technical factors may also play a role. In this regard, the comment made by Lubinska (1964) may be relevant, that preliminary experiments failed to reveal any accumulation of cholinesterase activity when the determinations of such activity were performed in pieces several mm long - of the isolated nerve segment.

Ranish and Ochs (1972) found that only about 10% of the total activity of an isolated nerve segment moves in the proximo-distal direction and 5% in the retrograde direction; the bulk of the enzyme seems to be stationary, at least so far as fast axoplasmic transport is concerned. It is known that cholinesterase is bound to membranes of endoplasmic reticulum, and to axonal surface membranes (Brzin et al., 1966; De Robertis, et al., 1962). The suggestion was put forward by Nimierko and Lubinska (1967) that cholinesterase which is transported in axons may be bound to endoplasmic reticulum, whereas that bound to axonal surface

TABLE 6-3

RATES OF AXOPLASMIC TRANSPORT OF CHOLINESTERASE IN mm/DAY

References	Preparation	Proximo-distal	Retrograde
Fonnum et al., (1973)	Vagus nerve, rabbit	425	123
Frizzel et al., (1970)	Vagus nerve, rabbit	15	-
	Hypoglossal nerve, rabbit	5	-
Jankowska et al., (1969)	Peroneal nerve, dog	120	-
Nimierko & Lubinska (1967), Lubinska & Nimierko (1971) Lubinska (1971)	Peroneal nerve, dog	260	134
Partlow et al., (1972)	Sciatic nerge, frog	99	19
Ranish & Ochs (1972)	Sciatic nerve, cat	430	220

remains stationary.

7. Axoplasmic transport of catecholamines

The histochemical fluorescent method specific for catecholamines (CA) was found to be a convenient way of visualizing axoplasmic transport in the present experiments, and is described in Part VI-B-1a. Several experimental approaches indicate that noradrenaline (NAdr) is contained in small and large granular vesicles, both with a dense core; see for example von Euler and Hillarp (1956); Hokfelt (1967); Bisby et al., (1971); Dahlstrom et al., (1971a) and Kapeller et al., (1967). It has been shown that proximal to the site where sympathetic nerve fibres were crushed, tied, or cut, there was an accumulation of a strongly fluorescent material when the histochemical fluorescent technique for CA was used (Dahlstrom 1967a,b, 1969, 1971a; Kapeller and Mayor, 1967; Dahlstrom and Haggendal, 1966, 1967, 1970; Geffen, Livett and Rush, 1969; Hokfelt and Dahlstrom, 1971). Ultrastructural studies showed that proximal to the site of nerve crushing there was an accumulation of dense-core granular vesicles (Banks et al., 1971a; Kapeller and Mayor, 1966, 1967, 1969a, b; Geffen and Ostberg, 1969). Similar results were obtained with immunofluorescent techniques used to demonstrate the presence of dopamine βhydroxylase, an enzyme associated with NAdr storage granules (Geffen, Livett, and Rush, 1969), and spectrofluorimetric determinations of NAdr have also revealed an increase of NAdr proximal to the site of nerve crush (Banks et al., 1971a; Laduron and Belpaire, 1968; Dahlstrom and Haggendal, 1966, 1967).

These experimental findings are all consistent with the hypothesis that NAdr storage granules are continually transported in a proximo-distal

direction in the axons, and that they accumulate proximal to the site of a nerve lesion because the latter has interrupted their normal transit. The rate of transport has been calculated from such rates of accumulation, and some of the values reported are given in Table 6-4.

Whatever the mechanism(s) for the transport of NAdr storage granules may be, it is localized within the axon itself, since the phenomenon still occurred along a segment of nerve ligated at both ends, and it appeared that no further synthesis of NAdr had occurred, since no increase in total NAdr content was detectable (Dahlstrom, 1967a). The time-course of the accumulation of NAdr at a nerve constriction has a very steep, fairly linear relationship during the first 48 hours, and after that time reaches a plateau (Dahlstrom and Haggendal, 1966; Kapeller and Mayor, 1967).

8. Effects of colchicine on axoplasmic transport and microtubules

The main experiments of the present research rest on the hypothesis that a blockade of axoplasmic transport (of a presumed trophic factor) caused either by nerve section or colchicine treatment provokes collateral sprouting in adjacent nerves. The evidence as to the action of colchicine on axoplasmic flow therefore will be reviewed now.

(a) Effect of colchicine on axoplasmic transport of proteins: Colchicine affects both the slow and fast phases of axoplasmic transport, but there are species variations with regard to the extent to which such phases are blocked, as the following examples will illustrate. James et al., (1970a) found that a dose of 10 ug colchicine injected in the lumbosacral spinal cord of chickens did not interfere with the incorporation

TABLE 6-4

RATES OF AXOPLASMIC TRANSPORT OF CATECHOLAMINES IN mm/DAY

Reference	Preparation	Rate mm/day
Banks, et al., (1971a)	Hypogastric n/inf.mesent. ganglion, rat (1)	26.4 - 21.6
Dahlstrom & Haggendal (1966, 1967, 1970)	Sciatic nerve, rat (1)	120
	Sciatic nerve, cat (1)	240
	Sciatic nerve, rabbit (1)	72
Geffen & Rush (1968)	Splenic nerve, cat (1)	33.6-101
Haggendal & Dahlstrom (1969)	Spinal cord, rat (1)	16.8
Leduron & Belpaire (1968)	Splenic nerve, dog (1)	24-48
Livett et al., (1968a, b)	Splenic nerve, cat (2)	120

Numbers in brackets refer to techniques used as follows:

(1) Spectrofluorimetric determinations of NAdr.

(2) Determinations of ^{14}C-NAdr.

of ^{3}H-a.a. into proteins, but the amount of labelled proteins which was transported down the sciatic nerve was dramatically reduced, that moving at 2 mm/day to a mere 6%, whereas that moving at 300 mm/day was less affected, but still reduced to about one third of the control value. In adult rats, an intraocular injection of 1 µg colchicine caused a decrease of about 30% of retinal protein synthesis, but such decrease did not occur with lower doses; nevertheless, 0.2 µg colchicine caused an almost complete blockade of the fast axonal transport of proteins (labelled with ^{3}H-a.a.) while a small but significant fraction of the protein transported at the slow rate of 2 mm/day was still being conveyed to the optic nerve terminals (Sjostrand et al., 1971). In the hypoglossal and vagus nerves of rabbits, an intracisternal injection of 100 µg colchicine provokes an almost complete blockade of the fast (up to 400 mm/day) transport of proteins labelled with ^{3}H-a.a., but the axonal transport of two enzymes (choline acetyl transferase and acetylcholinesterase) which moved at a rate of 5-26 mm/day was only partially affected (Sjostrand, 1970a,b). These findings show that a given colchicine dose may produce different degrees of blockade in the various components of the fast and slow axoplasmic transport system.

In the crayfish abdominal ganglia two sharp peaks of radioactive protein moving at 1 and 10 mm/day observed normally after the intraganglionic injection of ^{3}H-a.a. were both completely blocked by intraganglionic injection of 10^{-3}M colchicine (Fernandez et al., 1970, 1971). In chickens, the effects of 1 µg colchicine injected into the lumbar spinal cord are apparently long lasting, since even 27 days after such injection the slow (2 mm/day) was reduced to about half, and the fast

(up to 300 mm/day) to about one third of their control values in the sciatic nerve (McGregor et al., 1973). In rabbit motoneurons, there is morphological evidence that the pathological changes, e.g. decrease in microtubules, appearance of abnormal neurofilament bundles, etc., provoked by the injection of 100 ug colchicine into the lumbosacral spinal cord began to disappear by the end of the third post-operative week (Wisniewski et al., 1968).

(b) Effect of colchicine on the axoplasmic transport of cholinesterase: The accumulation of the products of cholinesterase activity above a sciatic nerve crush in rats disappeared when 100-900 ug colchicine were injected into the nerve prior to nerve crushing (Kreutzberg, 1969); smaller doses did not produce such effect. In the discussion of their results, Kasa et al., (1973) mention unpublished results like those above, but no details were given. These findings are consistent with the view that colchicine at the appropriate doses blocks the axoplasmic transport of cholinesterase. Similarly, Sjostrand (1970b) using biochemical determinations of the enzyme activity showed that the intracisternal injection of 100 ug colchicine resulted in a significant decrease in the amount of enzyme accumulating proximal to the site at which the vagus and hypoglossal nerves were crushed; these findings showed, however, only a partial blockade of the axoplasmic transport of cholinesterase by colchicine.

(c) Effects of colchicine on the axoplasmic transport of catecholamines: The application of colchicine at appropriate doses results in a significant reduction or in the complete absence of the accumulation of catecholamines (CA) which is normally observed above a nerve crush by

fluorescent histochemical techniques (Dahlstrom, 1969, 1971a,b; Hokfelt and Dahlstrom, 1970, 1971; McLean et al., 1972). In addition, the dense cored granular vesicles which almost certainly contain NAdr fail to accumulate after colchicine treatment (Banks et al., 1971a,b; Hokfelt and Dahlstrom, 1970, 1971). Similar results were obtained with spectrofluorimetric determinations of NAdr (Banks et al., 1971a, Dahlstrom, 1971a). All these findings are consistent with the view that colchicine causes a blockade of the axoplasmic transport of CA. Banks et al., (1971b) found this to be a dose-dependent phenomenon; at doses less than 0.3 ug/ml in vitro, colchicine did not significantly block the accumulation of NAdr above the ligated region of a nerve, but it did so at doses ranging from 1 to 10 ug/ml.

(d) Effects of colchicine on microtubules

(i) Characteristics of microtubules: These apparently cylindrical structures can be observed by electron microscopy after glutaraldehyde fixation, and have an outer diameter of 220-260 Å (Peters et al., 1970; Wisniewski et al., 1968). Their actual length is not known, but since they do not appear to branch in pre-terminal motor nerves, Weiss and Mayr (1971) suggested that microtubules may form a continuous structure from neuronal nucleus to nerve endings. Microtubules are formed of protein subunits, usually called tubulin, which has a molecular weight of about 60,000 (Schmitt and Samson, 1968, 1969; Davison and Huneeus, 1970; Feit et al., 1971).

