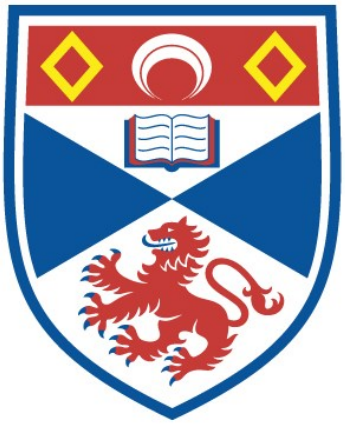
REGENERATION OF THE COCKROACH (PERIPLANETA AMERICANA) CENTRAL NERVOUS SYSTEM IN VITRO AND IN VIVO

Kathryn Ann Rand

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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A thesis submitted to the University
of St. Andrews for the degree of
Doctor of Philosophy

by

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ABSTRACT

The central nervous system of the cockroach is able to recover following nerve lesions, by specific re-innervation of targets. Some aspects of such regeneration were studied *in vitro* and *in vivo* to discover how this is achieved.

Neural explants from the cockroach central nervous system were maintained in culture for up to six months. The effect of various parameters, such as temperature and growth medium composition, upon the rate and extent of fibre outgrowth was studied.

There was no apparent selectivity in the formation of connection between co-cultured neural or muscular explants, suggesting that the cue necessary for specific re-innervation *in vivo* may be absent *in vitro*.

Under normal conditions, outgrowth usually began by about five d in culture. The delay before onset of fibre outgrowth, however, was reduced by prior section of nerves *in situ* two to three weeks before explantation of ganglia into culture. Outgrowth from these nerves which had received a 'conditioning lesion' was observed as soon as 12-24 hours after explantation into culture. Similarly, functional recovery occurred sooner *in vivo* after equivalent operations: normal function returned to animals more quickly after a second lesion to the same nerve, than after one lesion only.

The morphology of an identified motoneurone was studied by intracellular cobalt injection, to assess the effect of maintenance in culture, or of different nerve lesions performed *in vivo*. Nerve lesions caused changes in the branching pattern of this identified neurone. The extent of change appeared to be determined largely by the overall extent of damage to the nervous system. Direct damage to the cell was not a pre-requisite for changes to occur. In many cases, supernumerary branche entered territory which was normally foreign to the motoneurone. In some areas this growth appeared to be random, while in others sprouts gave th appearance of following paths of degenerating nerve fibres.

It is concluded that at least some adult insect neurones are capable of extensive regenerative growth, and of undergoing a high degre of structural modification, both *in vivo* and *in vitro*, indicating that some plasticity is a feature of the adult insect nervous system.

CERTIFICATE

I hereby certify that Kathryn A. Rand has spent nine terms engaged in research work under my direction, and that she has fulfilled the conditions of General Ordinance No. 12 (Resolution of the University Court, No. 1, 1967) and that she is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

DECLARATION

This is to certify that the thesis I have submitted in fulfillment of the requirements governing candidates for the degree of Doctor of Philosophy, in the University of St. Andrews, entitled "Regeneration of the Cockroach (*Periplaneta Americana*) Central Nervous System *In Vitro* and *In Vivo*" is my own composition and is the result of work done by me during the period of matriculation for the above degree. No part of this work has been previously submitted for a higher degree.

The research was conducted in the Department of Physiology and Pharmacology, United College of St. Salvator and St. Leonard, University of St. Andrews under the supervision of Prof. J.F. Lamb and Dr. R. M. Pitman.

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* * * * * * *

ACADEMIC RECORD

I was educated at the Newcastle-upon-Tyne Church High School, and the University of St, Andrews where I graduated in Physiology in June, 1976. The work described in this thesis was carried out in the department of Physiology and Pharmacology, University of St. Andrews, between October, 1976 and May, 1980.

"Large streams from little fountains flow,
Tall oaks from little acorns grow".

David Everett

CONTENTS

GENERAL INTRODUCTION

Damage to the human nervous system is usually followed by very limited functional recovery; any means by which this recovery could be improved would be of clinical value. Generally, recovery after nerve lesions is better in invertebrates and some lower vertebrates than it is in mammals. Despite extensive studies in a wide variety of animals, there are still many aspects of regeneration which are not understood. The processes which are known to be involved in nerve regeneration will now be considered.

(1) The Cell Body Response to Axonal Damage

Damage to a neurone causes a number of morphological and electrical changes in the cell. The structural changes seen in neurones of the vertebrate central nervous system (CNS) are collectively termed "chromatolysis" or the "axon reaction" (Lieberman, 1971). Some of the more obvious changes include a movement of the nucleus to an eccentric position within the soma, sometimes accompanied by a swelling of the soma, an increase in nucleolar volume, and a change in the cytoplasmic ribonucleic acid (RNA). Normally the RNA within vertebrate neurones is present in aggregates, known as Nissl bodies. After injury there is an increase in RNA synthesis and content in the soma. In the majority of vertebrate neurones studied, damage causes the Nissl bodies to break down so that the RNA becomes dispersed throughout the cytoplasm within 2-3 days; there are, however, some exceptions where Nissl bodies become more intensely stained for RNA. At this stage the neurone may begin to regenerate.

Central neurones of a number of invertebrates also show structural changes when damaged. As in vertebrates, swollen somata and eccentric nuclei are characteristic, but the RNA response superficially appears to be different. Normally the RNA of cockroach neurones, for example, is uniformly dispersed throughout the soma cytoplasm and does not form aggregates (Ashhurst, 1961). Axotomy leads to an aggregation of RNA which forms a dense shell around the nucleus (Cohen and Jacklet, 1965). It has been suggested that regeneration of the neurone does not start until this aggregate begins to break down (Cohen, 1967), and that the perinuclear aggregate is functionally equivalent to the Nissl aggregate in the vertebrate neurones. There is some dispute whether the RNA in the perinuclear shell is largely made up of newly synthesised RNA (Young, Ashhurst and Cohen, 1970; Cohen, 1970), or is formed by a redistribution of previously synthesised RNA (Byers, 1970).

The relationship between the axon reaction and regeneration in vertebrate neurones has been reviewed by Grafstein and McQuarrie (1978), while that for invertebrates has been discussed by Jacklet and Cohen (1967 b).

Damage to neurones also causes changes in the electrical properties of both vertebrate and invertebrate motoneurones. In both cases, increased excitability of the dendrites and cell body has been suggested. This is seen, for example, in cat spinal motoneurones (Kuno and Llinas, 1970) and in cockroach motoneurones (Pitman, Tweedle and Cohen, 1972 b).

The way in which a neurone 'recognises' that it has been damaged in unclear. It is likely that a number of mechanisms are involved since no one signal alone can totally account for the observed changes. These could include alterations in electrical properties, such as

transient depolarisation resulting from injury currents passing through the cut end of the axon. The signal could also be caused by physical changes within the neurone, such as an increased loss of axoplasm from the severed axon, or the deprivation of some trophic substance from the periphery. This subject has been reviewed critically by Cragg (1970).

(2) Nerve Regeneration and Restoration of Function

The primary role of nerve regeneration is presumably to restore appropriate function. Since mature neurones cannot proliferate, any restoration must result from adaptation of those neurones already present. There are two major ways in which this can be achieved. Specific re-innervation of a denervated cell or tissue by the original neurone, or neurones, is one such method; if such re-innervation is successful, then this should be the most effective form of recovery. A second way in which some function may be restored is by collateral sprouting. This involves growth of supernumerary branches from intact neurones to re-innervate targets which have been denervated by a nerve lesion (Moore, 1974). In either case, the responding neurone must first be induced to grow; the stimulus, or stimuli, responsible for causing this growth are not clear. It is possible that denervated cells may release a 'sprouting agent', or that normal tissue may cease to produce an 'anti-sprouting agent' when denervated (Brown and Holland, 1979).

These two types of neural response to damage are considered separately below:

(A) Collateral Sprouting

Partial denervation of mammalian muscle will frequently induce the remaining intact nerves to sprout and innervate the denervated muscle fibres. This has been demonstrated, for example, in the peroneus tertius muscle of the mouse (Brown and Ironton, 1976). However, this was followed by regeneration of the crushed nerve fibres which subsequently re-innervated their native muscle fibres. Some of these muscle fibres consequently became dually innervated

for several days, but 8-11 days after the return of the native nerve, sprouts from the foreign nerve began to withdraw. This would suggest that specificity of neuromuscular connections is present but graded, since muscle fibres will accept foreign innervation when there is no alternative.

Partial denervation of the cat superior cervical ganglion was followed by sprouting of the remaining intact nerve fibres which then made connections with the denervated neurones (Murray and Thompson, 1957). However, within six months, the sectioned nerve fibres regenerated and re-innervated their normal ganglion cells; foreign synapses produced by collateral sprouting became non-functional (Guth and Bernstein, 1961). Once again, specificity of connections appears to be graded.

Collateral sprouting is also seen within the CNS of mammals (see Edds, 1953; Moore, 1974 for reviews).

(B) Specific Re-innervation of Denervated Targets by the Original Neurones

One of the main problems faced by a regenerating axon is to locate its correct target. A number of factors are likely to come into play when guiding the growing tip.

During embryonic development neurones must have originally grown appropriately to form the correct connections; the guiding influences which operate during regeneration may be similar. The axon usually emerges from the cell body at a position such that it is directed towards its target. This emergence is genetically determined and occurs when the cell body becomes polarized.

Once axonal outgrowth has been initiated, further elongation may be guided by a number of factors in the environment. These can be broadly divided into electrical, mechanical and chemical

guidance (see Constantine-Paton, 1979 for review).

(a) Electrical Guidance

There is little evidence to indicate that electrical currents or fields play a major part in controlling the direction of axonal growth. However, there is some evidence for a limited effect.

Electric currents may produce electrophoretic movement of extracellular ions and molecules, resulting in either the formation of a chemical gradient, or orientation of dipoles in the direction of the lines of force. These asymmetries may guide growing neurites. Jaffe and Poo (1979) explanted fragments of embryonic chick dorsal root ganglia and maintained them in culture in the presence of a steady electric field. Neurites facing the cathode grew several times faster than those facing the anode. They suggested that the electric field had caused the electrophoresis of nerve growth factor (NGF) receptors along the plasma membrane of the neurites. Small d.c. currents have also been shown to increase the length, density and extent of branching of neurites growing from embryonic chick sensory ganglia (Sisken and Lafferty, 1978). Pulsating currents may affect the orientation of bipolar nerve cell bodies (Jaros, Sensenbrenner and Mandel, 1977) : the cells become orientated with their long axis less than 45° to the line of current flow. The pulsating current does not, however, affect the direction of neurite growth.

The application of a weak, steady electric current across the severed spinal cord of the lamprey has been shown to enhance regeneration of the severed neurones (Borgens, Roederer and Cohen, 1981). The direction of this applied current was opposite to that of the injury current developed at the cut axonal endings, and it was suggested that the applied current may reduce the

destructive effects produced by an injury current.

Whatever the effect of electric fields upon the direction of axonal growth, it is likely to be of minimal importance, since it is hard to imagine how electrical fields or currents alone could produce the complex patterns of growth seen in developing and regenerating nervous systems.

(b) Mechanical Guidance

There is much evidence indicating that mechanical guidance plays a large part in determining the route followed by a growing nerve fibre. It was shown several decades ago that axon growth in tissue culture will only occur when fibres are at a solid-liquid or liquid-air interface (Harrison, 1910). The phenomenon of contact guidance has been used as a partial explanation for the formation of nerve bundles. Many examples of "pioneer neurones" have been reported in invertebrates and vertebrates (e.g. Bate, 1976 a; Ho and Goodman, 1982; also see following for review : Baker, 1978; Anderson, Edwards and Palka, 1980). "Pioneer fibres" locate their target, often by-passing a number of obstacles to achieve this; subsequent fibres then follow the pioneers to reach their targets, so forming a bundle of nerve fibres. In at least some cases, the pioneering of specific pathways involves a number of different pioneer neurones in sequence, leading to the final target (Ho and Goodman, 1982), rather than a single pioneer neurone traversing a long distance.

Tensions produced within the environment of the growing axon also serve to direct fibre growth. Nerve fibres grown in a plasma clot, for example, follow the lines of tension in the substrate (Weiss, 1934). Nerve fibres themselves can produce mechanical

tension, and this is exerted by the mobile, expanded ending at the tip of the neurite known as the growth cone (Bray, 1979). (These structures will be discussed in more detail in Chapter 1). The tension developed within the fibre determines its direction of growth and its pattern of branching. Growth cones are known to show selective adhesiveness to different surfaces (Letourneau, 1975); this could affect the direction of neurite growth. The adhesion to the surfaces could be dependent on mechanical or chemical characteristics.

There seems little doubt that mechanical guidance does influence the direction of neurite growth, both in development and regeneration of the nervous system. It cannot, however, fully explain the behaviour of growing neurites. Groups of nerve fibres will, for example, often travel along the same route for some distance, and will then diverge and follow completely different routes. In many cases, mechanical guidance alone would still leave the fibre with a 'choice' of two directions in which to grow. Neither would it explain how nerve fibres can follow aberrant routes to reach their specific targets (Edwards and Sahota, 1968; Benjamin, 1976; Deak, 1976; Ghysen, 1978). There must therefore be some additional, perhaps chemical, factor which aids the nerve fibre in selecting the correct route.

(c) Chemical Guidance

Two ways have been suggested in which chemical factors can have an effect on nerve fibres. One is a long distance effect, termed chemotaxis, where some "alluring substances" (Cajal, 1928) are released from the degenerating distal stump of the nerve, or from the denervated target. The second mechanism is a short-range attraction between cells, termed chemoaffinity, and a

growing nerve fibre might undergo many of these "stepping stone" interactions with cells en route before finally making a functional contact with its target cell.

Regenerating nerve fibres from the proximal stumps of peripheral nerves show a preference for entering the peripheral stump (Cajal, 1928). However, evidence for an "alluring substance" released from the distal stump has proved negative, both in vitro (Weiss, 1934) and in vivo (Weiss and Taylor, 1944). Nor is there any direct evidence that denervated target cells attract nerve fibres from a distance. However, in many cases there does not seem to be a specific affinity between pre-synaptic and post-synaptic cells. This would explain why neurones may initially produce random outgrowth and transient connections, but ultimately form permanent connections with the correct target. Such transient, inappropriately directed growth has been demonstrated in regenerating cockroach neurones both morphologically (Denburg, Seecof and Horridge, 1977) and electrophysiologically (Whitington, 1979).

There is, however, evidence that certain specific chemicals can direct axonal growth. Gunderson and Barrett (1979) showed the β-nerve growth factor could actually direct the course of chick dorsal-root fibres in vitro as well as stimulating sprouting.

In conclusion, it seems that neither electrical, mechanical nor chemical influences alone can account for guidance of regenerating neurones. The complex interaction of these guiding factors may result in good functional recovery in some cases, but in others, may fail. Baker (1978) has reviewed a number of hypothetical mechanisms by which selective connections may be made

in vertebrates and invertebrates, and also outlines a number of illustrative experiments on the subject.

(3) Some Examples of Neuronal Specificity During Regeneration

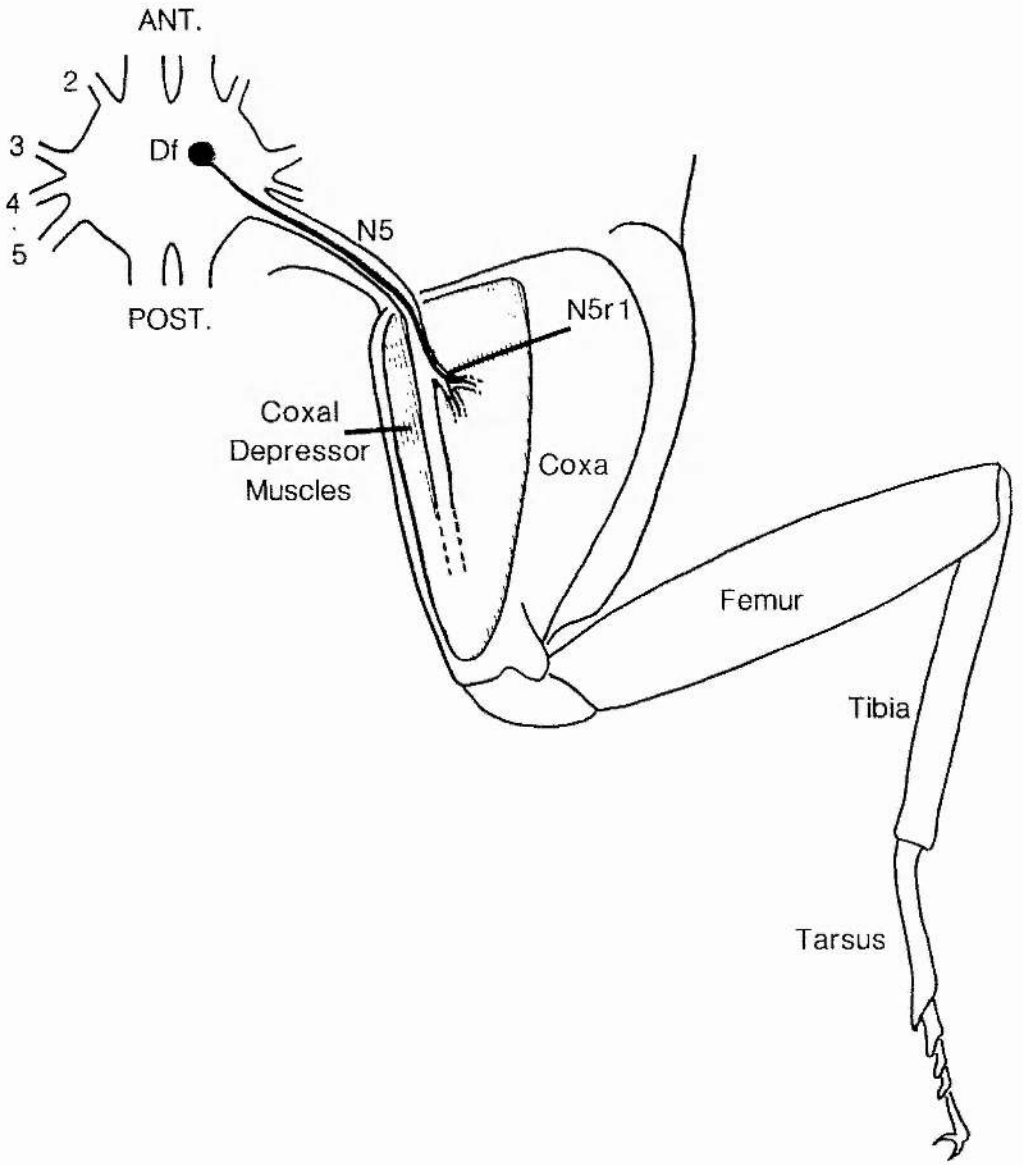
(a) Invertebrates

Many invertebrate nervous systems are capable of extensive regeneration and specific target re-innervation, leading to the restoration of function. The mechanisms by which specific re-innervation is achieved may, however, differ from one species to another; some examples will be discussed here. (See Young, 1973; Anderson, Edwards and Palka, 1980, for reviews).

Pearson and Bradley (1972) studied the regeneration of an identified motoneurone (the fast coxal depressor motoneurone, or D_f) following axotomy. The segmental nerve 5, through which the axon of this neurone runs, was sectioned and the animals then allowed to recover. After five months they were sacrificed; depolarising pulses passed into the soma of D_f caused phasic contractions of the coxal depressor muscles which this neurone would normally innervate (Fig. I.1). It was concluded that specific re-innervation of the muscles had occurred.

Denburg, Seecof and Horridge (1977) produced morphological evidence to support these electrophysiological results. Using cobalt back fills at various distances distal to the site of nerve 5 lesion, they examined the paths taken by a number of regenerating motoneurones including D_f. The time course for regeneration in these experiments indicates that cockroach motoneurones regain functional contact with their target cells by complete regeneration of the proximal stump to the target. There is no physical reconnection with the distal segment of the

Fig. I.1 Metathoracic ganglion and left leg of the cockroach. The cell body of the fast coxal depressor motoneurone, D_f, is shown in the ganglion; the axon leaves the ganglion through nerve 5 to innervate the coxal depressor muscles.


ANT.
2
3
4
5
Df
N5
POST.
N5r1
Coxal
Depressor
Muscles
Coxa
Femur
Tibia
Tarsus

axon. Since the distal axon segment degenerates rapidly (Jacklet and Cohen, 1967 a), it also seem unlikely that the proximal axon stump would make any functional connections with it. The remains of the nerve trunk may, however, persist for some time, and could conceivably act as a guide for regenerating axons.

Denburg (1975) has suggested that different muscles may contain different 'recognition' proteins which enable motoneurones to recognise their specific targets. Tyrer and Johnson (1977), however, analysed proteins from a number of different muscles in the cockroach. They found that the two 'recognition' proteins discussed by Denburg were present in many muslces. The spectrum of proteins present depended upon whether the muscle was a 'fast' or 'slow' type. These 'recognition' proteins were therefore not specific to individual muscles as suggested by Denburg.

Sensory fibres from the cercus of the cricket are able to re-establish specific contact with giant interneurones, even if contact is delayed by cercal removal at several successive moults (Edwards and Palka, 1971). However, if the giant fibres are permanently deprived of cercal input by amputation of the cercus at successive instars, they will accept foreign innervation from other axons, such as vibration receptors (Edwards and Palka, 1973; Palka and Edwards, 1974 b).

There is some evidence to suggest that re-innervation of insect muscle by motoneurones may not always be specific. Whitington (1977) claimed that removal of the coxal depressor muscles in the cockroach caused the fast coxal depressor motoneurone, which was now deprived of its normal target, to form inappropriate connections with 'foreign' muscles. The junctional

potentials recorded from the 'foreign' muscle, however, were very small, and it is possible that the recordings were actually movement artefacts caused by contractions of nearby muscles.

Murphy and Kater (1978, 1980) used an in vivo organ culture system to demonstrate specific re-innervation of the snail salivary gland by identified neurones. Their results suggested that re-innervation occurred by newly formed axonal sprouts making direct contact with the target. However, they also point out that, since the distal axon segment remains viable for several weeks, it would be possible for functional re-innervation to occur by reconnection of proximal and distal axon segments. Some axonal growth initially appeared to be random : sprouts entered foreign nerves, but later grew in hairpin turns, returning to the ganglion. Inappropriate connections may sometimes form between neurones. These are normally transient, being lost when appropriate connections are made, but may be maintained in circumstances where appropriate connections cannot form (Bulloch and Kater, 1981).

The effect of transection, or ablation of several segments of the earthworm ventral nerve cord has been studied (Birse and Bittner, 1981). Severed ends of both proximal and distal segments of identified giant axons regenerated towards each other. Specific electronic synapses were made between the axon stumps, and function was restored. This is similar to the mechanism seen in specific re-connection of an identified leech interneurone .

The leech nervous system has been fairly extensively studied (Coggeshall and Fawcett, 1964; Nicholls and Purves, 1970; Nicholls and Van Essen, 1974), and as a result a number of specific neurones are identifiable in terms of their position and connections with other cells. With such information on normal connectivity,

it has been possible to show that identified neurones re-establish specific connections (Baylor and Nicholls, 1971; Jansen and Nicholls, 1972; Van Essen and Jansen, 1976, 1977; Muller and Scott, 1979). Although Baylor and Nicholls found that regeneration was specific they stated that "recovery of function was not a reliable sign of regeneration", since a number of their experimental animals appeared to swim normally within a few weeks after the operation, when regeneration across the lesion had not occurred. They were unable to explain this restoration of function, but alterations in normal synaptic connectivity could be involved. Such altered, or unmasked, synaptic activity has been demonstrated between identified sensory and motoneurones in this animal after the sectioning of interganglionic connectives. Stimulation of the sensory neurone normally had an excitatory effect upon the motoneurone, but after lesions to the connectives, the effect became inhibitory. This inhibitory connection was normally present, but masked, before the CNS lesions. It was possible to show that this effect was mediated through an interneurone, by blocking chemical transmission in the presynaptic ganglion (Jansen, Muller and Nicholls, 1974).

The viability of isolated distal axon segments is essential for another form of regeneration seen in the leech CNS. The distal stump of an identified interneurone in this animal, like those of crayfish motoneurones, can survive for up to 5 months after isolation from the soma (Muller and Scott, 1979), and can remain functionally connected to its post-synaptic neurone for several weeks (Carbonetto and Muller, 1977). When the proximal region of a severed axon regenerates, it may form a basket of processes around the distal stump, establishing an intermediate

electrical synaptic connection. This results in rapid restoration of functional contact between the soma and the target cell. Regeneration of the axon then proceeds along the distal stump until the target cell is reached and a direct functional contact is made (Carbonetto and Muller, 1977; Muller and Carbonetto, 1979). It seems that the distal stump is an important factor in guiding these regenerating axons. Fernandez and Fernandez (1974) produced morphological evidence showing that synaptic contacts made within the connectives persist for up to 111 days. They suggest that these synapses are maintained, even when other sprouts from the severed axon regenerate to restore specific connections in the normal region of the ganglion neuropile.

Muller (1979) reviews work on the normal and regenerating leech nervous system.

The regenerating axon of crayfish motoneurones may regain specific functional connectivity with their targets by fusion with their own distal stump (Hoy, Bittner and Kennedy, 1967; Bittner, 1973). The distal stump may survive separated from the soma and remain morphologically and functionally intact for 150-250 days (Hoy et al, 1967; Nordlander and Singer, 1972; Bittner and Johnson, 1974). Axonal fusion during regeneration in the leech CNS has also been reported (Frank, Jansen and Rinvik, 1975).

Some crayfish interneurones deprived of their cell body are capable of receiving, conducting and transmitting electrical activity for months. If such cells are de-afferented, afferent neurones will regenerate and make functional contacts with the somaless interneurones within 2-3 weeks (Krasne and Lee, 1977). It therefore seems that the neurone is sufficiently viable without

its soma both to function, and to be recognized by regenerating neurones.

(b) Lower Vertebrates

Specific re-innervation by regenerating neurones may also lead to functional recovery in lower vertebrates, some examples are given below.

Re-innervation of cichlid fish eye muscles is apparently specific, since section of the oculomotor nerve is followed by a recovery of normal eye movements (Sperry and Arora, 1965). Individual eye muscles will accept foreign innervation by misdirected nerves if the native nerve is prevented from re-innervating the muscle. However, if the native nerve is subsequently allowed to return, connections made by this nerve become dominant over those made by the foreign nerve. The conclusions drawn from these experiments, however, were based largely upon behavioural observations and must therefore be treated with some caution.

Specific re-innervation of axolotl hindlimb muscle occurs after physical misdirection of spinal nerves (Cass, Sutton and Mark, 1973; Bennett and Raftos, 1977; Bennett, McGrath and Davey, 1979). If misrouted nerves were given sufficient time to form well established synapses with a foreign muscle before the return of the native nerve, then these synapses were not repressed by native re-innervation (Bennett et al, 1979). Cass et al. (1973) studied an axolotl muscle which is normally innervated by motor nerve fibres travelling in two separate peripheral nerves. If one of these nerves was severed, motor axons in the intact nerve formed collateral sprouts which innervated muscle fibres that had become denervated by the lesion. This process took about 1-2 weeks.

Unlike synapses formed by axons in experimentally re-directed foreign nerves, synapses formed by collateral sprouting were repressed by the return of the native nerve fibres. These synapses, however, may only become non-functional and not physically regress : re-sectioning the native nerve caused muscle fibres to respond to stimulation of the foreign nerve within only three days (Cass et al, 1973). The suppression of foreign innervation by collateral sprouts was not due to direct competition for synaptic sites (Wigston, 1979) : the native nerve re-innervated muscle fibres at the original sites, but could suppress foreign nerve terminals on other regions of the muscle fibre.

Similar repression of collateral sprouting has been reported in adult newts (Yip and Dennis, 1976): foreign neuromuscular synapses did not physically persist, however, on the return of the native nerve (Dennis and Yip, 1978).

The examples discussed above demonstrate specific peripheral re-innervation through competition between foreign and native nerves.

Specificity is also seen in regeneration of the central nervous system of lower vertebrates. In fish, amphibians and birds, there is, for example, specific regeneration of the pattern of retinotectal connections. Experiments in which the area of tectum available to regenerating retinal ganglion cells was expanded or reduced showed that this specificity was not on a "place to place" basis. Regenerating retinotectal connections were formed so that the normal projection pattern was restored and spread over the space available; connections were specific with regard to positions relative to other retinal cells rather than to absolute positions in the tectum.

These experiments on the regeneration of retinotectal connections have been reviewed by Gaze (1970).

Wood and Cohen (1979) found that transection of the lamprey spinal cord was followed by recovery of normal swimming movements, but this was not achieved for more than six months. Morphological studies of the regenerating neurones using intracellular dye injection showed that Mauthner and Muller cells had not specifically followed their normal routes, and that synapses were formed in atypical regions of the spinal cord. Windle (1980) has discussed the long time required for recovery in these experiments, and points out that if transection of the spinal cord is performed more caudally, recovery is seen in 20 days (Hibbard, 1973). Windle suggests that this superior rate of recovery is seen because less damage, and therefore less glial proliferation, occurs when more caudal transections are performed. More recently Wood and Cohen (1981) have repeated their earlier experiments more extensively, and found that in many animals, recovery occurred in three to four weeks. The authors, however, give no explanation for the differing time course of recovery reported in their two papers.

Successful spinal cord regeneration leading to a restoration of normal swimming has also been demonstrated in goldfish (Bernstein, 1964). Introduction of a Teflon barrier into the spinal cord for 30 days prevented such regeneration; subsequent removal of the barrier did not permit regeneration (Bernstein and Bernstein, 1967). Under these conditions, axons formed synaptic contact near the barrier and growth was arrested (Bernstein and Bernstein, 1969). Re-sectioning of the cord, however, allowed regeneration of axons through the glial-

ependymal scar formed at the site of the original lesion, and function was restored (Bernstein and Bernstein, 1969). This indicates that failure of growth seen after removal of the barrier is caused by some feature intrinsic to the neurones, and not to an environment which is unfavourable for growth. The authors suggest that the formation of synaptic connections causes a form of "contact inhibition" which stops further neuronal growth.

(c) Mammals

The adult mammalian CNS shows limited recovery from lesions (see Clemente, 1964, for review), although some central neurones are capable of considerable regenerative growth (Bjorklund and Lindvall, 1979; Kalil and Reh, 1979). A number of proposals have been put forward to explain why growth is abortive, or unsuccessful in re-establishing normal connections. They include the following:

(1) Disruption of Blood Supply. An injury causing a nerve lesion may also frequently result in damage to the surrounding tissues. The local blood supply is thus impaired, and poor growth in the affected area could result.

(2) Inappropriate Environment for Growth. It has been suggested that the absence of Schwann cells may be a factor contributing to poor CNS regeneration: central neurones are capable of more extensive regeneration if given a peripheral nervous system environment in which to grow (Richardson, McGuinness and Aguayo, 1980). The formation of glial scars at the site of injury may form a barrier to axonal growth. Puromycin is a protein synthesis inhibitor which has been shown to improve

CNS regeneration in mammals (Bernstein and Wells, 1980). It appears to act by suppressing the pathological changes which normally occur after spinal cord injury, so that glial scarring is less dense, while allowing normal growth processes, such as axonal elongation, to continue in the cord.

(3) Inability of Neurones to Recognise Target. Even if axonal growth proceeds successfully, there can only be a restoration of normal function if appropriate connections are made. In adult mammals this specificity is frequently lacking. Central neurones (Purves, 1975 a, 1976 b), and muscles (Frank, Jansen Lomo and Westgaard, 1974), may become innervated by both foreign and native nerves. Recently, however, some selectivity has been demonstrated in the re-innervation of rat intercostal muscles from different segments (Wigston and Sanes, 1982).

(d) Differences in Recovery Between Species

It has been suggested (Jacobson, 1970; Mark, 1974) that the differences in the specificity of re-innervation seen between lower and higher animals could be due to differences in the type of innervation. In higher vertebrates, individual muscle fibres are normally innervated by a single motoneurone, whereas many muscle fibres in lower animals are polyneuronally innervated. Denervated muscle fibres in higher vertebrates may accept re-innervation by any neurone, and be incapable of accepting innervation by further neurones because they are not normally polyneuronally innervated. If this were so, correct re-innervation would only occur if the native neurone was the first to make contact. This does not, however, explain the displacement of collateral sprouts by a regenerating native nerve. Moreover, as

discussed above, dual innervation of targets by foreign and native nerves has been observed in mammals.

In summary, we may see from the above discussion that the strategies used to restore normal contacts differ in different systems (e.g. leech CNS, crustacean motoneurones, cockroach motoneurones.) For reasons that are still unclear, return of function after damage to the mammalian nervous system is poor in comparison with that of many lower vertebrates and invertebrates.

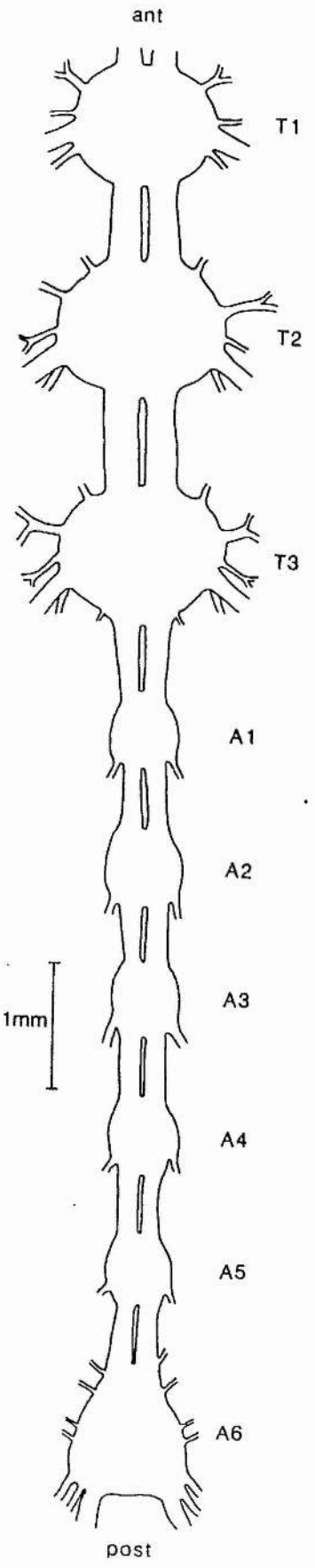
(4) The Choice of Experimental Animal

In order to study various aspects of regeneration, the cockroach, Periplaneta americana, was selected as an experimental animal for the following reasons:

(a) Good Capacity for Nerve Regeneration

As previously discussed, this nervous system shows a marked ability to regenerate and restore function following nerve lesions. Transection of the segmental nerve 5, for example, causes denervation of several leg muscles, but the coxal depressor muscles are specifically re-innervated by regenerating nerve fibres and normal movement is restored within 4-8 weeks (Bodenstein, 1957; Guthrie, 1967; Jacklet and Cohen, 1967 b; Pearson and Bradley, 1972). This specificity is not absolute, however, when considering homologous muscles from opposite sides of the animal (Bate, 1976 b; Fourtner, Drewes and Holzmann, 1978), or from different segments (Young, 1972; Westin and Camhi, 1975).

Fig. I.2 Diagram of the ventral nerve cord of the cockroach. Thoracic (T) and abdominal (A) ganglia are joined together in a chain through paired connectives. Segmental nerves (shown sectioned near the ganglia in this diagram) contain axons running to and from peripheral regions, such as muscle and cuticle.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(b) Small Numbers of Identifiable Neurones

The cockroach CNS is relatively simple by comparison with those of the vertebrates. It consists of a brain and suboesophageal ganglion in the head, and a chain of segmental ganglia in the thorax and abdomen (Fig. I.2). Essentially each segmental ganglion contains the neurones responsible for the control of one body segment. All motoneurone cell bodies are found within the CNS, whereas sensory neurone cell bodies are usually found peripherally. Maps of larger neurone cell bodies have been constructed for the mesothoracic (Young, 1969) and metathoracic (Cohen and Jacklet, 1967) ganglia of the cockroach. The peripheral nerves and somatic muscles have also been described and numbered (Carbonell, 1948; Dresden and Nijenhuis, 1953, 1958; Nijenhuis and Dresden, 1955; Pipa and Cook, 1959). This information, coupled with electrophysiological studies (Pearson and Iles, 1970, 1971; Iles, 1972) have made it possible to positively identify a number of specific cells, using parameters of size, position and function. A preparation in which specific identified neurones can be studied has the advantage that it eliminates the uncertainties inherent in using heterogeneous cell populations. The advantages presented by identifiable cells have been discussed by Horridge, (1973).

(c) Position of Neurone Cell Bodies

The neuronal somata are all situated in the cortex of the ganglion, surrounding the central neuropile area where synaptic connections are made between neurones. These cell bodies are consequently easily accessible for penetration with micro-electrodes. The superficial position of the cell bodies allows stimulation, recording and dye injection of cells without any major

disturbance to dendritic structure or interneuronal connections.

(d) Bilateral Symmetry of the Nervous System

The majority of cells within each ganglion are arranged in bilaterally symmetrical pairs, so that one side of the ganglion is a mirror image of the other. Homologous neurones control homologous muscles on the two sides of the animal. The majority of the motoneurones have their cell body and all processes confined to one half of the ganglion. This is usually ipsilateral to the segmental nerve through which the axon runs, and hence to the muscle it innervates. In many cases it is possible to have an " experimental cell" and a "control cell" within a single ganglion, and any difference seen between two homologous cells can be attributed to the operation performed on the experimental cell. Effects on the neurone due to genetic variability, age or nutritional state of animals can be eliminated using such a preparation. However, care must be taken in interpreting results from experiments where unilateral manipulations are performed on the nervous system. It is not possible to eliminate transneuronal effects that may be communicated to other contralateral cells.

(e) Economy

Although a small point, this is perhaps worth mentioning in view of the present financial climate for the scientist. Periplaneta americana is a relatively inexpensive animal to purchase and maintain when compared to most mammalian species. A large number of animals can be kept in a fairly small space, and breeding is rapid; animals reach maturity within about six months, although this varies depending on food supply and environmental temperature. These factors make it possible to study relatively large numbers of

animals if necessary.

The following three chapters describe features of the regenerating nervous system of Periplaneta americana *in vivo* and *in vitro*.

CHAPTER 1

THE ADULT COCKROACH CENTRAL NERVOUS SYSTEM IN TISSUE CULTURE

INTRODUCTION

While in vivo studies similar to those described above (see 'General Introduction') provide many convincing examples of neural specificity, they shed little light on the underlying mechanisms. Only in exceptional circumstances (e.g. nerve fibre growth in the tadpole tail fin, Speidel, 1933; 1935) is it possible to monitor neural growth continuously in vivo. Consequently, any observation on neuronal morphology usually requires the sacrifice of an animal. A relatively complete picture of the development of neurones can only be built up by sacrificing a number of individual animals at different time intervals. The in vitro approach, on the other hand, allows continuous observations to be made on the growth and distribution of neuronal processes in individual preparations. It is possible to follow the paths taken by growing neurites and to observe the behaviour of growth cones at the tips of these fibres (Wessells, Johnson and Nuttall, 1978; Bray, 1979).

Using the tissue culture system it is possible to alter various parameters of the growth conditions and study how neurite growth is affected, e.g. hormonal influences or mechanical guidance of nerve fibres. The substrate on which cells are grown will often have marked effects (Letourneau, 1975; Agranoff, Field and Gaze, 1976), and cells are also affected by the pH of the medium in which they are grown (Bear and Schneider, 1977). The effect of substances thought to influence neurite outgrowth can be tested; the following are some examples. Nerve growth factor (NGF) promotes neurite outgrowth from rodent sympathetic ganglion cells (Levi-Montalcini and Angeletti, 1968; Levi-Montalcini, 1979), and it has been suggested that this may result from an increase in the cell-substratum adhesion of these cells (Schubert, La Corbiere, Whitlock and Stallcup, 1978). NGF also stimulates neuritic outgrowth from chick embryo sensory ganglia (Sisken

and Lafferty, 1978). Haemin promotes neurite outgrowth from mouse neuroblastoma cells (Ishii and Maniatis, 1978).

In some tissue culture systems it is possible to use a chemically defined medium; such media are free from any constituents such as serum, which may vary from one batch to another and so produce variable results. Use of these defined media allows the effects of precise modifications in the growth conditions to be studied.

Nerve cells may be grown in tissue culture as dissociated cells or as multineuronal explants of neural tissue.

Dissociated cell cultures may be divided into two categories: primary and secondary. Primary dissociated cell cultures are obtained by the chemical and/or mechanical disruption of tissue. Such cultures will have a finite life span, but the cells should possess similar properties to those of the tissue from which they were derived. Secondary cultures are produced from cells which have been transformed into a state in which they will continue to divide and replicate (as with cancer cells). These cells may be passed indefinitely from one generation to another, but the similarity between these cells and the original tissue from which they were derived may be questionable. However, this allows the continuous production of an effectively unlimited number of homogenous cells, presenting an ideal system for studying some aspects of cell function such as ion fluxes and transport across membranes.

Tissues, or even whole organs, explanted directly from the living animal provide another form of primary culture. Methods for obtaining explants cause less tissue disruption, and so in this sense approximate more closely to the situation *in vivo*, since many of the cells remain in the appropriate cellular environment. Such a preparation may reflect more closely the conditions *in vivo*. Spinal cord explants from

foetal mice, for example, will maintain their normal organisation in culture for up to 54 days (Sobkowicz, Guillery and Bornstein, 1968).

The technique of tissue culture has been used extensively by investigators studying the vertebrate nervous system. Ross G. Harrison originally developed the technique using neural tube explants from early tail-bud frog larvae (Harrison, 1907; 1910) to demonstrate unequivocally that the axon is actually a cytoplasmic outgrowth from the nerve cell, and not a separate cell or cells.

Aspects of vertebrate tissue culture have been reviewed extensively by others (Fischbach, Fambrough and Nelson, 1973; Nelson, 1975; Bunge, 1975; 1976; Crain, 1976). Some characteristics of vertebrate nervous systems maintained in tissue culture will be briefly described.

Factors affecting neurite outgrowth have been studied using retinal explants from goldfish (Landreth and Agranoff, 1976; Heacock and Agranoff, 1977).

Synapse formation has been demonstrated between cells within explants of mouse sympathetic ganglia (Kim and Munkacsi, 1974) and rat hippocampus (Zhabotinski, Chumasov, Chubakov and Konovalov, 1979). Similarly, synaptic connections form in vitro between cells in separated explants of nerve and muscle tissue from rat or mouse, and chick embryos (for references see Shimada and Fischman, 1973) and between retinal and tectal explants (Lilien and Balsamo, 1978). Functional neuromuscular junctions have been reported between dissociated cells in culture (Shimada and Fischman, 1973; Schubert, Heinemann and Kidokoro, 1977; Koenig, 1979), and synapse formation between dissociated nerve cells has also been demonstrated morphologically and physiologically (Kim and Munkacsi, 1974; Gahwiler, 1977; Yavin and Yavin, 1977.

Spontaneous electrical activity has been recorded both from explants of rat hippocampus (Zhabotinski, et al 1979) and embryonic chick sensory ganglia (Rieske and Kreutzberg, 1978), and from dissociated nerve cells from the cerebellum and inferior olive (Gähwiler, 1977).

Using tissue culture techniques it was possible to show that Schwann cell proliferation is stimulated by the presence of a bare regenerating axon, and not, as was previously thought, by the degeneration products from damaged nerve (Wood and Bunge, 1975).

The information on invertebrate nervous systems in vitro is relatively sparse compared to that on vertebrate nervous systems. General reviews can be found in Levi-Montalcini, Chen, Seshan and Aloe, (1973) and Maramorosch, (1976).

Explanted invertebrate nervous tissue has been used to study various aspects of nervous system function. Leech segmental ganglia maintained in culture have been successfully used to study regeneration. If the connectives between these ganglia are crushed, the axons so severed regenerate across the lesion to make specific connections with their normal target cells, and impulse conduction between ganglia is restored. (Miyazaki, Nicholls and Wallace, 1976; Wallace, Adal and Nicholls, 1977).

Leech ganglia are spontaneously active in culture for up to 3 weeks. After this period they become silent, but remain responsive to electrical stimulation (Miyazaki et al, 1976). Spontaneous activity has been found in long term cultures of embryonic cockroach ganglia (Provine, Aloe and Seshan, 1973). Medial neurosecretory cell explants from nymphal and adult cockroaches are also spontaneously active (Seshan, Provine and Levi-Montalcini, 1974), whereas postembryonic non-neurosecretory neural explant cultures show no electrical activity.

Explants from embryonic cockroach nervous systems become physically interconnected in culture by outgrowing nerve fibres (Chen and Levi-Montalcini, 1969; Provine, Seshan and Aloe, 1976).

The same is true of corpora cardiaca and corpora allata from nymphal and adult cockroaches (Seshan and Levi-Montalcini, 1971). No functional connections were demonstrated, however. Levi-Montalcini and Chen (1971) claimed that there is some selectivity with which connections are made by embryonic cockroach ganglia in vitro. Fibres growing from thoracic ganglia became connected to leg explants in preference to other ganglia. On the other hand, fibres from brain or abdominal ganglia rarely made connections with leg explants. Chains of up to 8-12 abdominal and thoracic ganglia became interconnected by nerve fibres, irrespective of the relative sequence or orientation of the ganglia (Provine et al, 1976).

Different invertebrate cell types or groups of cells are often seen to interact with one another in co-culture. Aloe and Levi-Montalcini (1972a) for example, demonstrated that nymphal cockroach ingluvial and frontal ganglia will only survive in vitro if co-cultured with nervous tissue or foregut. Cockroach thoracic ganglia have a stimulating influence on the fibre outgrowth from leg nerve stumps, but the reverse is not true (Marks, Reinecke and Leopold, 1968). In this same series of experiments, Marks et al. also showed that blood and glial cells may form a bridge between explants which facilitates axonal growth. It has been suggested that glial cells may have a supportive role for nerve cells in the insect embryonic nervous system (Levi-Montalcini and Chen, 1969; Levi-Montalcini et al, 1973).

Further aspects of invertebrate nervous system function studied in vitro include neurohormone synthesis and storage (Marks, Holman and Borg, 1973; Pratt and Bowers, 1977) and protein synthesis in axotomized cells (Denburg and Hood, 1977). Schlapfer, Haywood and Barondes (1972) found that, as in vivo, whole explants of embryonic cockroach brain developed cholinesterase and choline acetyltransferase

activities *in vitro*. Such activity did not, however, develop in dissociated cell cultures of cockroach brain. This illustrates an advantage of using whole explants in tissue culture.

Dissociated cells from invertebrate nervous systems have also been successfully cultured. Geletyuk (1977) grew nerve cells from the circumpharyngeal nerve ring of the adult mollusk, *Limnaea stagnalis*. Some of these cells produced axonal processes which formed functional connections with other cells. These connections could be either excitatory or inhibitory, and remained functional *in vitro* for up to 4 months. A degree of selectivity of connections was evident; some cells which were physically close to one another did not become connected when more distant ones did.

Identified neurones can be studied in invertebrate nervous tissue in culture. This has the advantage that it eliminates variability which may result from studying heterogeneous samples of cells. Ready and Nicholls (1979) removed P cells (sensory cells which respond to pressure) and Retzius cells from leech segmental ganglia, and maintained them in culture for several weeks. During this time, normal action potentials and resting potentials were recorded. After 5-7 days *in vitro* the cells began to sprout processes, in apparently random directions. However, if two cells became interconnected by one of these processes, then a bundle of fibres subsequently formed between the cells, and relatively few processes extended from these cells in other directions. Many pairs of Retzius cells became connected in this way and showed electrical coupling similar to that seen *in vivo*. Chemical synapses also developed between some cells. The formation of connections between cells in culture showed some specificity: Retzius cells became coupled to other Retzius cells, but never to P cells. These interconnections are similar to those found *in vivo*.

Neurones dissociated from the embryonic cockroach central nervous system have been maintained in vitro (Marks, Reinecke and Caldwell, 1968; Chen and Levi-Montalcini, 1970 a and b; Beadle, Hicks and Middleton, 1982). Chen and Levi-Montalcini (1970 a) found that cells developed processes which formed connections with other neurones, and with explants of foregut. Cultures consisting only of dissociated nerve cells did not survive longer than a few weeks, and produced little or no fibre outgrowth (Chen and Levi-Montalcini, 1970 b). However, if co-cultured with explants of foregut, the cells survived for 4 to 5 months and grew many fibres. Cultures were more viable even if the nerve cells were prevented from making any connections with the foregut, indicating that this tissue may release some chemical factor, or factors, into the medium, or may absorb some substance which normally inhibits neurone growth. Beadle et al. (1982) have maintained actively growing dissociated neurones for over a month in the absence of any explants; the growth medium used, however, was not entirely chemically defined.

Neurones isolated from central ganglia of the snail, Helisoma, and maintained in vitro, are dependent upon the presence of brain, or a conditioning factor produced by the brain, for the development of electrical excitability and neuritic outgrowth (Wong, Hadley, Kater and Hauser, 1981).

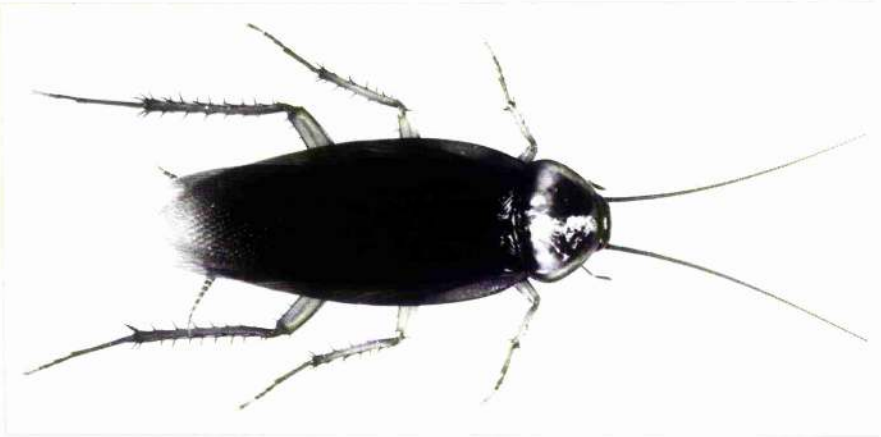
Dissociated nerve cells from embryonic locusts have also been maintained in culture, but again, their survival is dependent on the inclusion of established embryonic material (Giles, Joy and Usherwood, 1978).

Some invertebrate cell lines have been isolated (Kurtti and Brooks, 19777; Hink, 1979), and these may provide the same advantages as the vertebrate cell lines.

Although current knowledge on invertebrate nervous systems in culture is far less advanced than that of the vertebrate systems, their relative simplicity could prove to be useful for studying basic problems in neurobiology. Access to identified cells provides a potential which is not yet available with vertebrate material.