(ii) Binding of colchicine to tubulin: Borisy and Taylor (1967a, b), Shelanski and Taylor (1967, 1968) and Davison and Huneeus (1970) isolated from several sources, e.g. mitotic spindle of sea urchin eggs,

sperm tail, squid axoplasm, a protein which binds colchicine. In the case of the mitotic spindle (Borisy and Taylor, 1967b) colchicine treatment resulted in the disappearance of microtubules, which suggested that the protein which binds colchicine may be the microtubule protein. Davison and Huneeus (1970) extracted the protein from 900-1500 mg of freeze-dried squid axoplasm using small volumens of a solution (0.02M mercaptoethanol, 0.01M soldium phosphate, pH 6.3, 10^{-5}M GTP) containing 0.1 uc ^{14}C-colchicine. The clear centrifugal extracts were applied to gel filtration or ion exchange columns, the eluted portions containing the protein-bound ^{14}C-colchicine were grouped and subsequently processed for electrophor-esis in poly acrylamide gel; in this way a single protein band was obtained which contained about 75% of the protein-bound ^{14}C-colchicine. Nevertheless, the identification of the colchicine-binding protein as the microtubule protein is not yet completely proven however, these experiments strongly support the view that the isolated protein (tubulin) may be the microtubule protein.

Experiments in which subcellular fractionation has been combined with immunological studies involving antibodies against purified tubulin (Twomey et al., 1972) or with determinations of ^{3}H-colchicine bound to protein (Lagnado et al., 1971) suggest that tubulin might exist in a soluble pool from which subunits may be recruited for the assembly not only of microtubules, but also for the integration of membrane components of neurons. The finding that colchicine binding protein, diagnostic for microtubules, moves at the slow rate of 1.5-2.0 mm/day (Karlsson et al., 1970; James and Austin, 1970; Feit et al., 1971; McEwen et al., 1971; Cooper and Diamond, personal communication) lends support to the sugges-

tion made by Weiss and Mayr (1971) that microtubules may grow continually by adding up new subunits in the perikaryal region of the neuron.

(iii) Effect of colchicine on the number of axonal microtubules: Some of the values of the number of microtubules per cross-sectional area of axons (microtubule density) are given on Table 6-5. It has been found that colchicine at doses which blocked axoplasmic transport produced a significant decrease in the number of axonal microtubules (Banks et al., 1971b); in crayfish, however, such a decrease was not observed, and microtubules were morphologically identical in colchicine- and un-treated animals (Fernandez et al., 1970). At temperatures above 40°C or below 4°C microtubules disappear from axons (Hinkley et al., 1972; Samson, 1971), and when animals are brought again to room temperature microtubules re-appear; however, colchicine treatment prevented microtubule reassembly after similar temperature changes (Rodriguez et al., 1968; Samson, 1971; Hinkley et al., 1972). These findings suggest that colchicine may bind to the tubulin pool, but not to the tubulin present in organized microtub-ules; it is also possible that in crayfish colchicine-treated axons may show morphologically normal microtubules whose function may be impaired (see below).

Conclusion: Colchicine, at appropriate doses, blocks axoplasmic transport of a wide variety of substances; although there are variations from one species to another, it seems that fast axoplasmic transport is more severely affected than the slow one. An important characteristic of colchicine is its ability to bind to an axonal protein, most probably the microtubule protein; in addition colchicine provokes in some species a disruption of microtubules at doses which block axoplasmic transport.

TABLE 6-5

MICROTUBULE DENSITY

Reference	Preparations	Microtubule density
Banks, et al., (1971b)	Cat sympath. axons	16 per axon
Hinkley et al., (1972)	Crayfish ventral cord	40 per μ^2
Peters et al., (1967)	Newborn rat, optic nerve	4-10 per axon
Samson (1971)	Crayfish ventral cord	28 per μ^2
Smith et al., (1970)	Lamprey spinal cord	8-17 per μ^2

Some of the values reported of the number of microtubules per axon or per μ^2 of axonal cross area.

These findings suggest that microtubules may be involved in the mechanism of axoplasmic transport, and that the ability of colchicine to interfere with this transport is due to its ability to bind with the proteins which form the microtubules.

Part VI-B METHODS ADOPTED TO INVESTIGATE AXOPLASMIC TRANSPORT

The Falck-Hillarp fluorescent method for catecholamines and the acetyl-thio-choline method for cholinesterase were used with that aim in the present research. As has been discussed in the Historical Introduction above, the accumulation of catecholamines or cholinesterase activity proximal to the site of a nerve crush is consistent with the hypothesis that such substances are being transported along axons, and the investigations of other workers have shown that these move in the fast range of flow. It was predicted, therefore, that colchicine, as used in the present investigations, would block such axoplasmic transport, and so cause a decrease in such accumulations of catecholamines or cholinesterase.

1. The Falck-Hillarp fluorescent method for catecholamines

The fundamental reaction involves a condensation of monoamines with formaldehyde to yield a tetra-hydro derivative, which in a second reaction catalyzed by proteins is dehydrogenated to its corresponding di-hydro compound. Apparently most of the native proteins possess this property. The di-hydro compound is in a pH-dependent equilibrium (maximum between 6 to 10) with their tautomeric quinoidal forms, which absorb strongly between 360 and 420 mµ and are thus the source of strong fluorescence. Such reactions are represented below:

Monoamine + formaldehyde -→ dihydroxy-tetrahydroisoquinoline

$\xrightarrow{-2H}$ dihydroxy-dihydroisoquinoline $\underset{\xleftarrow{}}{\overset{\text{pH 6-10}}{\xrightarrow{\hspace{1cm}}}}$ quinoidal compounds
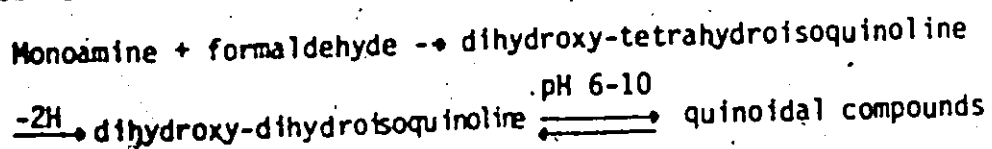
The condensation reaction above is given by 3 hydroxy-phenyl-ethyl

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amines like noradrenaline and dopamine, and also by indolyl-ethyl amines
like 5-hydroxytyrosine (5HT), and 5-hydroxytryptamine (5HTP). From the
histochemical point of view, it is of greatest interest that the bio-.
genic compounds closely related to catecholamines and 5-HTP, for in-
stance phenyl-alanine, tyrosine, tyramine, tryptophan, 5-hydroxy-indol
acetic acid, and deaminated 3-0-methylated metabolites of catecholamines
do not react at all or too little to be of any significance for the
histochemical localization of biogenic monoamines.

The most important variables in the fluorescent method for cate-
cholamines are the humidity of formaldehyde gas (generated from para-
formaldehyde), incubation time, and temperature. The histochemical
fluorescent method for catecholamines is not a quantitative one, owing to
local differences in concentration of the amines in different parts of
the neuron, and to differences from neuron to neuron.

(a) <u>Description of the method used in the present research</u>: The gen-
eral procedure involved the following steps: (1) freeze-drying, (2)
reaction with formaldehyde, (3) embedding, and (4) sectioning and
mounting. The specimen (see below) was frozen by rapid dipping into
liquid propane cooled by liquid nitrogen and the drying was performed <u>in</u>
<u>vacuo</u>, the latter step usually taking from 1 to 3 days. The critical
factor in the formaldehyde gas exposure is the water content of paraformal-,
dehyde generating the formaldehyde gas; the optimal humidity is between
50 and 97.5%, and to find the best condition required several trials. The
formaldehyde reaction was carried out in a well-closed jar containing 5 to
10g of paraformaldehyde at 80°C for 1 hour. After the formaldehyde
reaction, the tissue specimens were embedded in paraffin and longitudinal

sections 8 u in thickness were cut from the paraffin blocks. The sections were mounted on well cleaned glass slides and enclosed in Entellan containing a small amount of xylene to dissolve the paraffin. The sections were examined with a fluorescent microscope. Further details about the method can be consulted on Fuxe et al., (1970), or Falck and Owman (1965).

2. The acetyl-thio-choline method for cholinesterase

The behaviour of cholinesterase can be explained by postulating that the active site of the enzyme contains two subsites. The first of these is an anionic site which binds, and thus orientates the basic group of acetylcholine (ACh), and of related compounds. The second subsite containing a basic group is considered to react with the ester bond of ACh during the hydrolytic process. The formation of the enzyme substrate complex is the first stage in the proposed reaction which proceeds as follows:

$$ACh + enzyme \longrightarrow acetyl\text{-}enzyme + choline$$

$$Acetyl\text{-}enzyme + H_2O \longrightarrow CH_3\text{-}COOH + enzyme$$
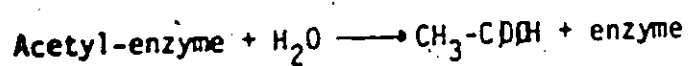
Acetyl-thio-choline (Ac-Th-CH) is hydrolyzed by cholinesterase at a more rapid rate than ACh itself. It is presumed that the increased rate of hydrolysis is due to the CO-S-linkage being weaker than the CO-O-linkage of ACh.