Fig. 1.1 Adult male cockroach, viewed from the dorsal surface (A) and from the side (B). The animal was anaesthetized with carbon dioxide before photographing; as a result, the prothoracic legs are held in a slightly abnormal position, and tarsi are upturned. Calibration : 2 cm.

A



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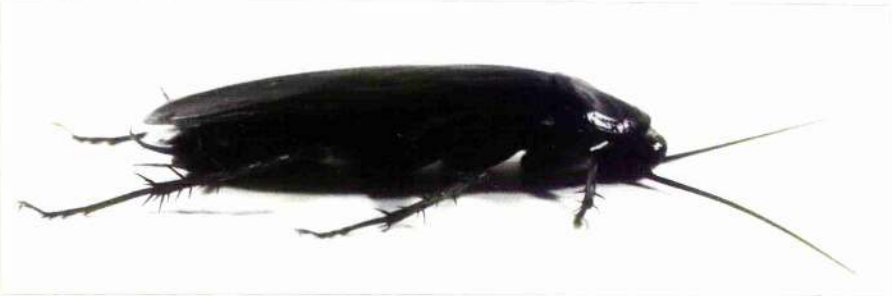


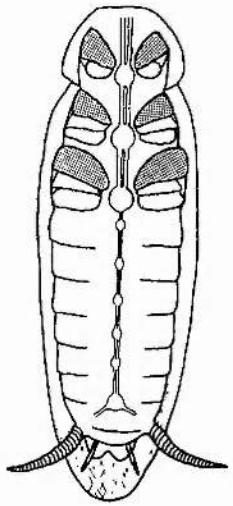
METHODS

PREPARATION OF EXPLANT CULTURES

Primary cultures of explanted nervous tissue, and occasionally muscle were established from male adult or nymphal cockroaches, Periplaneta americana (obtained from Gerrard and Haig, East Sussex) shown in Fig. 1.1.

Fig. 1.2 summarizes the method adopted. All surfaces and equipment within a Bassaire laminar air flow cabinet were cleaned with 70% ethanol. Dissecting instruments were soaked in 95% ethanol and then flamed in a bunsen burner immediately before use. Animals were anaesthetized in a humidified atmosphere of 100% carbon dioxide for about 5 minutes, removed, totally immersed in 70% ethanol and then blotted dry with a clean tissue. The head, legs and wings were removed, and the body secured, ventral surface uppermost, to a dissection board with clean stainless steel pins. Viewing with a Kyowa (X15-X20) binocular microscope, the cuticle overlying the ventral nerve cord was carefully cut away and the cord from the first thoracic ganglion to the sixth abdominal ganglion removed. The nerve cord was placed in a sterile vented 35mm plastic Petri dish (Flow) containing 1ml of sterile growth medium. Unless otherwise stated the medium used was that of Chen and Levi-Montalcini (1969), which consists of a mixture of Basal Medium Eagles with Earle's salts and Schneider's Drosophila medium, to which Penicillin, Streptomycin and Fungizone is added. (See Table 1.1 for composition of medium). The nerve cord was cleaned of superficial fat and divided into short chains or single ganglia. Each segment was then passed through several changes of sterile medium to further reduce risks of bacterial or fungal contamination. Several ganglia were transferred to the final Petri dish which contained 0.5ml of growth medium. Occasionally cultures were grown on glass coverslips or collagen coated coverslips (Bornstein 1958), and also sometimes Falcon or Nunc plastic Petri dishes were used. The ganglia were placed ventral surface

Fig. 1.2 Method used for setting up neural explants in culture. The entire procedure was carried out under aseptic conditions in a laminar air flow cabinet. All dissecting instruments were soaked in 95% ethanol and flamed dry before use. The ventral nerve cord was exposed in (1) by carefully removing the overlying cuticle. It was then removed from the animal by cutting the segmental nerves from all ganglia, and quickly placed into a Petri dish containing growth medium. Pieces of nerve cord were prepared for culture as described in the Figure.



1. ANIMAL PREPARED FOR DISSECTION

ADULT ANIMAL ANAESTHETISED IN 100% CO_2, IMMERSED IN 70% ETHANOL AND BLOTTED DRY. HEAD AND LEGS REMOVED AND ANIMAL PINNED OUT, VENTRAL SURFACE UPPERMOST.

VENTRAL NERVE CORD REMOVED AND TRANSFERRED TO PETRI DISH CONTAINING GROWTH MEDIUM.

2. NERVE CORD CLEANED AND DIVIDED

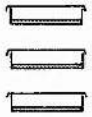
SUPERFICIAL OVERLYING TISSUE CLEANED FROM NERVE CORD, WHICH IS THEN DIVIDED INTO SINGLE OR CHAINS OF 2-3 GANGLIA.

EACH SEGMENT OF NERVE CORD TRANSFERRED TO SEPARATE PETRI DISHES.

3. GANGLIA WASHED

THE GANGLIA ARE TRANSFERRED TO PETRI DISHES CONTAINING FRESH MEDIUM TO REDUCE RISK OF CONTAMINATION BY MICR-ORGANISMS.

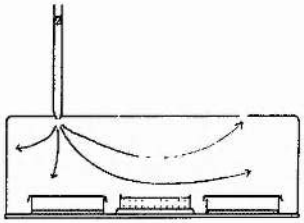
GANGLIA TRANSFERRED TO FINAL PETRI DISHES.



4. FINAL ARRANGEMENT OF GANGLIA

0.5 ML MEDIUM PLACED IN DISH IMMEDIATELY BEFORE TRANSFERRAL OF GANGLIA. (THIS IMPROVES ADHESION OF THE EXPLANTS TO THE BOTTOM OF THE DISH.) ONE OR MORE SEGMENTS OF NERVE CORD PLACED IN EACH DISH, LEAVING ABOUT 1 MM BETWEEN EACH.

FINAL DISHES TRANSFERRED TO BOX.



5. CULTURES MAINTAINED IN A HUMIDIFIED ATMOSPHERE OF 95% AIR, 5% CO_2 AT 28°C.

DISHES CONTAINING CULTURES PLACED IN CLEAN PLASTIC BOX CONTAINING OPEN PETRI DISH FILLED WITH STERILE DISTILLED WATER. BOX SEALED WITH TAPE AND FLUSHED WITH FILTERED 95% AIR, 5% CO_2 MIXTURE. THIS ENSURES PH OF MEDIUM MAINTAINED AT ABOUT 7.3. MEDIUM IS CHANGED EVERY 4 OR 5 DAYS. CULTURES HAVE BEEN MAINTAINED FOR UP TO 6 MONTHS USING THIS SYSTEM.

TABLE 1.1

COMPOSITION OF CHEN AND LEVI-MONTALCINI MEDIUM

Quantity	Component	Source
11 ml	Schneider's Drosophila Medium (Revised) with L-Glutamine	Gibco Bio-Cult (Cat. No. 172)
9 ml	Basal Medium Eagles (Modified) with Earle's Salts	Flow Laboratories (Cat. No. 12-006-54)
0.2 ml	Fungizone (250 µg/ml) diluted to 25 µg/ml with sterile distilled, deionized water.	Squibb (Cat. No. 7-016B)
0.4 ml	Penicillin - Streptomycin (5000 I.U. of each/ml)	Flow Laboratories (Cat. No. 7-010C)

uppermost, about 1mm apart in the dish.

Occasionally nervous tissue was co-cultured with explants of coxal depressor muscles 177d and e and 178 (Carbonell, 1948), by removing the entire coxa from the animal, placing it in a Petri dish containing sterile medium, and carefully removing the surrounding cuticle. Pieces of coxal depressor muscle were gently pulled away from other coxal muscles and, to minimise diffusion problems, pieces were used which were less than 1mm thick in at least one dimension. Muscle explants were placed approximately 0.5mm away from explanted ganglia in the culture dish.

In early experiments, explants were surrounded by a ring of Millipore filter to reduce turbulence caused by moving the dish. These rings were made by cutting a hole in the centre of the filter (pore size 0.22 μm) with a sharp blade. Their inclusion in the dish also seemed to reduce precipitation of the medium around the culture. In later experiments, however, the Millipore rings were found to be unnecessary if dishes were handled carefully and a minimum volume of medium was used.

Once the explants had been arranged in the Petri dish, dishes were transferred to a clean plastic box. An open Petri dish of sterile distilled water was also placed in each box to ensure that a humidified atmosphere was maintained within the box. Water alone was found preferable to moistened sterile gauze or cotton wool as it was less susceptible to contamination by micro-organisms. The boxes were sealed with plastic insulating tape, and a mixture of 95% air, 5% CO_2 passed in through a hole in the box via a sterile glass pipette. A second hole in the box served as an outlet. The gas mixture was allowed to flow in gently (about 1.0 - 1.5 lb ft/in^2) so that the dishes were not disturbed. When the box was completely flushed with the gas mixture,

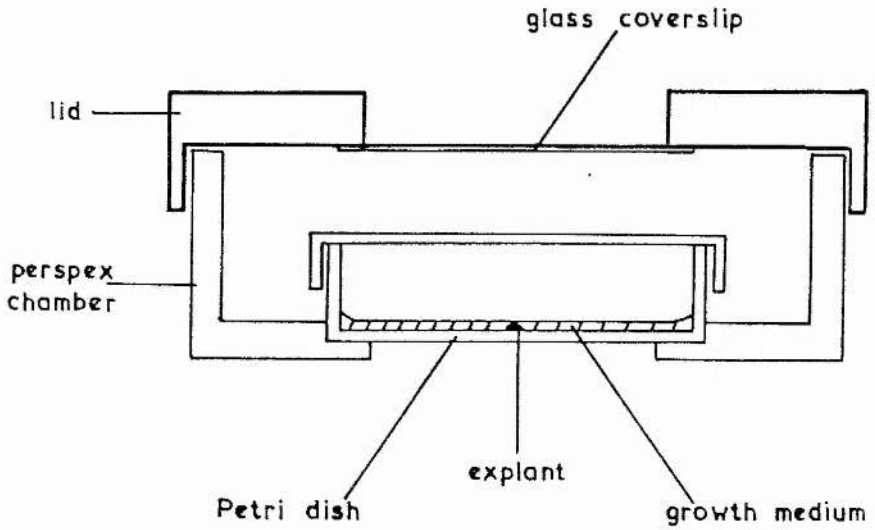


Fig. 1.3 Perspex chamber used to house Petri dish containing cultures. The chamber was cleaned with 95% ethanol before use, and reduced the risk of microbial contamination of cultures during observations under the inverted microscope. The glass coverslip incorporated into the lid allowed viewing of the preparations with minimal optical distortion. It was necessary to remove the chamber lid, however, when using high power objectives with a short working distance.

the pipette was removed and the holes in the box immediately sealed with plastic tape. This atmosphere ensured that the medium maintained a pH of 7.3 If the medium was allowed to stand in pure air, the pH rose to 7.7. The change in colour of the phenol red in the medium produced by this pH shift indicated if the box had remained airtight.

Cultures were maintained at either 37°C or 28°C. Usually the growth medium was replaced with fresh medium every 3-4 days under sterile conditions. Cultures could, however, survive with no apparent detrimental effects when the medium was only replaced once every 7-8 days.

Explants were routinely studied under bright field or phase contrast illumination using a Reichert inverted microscope with a long range condenser. Any interesting features were recorded photographically using a Reichert Kam ES - Electronic Camera System. Culture dishes were placed in a clean perspex chamber (Fig. 1.3) for observation to reduce the risk of bacterial contamination. There was inevitably a gradual shift in pH of the medium during this period but this did not appear to have any detectable effects upon the culture; those viewed every day appeared no different from those observed only once a week. Similar observations have been made on cultures of dissociated mouse cerebellar cells (Messer, 1977).

Usually cultures were terminated within 8-12 weeks, but they could be maintained for up to 29 weeks at 28°C.

ESTIMATION OF FIBRE OUTGROWTH

In the estimation of increase in outgrowth produced by a culture, two parameters were considered:

(1) increase in length of fibres.

(2) increase in number of fibres.

The rate of outgrowth was assessed by measuring fibre outgrowth from

individual segmental nerve stumps or connectives at successive time intervals.

Method 1

Photographs of individual nerve stumps and their emergent processes were used to draw concentric circles centred on each stump. (Fig. 1.4). The distance between each circle was equivalent to either 0.05mm or 0.1mm, depending on the magnification of the photograph. The length of the longest fibre was measured. Using the difference in length at successive time intervals, it was possible to calculate the approximate rate of growth.

e.g. at 4 days, longest fibre is 0.2mm

at 7 days, longest fibre is 0.6mm

therefore, in 72 hours, there has been an increase of 0.4mm.

Thus the growth rate is $\frac{400}{72}$ μm/h = 5.56 μm/h

This method would tend to give a low value for the maximum rate of growth, since the longest fibre need not necessarily be the same fibre each time. Any 'overtaking' of the original leading fibre by another would not be taken into account using this method. However, since outgrowth rapidly became very prolific, it was impossible to follow the path of an individual fibre for any reasonable length of time, as its origin became masked by the vast number of fibres originating from the segmental nerve stump. The method may also give a false impression of cessation of any growth from the nerve trunk under observation, as any elongation of shorter fibres would not become apparent unless they exceeded the previous maximum fibre length.

Method 2

Photographs with concentric circles centred on nerve stumps were prepared as in method 1. The number of fibres crossing each

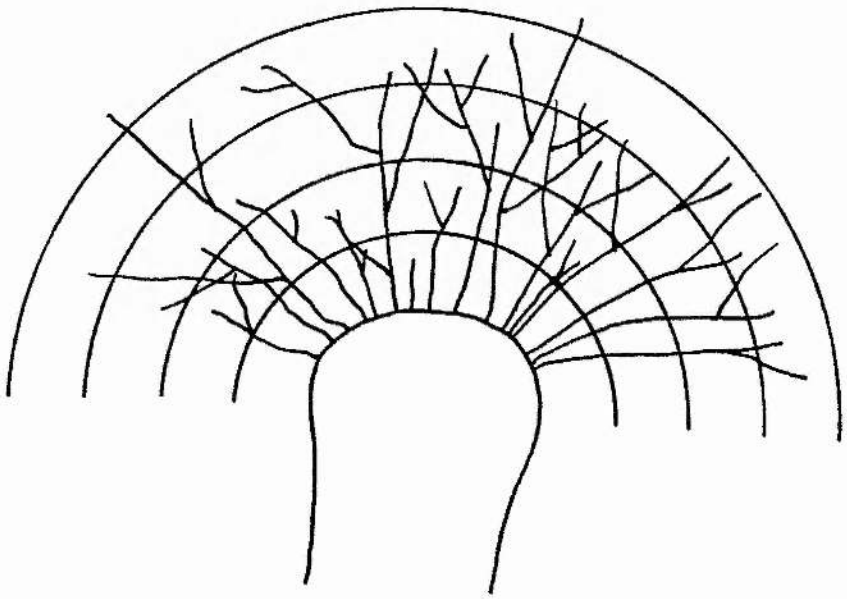


Fig. 1.4 Method used to assess fibre growth from nerve stumps. Concentric circles, centred on the nerve stump, were drawn with radii differing by 0.05 mm or 0.1 mm. The number of fibres crossing each circle was counted, and the length of the longest fibre was noted. Comparisons of such data collected from a given nerve stump over different periods of time in culture were used to assess the extent and rate of fibre outgrowth from that nerve, as described in the text.

successive ring was counted.

This method gave an estimate of the extent of outgrowth of the nerve fibre population. It was, however, more difficult to use on older cultures. Fibre outgrowth soon became too prolific near the nerve stump to be able to distinguish individual fibres; a closely opposed group of fibres could appear like one thicker fibre.

Although neither of these methods were ideal for assessing fibre outgrowth, it was possible to use them to obtain a general idea of the rate and extent, and to show up any differences between different groups of cultures.

CULTURE OF SINGLE CELLS

Preliminary attempts were made to grow single, or small groups of motoneurones from the metathoracic ganglia of cockroaches. It was not possible to remove the cells from ganglia under aseptic conditions as the equipment required to visualise individual cell bodies in whole ganglia would not fit into a laminar air flow cabinet. For this reason, isolated nerve cell bodies were transferred to a drop of growth medium, then immediately transferred to a laminar air flow cabinet and subsequently passed through about 20 changes of sterile medium to reduce risks of microbial contamination.

Selected cell bodies were removed from the metathoracic ganglion by "lassoing" their axon hillock with a piece of nylon monofilament (20 μm diameter) then gently pulling the soma free from the ganglion. The nylon monofilament could be gripped with sterile forceps for transferring the cell or cells. The culture of non-neurosecretory isolated nerve cells from nymphal or adult insects is often unsuccessful unless co-cultured with established explants from the same species (Giles, Joy and Usherwood, 1977). The explant presumably releases a

substance or substances which condition the medium. Isolated cell bodies from the metathoracic ganglion were therefore co-cultured with thoracic ganglia explanted from a different animal under aseptic conditions. Ganglia explants were either put in Petri dishes at the same time as the isolated cells, or established in culture a number of days before addition of the single cells.

Once set up, these cultures were treated in an identical way to the explant cultures.

MEDIA

Explants were cultured in several different media to establish optimal growth conditions for the nervous tissue. All cultures were first of all established in medium (1) except some grown in medium (4).

(1) Medium of Chen and Levi-Montalcini:

This medium had four components and was made up freshly each time immediately before use. It consisted of 11ml Schneider's Drosophila Medium, 9ml Basal Medium Eagles, 0.2ml Fungizone (25 µg/ml) and 0.4ml Penicillin-Streptomycin (5000 I.U. of each/ml). Details of the components are given in Table 1.1. The compositions of the two constituent media are given in Tables 1.2 and 1.3.

The Chen and Levi-Montalcini medium proved the most successful of those tried, and was therefore used for the vast majority of experiments.

(2) Mitsuhashi and Maramorosch Basal Medium:

The composition is given in Table 1.4.

(3) Grace's Medium with L-Glutamine:

The composition is given in Table 1.5.

(4) Modification of Marks' M-18 Medium:

The composition of this medium was obtained by personal

TABLE 1.2

EAGLE'S MEDIA AND MODIFIED EAGLE'S MEDIA

Component	mg/litre
$CaCl_2\ 2H_2O$	264.9
KCl	400.0
$MgSO_4\ 7H_2O$	200.0
NaCl	6800
$NaHCO_3$	1680
$NaH_2\ PO_4\ 2H_2O$	158.3
Glucose	1000
Sodium phenol red	17.00

TABLE 1.3

SCHNEIDER'S DROSOPHILA MEDIUM (Revised)

Component	mg/litre
Inorganic Salts	
$CaCl_2$ (anhyd.)	600.0
KCl	1600.0
KH_2PO_4	450.0
$MgSO_4\ 7H_2O$	3700.0
NaCl	2100.0
$NaHCO_3$	400.0
Na_2HPO_4	700.0
Other Components	
Alpha-Ketoglutaric acid	200.0
Fumaric acid	100.0
Glucose	2000.0
Malic acid	100.0
Succinic acid	100.0
Trehalose	2000.0
Yeast hydrolysate	2000.0
Amino Acids	
Beta-Alanine	500.0
L-Arginine	400.0
L-Aspartic acid	400.0
L-Cysteine	60.0
L-Cystine	100.0
L-Glutamic acid	800.0
L-Glutamine	1800.0
Glycine	250.0
L-Histidine	400.0
L-Isoleucine	150.0
L-Leucine	150.0
L-Lysine HCl	1650.0
L-Methionine	800.0
L-Phenylalanine	150.0
L-Proline	1700.0
L-Serine	250.0
L-Threonine	350.0
L-Tryptophane	100.0
L-Tyrosine	500.0
L-Valine	300.0

TABLE 1.4

MITSUHASHI AND MARAMOROSCH BASAL MEDIUM FOR MOSQUITO CELL CULTURE

Component	mg/litre
Lactalbumin hydrolysate	8125
Yeastolate	6250
$CaCl_2\ 2H_2O$	250.0
KCl	250.0
$MgCl_2\ 6H_2O$	125.0
NaCl	8750
$NaHCO_3$	150.0
$NaH_2PO_4\ 2H_2O$	282.6
Glucose	5000

TABLE 1.5

GRACE'S INSECT TISSUE CULTURE MEDIUM

Component	mg/litre
L-Isoleucine	50.00
L-Phenylalanine	150.0
L-Tryptophan	100.0
L-Leucine	75.00
L-Histidine HCl H_2O	3378.0
L-Methionine	50.00
L-Valine	100.0
L-Arginine HCl	700.0
L-Lysine HCl	625.0
L-Threonine	175.0
L-Asparagine H_2O	397.7
L-Proline	350.0
L-Glutamine	600.0
DL-Serine	1100
Glycine	650.0
L-Alanine	225.0
α-Alanine	200.0
L-Cystine	19.18
L-Tyrosine	50.00
L-Glutamic acid	600.0
L-Aspartic acid	350.0
Sucrose	26680
Fructose	400.0
Glucose	700.0
L malic acid	670.0
α-Ketoglutaric acid	370.0
D Succinic acid	60.00
Fumaric acid	55.00
p-Aminobenzoic acid	0.02
Folic acid	0.02
Riboflavin	0.02
Biotin	0.01
Thiamin HCl	0.02
D-Calcium pantothenate	0.02
Pyridoxine HCl	0.02
Nicotinic acid	0.02

TABLE 1.5 (Continued)

Component	mg/litre
i-Inositol	0.02
Choline chloride	0.2
NaH_2PO_4 $2H_2O$	1140
$CaCl_2$ $2H_2O$	1325
$MgCl_2$ $2H_2O$	2280
$MgSO_4$ $7H_2O$	2780
KCl	2240
$NaHCO_3$	350.0

1X liquid contains L-glutamine but contains no insect haemolymph.

TABLE 1.6

MODIFIED MARKS' M-18

Component	grams/litre
1. Salts*	
$CaCl_2$ 2HOH	0.450
KCl	0.400
$MgCl_2$ 6HOH	0.400
NaCl (J.T. Baker pref.)	0.790
Na_2SO_4	0.300
NaH_2PO_4 HOH	0.200
Phenol red	0.0025
Hepes 10mM	2.38
2. Amino Acids I	
L-glutamic acid	1.00
0.5 N NaOH	40 drops
L-lysine	0.200
L-histidine NCl HOH	0.661
L-arginine HCl	1.00
L-aspartic acid	0.400
L-threonine	0.100
L-asparagine HOH	0.341
L-serine	0.200
L-proline	0.500
glycine	0.600
L-alanine	0.100
beta-alanine	0.200
L-valine	0.300
L-methionine	0.300
L-isoleucine	0.100
L-leucine	0.200
L-phenylalanine	0.100
phenol red	0.0025
3. Amino Acids II	
L-tyrosine	0.166
L-cystine 2HCl	0.113
L-glutamic acid	1.00

* dissolve each component, in sequence given, before adding the next one.

TABLE 1.6(Continued)

Component	grams/litre
4. Organic Acids	
L-malic acid	0.500
alpha-ketoglutaric acid	0.300
succinic acid	0.300
fumaric acid	0.100
citric acid HOH	0.109
phenol red	0.005
5. Vitamins + ?	
Difco Bacto Yeast Extract, 0127-01	1.5
6. Antibiotics	
Penicillin K^+ (1590 U/mg. ppn.)	0.0314
Streptomycine sulphate	0.050
7. $NaHCO_3$	
$NaHCO_3$	0.600
8. Sugars	
glucose	20.0
sucrose	10.0
trehalose 2HOH	3.12
9. Glutamine - Cysteine-Tryptophan	
L-glutamine	1.00
L-cysteine $\frac{1}{2}$HOH	0.065
L-Tryptophan	0.100
10. Petal Calf Serum	ml/litre
GIBCO 614 fetal calf serum	75

communication from J. F. Vanable (Purdue University) to Dr Pitman. The composition is given in Table 1.6. It was made up from its individual constituents.

HISTOLOGICAL EXAMINATION OF GANGLION STRUCTURE

I LIGHT MICROSCOPY

Transverse sections through wax or Araldite embedded cultured ganglia were made to study their overall structure.

(A) Wax Embedded Preparations

The specimens were embedded in wax and sectioned by standard procedures. The sections were firmly adhered to microscope slides, wax was dissolved from them and they were then stained and mounted in DPX.

The following staining procedures have been used:

(a) Pyronine - Malachite Green

This relatively specifically stains ribonucleic acid pink. The method used was that of Baker and Williams (1965) where details of the procedure and solutions can be found. The stains used were:

Pyronine Y (Gurr 25200)

Malachite Green (Gurr 16900)

(b) Toluidine Blue

Ganglia were fixed in formol saline for 24 hours at room temperature. They were then prepared for embedding in Paraplast, sectioned and stained by standard procedures. The sections were stained for 10-15 minutes in a 0.1% solution of Toluidine Blue (Toluidine Blue Vital - Gurr 14062) in 30% ethanol.

(c) Osmium Tetroxide (OsO_4)

This technique has the advantage of staining all elements of the tissue black and is thus ideal for showing up clearly all

the general features. The usual procedure used is to fix in 2·5% glutaraldehyde in 0.2M sucrose/0.1M sodium cacodylate buffer (pH 7.4), wash in this buffer, and then post-fix in 1% OsO_4. The material is then taken through the wax embedding procedure. Although this method gave very good results when used on ganglia removed directly from the animal and fixed immediately, when used on cultured ganglia, tissue preservation was very poor and sections appeared extremely chattered. Better sections could be obtained if growth medium replaced sucrose/buffer in the fixative and if the tissue was cut more slowly and thinly (6 µm or 8 µm instead of 10 µm). The final technique used was as follows:

(1) Fix in 2·5% glutaraldehyde in medium for 2 hours at 4°C.