(a) Description of the Ac-Th-Ch method used in the present research: AcThCh is hydrolyzed by both "true" acetylcholinesterase and by non-specific cholinesterases. Acetyl-thio-choline iodide (20 mg) was dissolved in 10 ml of a stock solution containing copper sulphate (0.3g), glycine (0.375g), magnesium chloride (1g), maleic acid (1.75g), 1N NaOH (30 ml),

and a saturated solution of disodium sulphate (170 ml). Longitudinal frozen sections of the crushed nerve, 15 u thickness, were incubated in the above medium at 20^oC for 3 hours. After that time, the sections were rinsed in 2 or 3 changes of saturated Na_2SO_4 and immersed in a dilute solution of yellow ammonium sulphide. On hydrolysis of AcThCh the liberated ThCh presumably reacted with the copper salts to give a relatively insoluble product, i.e. copper thio-choline, which subsequently reacted with ammonium sulphide to yield copper sulphide. The latter appeared as dark brown spots. Thus sites of cholinesterase activity were stained dark-brown. For further details see Gomory (1952).

3. Description of the operations performed

Salamanders were anesthetized in 0.1% MS-222 as described previously (Part II-A4). An incision was made just in front of the ilium bilaterally, and a trough was made in the muscles until both nerves 16 were exposed and carefully freed from the surrounding connective tissue with a glass rod. The right nerve 16 was exposed to colchicine solutions (ranging from 0.025M to 0.1M) for 30 minutes; after that time, the excess solution was washed with 2 ml of amphibian Ringer. The left nerve 16 was exposed to amphibian Ringer only. One hour later both nerves 16 were crushed about 1 cm from the midline with a fine watchmaker forceps, the wounds were sewn up, and the salamanders were allowed to recover from anesthesia. Four days later the salamanders were anesthetized again with 0.1% MS-222 and both nerves were removed and processed either to demonstrate catecholamines or cholinesterase as described above. The portion of nerve 16 included about 1 cm on each side of the site of crushing.

Eight salamanders were used for demonstrating catecholamines and 6 sala-
manders for cholinesterase.

Part VI-C : <u>RESULTS</u>

1. <u>Observations on axonal transport of catecholamines</u>

In untreated nerves processed according to the Falck-Hillarp technique an accumulation of a strongly fluorescent material was observed proximal to the site of the nerve crushing (Figure 6-1). The fluorescence was present presumably, in unmyelinated axons of noradrenergic neurons. The accepted interpretation of this phenomenon is that catecholamines (CA) are continually transported from the neuronal body towards the periphery, and the disturbance of this transport system caused by nerve crushing resulted in the piling up of the fluorescent CA.

In nerves that were treated with 0.025M colchicine there was a considerable reduction in the amount of fluorescent material that accumulated proximally to the site of nerve crushing (Figure 6-1), but some fluorescence could still be observed. In nerves treated with 0.05M colchicine there was a further reduction in the accumulation of the fluorescent material, but a small amount of it could still be detected. Treating nerve 16 with 0.1M colchicine resulted apparently in a complete absence of the fluorescent material. In the photographs a certain amount of non-specific fluorescence can be seen. This is a characteristic phenomenon, and easily distinguished in the microscope because of its quite different colour appearance.

Conclusion

The results showed that in untreated nerves there was an accumulation of a strongly fluorescent material, i.e. catecholamines proximal to the site of nerve crushing, and this is consistent with the view that CA

FIGURE 6-1

Effect of acute application of colchicine solutions for 30 minutes
to nerve 16 (see Methods), on axoplasmic transport of CA. The proximal
end of the nerve is at the left in each preparation; the narrowed zone
the nerve indicates the site of nerve crushing. The untreated or contr.
nerve shows an accumulation of a strongly fluorescent material, i.e. cat-
cholamines, proximal to the site of nerve crushing. Such an accumulat.
decreased progressively when the nerve was treated with the various
different concentrations of colchicine shown in mM below each photomicro-
graph. The fluorescence proximal to the site of nerve crushing was neg-
ligible in the case of 100 mM (see text for description).

Control   Colchicine   25   50   100mM
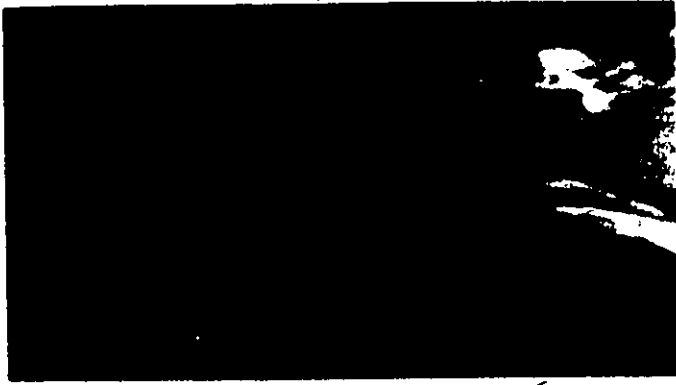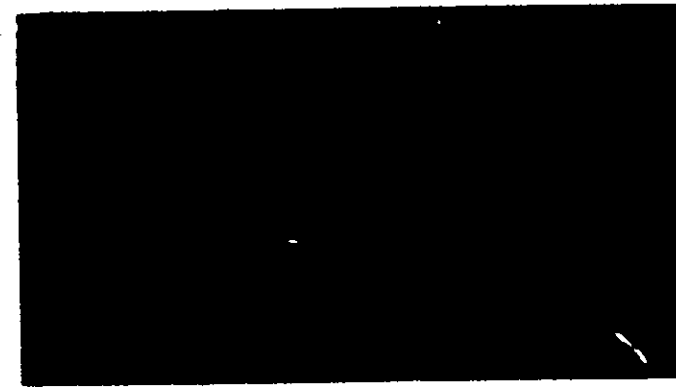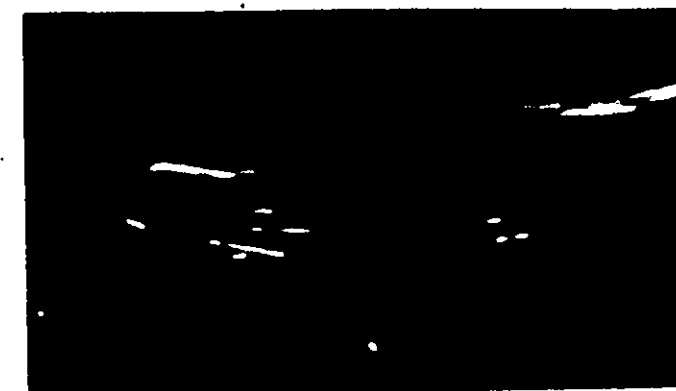
100μ

are continually transported along nerve axons, presumably sympathetic ones, and the piling up of CA resulted from the block of transport caused by nerve crushing. Colchicine, in doses ranging from 0.025M to 0.1M, caused a progressive reduction in the amount of fluorescent material which accumulated above the site of nerve crushing. With the highest dose (0.1M colchicine) the fluorescent material failed to accumulate at all. Thus, colchicine in a fairly dose-dependent manner, blocked the axoplasmic transport of CA and presumably, therefore, of other substances carried by the axonal transport system.

2. Observations on axonal transport of cholinesterase

In untreated nerves processed by the acetyl-thio-choline method for cholinesterase, the accumulation of a reaction product giving a deep brown staining was observed proximal to the site of nerve crushing (Figure 6-2). This staining, as discussed above, represents the presence of cholinesterase. As with the CA results, this indicates that cholinesterase is normally transported along nerve fibres in a proximo-distal direction, and the block caused by nerve crushing resulted in the accumulation of cholinesterase above the region of the crush.

Treating the nerve with 0.05M colchicine resulted in a considerable reduction in this accumulation of cholinesterase activity, as can be seen from Figure 6-2. The results of experiments in which various doses of colchicine were used showed that 0.025M colchicine was ineffective, and 0.1M colchicine was not significantly more effective than 0.5M, as observed with this technique. However, it should be noted that this test, even more than the fluorescent one for CA, was essentially qualitative, and in

FIGURE 6-2

Effect of acute application of 0.05M colchicine on the axonal transport of cholinesterase. The proximal end of the nerve trunk is at the left in each photomicrograph; the narrowed zone indicates the site of nerve crushing. The control or untreated nerve (lower picture) shows the accumulation of a dark-staining material proximal to the site of the nerve crush, indicating an accumulation of cholinesterase activity. Treating the nerve with 0.05M colchicine for 30 minutes resulted in a considerable decrease in this accumulation (upper picture).

200µ

this instance it would be over-interpreting the results to suggest that a dose-dependent effect of colchicine was detectable.

Conclusion

The acetyl-thio-choline method for cholinesterase showed an accumulation of a dark staining material, i.e. products of cholinesterase activity, above the site of nerve crush in control nerves. In nerves treated with 0.05M or 0.1M colchicine, for 30 minutes, 1 hour previous to crushing such accumulation was significantly decreased; 0.025M colchicine did not produce such effect. These data are interpreted as evidence of the blockade of axoplasmic transport of cholinesterase by concentrations of 0.05M colchicine or more, and presumably of other substances carried also by the fast axoplasmic transport system.

Part VI-D DISCUSSION ON THE HISTOCHEMICAL INVESTIGATIONS ON THE AXOPLAS-

MIC TRANSPORT OF CATECHOLAMINES AND CHOLINESTERASE

The most acceptable interpretation of the findings that there was

an accumulation of catecholamines and cholinesterase proximal to the site

of nerve crushing is that these substances are continually transported

from the cell body toward the nerve terminals. Similar results have been

reported by Dahlstrom (1971a) using the fluorescent method for catechola-

mines in rat sciatic nerve, and by Banks et al., (1971a) in the inferior

mesenteric ganglion/hypogastric nerve of rats using spectrofluorimetric

determinations for catecholamines. Likewise, Lubinska and Nimierko (1971)

using determinations of cholinesterase activity, detected an increase of

such activity at both ends of isolated nerve segments. These authors con-

cluded also that the accumulations of catecholamines or cholinesterase at

the site of nerve section was evidence of the axonal transport of such

substances.

In the present studies, the application of colchicine solutions

(0.03M to 0.1M) resulted in an approximately dose-dependent decrease in

the amount of catecholamines which accumulated proximal to the site of a

nerve crush, and although not so evident, there was some suggestion that

this applied also to cholinesterase.