(2) Wash in sucrose/buffer solution - two washes, 15 minutes each at 4°C.

(3) Post-fix in 1% or 0.5% OsO_4 in sucrose/buffer solution for 1 hour at 4°C.

(4) Wash in sucrose/buffer solution - two washes, 15 minutes each at room temperature.

(B) Araldite Embedded Preparations

(i) Fixation and Embedding

It was necessary to modify the standard Araldite embedding technique in the following way to achieve satisfactory results with cultured ganglia:

(1) Fix Pour fixative solution (Table 1.7) at room temperature into the Petri dish containing the preparation, also at room temperature. Transfer the Petri dish to the refrigerator and maintain at 4°C for the remainder of the fixation period (8 hours). Replace the fixative solution with fresh fixative at 4°C once during this period.

(2) Wash - two washes in buffer solution, 20-30 minutes each at 4°C.

(3) Post-Fixation in 1% OsO_4 for 2-3 hours at 4°C.

(4) Allow specimens to reach room temperature.

(5) Wash - two washes in distilled water, 20 minutes each at room temperature, and transfer to a glass vial.

(6) Dehydrate through a series of ethanols from 30% to 100% - 30 minutes in each solution.

(7) Transfer to 1:1 mixture of 100% ethanol and epoxypropane - 30 minutes.

(8) Clear in pure epoxypropane - three changes - 20 minutes each.

(9) Transfer to 1:1 mixture of epoxypropane and Araldite mixture ABCD (Table 1.7). Leave overnight at room temperature with caps off the vials to allow the epoxypropane to evaporate. The solution should only just cover the preparation so that complete evaporation is possible.

(10) Transfer to fresh vials containing pure Araldite ABCD mixture. Leave for 4-5 hours.

(11) Embed at the required orientation in a silicone embedding tray containing fresh Araldite ABCD. Leave for at least 48 hours at 60°C.

(ii) Sectioning and Staining

Semi-thin sections (1.5 μm) were cut on an ultramicrotome (Reichert Om U2) and spread with xylene vapour. Each section was then transferred to a separate drop of distilled water on a clean microscope slide. The distilled water was dried off

TABLE 1.7

SOLUTIONS FOR ARALDITE EMBEDDING AND STAINING

(a) Buffer Solution

0.2M sodium cacodylate in 0.25M sucrose solution. pH 7.4

Store at 4°C.

(b) Fixative in Buffer

2.5% glutaraldehyde in solution (a). Make up freshly before use.

(c) 1% Osmium Tetroxide Solution

0.1g ampoule of solid osmium tetroxide dissolved in 10 ml of solution (a).

(d) Araldite

'AB' Mix

10g of resin (CY 212) and 10g of hardener (DDSA) mixed by rotation for 1 hour.

'CD' Mix

3g of accelerator (DMP 30) and 3 drops of plasticizer (di-butyl phthalate) mixed by rotation for 1 hour.

'ABCD' Mix

20g of 'AB' and 0.5g of 'CD' mixed by rotation for 1 hour.

All mixes can be stored in the deep freeze.

(e) Toluidine Blue

0.5g sodium tetraborate and 0.25g Toluidine Blue dissolved by heating in 100 ml distilled water. The solution is filtered when cool.

(f) 2% Uranyl Acetate

The uranyl acetate is dissolved in 50% ethanol, keeping the solution in the dark.

(g) 0.4% Lead Citrate

Lead citrate dissolved in 0.1N sodium hydroxide which is free of CO_2. This solution must be made up about 15 minutes prior to use. Just before use the solution is filtered into a CO_2 free atmosphere.

on a hot plate, taking care not to allow the water to boil, and then allowed to cool. Sufficient Toluidine Blue (Table 1.7) was applied to cover the sections and the slide heated, again avoiding boiling, until a green ring started to form around the edge of the stain (about 3 seconds). Excess stain was drained off, the slide allowed to cool for a few seconds, and thenbriefly washed with distilled water. Once dry, the sections were mounted in DPX.

II ELECTRON MICROSCOPY

(A) Transmission Electron Microscopy

Araldite embedded material was prepared as for semi-thin sections for light microscopy. Thin sections (gold) were cut on a Reichert OmU2 ultramicrotome and collected on grids (150 mesh) and dried. They were then taken through the following:

(1) Stain in uranyl acetate for 30 minutes in the dark.

(2) Rinse thoroughly in 2 changes of 50% ethanol.

(3) Rinse in distilled water.

(4) Rinse in 0.1N NaOH.

(5) Immerse in lead citrate solution in a carbon dioxide-free atmosphere for about 10 minutes.

(6) Rinse in 2 changes of 0.1N NaOH.

(7) Rinse 3 times in distilled water.

(8) Dry on filter paper.

Sections were observed using a Phillips 301B transmission electron microscope, at 60 kV.

(B) Scanning Electron Microscopy

Ganglia were cultured on small, sterile glass coverslips, 1cm in diameter, in plastic Petri dishes. The glass coverslips

provided a convenient way of removing the cultures from the plastic dishes without disturbing the finer nerve processes. After 2-3 weeks the fibre outgrowth from the ganglia was fairly extensive, and the glass coverslips were removed from the culture dishes. Cultures were prepared for observations under the scanning electron microscope as follows:

(1) Fix for $1\frac{1}{2}$-$2\frac{1}{2}$ hours in the same fixative used for Araldite embedded preparations (Table 1.7)

(2) Wash several times in buffer (Table 1.7)

(3) Dehydrate through an ascending series of ethanols.

(4) Transfer to amyl acetate.

(5) Critical point dry in liquid CO_2. This drying method is used since it should generate minimal pressures and tensions on the specimen, and therefore preserve any delicate features.

(6) Glue glass coverslips containing cultures to metal stubs using Gestetner Corrector Fluid.

(7) Paint junction between the coverslip and stub with Colloidal Silver (M154) to ensure electrical continuity between the two.

(8) Gold coat the preparations.

The cultures were observed using a Cambridge S 600 Scanning Electron Microscope (Gatty Marine Laboratory).

RESULTS

(A) GENERAL FEATURES OF EXPLANT CULTURES

Thoracic and abdominal ganglia explanted from adult and nymphal cockroaches produced fibre outgrowth within the first 1 - 2 weeks in culture. This outgrowth varied greatly in extent and form between one culture and another, although it was generally more extensive from thoracic ganglia than from abdominal ganglia. Such unpredictability and inconsistency in growth was also observed by Levi-Montalcini and Chen (1969). This great variability makes it a difficult task to quantify many of the results, and for this reason, the general features will be discussed largely qualitatively.

(1) EXPLANTS FROM ADULT ANIMALS

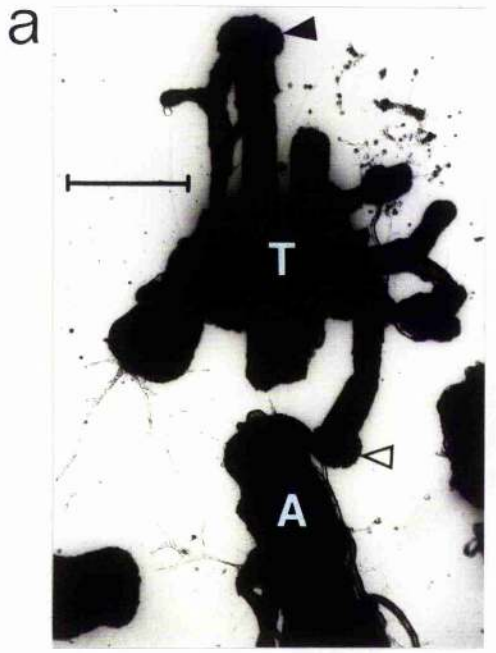
(a) General Features of the Entire Explant

When ganglia had been explanted into culture, the cut ends of the nerve stumps were sometimes swollen (Fig. 1.5 a & b). The cause was unknown, although occasionally similar swollen endings were seen on nerve stumps *in vivo* following nerve lesions, indicating that this is probably a response to damage rather than one produced by the culture environment. These swollen endings were most commonly seen in the larger nerve stumps, such as nerve 5 and the connectives. They were rarely, if ever, observed in small nerve trunks. Sometimes clear, vesicular structures were seen at the cut surface of such swollen nerve stumps *in vitro* (Fig. 1.5 b). The nature and reason for the appearance of these features is unknown; they did not persist for more than a few days.

Fibres resembling neuronal processes originated from the cut ends of nerve trunks (Fig. 1.6). Although occasionally seen within 24 hours of ganglion explantation, such outgrowth did not

Fig. 1.5a Micrograph of an adult metathoracic ganglion (T) and abdominal ganglion (A) after 19 days in culture. Swollen ends are particularly noticeable on an anterior connective (solid arrowhead) and nerve 5 (open arrowhead) of the metathoracic ganglion. This, and all following micrographs of cultures, were taken of living explants from cockroaches. Calibration : 1 mm.

Fig. 1.5b Swollen end of anterior connective from an adult thoracic ganglion after 5 days in culture. The normal width of the connective can be seen at the bottom of the photograph (between white arrowheads). Note the vesicular structure (solid arrowhead) at the sectioned surface of the connective.
Calibration : 200 μm


a
T
A

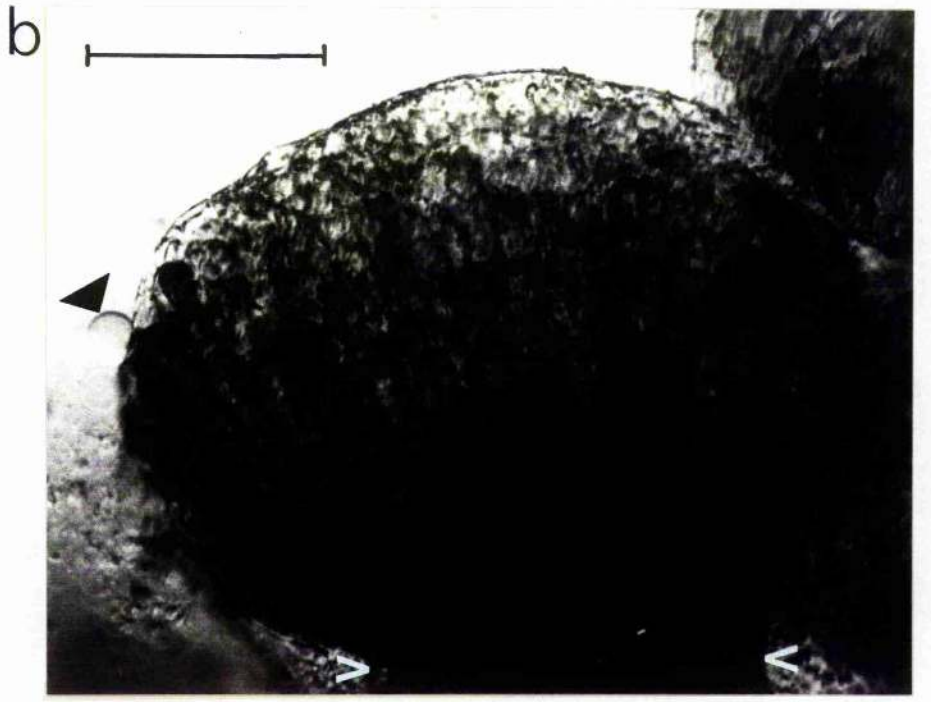

b

Fig. 1.6 Adult mesothoracic ganglion after 13 days in culture. Fibre outgrowth (arrows) is seen mainly from the cut ends of nerve trunks after this short period in vitro. Calibration : 1 mm

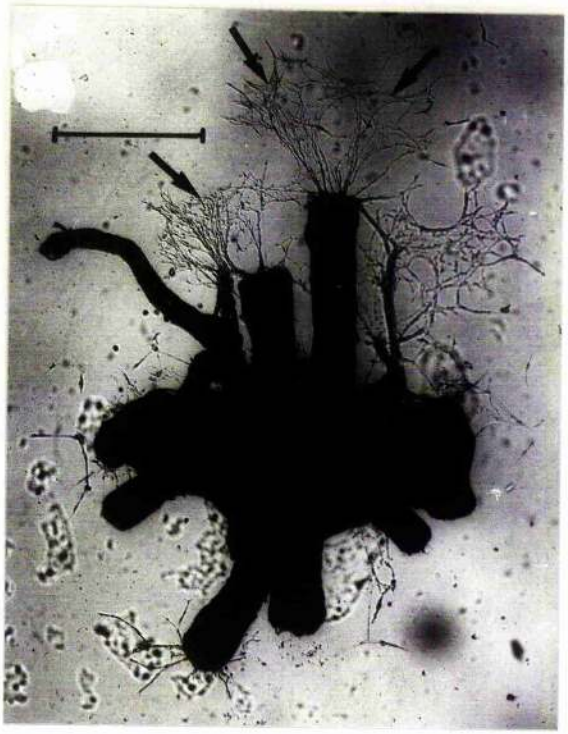


Fig. 1.7 The initiation of fibre outgrowth from thoracic and abdominal ganglia maintained in culture at 28°C and 37°C. The graph shows the percentage of explants producing fibre outgrowth at given times in culture. Any explants showing no outgrowth by 21 days were discarded, and data from such explants were not included in this graph; therefore, in all categories, 100% of the explants represented here showed outgrowth by 21 days. (The percentage of the total number of explants showing outgrowth by 21 days can be seen in Fig. 1.21).

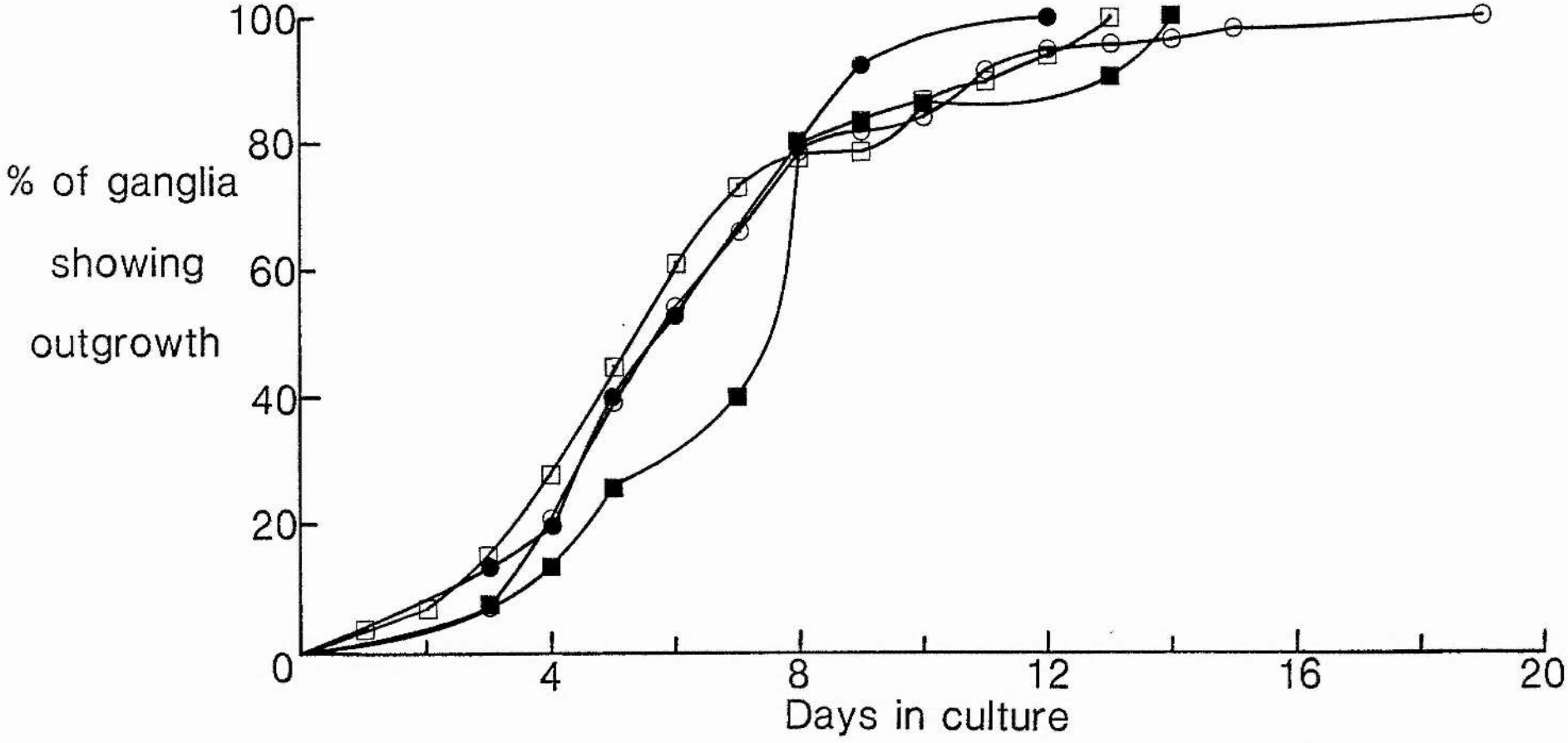


Figure 1.7

—○— Abdominal ganglia at 28°C (n=105)

—□— Thoracic ganglia at 28°C (n=116)

—●— Abdominal ganglia at 37°C (n=15)

—■— Thoracic ganglia at 37°C (n=30)

Fig. 1.8 Adult metathoracic (T_3) and first three abdominal (A_1, A_2 and A_3) ganglia after 22 days in culture. After this longer period in vitro, fibres radiate from all around the ganglia, as well as from cut nerve trunks (c.f. Fig. 1.6). Calibration : 1 mm.

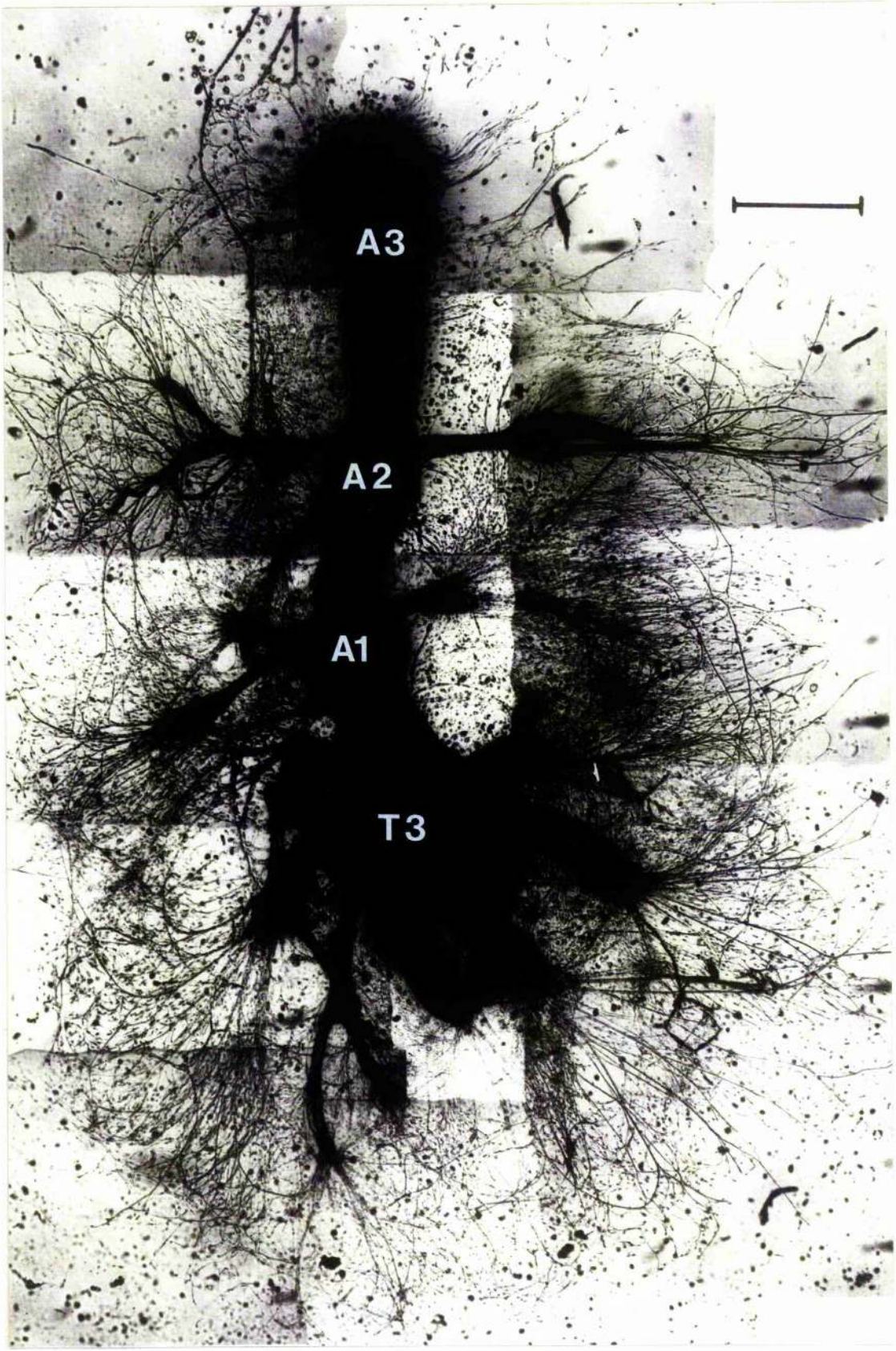
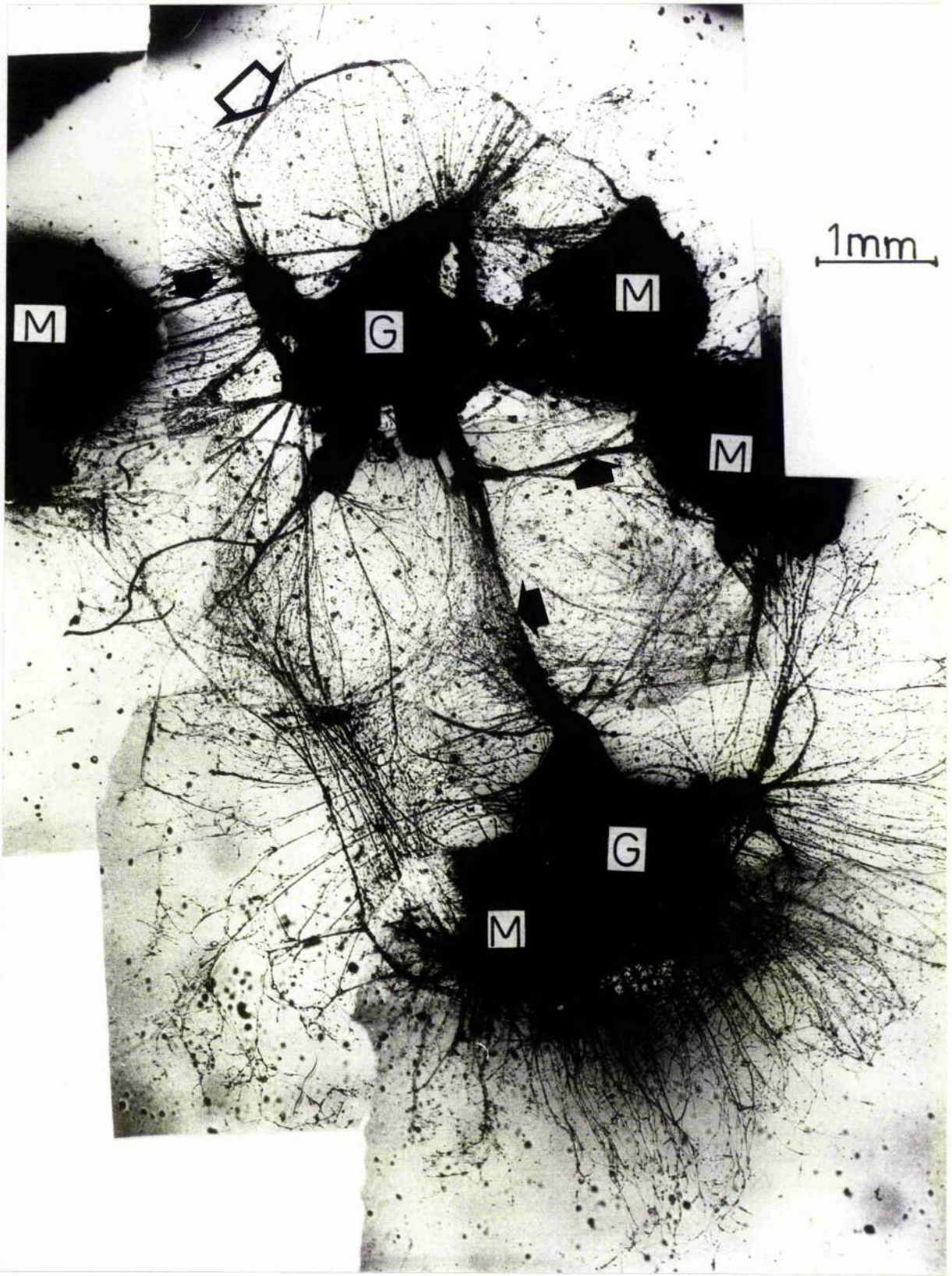

A3
A2
A1
T3

Fig. 1.9 Two adult thoracic ganglia (G) and four coxal depressor muscle (M) explants after 177 days in co-culture. Fibre outgrowth is still prolific, forming a complex, interconnecting network. Some fibre bundles bridge gaps between explants (solid arrows), while others form long fasicles (open arrow) encircling the explants.


M
G
M
M
1mm
G
M

occur before 5 - 8 days in the majority of thoracic or abdominal ganglion explants. The delay was similar in ganglia maintained at 37°C or 28°C. Fig. 1.7 graphically represents the time at which the first processes can be observed emerging from abdominal and thoracic ganglia maintained at either 37°C or 28°C. Any cultures which showed no signs of outgrowth by 21 days were discarded.

Although early fibre outgrowth from young cultures was usually seen to originate from the cut ends of nerve trunks, after about 2 - 3 weeks in culture, fibres appeared to radiate from all around ganglia (Fig. 1.8). As fibre growth became more extensive, a complex network was formed around the ganglion (Fig. 1.9).

Often the ends of ganglionic nerve stumps became fused together, especially if fibre outgrowth from them was extensive, and as the culture aged the features of the ganglion became hard to distinguish (Fig. 1.10).

In the first 3 - 4 weeks after explantation, fibre outgrowth was rapid and prolific; it was in this period that the most prominent features of the culture developed. After about 4 weeks the changes were less marked, but this did not indicate the onset of a deterioration of the cultures as a number were maintained for up to 29 weeks with little signs of fibre regression or thinning.

The orientation of fibre growth appeared to be largely random, although fibres often grew close together for fairly long distances, forming bundles (Fig. 1.11 and 1.12) which ranged from about 10 µm to 80 µm thick. Such bundles usually formed connections between explants, or long fasicles running along a curved path partially encircling the explants (Fig. 1.9). 'Free ended' fibres (i.e. fibres which remained unconnected at their end to any part of the explant from which they originated, or to any other explant)

Fig. 1.10 'Rounding up' of ganglia in culture. The photograph shows an adult sixth abdominal ganglion (G) and coxal depressor muscle explants (M) after 47 days in co-culture. The cercal nerves (C) and the ganglion have become almost indistinguishable as separate features. Note the strong connections made by nerve fibres (arrowheads) between the neural and muscle explants.

Calibration : 1 mm.

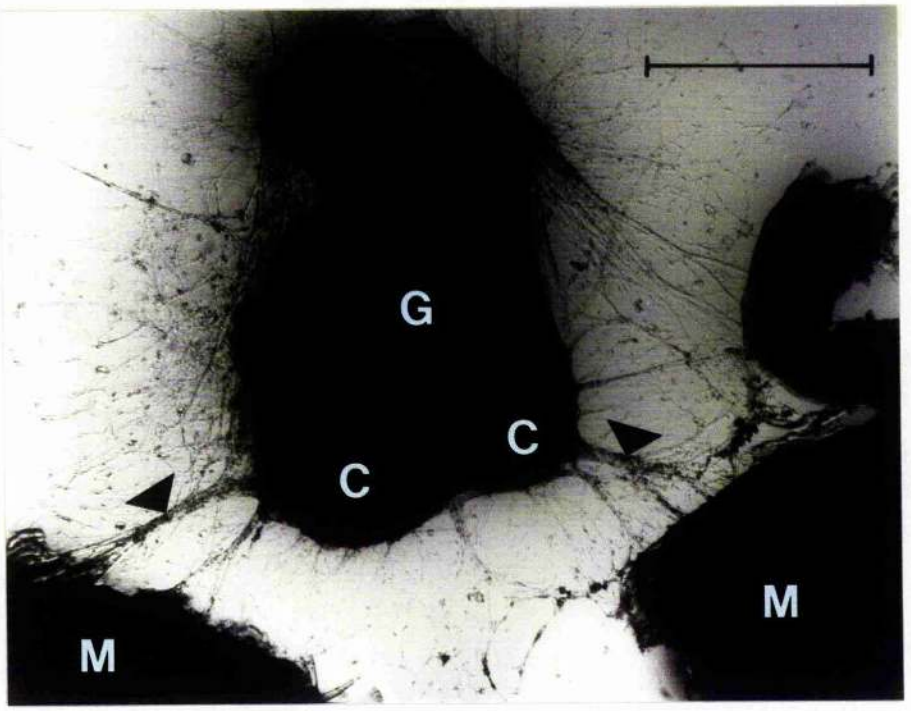

G
C
C
M
M

Fig. 1.11 Connections formed between adult ganglia after 19 days in culture. Nerve fibres connect the posterior connectives of the prothoracic ganglion (bottom of photograph) to the anterior connectives of the third abdominal ganglion (top of photograph). The majority of the fibres grow together forming a parallel bundle (arrow). Calibration : 0.5 mm.

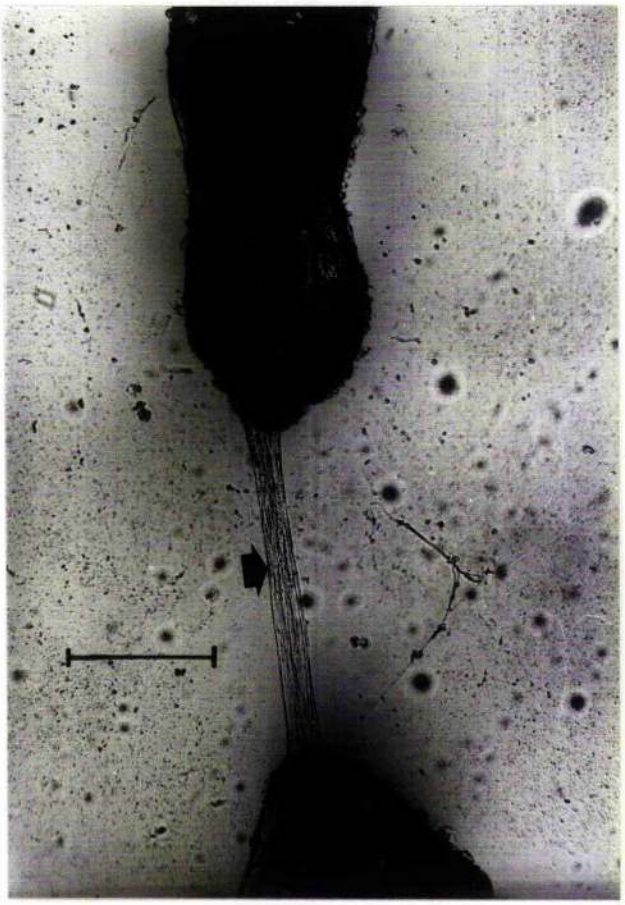
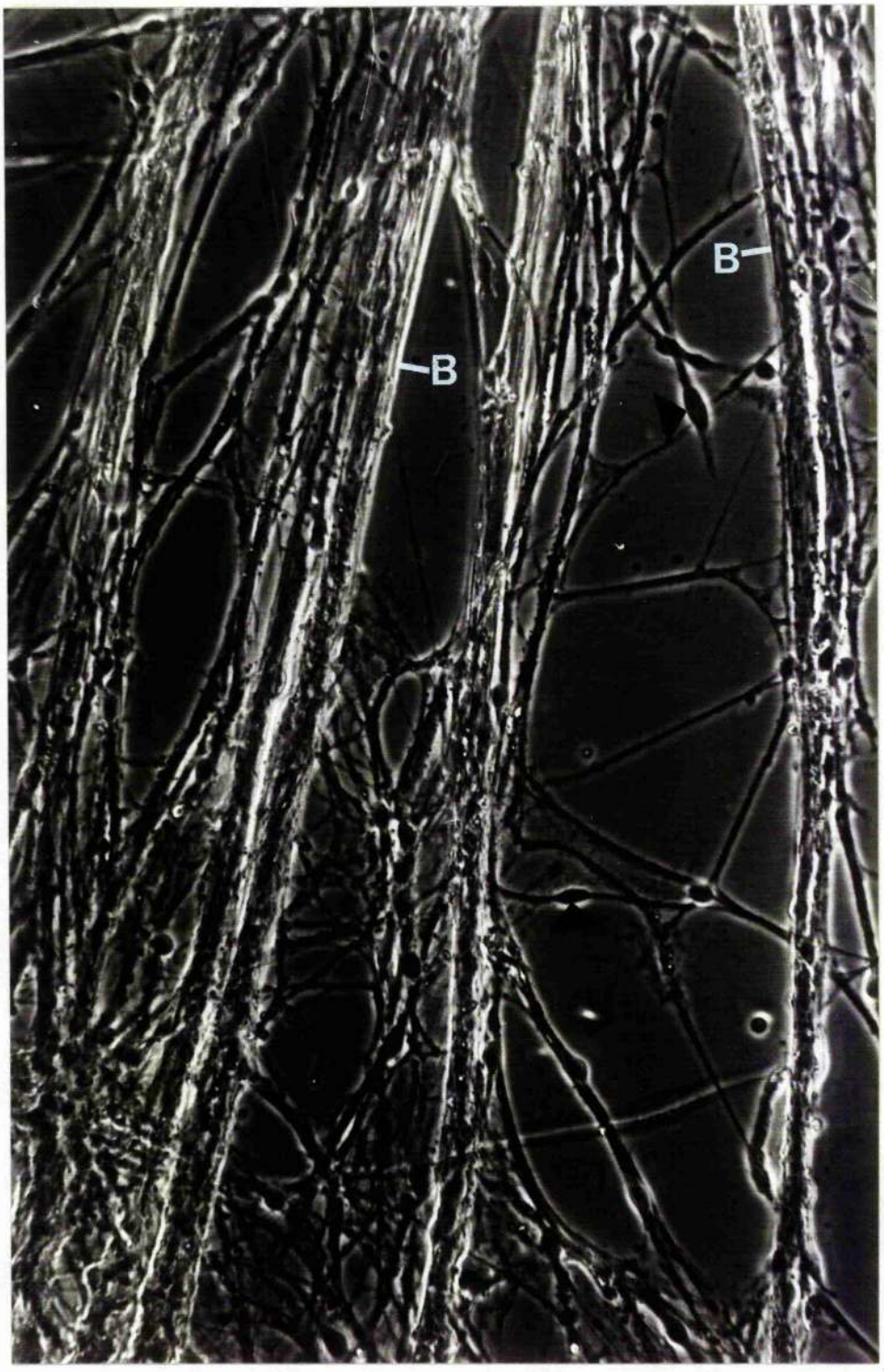


Fig. 1.12 Detailed appearance of nerve fibres from an adult thoracic ganglion after 52 days in culture. A number of fibres run in parallel groups forming bundles (B). Note also the varicosities (arrowheads) within many fibres. Photographed under phase contrast illumination. Calibration : 100 μm


B
B

did not develop into bundles, although they could attain a length of several millimetres and persist without showing any major signs of regression.

(b) Features of the Nerve Fibres

Fibres growing from the explants commonly showed frequent branching (Fig. 1.13), and were often varicose along their lengths (Fig. 1.14). Fibre diameters ranged from less than 1 μm to about 5 μm; the diameter of varicosities ranged from 2 μm to 17 μm.

Many fibres could be seen to terminate in structures which appeared to be growth cones, since they had the following characteristic features:

a) microspikes and veil-like expansions around their periphery,

b) contained granular structures

c) measured approximately 10 - 30 μm across.

All of these features can be seen in Fig. 1.15.

Single fibres, and sometimes bundles of fibres, formed points of anchorage to the Petri dish. This became apparent when the dish was gently disturbed, and fibres or fibre bundles were seen to move freely along part of their length, but remained attached to the dish at distinct points. Fibres could form points of attachment along their length or at the ends. When such attachments formed along the fibre length, they were often followed by an abrupt change in direction of fibre growth (Fig. 1.16). Fibre bundles sometimes became indirectly held in place by a number of fine 'off-shoot' fibres which grew at right angles to the main bundle, and were attached to the Petri dish at their ends (Fig. 1.17).

Disturbance of fibres which were free along part of their length illustrated their elastic properties, as they became bowed and then returned to their original straight form once turbulence

Fig. 1.13 Branching of nerve fibres in culture. The photograph shows a nerve fibre from an adult abdominal ganglion after 42 days in culture. Note the branch point (arrow), varicosities (solid arrowheads), and growth cones (open arrowheads) at the tips of the two branches. Photographed under phase contrast illumination.

Calibration : 100 µm

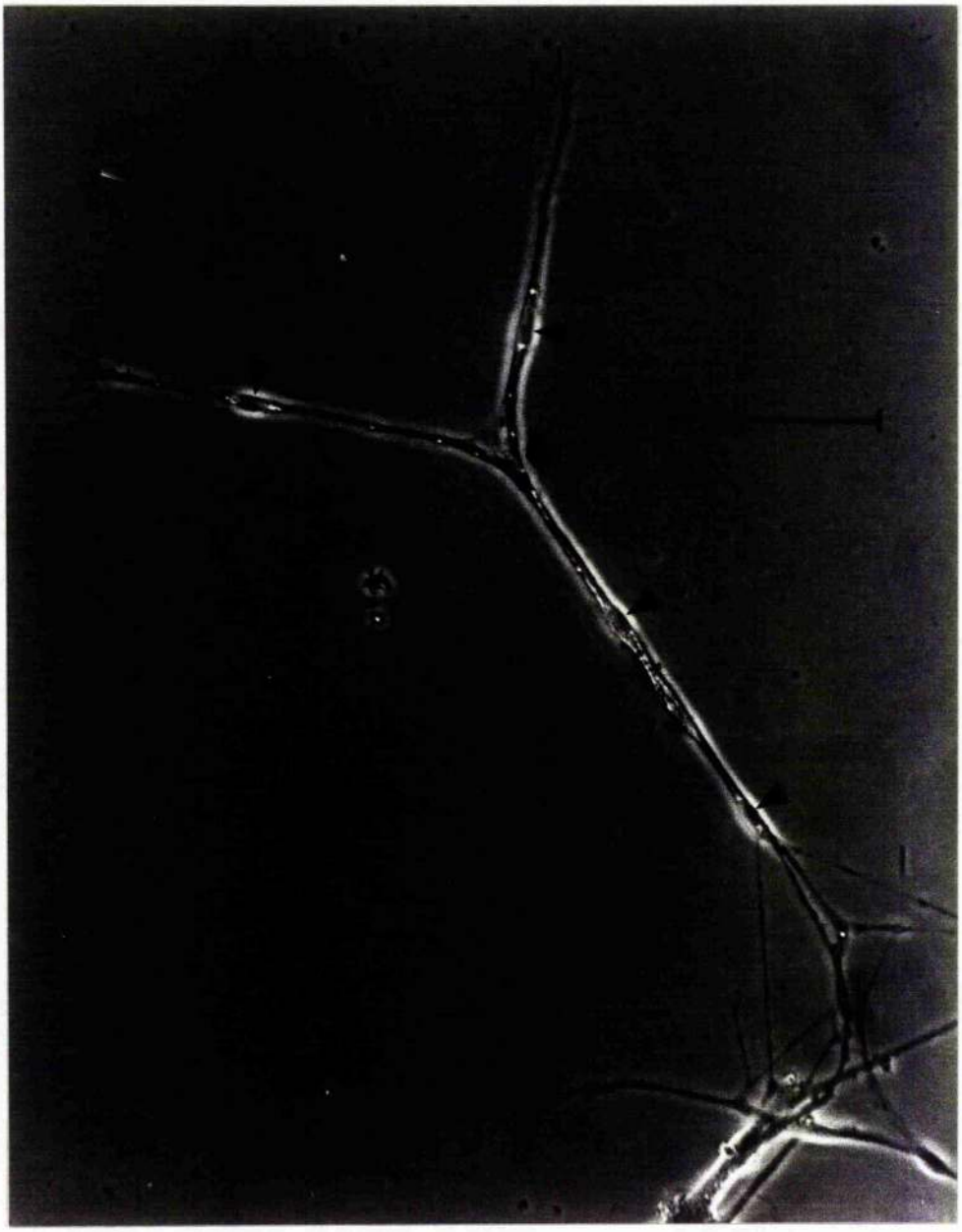


Fig. 1.14 Fibre outgrowth from anterior connective (C) of an adult mesothoracic ganglion after 8 days in culture. Many varicosities (arrowheads) can be seen along the length of the fibres. Photographed under phase contrast illumination. Calibration : 100 µm

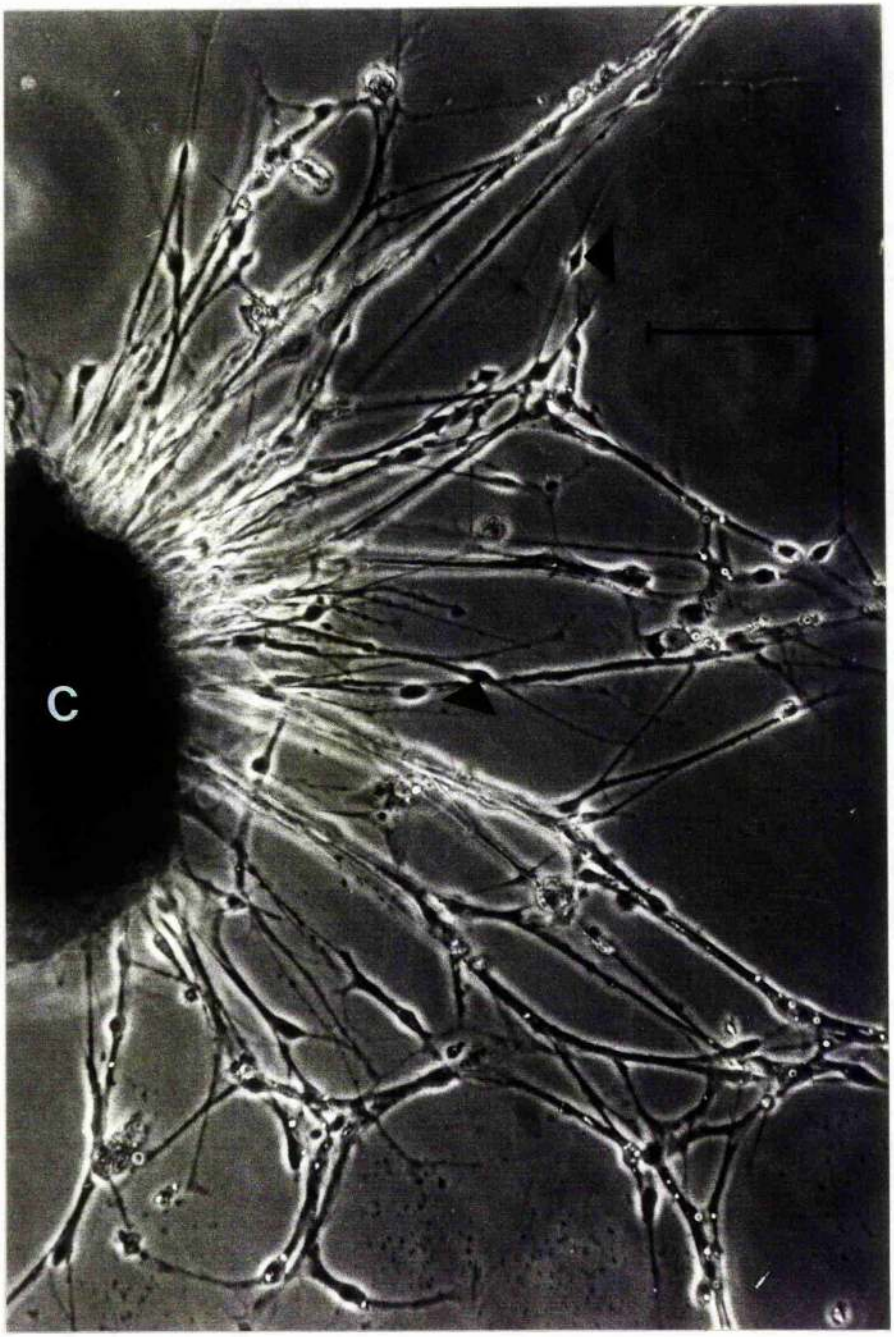

C

Fig. 1.15 Nerve fibre terminating in growth cone. The fibre is from an adult thoracic ganglion after 5 days in culture. The growth cone (large open arrowhead) contains granules; several microspikes (solid arrowheads) extend from the margins, some of which support characteristic veil-like expansions (small open arrowheads). Photographed under phase contrast illumination. Calibration : 100 µm.

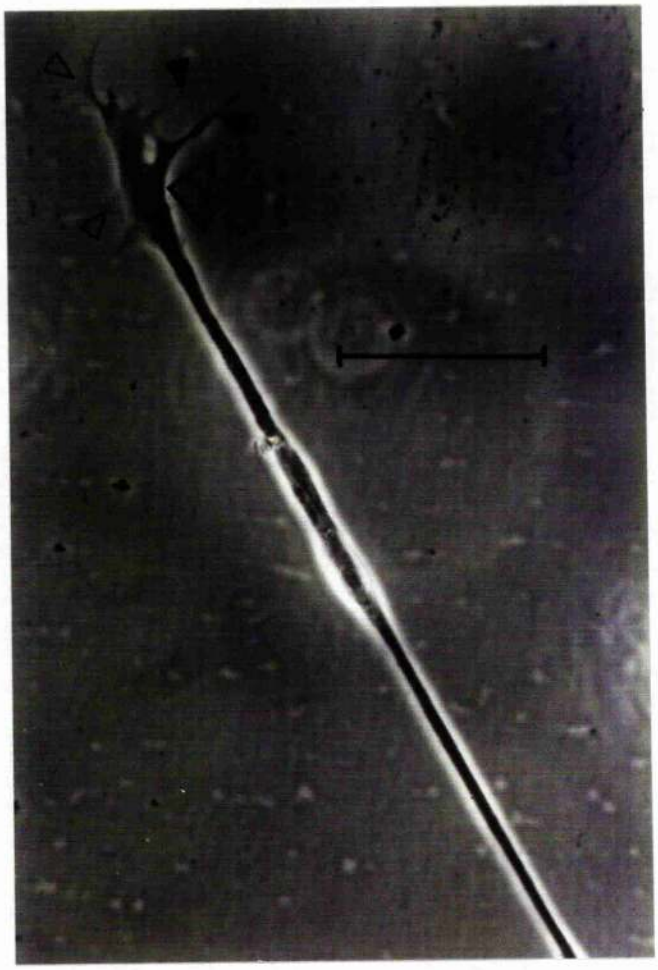


Fig. 1.16 Adhesion of nerve fibres to substratum. The photograph shows nerve fibres from an adult thoracic ganglion after 64 days in culture. One fibre has formed a point of attachment to the Petri dish (arrow), after which it abruptly changes direction of growth. Photographed under phase contrast illumination.

Calibration : 100 µm

Fig. 1.17 'Anchoring' of nerve fibres in culture. The photograph shows nerve fibres from an adult thoracic ganglion after 177 days in culture. A bundle of fibres (solid arrow), which is not directly attached to the surface of the Petri dish, is indirectly held in place by a group of fine fibres (arrowheads) attached to the dish at their ends. Some unidentified cells (open arrows) can be seen. Photographed under phase contrast illumination.