It is conceivable, then, that the proximo-distal movement of other

substances carried by the axonal transport system was also blocked. From

our data it is not possible to calculate the transport rate for cate-

cholamines and cholinesterase; however, this has been reported in several

experimental models (see Part VI-A-6 and 7), and it is accepted that cate-cholamines and cholinesterase are both carried in the fast phase of axonal transport. It is inferred, therefore, that colchicine at doses ranging from 0.03M to 0.1M blocked the fast axoplasmic transport of materials in the right nerve 16 of salamander which received such a treatment. While these results do not show whether the slow (2 mm/day) axoplasmic transport of materials was also blocked by colchicine, pre-liminary experiments in this laboratory (Cooper and Diamond, personal communication) have shown that after treating right nerve 16 with ^{3}H-colchicine for 30 minutes, a wave of radioactivity moves down the axon at a rate of 1-2 mm/day. Such finding suggests that colchicine, by its known property of binding to microtubule protein (Davison and Huneeus, 1970) may have labelled the microtubule protein which was present in the region of nerve which was exposed to ^{3}H-colchicine, and that subsequently such labelled protein or perhaps even tagged microtubules moved along the axons with the bulk of axoplasm at a "slow" rate of 1-2 mm/day. Also, since the ultrastructural studies on colchicine-treated nerves (see Part V-B-4) showed that such treatment resulted in a significant decrease in the number of microtubules, it is conceivable that these structures are continually dissembling and reassembling, and that colchicine-bound microtubule protein is prevented from forming microtubules again (c.f. Rodriguez et al., 1968). Taken as a whole, the results support the suggestion that microtubules are involved in the mechanism of fast axo-plasmic transport, certainly of catecholamines and cholinesterase (Dahl-strom, 1971a). To summarize, the data presented in this section show that the colchicine doses used to bring about the increases in touch-receptive

fields of nerves neighbouring the treated one, were in the range which caused interference with the fast axoplasmic transport of materials in the affected nerve. The significance of this will be dealt with in the Final Discussion.

Part VII   EXPERIMENTS IN WHICH NERVE 16 WAS SECTIONED

AND NERVE 15 WAS TREATED WITH COLCHICINE

It was observed in a few individual experiments that after treat-
ing nerve 16 with colchicine, nerve 15 increased its sensory field of
innervation but nerve 17 did not. It seemed conceivable that colchicine
may have diffused to nerve 17 but not to nerve 15, and that the colchi-
cine which may have reached nerve 17 may have been causing another phenom-
enon to be revealed, namely that colchicine may inhibit nerves from
sprouting. That possibility seemed likely since the distance between
nerves 16 and 17 is shorter than the distance between nerves 16 and 15.
Also between nerves 16 and 17 there is only a loose connective tissue,
whereas between nerves 16 and 15 there is a considerable muscular mass.

In order to test the possibility that colchicine may have diffused
in significant amounts to nerve 17, a small group (n = 3) of salamanders
was prepared as if to receive a colchicine treatment of nerve 16 as des-
cribed in Part II-A-2, but instead of a colchicine solution, the nerve
was exposed to amphibian Ringer containing 0.01% methylene blue for 30
minutes. After that time, a careful dissection of the limb plexus was
performed. It was found that the tissues in the immediate vicinity of the
trough made to expose nerve 16 were stained with a deep blue colour.
Toward the place where the branches from nerves 16 and 17 joined to form
the hind limb plexus it was found in 2 out of the 3 cases that the
surrounding connective tissue had a very faint blue colour. However, no
such staining was observed in the vicinity of nerve 15.

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It was possible, therefore, that in some cases a small but indeterminate amount of colchicine may have reached nerve 17 rather than nerve 15. In order to test the possibility that colchicine might inhibit sprouting, experiments were performed in which nerve 16 was sectioned, and in addition, nerve 15 was treated with colchicine. Normally nerve 15 would respond by sprouting in such a situation. The experiments therefore tested the ability of colchicine, applied directly to nerve 15, to interfere with this response.

Part VII-A **METHODS**

Nerve 16 was sectioned and tied following a procedure identical to that described in Part II-A-2. A second medio-lateral incision was performed in front of the wound made to expose nerve 16, and a trough was made in the muscles so that nerve 15 was exposed, and then carefully freed of connective tissue using a glass rod. Nerve 15 was then bathed in 0.05M or 0.1M colchicine for 30 minutes. After that time, the excess solution was washed with approximately 2 ml of amphibian Ringer and both wounds were sewn up.

Part VII-B   RESULTS

1. Behavioural observations

These salamanders were observed during a period ranging from 12 to 23 post-operative days. It was observed that they showed either moderate (3) or severe (4) degree of limping, and in contrast to all other types of operated and/or treated salamanders, they never recovered a normal pattern of walking during this observation period. Furthermore, from Figure 7-1 it would seem that limping may indeed have increased with time, at least in some of these animals, since during the third week of observation these salamanders showed either moderate (3) or severe (4) degree of limping.

In all other groups, for instance salamanders in which nerve 16 was cut, some recovery from limping always was apparent from the 14th post-operative day onwards. These behavioural observations therefore strongly suggested that certain compensatory mechanism(s) were prevented from operating in the present group of animals. Clearly, this must be related to the colchicine treatment of the nerve adjacent to the sectioned one.

2. Mapping experiments

Salamander S-32 is an example from this group. The mapping was performed 14 days after section of right nerve 16 combined with treatment of right nerve 15 with 0.05M colchicine for 30 minutes. The areas of skin innervated by nerve 15 were almost identical on both sides, i.e. 212 mm^2 on the right side and 208 mm^2 on the left side. The surgical procedure

FIGURE 7-1

Behavioural observations on a salamander (5-10) in which right nerve 16 was cut and right nerve 15 treated with 0.05M colchicine for 30 minutes as described in Methods. Note that the degree of limping became worse at beginning of the 3rd post-operative week; by that time in salamanders in which only nerve 16 was cut, normal walking was restored even after an initial "severe" limping (compare Figures 2-8 and 2-9). The ordinate represents the degree of limping (see Methods) and the abscissa the post-operative period in days.

Limping

0 1 2 3 4

0 6 12 18 24 30 36 42 Days
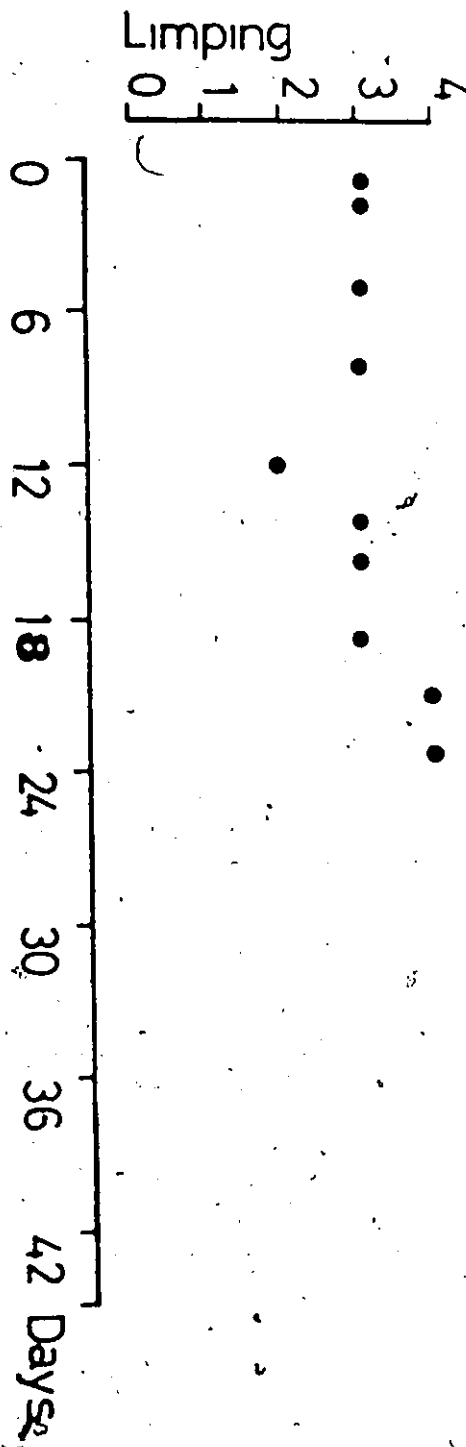
of cutting and tying the right nerve 16 had totally eliminated its field of innervation. Nerve 17 had increased its area of innervation on the right hind limb; it now innervated one toe more on the right side plus a slightly wider area of hind limb skin than did the left 17th nerve. The actual area innervated by the right 17 nerve was 408 mm^2, and the left supplied an area of 376 mm^2. No change was detected in the motor fields, except of course that the right nerve 16 did not innervate any muscle.

In this example, then, after partial denervation of the hind limb by cutting nerve 16, nerve 15 (which was treated with colchicine) did not enlarge its peripheral fields, while nerve 17 (untreated) did appear to have sprouted. This experiment strongly suggested that colchicine did indeed inhibit nerve 15 from sprouting in response to the adjacent denervation.

3. Statistical analysis

The data corresponding to the set of experiments in which nerve 16 was sectioned and nerve 15 was treated with colchicine has been assembled in Table 7-1, and has been plotted in Figure 7-2.

It is apparent from the data that the only significant change was an increase in the sensory field of right nerve 17 after section of right nerve 16 and treatment of right nerve 15 with colchicine. The colchicine treated nerve (15) did not increase its area of skin innervation.

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TABLE 7-1

CHANGES IN TOUCH-RECEPTIVE FIELDS

Nerve 15	Nerve 16	Nerve 17
-4.32 ± 3.68	-	25.84 ± 11.52*

CHANGES IN MOTOR FIELDS

0.0 ± 0.44	-	0.60 ± 0.40

Changes in peripheral nerve fields after section of right nerve 16, and at the same time treating right nerve 15 with colchicine. This set of experiments includes the results from 13 salamanders. The values given are the average (± SEM) of the difference of innervation fields between right and left nerves of any given pair. The sensory fields are given in mm^2 of innervated skin, and the motor fields as the number of muscle groups. Significance of the difference is indicated by * $P < 0.05$.
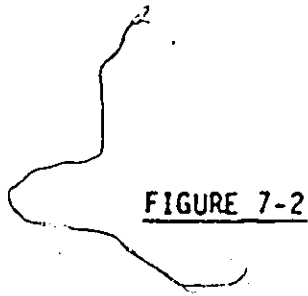
FIGURE 7-2

Effects of cutting right nerve 16 and at the same time treating
right nerve 15 with 0.1M colchicine for 30 minutes on the size of peri-
pheral innervation fields. In (a) ordinate shows the difference in the
size of touch-receptive fields in mm^2 of skin (right side minus left),
and in (b) the difference (right minus left) in the number of muscle
groups innervated. The nerves are identified by their respective numbers.
The height of the bars represents the average ($\pm$ SEM) of such values;
the broken lines indicate that the fields of right nerve 16 were present.
Significance of difference is indicated by * $P < 0.05$. Compare this Figure
with Figures 3-11 and 4-7.
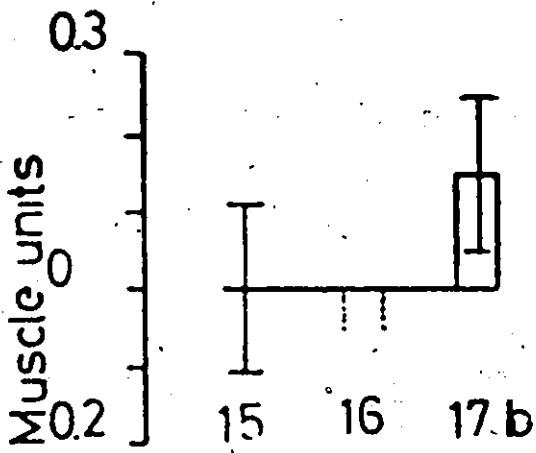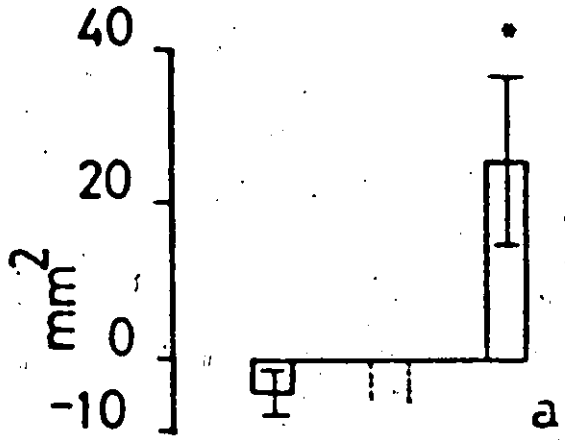
Part VII-C   DISCUSSION

Colchicine prevented the increase in field size of nerve 15 which normally would be found after section of nerve 16. A similar prevention of sprouting of nerves in response to the stimulus provided by adjacent denervation was made by Hoffman (1952). Related findings have been made in the goldfish (Grafstein, 1967, 1971; Grafstein and Murray, 1969). In the optic nerve the rate of axoplasmic transport of proteins is normally 0.4 mm/day ("slow") and this rate increases 2 or 3 times by the 8th day after optic nerve section, when the regenerating axons reconnect with the tectum. Presumably this increase is important in providing materials for laying down new axoplasm. These workers found that a dose of 6 ug of colchicine applied intracranially (about 0.2M) was able to abolish both the nerve outgrowth, and to decrease the rate of axoplasmic transport below normal levels. Surprisingly, with a dose of 3 ug intracranially, outgrowth was not blocked in any of the 5 animals examined, but axoplasmic transport rate was reduced in 3 out of 5 animals to about the same degree as with the 6 ug dose. It would have been interesting to have known for how long outgrowth could continue in the absence of supply of materials transported by axoplasmic flow.

The conclusion from these studies is that colchicine, in addition to creating a situation whereby adjacent untreated nerves sprout, also prevents sprouting of a treated nerve in response to section of a neighbouring nerve. This will be referred to again in the Final Discussion.

Part VIII   EXPERIMENTS INVOLVING PARTIAL DAMAGE TO NERVE 16

As has been shown, the procedures both of complete section of
nerve 16, and of treatment of nerve 16 with colchicine, caused nerves
15 and 17 to increase their cutaneous fields of innervation, presumably
by collateral sprouting. The morphological and electrophysiological
investigations showed that the particular colchicine treatment used did
not cause Wallerian degeneration of the treated nerve. It was postulated
that the increases of peripheral nerve fields was related to the blockade
of axoplasmic transport of materials which certainly occurred in the
affected nerve, and which presumably included a "trophic" substance
normally produced in the neuronal soma and continually supplied to the
periphery.

This "trophic" factor then would be concerned with the regulation
of nerve fields, and when the supply of this factor is reduced, adjacent
nerves sprout and invade the territory of the treated nerve. It can be
argued, however, that while there was no obvious Wallerian degeneration
in the nerve trunk of the colchicine-treated nerve, perhaps some degen-
erative changes may have occurred at nerve terminals; results which could
be interpreted in this way have been reported in the visual system of
pigeons after injection of colchicine by Cuenod et al., (1972). This
postulated degeneration could be the cause of the sprouting in the adjac-
ent intact fibres.

These considerations suggested the need to do experiments in which
some of the fibres of nerve 16 would be deliberately damaged, and the

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resultant changes, if any, in the peripheral nerve fields compared with those found after the experimental procedures already studied. A group of 6 salamanders was studied, therefore, at the 3rd post-operative day, when sprouting has not yet occurred, and another group of 8 salamanders between the 12th and 22nd post-operative days (14 days on the average) when Wallerian degeneration would be expected to be well advanced, and collateral sprouting, if any, well established.

Part VIII-A   METHODS

A medio-lateral incision was performed just in front of the right ilium, the muscles in front of the bone were carefully dissected with watch-maker forceps until spinal nerve 16 was exposed and the nerve trunk was then carefully freed from the surrounding connective tissue using a glass rod. The nerve was then deliberately damaged in one of 2 ways: (1) by tying a thread loosely around it until obvious damage was apparent, or (2) a partial section of the nerve was performed, in which 30-50% of the diameter of the nerve trunk was sectioned.

These operations were performed after anesthetizing the salamanders with 0.1% MS-222; the wound suturing and post-operative care of the salamanders was identical to that described previously in Part II-A-2.

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Part VIII-B   RESULTS

1. Behavioural observations

        During the first 3 post-operative days most salamanders (11 out
of 14) presented slight (2) degree of limping; of the remaining animals,
one did not limp at all, one presented only minimal limping, and the
third, a moderate or (3) degree of limping. By the 5th post-operative
day those animals which were kept for 14 days mostly showed minimal (1)
limping and after the 12th post-operative day only minimal (1) or no
limping at all. An example from the latter experimental group is shown
in Figure 8-1, and it is apparent that this salamander showed a faster
recovery from moderate to minimal limping over the 1st post-operative
week than salamanders in which nerve 16 was completely cut and tied (com-
pare with Figures 2-8 and 2-9); also by the 18th post-operative day had
recovered normal walking.

2. Mapping experiments

    (a) Individual examples: Salamander (D-8) is an example of the group
examined on the 3rd post-operative day. Both sensory and motor fields of
nerves 15 and 17 were bilaterally symmetrical. Right nerve 16 which
underwent section of approximately 40% of the diameter of its nerve trunk[4]
showed right sensory and motor fields which were markedly smaller than
those on the left, unoperated side. On that side, nerve 16 innervated the
whole skin of the hind limb. On the right side nerve 16 innervation was
absent from an area comprising the posterior third of calf skin and the

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FIGURE 8-1

Behavioural observations in a salamander (D-1) in which about 50% of the 16th nerve trunk was sectioned. Note that during the first post-operative week there was a dramatic recovery from "moderate" to "minimal" limping, and that at the 18th post-operative day the animal had recovered normal walking. Compare this result with a typical one from a salamander in which right nerve 16 was completely cut and tied (Figures 2-8 and 2-9). Ordinate represents degrees of limping, and abscissa, post-operative period in days.
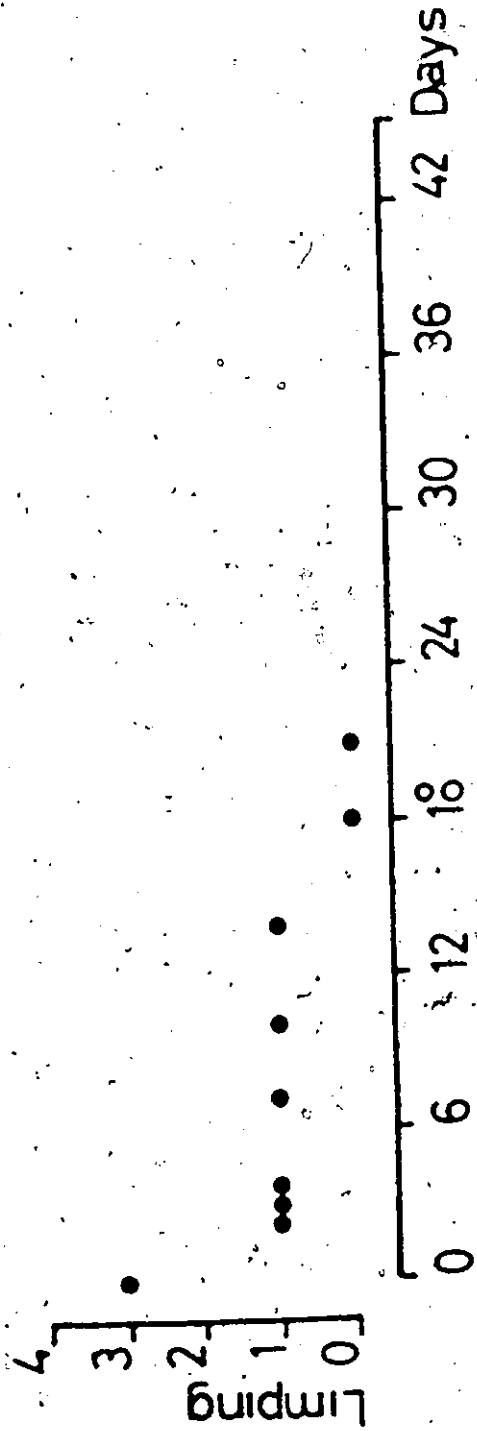
4th and 5th toes. This amounts to approximately 20% of the area inner-
vated by the control side. On the motor side, left nerve 16 innervated
all 7 muscles that were sampled, whereas right nerve 16 innervated only
4 of them. This is equivalent to a loss of 43% of the muscle groups
innervated by the control side.