Calibration : 100 μm

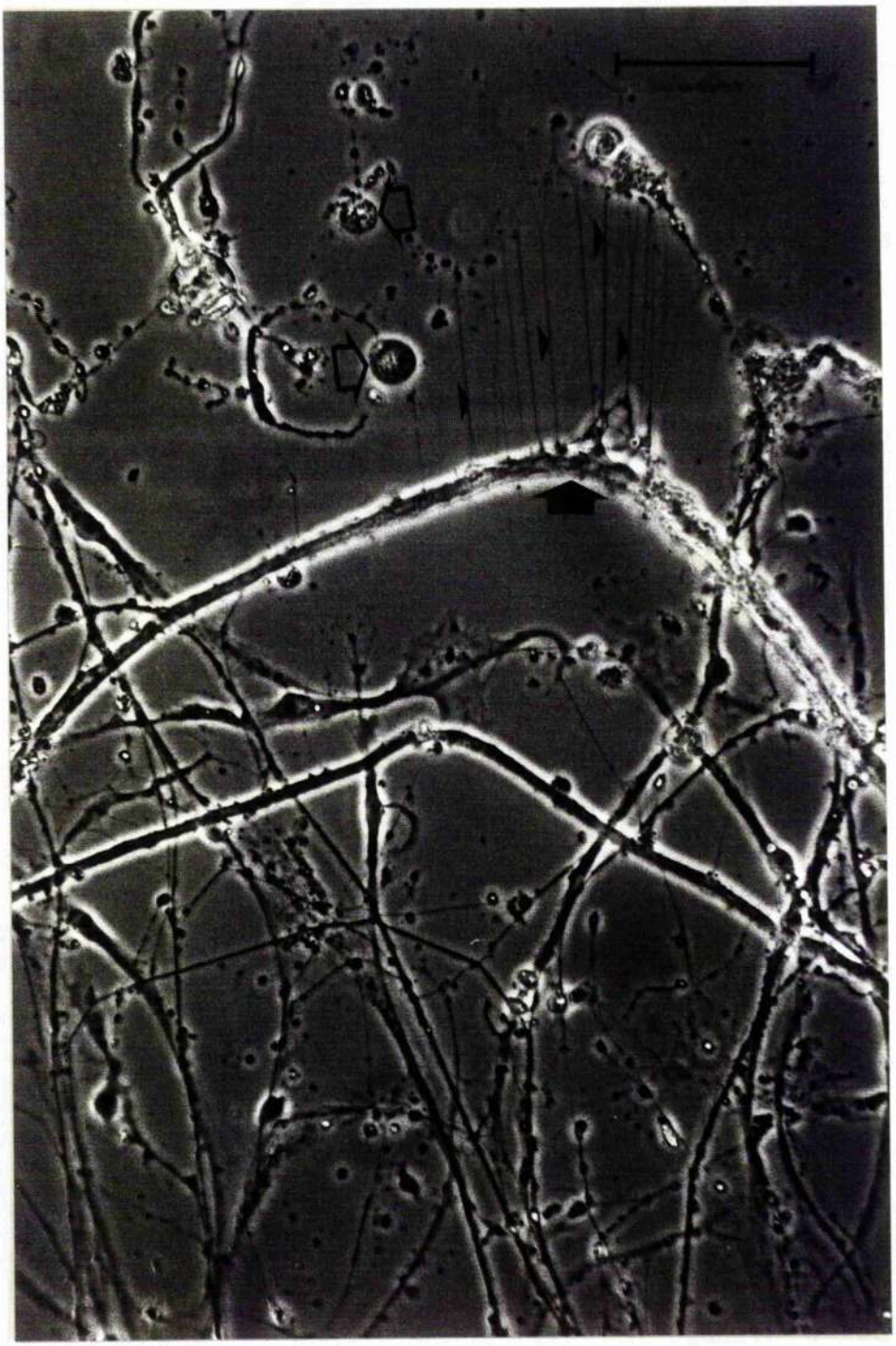
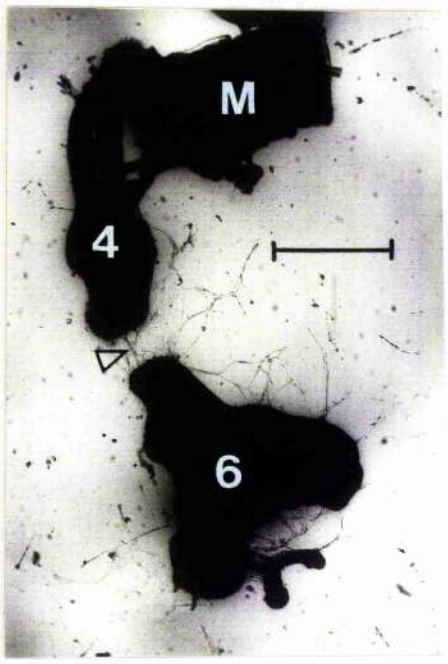


Fig. 1.18 The development of tension in fibres joining explants

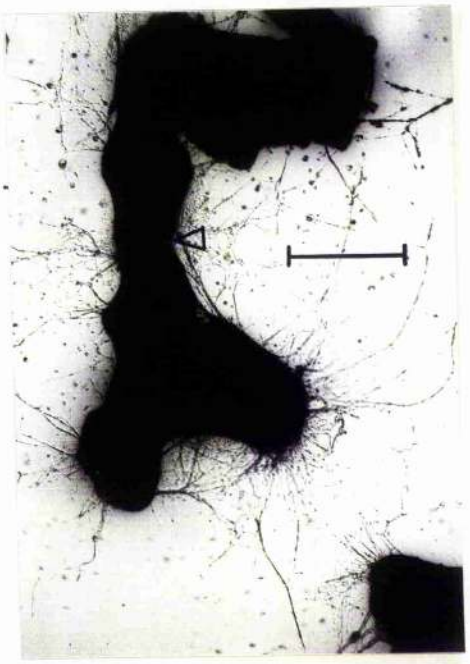
(a) Adult sixth abdominal (6) and fourth abdominal (4) ganglia after 17 days in culture. A number of fibres (open arrowhead) form connections between the connectives of the two ganglia. A coxal depressor muscle explant (M) is also present.
Calibration : 1 mm

(b) The same preparation as in (a), after 56 days in culture. The two ganglia have become drawn together, and their connectives appear to have fused at the point indicated by the open arrowhead.
Calibration : 1 mm

a



b



in the medium ceased. There were also indications that these fibres were under tension, since explants which became physically connected to one another by fibres were often drawn closer together with time; it was not uncommon to see such explants become drawn so close together that they became fused, so obscuring fibres connecting the ganglia (Fig. 1.18).

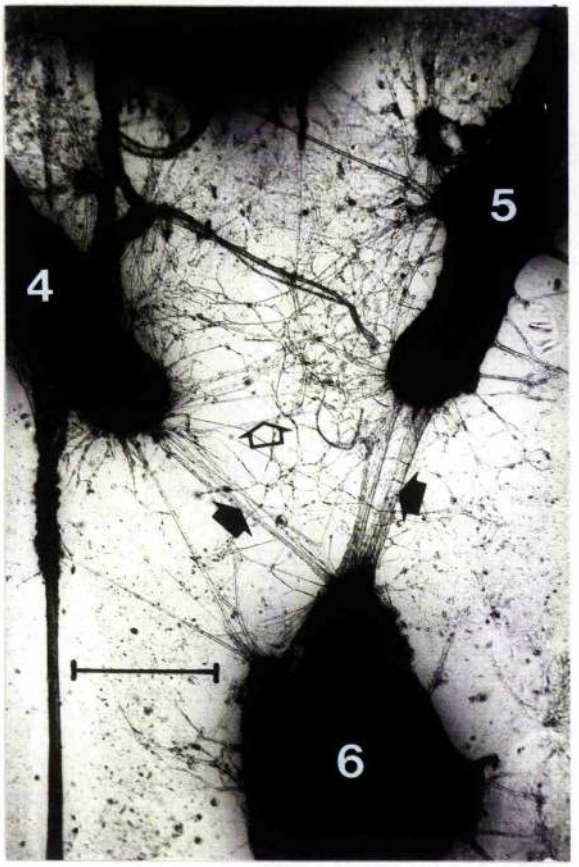
(c) Connections Between Explants

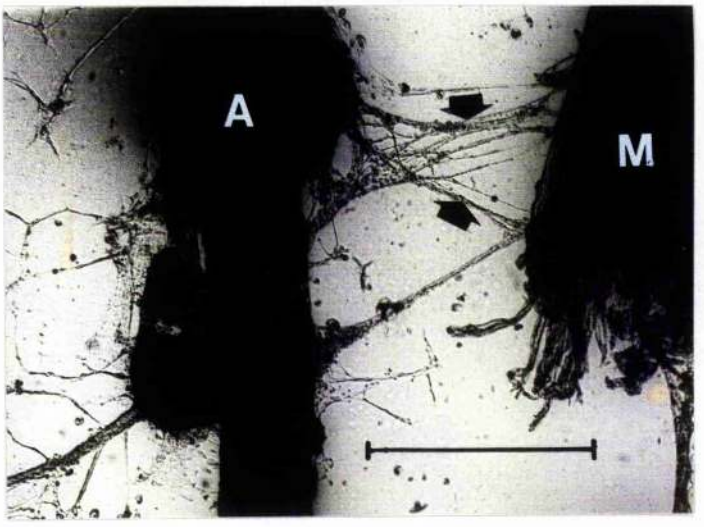
Ganglia in vitro frequently became physically linked to one another by interconnecting fibres. Connections could be made between ganglia separated by more than 1 mm. Although ganglia became physically connected no evidence was obtained to indicate that these connections were accompanied by synaptogenesis. There was no apparent selectivity in connections. Many fibres from a single ganglion were often interconnected in a very complex way. Ganglia given a choice between their normal neighbouring ganglion and a more distant one, usually formed connections of similar density in each case. Some exceptions to this were seen between abdominal ganglia where the large terminal abdominal ganglion formed more connections with other smaller abdominal ganglia than did the smaller ones with each other (Fig. 1.19). This, however, may only reflect more extensive outgrowth from the larger ganglion which would contain more cell bodies than the small abdominal ganglia.

Connections could be made between ganglia and coxal muscle explants. Once again, there appeared to be little or no selectivity, since both abdominal (Fig. 1.10 and 1.20) and thoracic (Fig. 1.9) ganglia were capable of forming such connections. Those made by thoracic ganglia, however, tended to be somewhat more dense, but this again may only reflect more profuse outgrowth seen from

Fig. 1.19 Selectivity of connections between ganglia.
Fourth, fifth and sixth adult abdominal ganglia (labelled 4,5, and 6 respectively) after 11 days in culture. Numerous fibres (solid arrows) connect the terminal (sixth) ganglion to the smaller ganglia; only a few processes are seen between the connectives of the smaller (fourth and fifth) ganglia (open arrow). Calibration : 1 mm

Fig. 1.20 Connections between adult abdominal ganglion and muscle explant after 29 days in co-culture. Fibres (solid arrows) from the abdominal ganglion (A) form connections with the coxal depressor muscle explants (M).
Calibration : 1 mm


4
5
6


A
M

the thoracic ganglia.

Occasionally contractions of the muscle explants were observed. However, attempts to record post-synaptic potentials were unsuccessful, and therefore it is not possible to be sure whether contractions reflected pacemaker activity in the muscle fibres or functional synaptic connections between nerve and muscle explants.

(d) The Effect of Temperature on Fibre Outgrowth

The percentage of thoracic and abdominal ganglion explants developing fibre outgrowth by 21 days at 28°C or 37°C is shown in Fig. 1.21. A very small percentage of explants failed to show any outgrowth at 28°C, whereas at the higher temperature, a much higher proportion of explants showed no outgrowth. However, among those ganglia which did produce nerve processes, the timing of initial outgrowth was similar whether they were maintained at 28°C or 37°C. This suggests that maintenance at the higher temperature has an all-or-none effect on the initiation of growth, rather than simply slowing down this stage in the regenerative processes. Fibres extending from explants maintained at 37°C tended to be less profuse than from those maintained at 28°C. The rate of fibre extension was also slower at 37°C, ranging from 2 μm/h to 6 μm/h, and usually averaging approximately 2.5 μm/h, while at 28°C, the growth rate ranged from 2 μm/h to 11 μm/h, and was usually approximately 6.5 μm/h.

A number of cultures were inadvertently subjected to a fall in temperature from 28°C to about 19°C for seven days. The temperature was then raised again to 28°C. The appearance of the cultures was recorded photographically before, and for several weeks following the changes in temperature. Analysis of these photographs revealed that the cultures had survived the fall in

Maintenance Temperature	Ganglion Type	Total No. of Explants Observed	Total No. Showing Outgrowth by 21d.	% Showing Outgrowth by 21d.
37°C	Abdominal	21	15	72%
37°C	Thoracic	49	30	61%
28°C	Abdominal	108	105	97%
28°C	Thoracic	117	116	99%

Fig. 1.21 The effect of temperature on the number of explants showing fibre outgrowth. The table shows the percentage of the total number of explanted ganglia producing fibre outgrowth by 21 days in culture at either 28°C or 37°C.

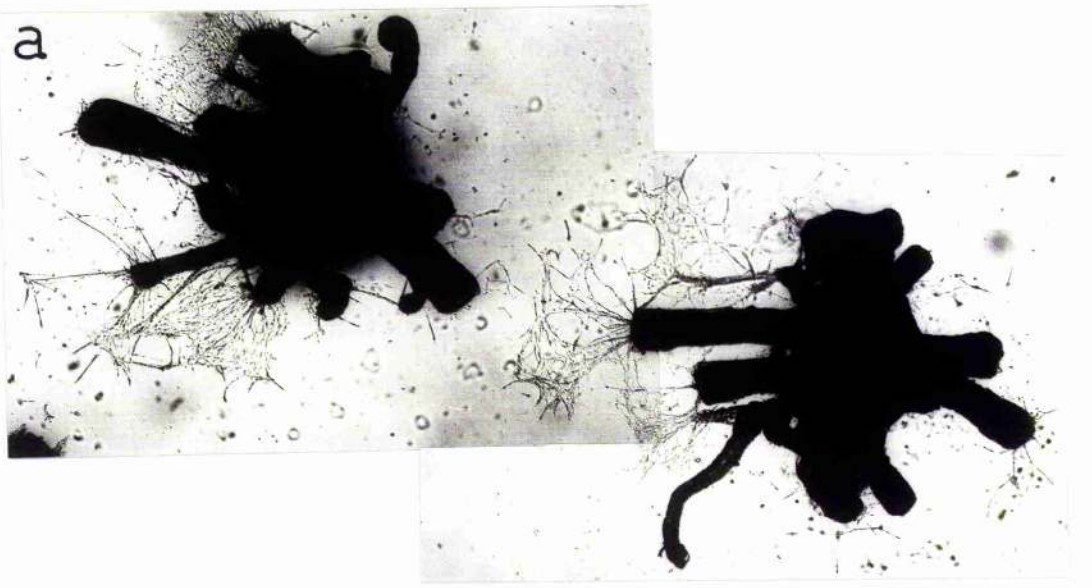
Fig. 1.22 The effect of temperature on fibre outgrowth.

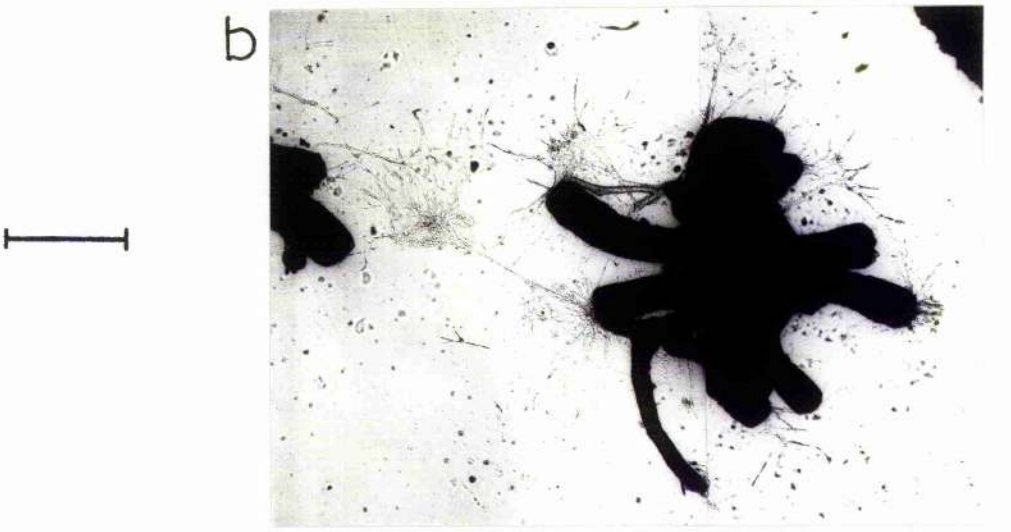
(a) Adult prothoracic (left of picture) and mesothoracic (right of picture) ganglia after 13 days in culture at 28°C. Fairly extensive fibre outgrowth can be seen from several nerve stumps.

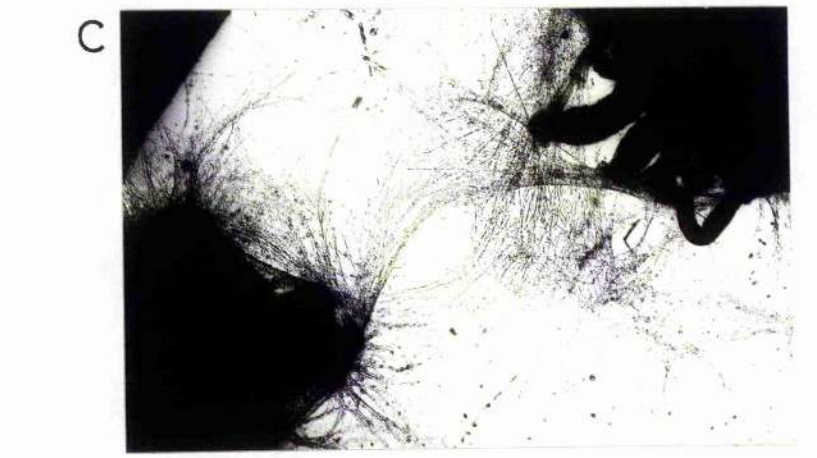
(b) The same preparation as in (a) after 19 days in culture. The maintenance temperature fell from 28°C to 19°C between day 14 and day 19. Little, if any, fibre regression is evident as a result of this fall in temperature, although some fibre groups appear to have become somewhat disconnected from their origin.

(c) The same preparation as in (a) after 162 days in culture. The maintenance temperature was restored to 28°C on day 19 and remained at this thereafter. Fibre outgrowth has become extensive between ganglia, indicating that the explants survived the fall in temperature to 19°C.

Calibration : 1 mm


a


b


c

temperature, and continued to produce further fibre outgrowth after return to 28°C (Fig. 1.22 a - c). These cultures are therefore resistant to quite large temperature changes. The significance of this is considered in the Discussion to this chapter.

(e) Muscle Explants in Vitro

As described previously, muscle explants were included with some of the neural cultures. Contractions of muscle explants were very infrequent. On only three occasions out of more than 100 were co-cultured explants seen to contract. In two of these cases, the muscle was obtained from the body wall of the animal, while in the third it was inadvertently included in the culture and was therefore unidentifiable. Contractions were never seen in coxal depressor muscle explants. After 10 or more days in culture, it was not possible to record resting potentials with certainty from contractile or non-contracting muscle. Microelectrodes only recorded slow negative potential shifts; these potentials ranged from 4mV to 22mV and averaged about 11mV, and may have been pressure potentials. In contrast, coxal depressor muscle which was freshly dissected from an animal and placed in culture medium gave responses similar to those obtained by other workers using acute preparations. Rapid, sudden potential changes were observed on penetration of the fibres, and resting potentials ranged from 24mV to 53mV (average 37.75mV). This same piece of muscle was left in culture medium overnight at 28°C. Attempts to record resting potentials the following day gave similar results to those obtained from muscle maintained in culture for 10 days.

Occasionally, fibres extended from muscle explants in culture. This outgrowth often developed from the cut surface of nerve which remained attached to the muscle. Assuming that the cell

body is necessary for fibre growth, and that these fibres are of neural origin, they must originate from sensory neurones, since all motoneurone cell bodies are found within the central nervous system.

(2) EXPLANTS FROM SMALL NYMPHAL ANIMALS

Ganglia cultured from very young nymphs (3 - 5 mm long) showed growth which was essentially similar to that seen in cultures of ganglia from adults. (Fig. 1.23, c.f. Fig. 1.8). Preliminary experiments suggest that these smaller ganglia may be superior in short term cultures. However, there appeared to be little difference between these and adult ganglia at culture times above 3 - 4 weeks; a more extensive study is necessary to confirm this.

Fibre outgrowth from small nymphal ganglia became established more quickly than did that from adult ganglia. Out of 14 cultures set up, 8 produced fibre outgrowth which was fairly well established by the fourth day in culture.

(3) CULTURE OF SINGLE NERVE CELLS

Very limited success was achieved in growing single, or small groups of motoneurones in culture. On one occasion a number of spiked processes grew from a small group of cells (Fig. 1.24), but these did not reach any appreciable length and, gradually regressed. The number of such cultures prepared, however, was relatively few, and it is possible that more successful results may be obtained using refined techniques, and optimised growth conditions.

(B) THE EFFECT OF DIFFERENT GROWTH MEDIA

Cultures produced markedly more extensive fibre growth when maintained in the medium of Chen and Levi-Montalcini than did those maintained in other media. Cultures did, however, continue to produce some additional outgrowth when this medium was replaced

Fig. 1.23 Part of the ventral nerve cord from a young nymphal cockroach (5 mm in length) after 5 days in culture. The prothoracic (T_1) mesothoracic (T_2), metathoracic (T_3) and first two abdominal (A_1 and A_2) ganglia are seen. Fibre outgrowth is already fairly well established.

Calibration : 0.5 mm

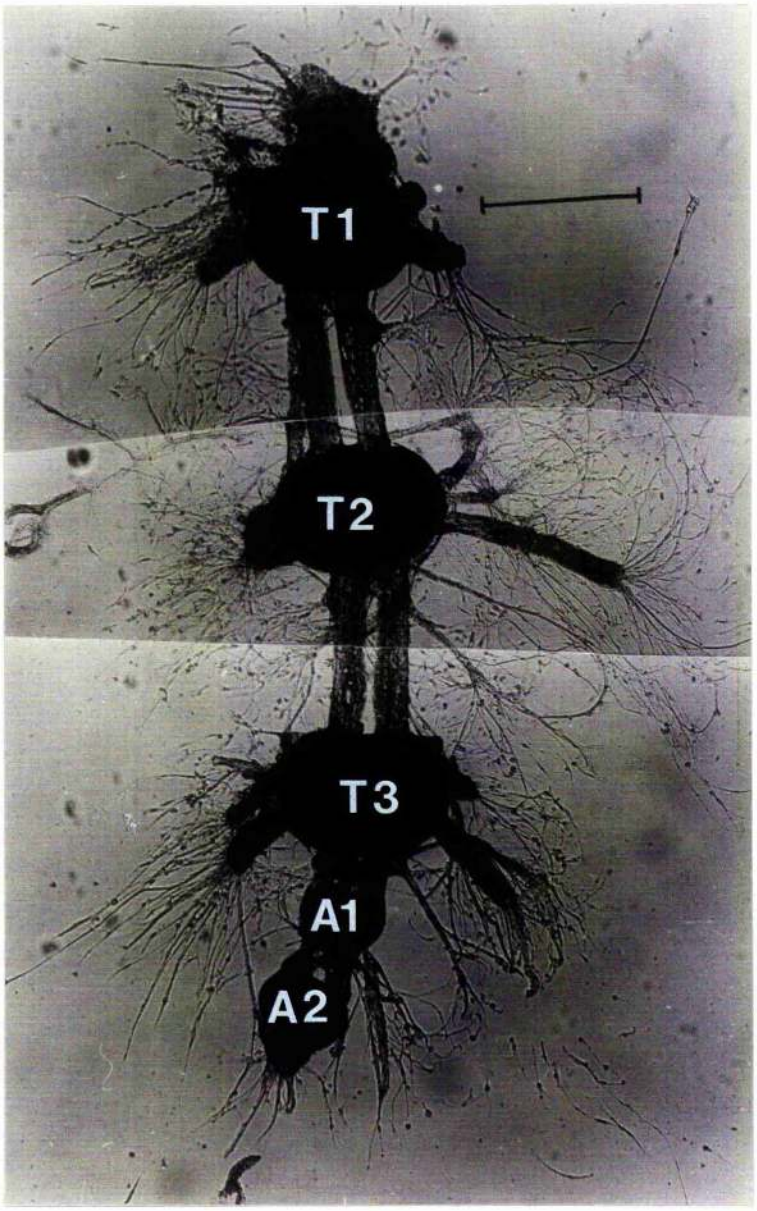
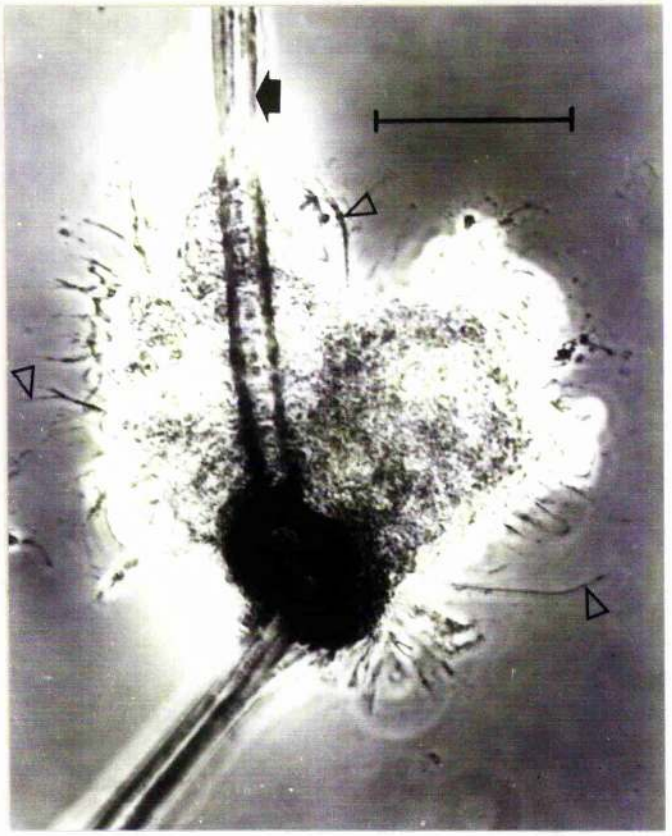

T1
T2
T3
A1
A2

Fig. 1.24 Fibre outgrowth from isolated neurone cell bodies. This small group of somata was removed from an adult metathoracic ganglion using a piece of nylon monofilament (arrow) to which they are still attached. They are seen here after 4 days in co-culture with a neural explant which was established 3 weeks previously. A number of fine processes (open arrowheads) can be seen extending from the cell bodies. Photographed under phase contrast illumination.
Calibration : 100 µm



with Mitsuhashi and Maramorosch Basal Medium. Replacement with Grace's Medium, on the other hand, resulted in a marked deterioration in the fibres. Neither Grace's nor Mitsuhashi and Maramorosch media were used when initially setting up cultures, as cultured embryonic cockroach ganglia produce no outgrowth in these media unless first maintained in the medium of Chen and Levi-Montalcini (personal communication from D. Hicks, Thames Polytechnic). Explants grown from the outset in modified Marks' M-18 medium (see methods) rarely produced any fibre outgrowth. The potential of modified Marks' M-18 medium to maintain outgrowth initiated in the medium of Chen and Levi-Montalcini was not tested.

(C) THE EFFECT OF SUBSTRATE ON FIBRE OUTGROWTH

Although no quantitative study was made, explanted ganglia seemed to produce fibre outgrowth equally successfully on glass coverslips, or on Flow, Falcon or Nunc plastic Petri dishes; growth on collagen coated surfaces was, if anything, inferior. Since collagen produced optical problems for light microscopy, this substrate was not used routinely.

A number of other substrates were tested to determine which produced optimal adhesion of explants. This was necessary since explants could be easily dislodged during the first few days in culture. Adhesion to agar was poor. Attempts to grow cultures on "Snappies Clear Roasting Wrap" were also unsuccessful, since adhesion was once again poor, and the material was repellent to the medium. Untreated plastic Petri dishes were routinely used, since they produced optimal adhesion, and avoided the risk of microbial contamination, inherent in other substrate preparation procedures.