It is evident from this example that at the 3rd post-operative
day after partial section of right nerve 16 there was a considerable de-
crease in the size of the peripheral fields of innervation of the
partially damaged nerve.

Salamander (16-83) is one example from the group of experiments
performed between 12 and 22 days after partial damage of nerve 16. In
this salamander about 50% of the diameter of the nerve trunk was sectioned.
This example is particularly illustrative because nerve 15 on the
unoperated side did not innervate any toes at all, which falls into the
category of "small" touch-receptive field of innervation of nerve 15. It
would be expected, therefore, that this salamander would have shown a
clear cut increase in the area of skin innervated by nerve 15, if the
latter would have sprouted. A comparison of the mappings of right and
left sides, however, showed that neither nerve 15 nor nerve 17 had
increased their areas of cutaneous innervation. Right nerve 16 showed an
area of skin innervation that was 28 mm^2 smaller than that of the left
side, which may indicate a small reduction in its touch-receptive field.
The motor fields of innervation were identical on both sides for all
given pairs of spinal nerves.

These experiments showed that if nerve 16 was partially damaged,
it appeared to regain its field of innervation, while the adjacent nerves

(15 and 17) did not take over the initially denervated territory.

(b) Statistical analysis

The data concerning the groups of experiments in which nerve 16 was deliberately damaged has been plotted in Figures 8-2 and 8-3.

It can be seen from these data that at the 3rd post-operative day after partial section of nerve 16 there was a significant decrease in both its touch-receptive and motor fields; the adjacent nerves, however, did not show any significant changes at that time. By the 14th post-operative day, however, nerve 16 had recovered symmetrical fields of innervation, and the difference between right and left sides was not significant (level of significance $P > 0.05$), neither in the motor nor the sensory fields. Only the touch-receptive field of nerve 15 showed a small though significant ($P < 0.05$) increase in the size of its field. On the motor side, at 14 days (on the average) after partial section of right nerve 16, all three nerves innervating the hind limb showed bilaterally symmetrical fields of innervation.

FIGURE 8-2

Effects of partial damage to right nerve 16 on the size of touch-
receptive fields of hind limb nerves. (a) shows the changes found at the
3rd post-operative day (n = 6), and (b) the changes found at the 14th post-
operative day (n = 8). It is apparent that the nerve lesion caused a con-
siderable decrease initially in the touch-receptive field of right nerve
16, but that this had recovered its normal size by the 14th post-operative
day. On the other hand, nerve 15 showed a small though significant
increase in the size of its touch-receptive field at the 14th post-
operative day. even more important, 16th nerve regained a bilateral
symmetry of its peripheral nerve fields. Ordinate represents the differ-
ence in area of skin (mm^2) innervated between right and left nerves; the
bars represent the average ($\pm$ SEM) of such values, and nerves are identi-
fied by their respective numbers. Difference of significance is indicated
by $\cdot$ P <0.05.

mm^2

a

b
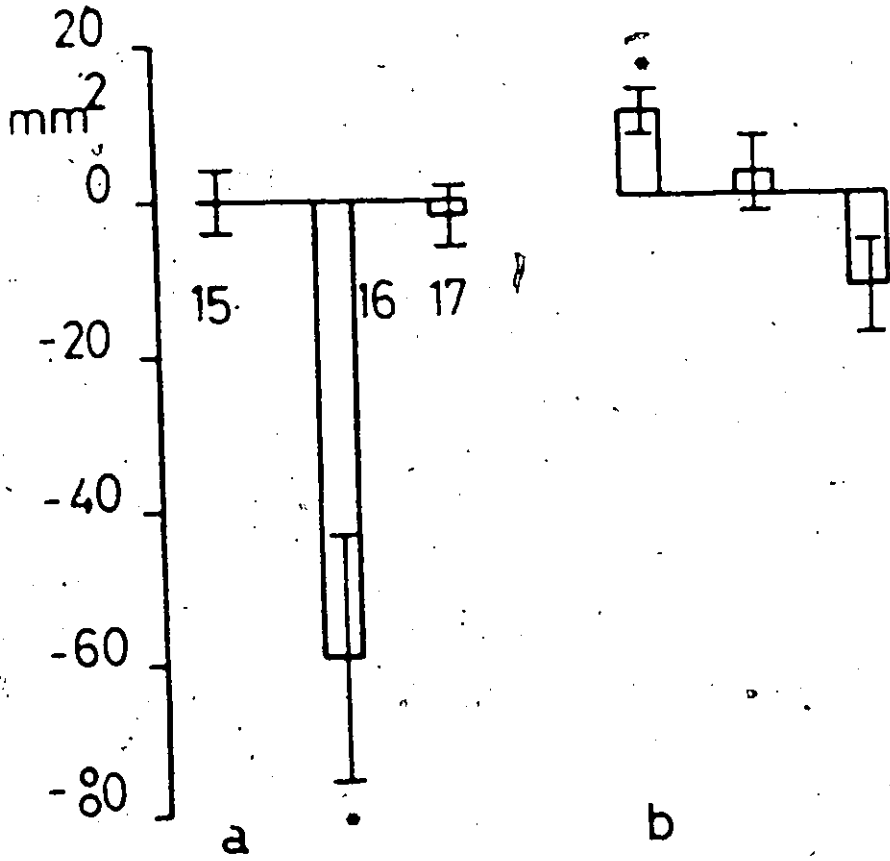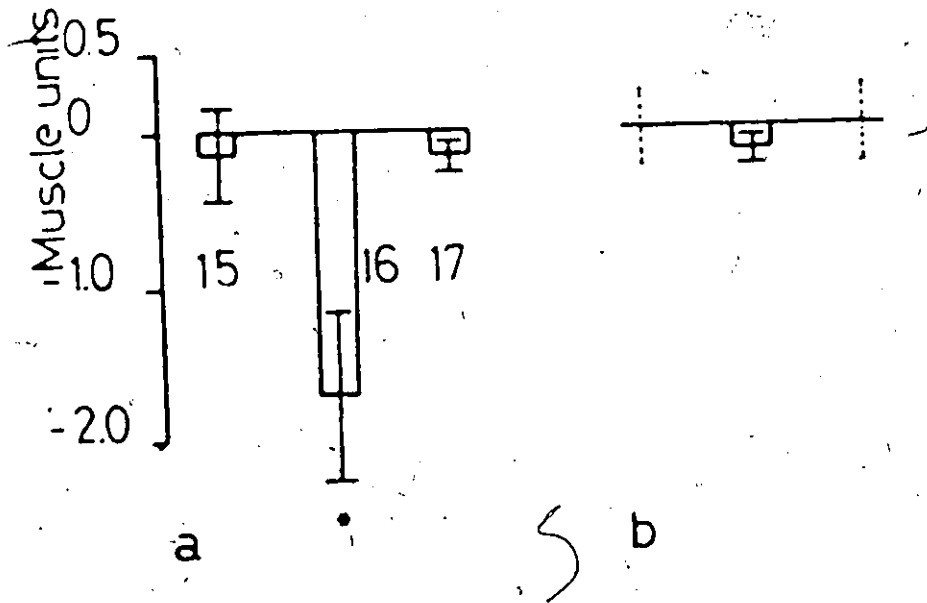
FIGURE 8-3

Effects of partial damage of right nerve 16 on the size of motor fields of innervation of hind limb nerves. (a) shows the changes found at the 3rd post-operative day (n = 6), and (b) the changes found at the 14th post-operative day (n = 8). It is apparent that the nerve lesion caused a considerable decrease in the motor field of innervation of right nerve 16 initially, but by the 14th post-operative day the pattern of motor innervation was similar to that of unoperated salamanders. Ordinate represents the difference in the number of muscle groups innervated between the right and left nerves; the bars represent the average ($\pm$ SEM) of such values, and nerves are identified by their respective numbers. The broken lines indicate that the average ($\pm$ SEM) had a value of zero. Difference of significance is indicated by * $P < 0.05$.

Part VIII-C   DISCUSSION

A comparison of the results obtained after complete section of nerve 16 with those obtained after elimination of an estimated 30-50% of its fibres showed striking differences. As was described in Parts III and IV, after complete section of nerve 16 (which was prevented from regenerating because a tie was applied to the proximal stump) both nerves 15 and 17 increased their cutaneous and motor fields of innervation; these changes were of considerable magnitude and highly signifi-cant. Also the pattern of changes in the cutaneous peripheral fields of innervation were very similar after complete section of nerve 16 or after treating nerve 16 with cholchicine. On the other hand, after partial elimination of nerve 16, only the sensory field of nerve 15 showed a moderate increase in its area of innervated skin. Of the great-est significance is that total section of nerve 16 (with tying of the proximal stump) caused a continued total loss of its peripheral motor and sensory fields, while partial elimination of 30-50% of its fibres, though causing an initial significant decrease in the peripheral fields, was followed within 2 weeks by virtually complete recovery of the normal motor and sensory innervation pattern.

This would imply then, that the factors responsible for the appor-tioning of territory may be qualitatively different for each nerve, which would allow a preferential matching between source of the nerve ending and target organ. In this respect, relevant observations have been made in A. mexicanum by Cass et al., (1973) in which a denervated muscle group

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received innervation from adjacent nerves in a "patchy" fashion; However, when the regenerating fibres reached the partially denervated area, the "patches" of foreign innervation diminished in size and eventually disappeared.