(D) MORPHOLOGY OF CULTURED GANGLIA

The technical problems encountered during preparation of cultured ganglia for morphological examination have already been referred to under 'Methods'; some property, or properties, of the ganglia changed when in culture, rendering them unusually difficult to fix and section successfully. Modifications in the procedures did, however, reduce this problem.

The general features of these ganglia cultured from adult animals are described in the following sections.

(a) LIGHT MICROSCOPE OBSERVATIONS

Although most of the general features of ganglionic internal structure could be observed using any of the histological staining techniques described under 'Methods', particular aspects could be observed better by using one method rather than another.

The following features were observed in cultured ganglia from adult animals:

(i) Change in Ganglion Shape

Ganglia almost invariably became dorso-ventrally flattened after a period in culture; the ventral surface, which was uppermost in the culture dish, remained fairly convex, while the dorsal surface, which was opposed to the surface of the dish, became flattened (Fig. 1.25 b - g).

(ii) Changes in Cell Bodies

Neuronal somata were present in ganglia in both short and long term cultures (Fig. 1.27 - 1.30). There was a decrease in the size of the larger somata, resulting in the population of cell bodies becoming more homogeneous in size as cultures aged. This made identification of specific cells more difficult than it would be in a non-cultured ganglion. The cell bodies sometimes appeared to

occupy a somewhat more central position in the ganglion than normal. This resulted from an increase in thickness of the ganglion nerve sheath (see below) and from the general flattening of the ganglion, which reduced the distance between cells on the dorsal and ventral surfaces. After about 4 weeks in culture, the nuclei moved to an eccentric position within the cell body. They sometimes moved towards the axon hillock, but sometimes in the opposite direction, causing bulging of the soma (Fig. 1.29 c). Sections stained with Pyronine Malachite-Green showed that a number of cells possessed a dense aggregate of RNA forming a ring around the nucleus. This was seen in ganglia maintained in vitro for 1 - 6 days (Fig. 1.25 b - g, c.f. Fig. 1.25 a - normal). In older cultures, the intensity of this perinuclear ring was reduced, until at about 12 days in culture, very little sign of any rings remained.

(iii) Changes in the Sheath Surrounding the Ganglion

The ganglionic sheath increased in thickness after ganglia had been maintained in vitro. The thickness varies greatly from one region to another, in both normal and cultured ganglia, and it was consequently difficult to obtain quantitative values for the extent of thickening. The sheath is particularly thick in the section shown in Fig. 1.25 g of a 6 day cultured ganglion, ranging between approximately 7 μm and 28 μm (c.f. section through normal ganglion, Fig. 1.25 a, where the thickness ranges from approximately 3.5 μm to 14 μm)

(iv) Changes in Neuropile

The neuropile of normal ganglia is highly ordered and bilaterally symmetrical. Transverse sections through the ganglia show fibre tracts with a number of different orientations; some

are transverse, some longitudinal, and some oblique (Fig. 1.26). The appearance of the neuropile varies in sections taken from different regions of the ganglion, but the bilateral symmetry is maintained throughout. The fibre tracts forming the neuropile have been described by Pipa, Cook and Richards (1959), and Gregory (1974) (Fig. 1.26 a).

Transverse sections through cultured ganglia revealed that the neuropile undergoes a succession of dramatic changes with time. First of all, the highly organised and symmetrical appearance seen in vivo is lost, and then later it seems to become re-organised.

During 1 - 9 days in culture there is a dramatic increase in the number of fairly small diameter circular profiles seen within the transverse sections. These circular profiles could represent:

a) tubular structures sectioned transversely, or

b) spherical structures sectioned at any angle, or

c) spaces within the neuropile

At this stage the organisation of the neuropile appears to be lost (Fig. 1.27).

After 10 - 20 days in culture, the neuropile appears to be more organised; the number of circular profiles decreases, but a higher proportion of those present are larger (Fig. 1.28).

After 21 - 30 days, there is a further decrease in the number of circular profiles (Fig. 1.29).

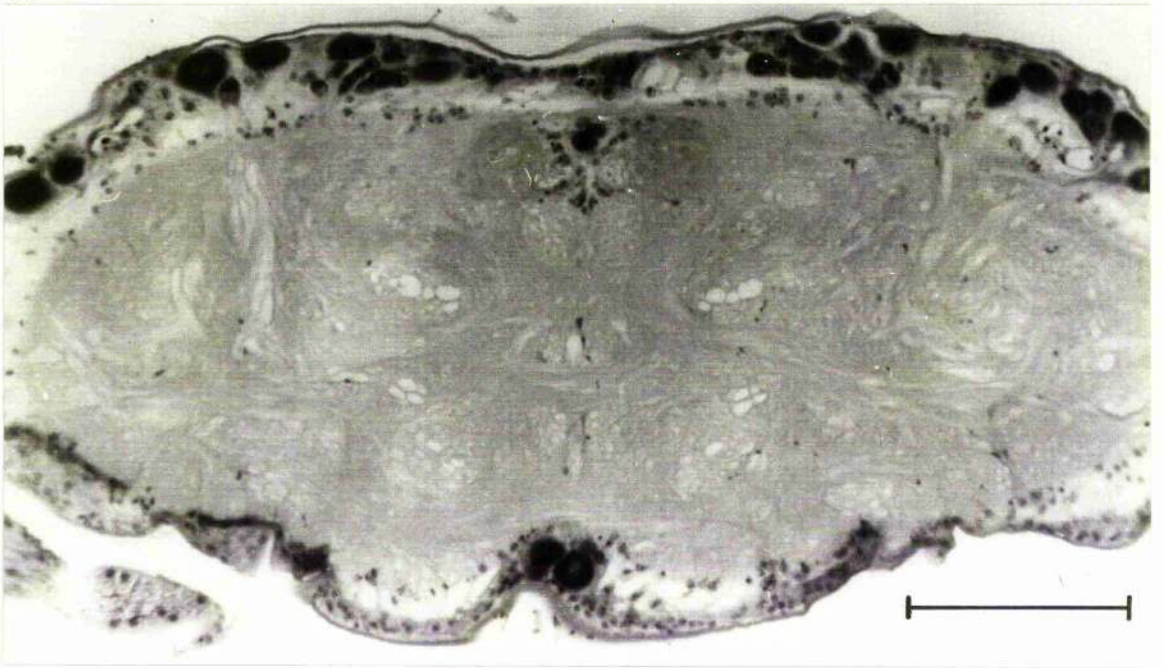
By 85 days or more in culture, the neuropile is well organised, with only a few circular profiles seen in transverse sections. (Fig. 1.30).

Fig. 1.25 Transverse sections through adult cockroach thoracic ganglia stained with Pyronine-Malachite Green: deeply stained areas contain a high concentration of RNA. The sections are shown with the ventral surface at the top of the page.

(a) Normal ganglion, prepared for histological studies immediately after removal from the animal. The central region (or neuropile) appears densely packed and symmetrical. The area containing the majority of the cell bodies has been re-printed less intensely to show the depth of staining surrounding the nuclei. These normal somata show no perinuclear shell of RNA. Calibration : 200 µm

(b) Ganglion after 1 day in culture. An accumulation of RNA has already begun around the nuclei of some cell bodies (arrowheads). The neuropile appears to be less densely packed. Calibration : 200 µm

a



b

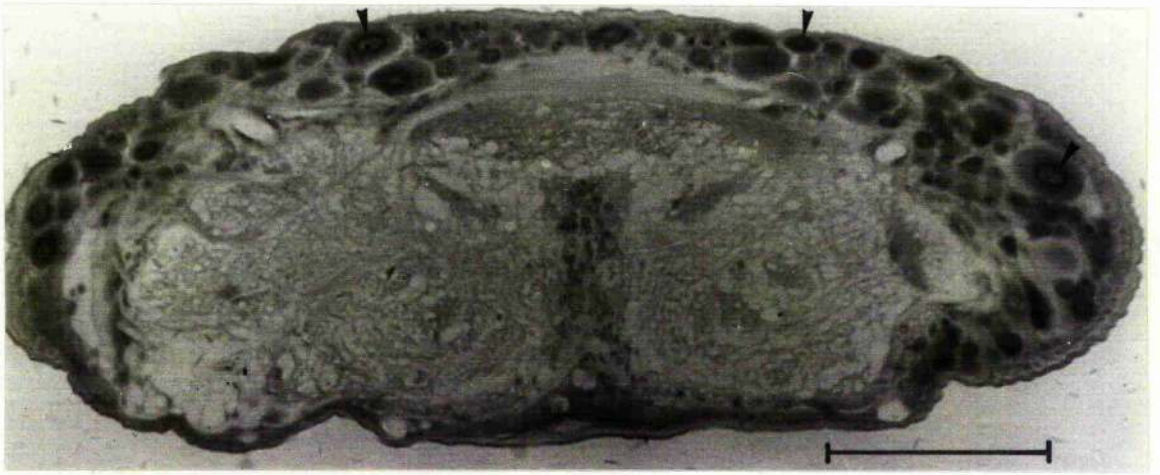
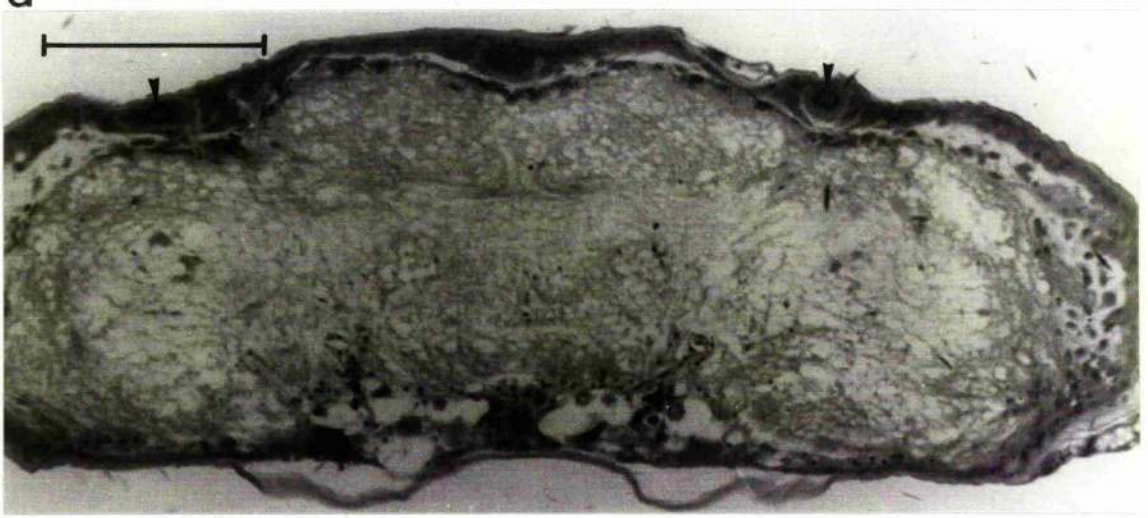


Fig. 1.25 (c), (d) and (e) Ganglia after 2, 3 and 4 days in culture, respectively. An intensely stained shell of RNA can be seen surrounding the nuclei of many cell bodies (arrowheads). The neuropile has become progressively less densely packed. Calibration : 200 μm

c

d



e

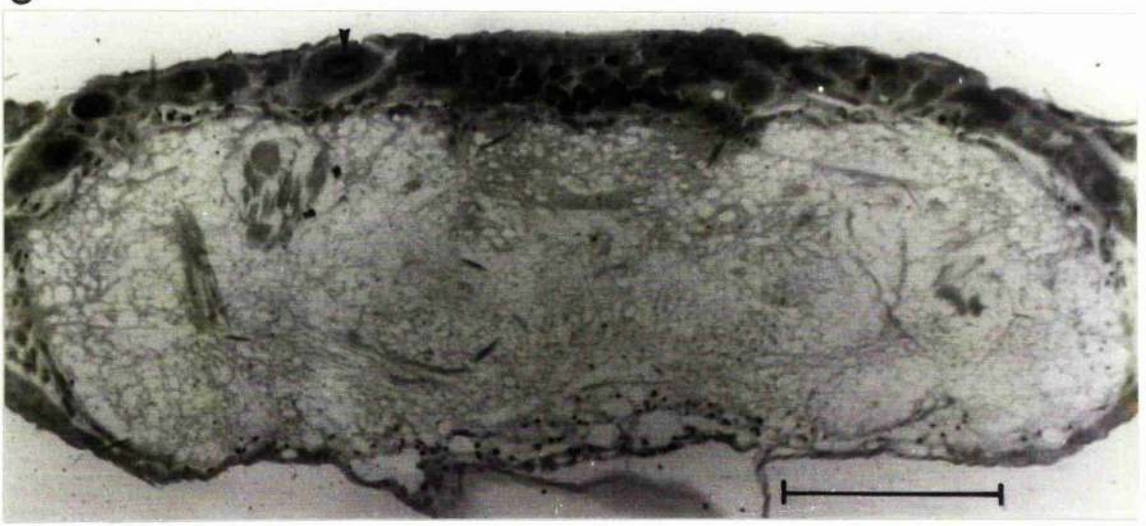
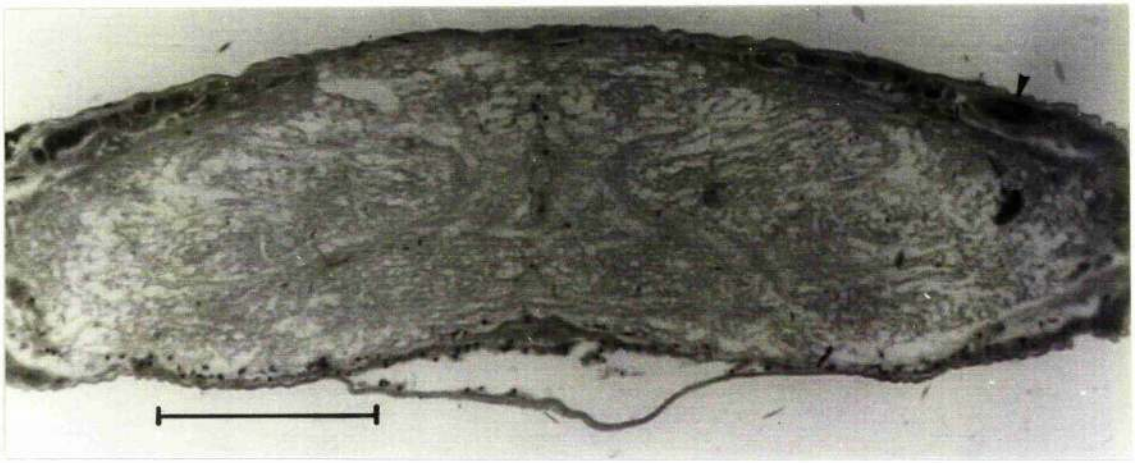


Fig. 1.25 (f) and (g) Ganglia after 5 and 6 days in culture, respectively. A high concentration of perinuclear RNA (arrowheads) is still evident. The neuropile has a number of large spaces within it. The ganglia have undergone a progressive dorsoventral flattening with increased time in culture. (c.f. Fig. 1.25 a and b). Calibration : 200 μm

f



g

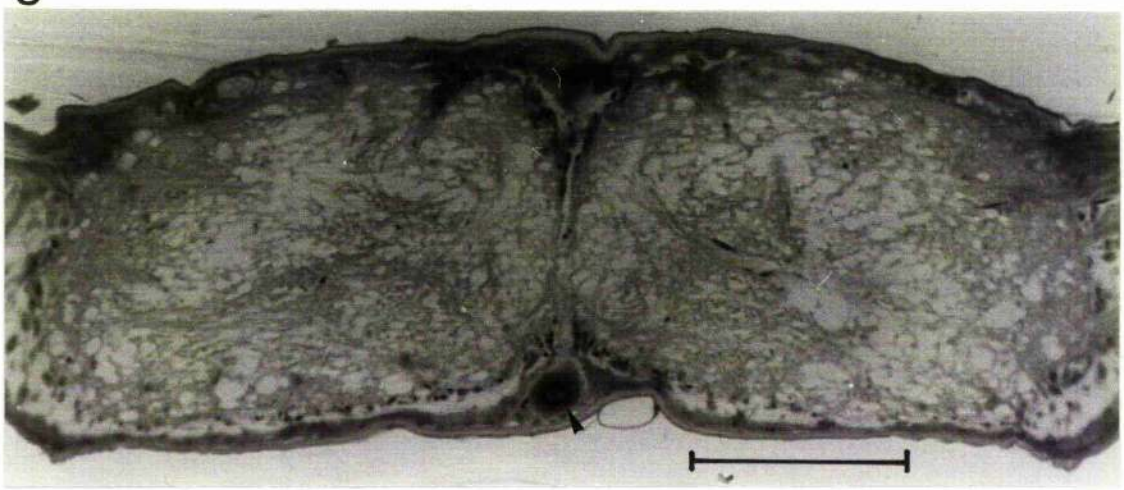


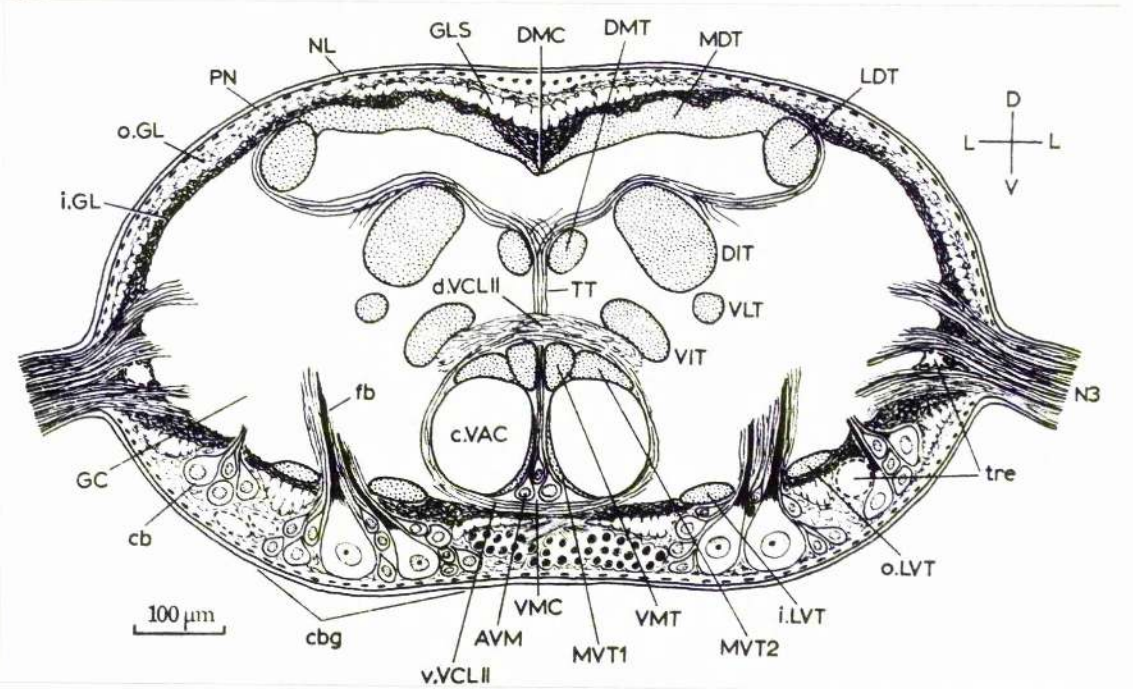
Fig. 1.26 The appearance of normal adult thoracic ganglia in transverse section.

(a) Transverse section of mesothoracic ganglion at the level of nerve 3, showing the symmetrical arrangement of both the central neuropile and the surrounding cell bodies. (Taken from Gregory, 1974).

(b) Transverse section through a thoracic ganglion stained with osmium totroxide. The section has been taken from a similar position in the ganglion to that seen in (a). Fibres within the neuropile run in different directions so that some are cut transversely (t), some longitudinally (L) and others obliquely. Calibration : 200 μm

(c) Transverse section through a thoracic ganglion stained with osmium totroxide. This section has been taken from a region of the ganglion close to the connectives. Many cell bodies (arrows) can be seen, while the area occupied by the neuropile (n) is relatively small, containing only fibres which run in a rostro-caudal direction. A segmental nerve on each side of the ganglion (arrowheads) has been sectioned transversely.
Calibration : 200 μm

a


NL
GLS
DMC
DMT
MDT
LDT
PN
o.GL
i.GL
D
L
L
V
DIT
d.VCL II
TT
VLT
VIT
fb
c.VAC
N3
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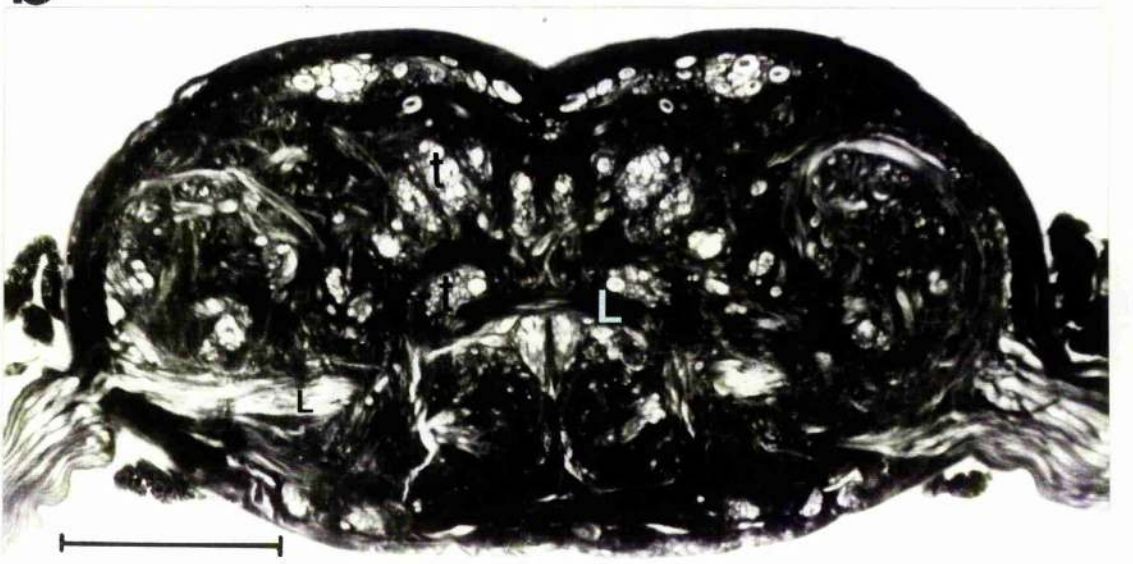
i.LVT
v.VCL II

b


t
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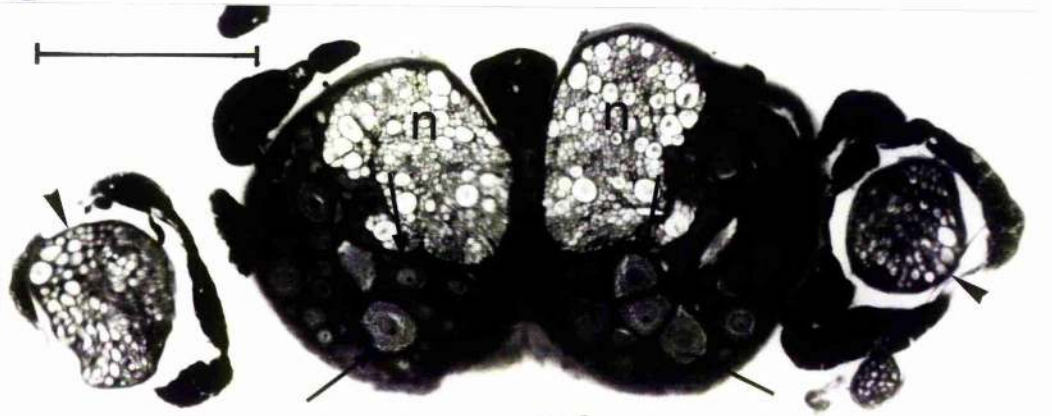
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n

Fig. 1.27 - 1.30 Changes in the neuropile organisation of ganglia maintained in culture for 5 - 155 days.

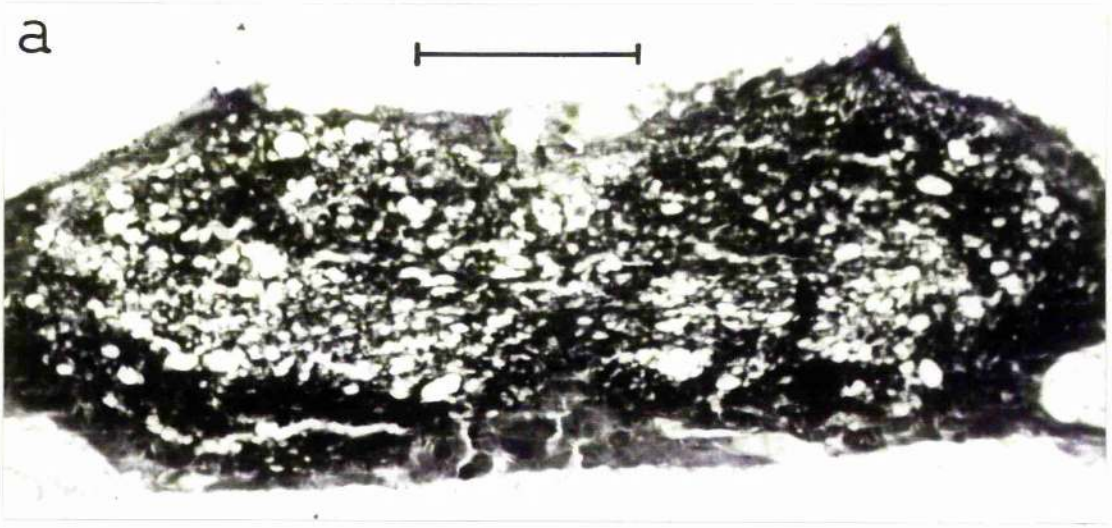
Fig. 1.27 Transverse sections through adult thoracic ganglion after 5 days in culture. Osmium tetroxide stain. The neuropile is filled with numerous small spaces and the ganglion has become dorsoventrally flattened (c.f. Fig. 1.26 b).