Similar changes have also been observed in the superior cervical ganglion of the cat after partial section of a number of the pre-ganglionic branches (rami communicantes T1-T3) by Guth and Bernstein (1961). In that case, the intact pre-ganglionic fibres (r.c.T4) sprouted and formed functional connections, since now electrical stimulation of these pre-ganglionic fibres evoked pupil-dilatation on the partially denervated side, but not on the control side. When the axotomized pre-ganglionic fibres were allowed to regenerate, the collateral sprouts from the undamaged pre-ganglionic fibres apparently underwent regressive changes, since electrical stimulation of their pre-ganglionic branches (r.c. T4) did not evoke anymore a pupil dilatation response. On the other hand, pupil dilatation was evoked when the regenerated fibres (r.c. T1-T3) were stimulated, i.e. regained control over those ganglion cells mediating pupil dilatation.

There are two possible explanations of these results, both of which, however, require that in the face of competition from nerve 16 sprouts, the potential sprouting and take-over by nerves 15 and 17 is apparently not realized: (1) the cut and damaged fibres of nerve 16 regenerate to innervate their original target areas; or (2) the intact remaining fibres of nerve 16 sprout and take over the full original fields of nerve 16. Possibility (1) is unlikely, since in similar experiments Stirling (1970a) found that by the time regeneration in the

periphery had occurred, the fields of the adjacent nerves 15 and 17 had already enlarged to fill the region denervated by the procedure of crushing the nerve 16. It seems, therefore, that in the present experiments, the intact fibres of nerve 16 sprouted in the periphery, and successfully prevent the neighbouring nerves from forming sprouts. It is conceivable that the latter sprouts were not even developed, or alternatively they never became functional. .

To summarize, the pattern of changes observed in the size of peripheral fields after colchicine treatment and after _partial_ section of nerve 16 are so different that it seems unlikely that the collateral sprouting after colchicine could be attributed to a degeneration of nerve fibres equivalent to surgical section of about one half of the axons of the nerve trunk. However, an ultrastructural study of sensory nerve endings and endplates after colchicine-treatment needs to be done in the future.

Part IX   <u>FINAL DISCUSSION</u>

The investigations described in this thesis have shown that acute
application of colchicine solutions to the principal nerve of the
salamander hind limb mimics the effects of cutting that nerve, in that
the adjacent nerves increased the size of their touch-receptive fields;
this was assumed to occur by collateral sprouting (see Part III). This
response was not due to a functional deficit (Part II) or to Wallerian
degeneration in the treated nerve (Part V). The colchicine treatment
caused a significant decrease in the axoplasmic transport of catechola-
mines and cholinesterases (Part VI), and presumably of other substances
carried by the fast phase of axoplasmic transport.

Part IX-A   THE NEUROTROPISM HYPOTHESIS IN RELATION TO COLLATERAL

SPROUTING

Taken as a whole, these experimental findings are consistent with the concept that trophic factor(s) are continually supplied to the periphery by means of fast axoplasmic transport system; their role would be in the regulation of the size of peripheral nerve fields. This hypothesis, of course, rests on circumstantial evidence, but offers a reasonable explanation of the known facts, and it is amenable to further experimentation. It is attractive also in that it is consistent with an explanation for the initial development of epithelial nerve fields in the embryo which was originally proposed by Ramon y Cajal (1919).

Outstanding features of the phenomenon of collateral sprouting are its local nature, and the early contact which can sometimes be established between the outgrowing nerve collaterals and nearby cordons of proliferating Schwann cells (Edds, 1953). These observations suggest (see for instance Hughes, 1968) that Schwann cells may release a substance that stimulates nerve growth, and which would direct nerve sprouts toward the target tissues; it seems that at least the stimulus for sprouting must be of a chemical nature; the direction of subsequent growth, however, can in many instances also be explained by a contact guidance phenomenon (Weiss, 1955).

Since collateral sprouting is a self-limiting process, it is conceivable that nerve sprouts upon contact with proliferating Schwann cells or denervated tissues "turn off" the stimulus-producing mechanism(s).

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(see for instance Edds, 1953). The nature of the mechanism(s) which control the start and end of collateral sprouting are still highly hypothetical. However, the slime molds (Dictyostelium sp.) which aggregate and form a sporophore under the action of cAMP might be a relevant model in this regard (Bonner et al., 1969). It is conceivable that the mechanisms which have been proposed to explain the development of the pattern of cutaneous innervation in the embryo, (Cajal, 1919), may persist in adult animals. In that case, sprouting after partial denervation would come to an end because the effect of a hypothesized stimulant substance released by the target tissues becomes neutralized by factors released from the nerve fibres; when a critical equilibrium is reached between these two substances no further nerve growth would occur, and the state would then correspond to the appropriate density of innervation needed for normal behaviour.

There is also an alternative hypothesis that the observed increases in peripheral fields may be due not to collateral sprouting, but to the emergence of function in nerve endings which were there from the beginning, but in a non-functional state (see Part I). However, this hypothesis rests on indirect evidence; in the recent experiments of Mark and his collaborators for example, the critical experiment of studying the neuromuscular transmission (of the presumed functionless endplates) under appropriate conditions of nerve stimulation was not performed. Their experiments are thus inconclusive, although consistent with a very interesting hypothesis. For that reason, I favour the interpretation that increases in nerve fields after partial denervation are due to

collateral sprouting, since the latter phenomenon has been actually and unambiguously demonstrated by histological methods (Edds, 1950; Weddell et al., 1941). As mentioned in Part VIII-C, Mark's findings, as well as the present ones, that after partial damage of nerve 16 the intact fibres of the same 16th nerve reinnervate the denervated area in preference to nerves 15 and 17, can be interpreted on the basis of competition for appropriate end-sites; inappropriate or foreign nerve endings are "disallowed". Mark's interpretation requires the additional assumption that the foreign nerve endings do reach appropriate locations but remain non-functional, although morphologically indistinguishable from normal nerve endings.

THE CONCEPT THAT "TROPHIC EFFECTS" MAY DEPEND ON NERVE SUPPLY

The observation that after nerve section the distal stump of the nerve and the denervated organs underwent degenerative changes (for a review of the early literature see Cajal, 1928), suggested that the neuronal body was a "trophic centre" which supplied the periphery with substance(s) which were essential for maintaining the normal structure and function of axonal processes and effector organs. The results of the present investigation give a striking support for such a concept. The following examples of "neurotrophic influences" lend further weight to the suggestion that trophic factors transported along axons may be involved in so-called "trophic" effects.

1. Limb regeneration in lower vertebrates

Lower vertebrates like newts, regenerate amputated limbs. Singer (1952) observed that very early on, nerve fibres established contact with the regenerating blastema and invaded the overlying skin. However, regeneration did not occur in limbs which were denervated at the time of amputation. Also, when denervation was performed at early stages of regeneration, the latter process ceased, and resumed only if regenerating fibres were allowed to reinnervate the limb. Limb regeneration was initiated and maintained by either motor or sensory fibres, which indicate that such fibres do not differ in their "trophic" capabilities in this situation (Singer, 1952). Similar phenomena were also observed in newts in which ventral and dorsal roots had been cut (Sidman and Singer, 1951),

showing that central connections are not necessary for the manifestation of this trophic function. Limb regeneration does not normally occur in frogs, e.g. Rana sp.; it does, however, when extra nerves are brought into the amputation site, or in those anurans, i.e. Xenopus, which have a larger amount of axoplasm per cross-sectional area of the limb. (Rzehak and Singer, 1966; Singer et al., 1967). These results strongly suggested that the axons supply the peripheral tissues with a factor which induces limb regeneration. It should be noted, however, that limb regeneration does occur in limbs which had never had an innervation, i.e. aneurogenic limbs (Yntema, 1959). This result suggests that such limbs retain a capability of producing growth factors, or have a lower threshold to normally present ones emanating from other tissues. When an aneurogenic limb is allowed to acquire an innervation for the first time, and 10 days later is amputated, limb regeneration still occurred in about 75% of cases; however, no regeneration was observed when amputations were performed after the 13th day of innervation (Thornton, 1968). These results indicate that over the short period of a few days, the target tissues become nerve dependent insofar as limb regeneration is concerned. It seems, therefore, that nerves produce a trophic substance which induces limb regeneration, and in addition, nerves may suppress in the target tissues their ability to produce or respond to other growth promoting factors.

2. The ontogenetic development of muscle spindle

In the hind limbs of rats, the first differentiation of muscle spindle was observed at the 19th day of gestation, and morphogenesis was

completed by the 25th day after birth. Although proprioceptive fibres are observed at the 19th day of gestation, motor nerve endings are not usually apparent until the 5th day after birth in the hind limb muscles e.g. soleus. When nerve ischiadicus was sectioned in 19-20 day old rat fetuses (Zelena, 1957) differentiation of muscle spindles did not occur in soleus muscle; this finding suggested that differentiation of muscle spindles depends upon an influence exerted by the proprioceptive nerve fibres.

3. Trophic effects on dendritic organization

One of the main afferent inputs to the rat pre-piriform cortex arises from cells in the olfactory bulb. Section of the latter resulted in a significant reduction in the density of the dendritic network of pyramidal neurons, particularly in causing a decrease among dendritic branches of higher orders, without any obvious reduction in the number of primary dendrites (Jones and Thomas, 1962). It seems, therefore, that deafferentation leads to atrophy of the dendritic tree. However, lack of function may also play a role, since rabbits reared in complete darkness from birth showed morphological changes in the dendritic spines of neurons of the visual cortex, (Globus and Scheibel, 1967), and in rats reared under similar conditions, there was a significant reduction in the number of dendritic spines in the apical dendrite of pyramidal cells of the visual cortex (Valverde, 1967).

4. Trophic effects on taste buds

In mammals and fishes, nerve section results in the disappearance of taste buds, which re-differentiate when regenerating fibres of the

sectioned nerve reinnervate the papillae of the tongue (Oakley and Benjamin, 1966). In rats, fungiform papillae (anterior end of the tongue) are innervated exclusively by the chorda tympani nerve, the vallate papilla by the glossopharyngeal ner.e, and taste-buds of the folliate papillae by either one or the other of these nerves (Zalewski, 1969b). When these nerves are cut and the proximal stumps are cross sutured with the distal stumps, taste-buds reappear (Zalewski, 1969a). However, no taste-buds are found following reinnervation of the anterior or posterior end of the tongue with predominantly sensory non-gustatory nerves (lingual), motor (hypoglossal), or mixed, e.g. myohyoid) (Guth, 1958; Zalewski, 1969b). These results indicate that in mammals only nerves which contain gustatory fibres induce the formation of taste buds in denervated papillae. However, in newts when the tongue is transplanted to the orbit, taste buds disappear within 3 weeks, and subsequently reappeared (Poritsky and Singer, 1963), which indicates that in amphibians non-gustatory nerves can exert this trophic function on the tongue epithelium. Moreover, when the tongue is transplanted to the liver in newts, taste buds were found in the absence of any demonstrable innervation (Wright, 1964); it should be noted, however, that the liver is an organ which possesses a remarkable capacity of regeneration, even in mammals (IRP Bull., 1969). Nevertheless, these results indicate that the trophic factor(s) necessary for inducing and maintaining taste buds are not exclusively produced by gustatory nerves in newts, in contrast to the situation in mammals.

5. Trophic effects of nerve on muscle

The trophic effects of nerve on muscle have received considerable attention over the past years, reviewed recently by Harris (1974). It seems that in many experiments it is difficult to distinguish effects which could be attributed to the lack of a presumed trophic factor, with those due to disuse of the muscle. The most important advance in this field, and relevant to the present studies, has been the demonstration that blockade of axoplasmic transport using either colchicine or vinblastine resulted in phenomena which are also observed after nerve section, such as acetylcholine supersensitivity (Hoffman et al., 1972; Albuquerque et al. 1970, 1971, 1972), TTX-resistant action potentials (Albuquerque et al., 1972; Cangiano, 1973), and lowered membrane resting potential (Albuquerque et al., 1972); in these experiments neuromuscular transmission was retained, although muscular atrophy was observed in some cases. However, (Cangiano, 1974; Lømo, 1974) the situation is still uncertain since colchicine may act directly on the muscle membrane to cause these changes, independently of its action in blocking axoplasmic transport.

Part II. THE POSSIBLE ROLE OF TROPHIC FACTORS IN COLLATERAL SPROUTING

It is apparent from the list of examples considered in Part 1 that collateral sprouting is a phenomenon of widespread occurrence in response to partial denervation, except for possible in a few cases noted for the CNS (see Part I-C-3(a)). Collateral sprouting could be considered as a mechanism of evolutionary advantage for the animal, in that it would allow compensation after traumatic losses of innervation. In female rats the peripheral fields of the pudendal nerve have been observed to change in response to injections of estrogens (Komisaruk et al. 1972). This suggest that collateral sprouting may occur in healthy adult animals as a means of controlling peripheral nerve fields according to functional states of the whole animal, in this particular example, hormonal levels. This idea is further strengthened by the observation that a small percentage of motor nerve endings (Baker and Ip, 1966) as well as sensory ones (Tello, 1932; Fitzgerald, 1961, 1962) have been observed to undergo obvious degenerative changes in normal animals including humans, while nearby axons emitted sprouts; these findings suggest that normally there is a slow and continual degeneration of some nerve endings which are replaced by sprouts from nearby healthy axons.

Moreover, possibly "adaptive" changes in the CNS such as, the shortening in reflex latency between salamander nerves 15 and 17, when the former nerve increases the size of its sensory field after section of nerve 16 (Stirling, 1973) may involve collateral sprouting in the CNS.

This kind of result suggests in general that neurons which have a larger afferent input may acquire a greater control over central neuronal circuitry, possibly by <u>central</u> sprouting. It is a matter of speculation, but it can be suggested that learning, in the sense of acquiring knowledge by experience, may involve similar mechanism(s).

According to the hypothesis proposed to explain the findings of the present research, reduced axoplasmic transport disturbs the equilibrium between a presumed stimulant substance released by the target tissues and a trophic nerve factor which would be inhibitory to any further nerve growth; the result is the appearance of a signal for sprouting. Such a stimulant substance may act locally on nerve endings, or it may be picked up by nerve terminals, transported in retrograde direction to the neuronal body where it would trigger the events leading to collateral sprouting. The experiments in which colchicine-treated nerve 15 did <u>not</u> increase its touch-receptive field in response to the stimulus provided by section of the adjacent nerve 16, may be interpreted as evidence of colchicine blocking the retrograde axoplasmic transport, and hence the signal to sprout. This suggestion is supported by observations in which the chromatolytic responses observed in cockroach motoneurons after section of the motor nerves (Pitman <u>et al</u>., 1972) or in ciliary ganglion of chickens after axotomy of the post-ganglionic fibres (Pilar <u>et al</u>., 1972) was mimicked by local application of colchicine; in these cases it seems that colchicine blocked the retrograde transport of a substance which normally would prevent the chromatolytic reaction.

An estimate of the minimum rate of transport of the postulated nerve trophic factor can be made, assuming that sprouting starts after

all substance contained in sectioned (or treated) nerves has flowed to terminals and been effective there (Miledi and Slater, 1970; Harris et al., 1972). Since collateral sprouting was observed as early as the 5th day after colchicine treatment, and the distance between the site of drug application and the tip of the toes ranges from 30 to 50 mm, the neurotrophic factor(s) can be estimated as being transported at a rate of about 10 mm/day. Measurements of the rate of transport of ^{3}H-labelled material in hind limb nerves of salamander have been found to be 35-40 mm/day for the fast transport system, and 0.5-1.0 mm/day for the slow flow at 20°C (Cooper, Diamond, Fried and Turner, personal communication) slow flow is usually of this value in most animals (see Table 6-1). Thus it seems that the findings are consistent with the assumption that the neurotrophic factor(s) may be carried by the fast axoplasmic transport system.

Finally, experiments like those of Cuenod et al., (1972) in which intraocular injection of colchicine did not provoke impairment in the conduction of nerve impulses in the optic nerve but caused early degenerative changes in the optic nerve terminals raises the question of whether in the present studies colchicine may have caused degeneration in some terminals in such a way that the total area innervated by nerve 16 did not differ significantly from that of untreated nerve 16, but that the density of nerve 16 endings was decreased. Preliminary experiments in this laboratory (Cooper and Diamond, personal communication) would indicate that this phenomenon did not occur after colchicine treatment.

In conclusion, the results of the present research are consistent with the hypothesis that neurotrophic factors carried by fast axoplasmic

transport are concerned with the regulation of the size of peripheral nerve fields. On the other hand, they are inconsistent with the hypothesis which assumes that collateral sprouting depends on the release of products of nerve degeneration, or functional loss due to absence of nerve impulses, or products of Schwann cell reaction, etc. Since afferent nerve impulses travel in the opposite direction to that of proximodistal axoplasmic flow, and it is hard to imagine for the skin something analagous to disuse atrophy observed in muscle, it may be that the cutaneous nerves of salamander constitute the best example for the existence of neurotrophic influences of nerve, probably involving the (fast) axoplasmic transport of the hypothesized trophic factors.

Part X   SUMMARY

1.   In the present research, the effects of colchicine-induced block
of axoplasmic transport have been compared with those of partial dener-
vation on the peripheral side of nerves (15, 16 and 17) innervating the
hind limb of salamanders.

2.   The electrical responses in nerves and muscles to either mechan-
ical stimulation of the skin, or electrical stimulation of the nerve,
showed that both the touch-receptive and motor fields of hind limbs of
normal salamanders were bilaterally symmetrical. Therefore, changes in
such symmetry could be ascribed to the experimental procedure performed
on the treated side.

3.   In vitro experiments showed that acute application of colchicine
solutions (up to 0.1M) for 30 minutes to a nerve trunk did not provoke any
immediate impairment in its ability to conduct nerve impulses, although
higher doses (0.2M) did. Doses higher than 0.1M, therefore, were not
used in the following experiments.

4.   Both after total section or colchicine treatment of nerve 16, the
size of the touch-receptive fields of the adjacent nerves 15 and 17 were
significantly increased. In both cases the earliest detectable increase
occurred at the 5th post-operative day, and the effect was maximal by the
8th to the 10th post-operative day. The colchicine effect was dose-
dependent.

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Similar increases in motor fields were significant only after nerve section, but not after colchicine treatment, although some individual experiments strongly suggested that increases in motor fields may also occur in colchicine-treated salamanders. The increases in peripheral nerve fields have been attributed to collateral sprouting.

5. Section of right nerve 16 resulted in an immediate severe functional deficit. e.g. severe limping; however, normal walking recovered on the average by the 5th post-operative week. Colchicine- or amphibian Ringer-treated salamanders showed only minimal or no limping at all.

6. Morphological and electrophysiological studies showed that the acute application of colchicine solutions did not provoke subsequent nerve degeneration, and the size of the peripheral fields of the treated nerve were unaffected.

7. Colchicine at doses which provoked collateral sprouting (0.03M to 0.1M) diminished significantly the axoplasmic transport of catecholamines and cholinesterase, as shown by histochemical methods, and reduced significantly the number of axonal microtubules.

8. A similar application of colchicine to nerve 15 prevented it from sprouting in response to the stimulus provided by section of the adjacent nerve 16.

9. These findings are consistent with the hypothesis that nerve terminals are continually supplied by fast axoplasmic transport with a

trophic factor concerned with the regulation of the size of peripheral nerve fields. When this supply is reduced, either by nerve section or by pharmacological blockade of axoplasmic transport, adjacent nerves sprout and invade the territory of the treated nerve. In addition, the ability of the nerves to sprout is dependent upon the maintenance of axoplasmic transport.

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