(a) Section through whole ganglion. Calibration : 200 μm

(b) Detailed appearance of neuropile region. The majority of the fibres have been sectioned transversely.

(c) Detailed appearance of cell bodies.

Calibration for (b) and (c) : 50 μm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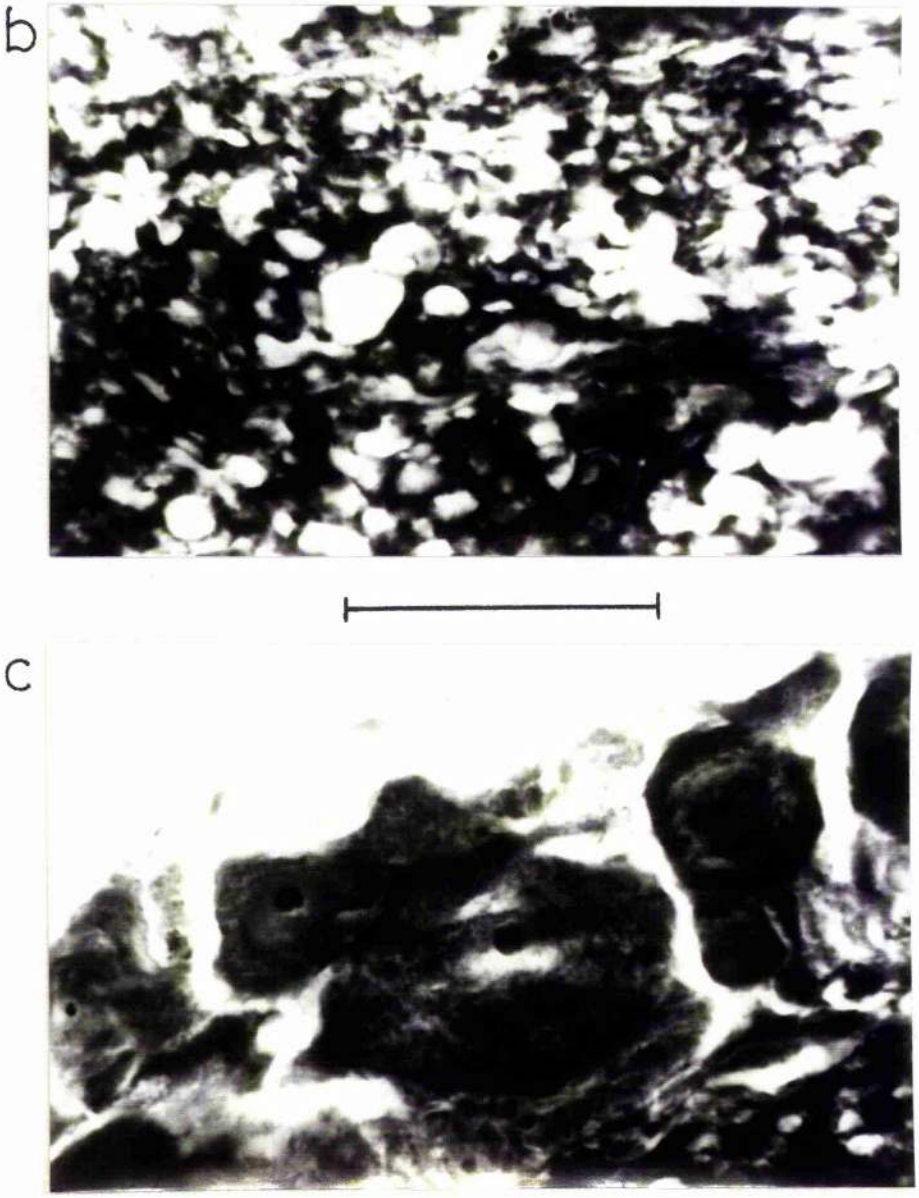

b
c

Fig. 1.28 Thoracic ganglion after 10 days in culture. Some slightly larger spaces have developed within the neuropile which still remains largely disorganised. (Sections prepared as for Fig. 1.27)

(a) Section through whole ganglion. Calibration : 200 μm

(b) Detailed appearance of neuropile region. The majority of fibres are still seen in transverse section.

(c) Detailed appearance of cell bodies.

Calibration for (b) and (c) : 50 μm

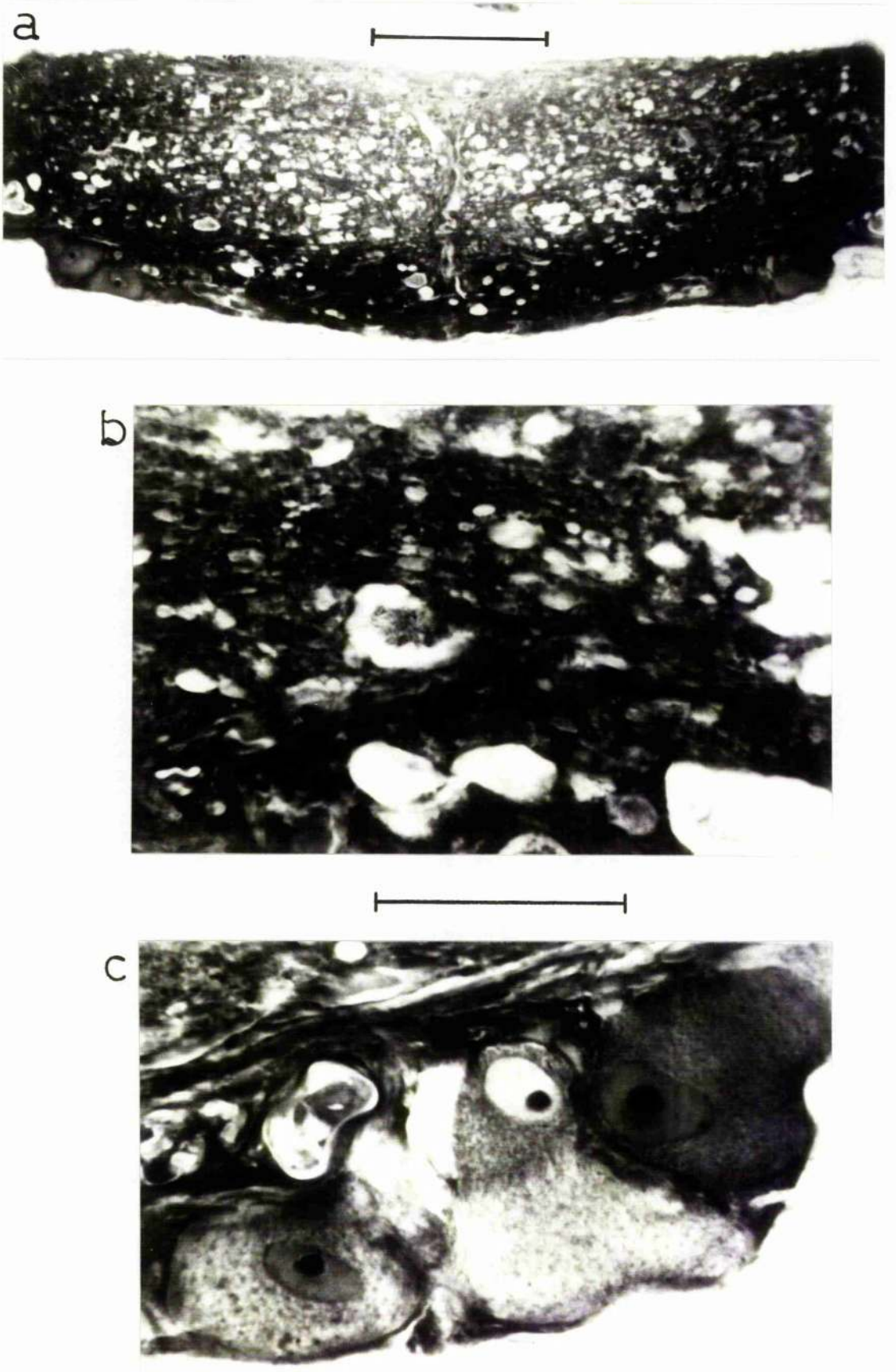

a
b
c

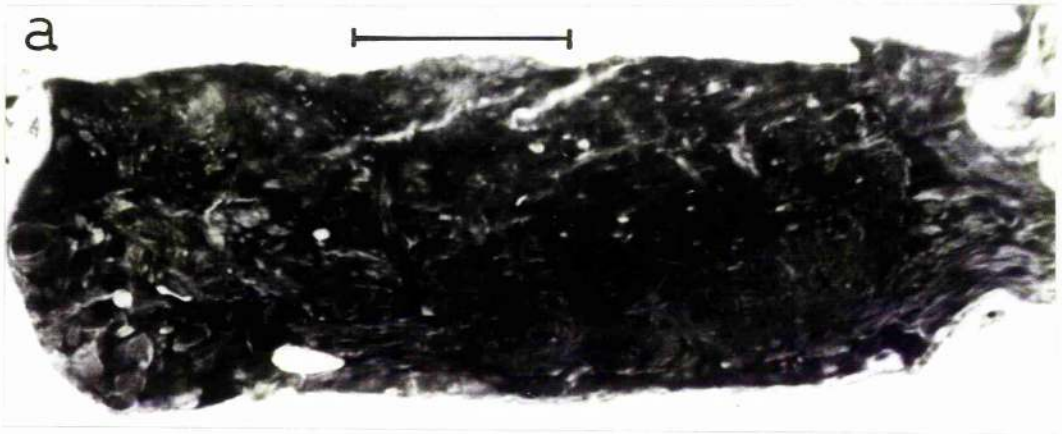
Fig. 1.29 Thoracic ganglion after 30 days in culture. Fewer spaces are present in the neuropile and fibres now run in more diverse directions, although there still appears to be little organisation. (Sections prepared as for Fig. 1.27).

(a) Section through whole ganglion. Calibration : 200 μm

(b) Detailed appearance of neuropile region. The region between the two arrows contains a number of fibres which have been sectioned longitudinally.

(c) Detailed appearance of cell bodies.

Calibration for (b) and (c) : 50 μm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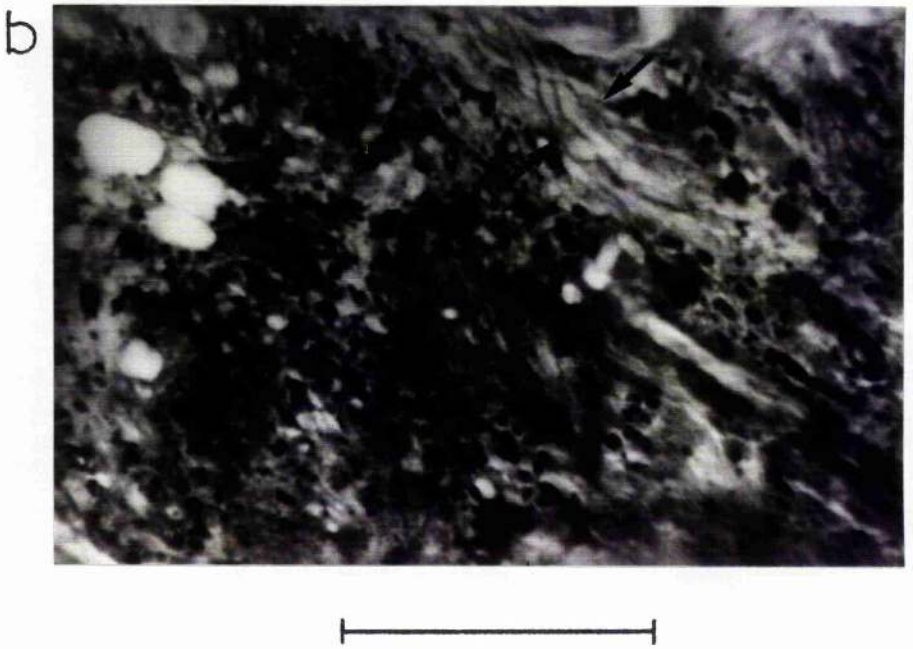

b

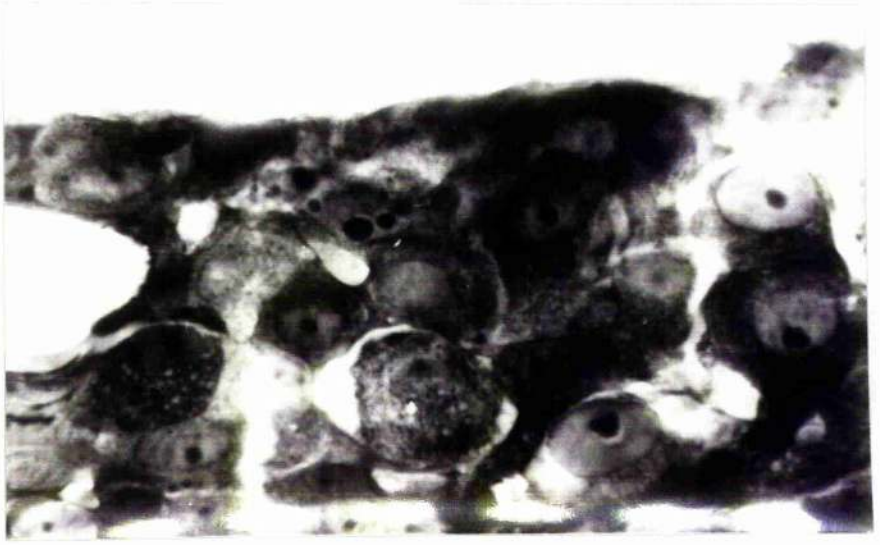

c

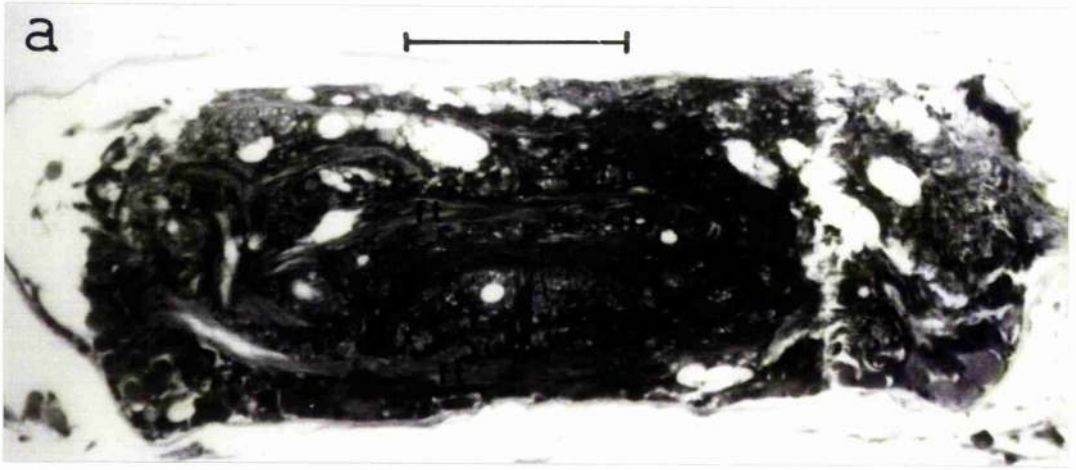
Fig. 1.30 Thoracic ganglion after 155 days in culture. The neuropile appears to have regained much of its ordered, bilaterally symmetrical pattern, and a number of longitudinally sectioned fibre tracts (ft) can be seen. (Sections prepared as for Fig. 1.27).

(a) Section through whole ganglion. Calibration : 200 μm

(b) Detailed appearance of neuropile region. Fibres are sectioned at a number of different orientations.

(c) Detailed appearance of cell bodies.

Calibration for (b) and (c) : 50 μm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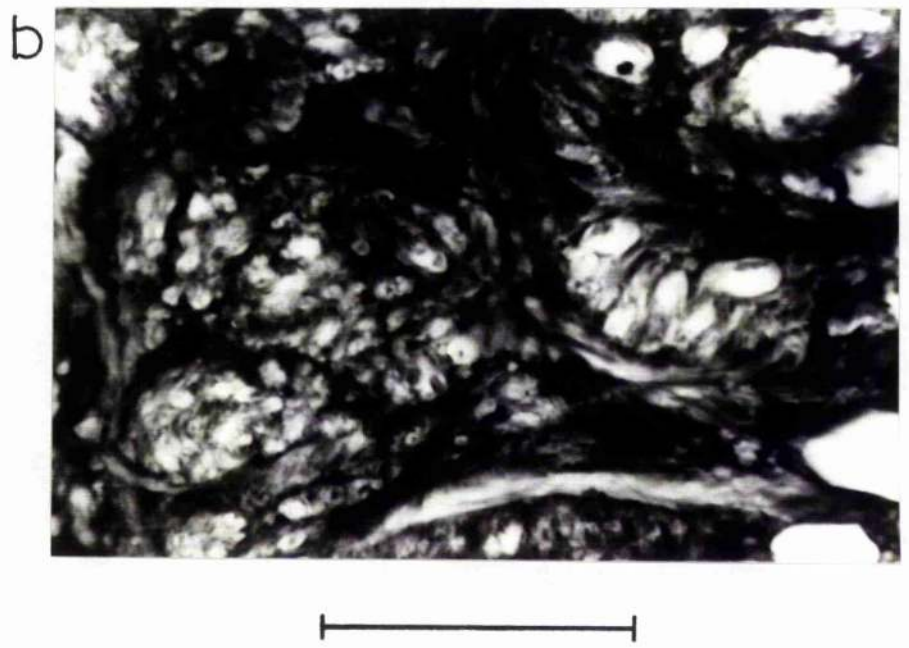
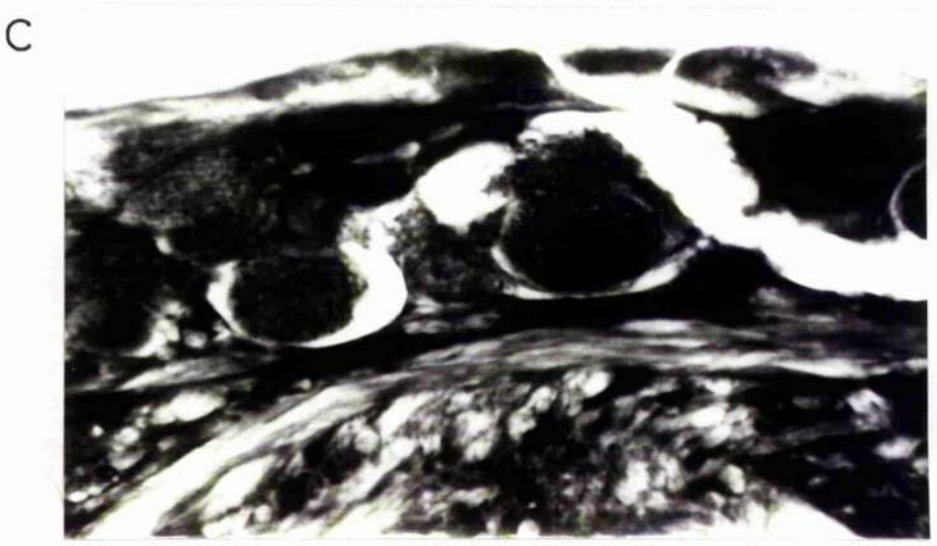

b


c

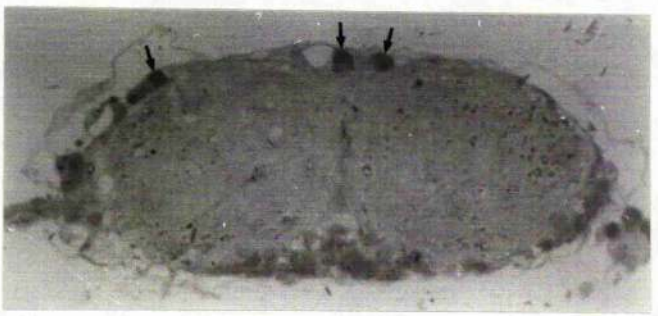


Fig. 1.31 Transverse section through a small nymph thoracic ganglion after 36 days in culture. The neuropile appears to be well organised and cell bodies (arrows) are present. The ganglion has not become markedly dorsoventrally flattened. (c.f. adult ganglia.) Section stained with Pyronine Malachite Green. Calibration : 200 µm

Transverse sections through ganglia cultured from small nymphs revealed a structure similar to that of adult ganglia. The neuropile was perhaps less radically altered, and the degree of flattening less marked (Fig. 1.31) (c.f. Fig. 1.25 - 1.30).

(b) ELECTRON MICROSCOPE OBSERVATIONS

In a relatively brief ultrastructural survey, the following structures were seen in cultured ganglia:

(1) TRANSMISSION ELECTRON MICROSCOPE

Observations were made on sections from ganglia which had been maintained in culture for 7 weeks.

(i) Degenerative Whorls

These were seen at various places through the neuropile (Fig. 1.32 and 1.33) indicating that a number of nerve fibres were degenerating within the ganglia.

(ii) Microtubules

A large number of microtubules could be seen clustered in groups at certain areas through the neuropile (Fig. 1.34). They had a characteristic appearance in transverse section (Fig. 1.35).

(iii) Tracheal Tubes

At least part of the tracheal system had been structurally maintained through the ganglia (Fig. 1.36).

(iv) Mitochondria

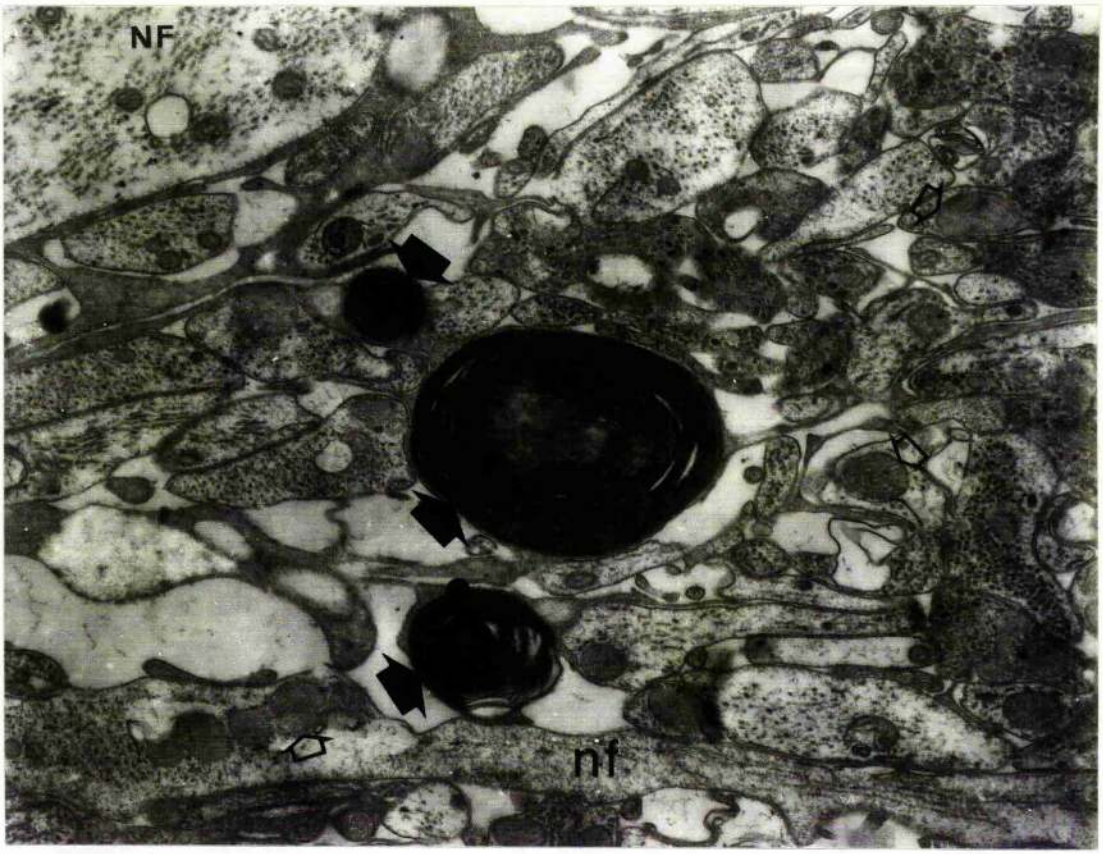
A number of large, complex and also small mitochondria were seen throughout the ganglia (Fig. 1.32, 1.34, 1.35, and 1.37).

(v) Cell Bodies

No cell bodies were seen in the particular sections studied, but this was not a reflection of the number of somata present, since light microscope examinations showed that cell

Fig. 1.32 Electron micrograph of a transverse section through an adult cockroach ganglion after 27 days in culture. Three degenerative whorls (solid arrows), and numerous mitochondria (open arrows) can be seen. A transversely sectioned nerve fibre (NF) is present in the top left hand corner of the photograph, and a nerve fibre in longitudinal section (nf) runs along the bottom of the photograph. Magnification : x 18,070.

Fig. 1.33 Electron micrograph of a degenerative whorl. A cluster of nerve fibres (f) is surrounded by a myelin-like sheath (arrow). Magnification : x 72,844


NF
nf

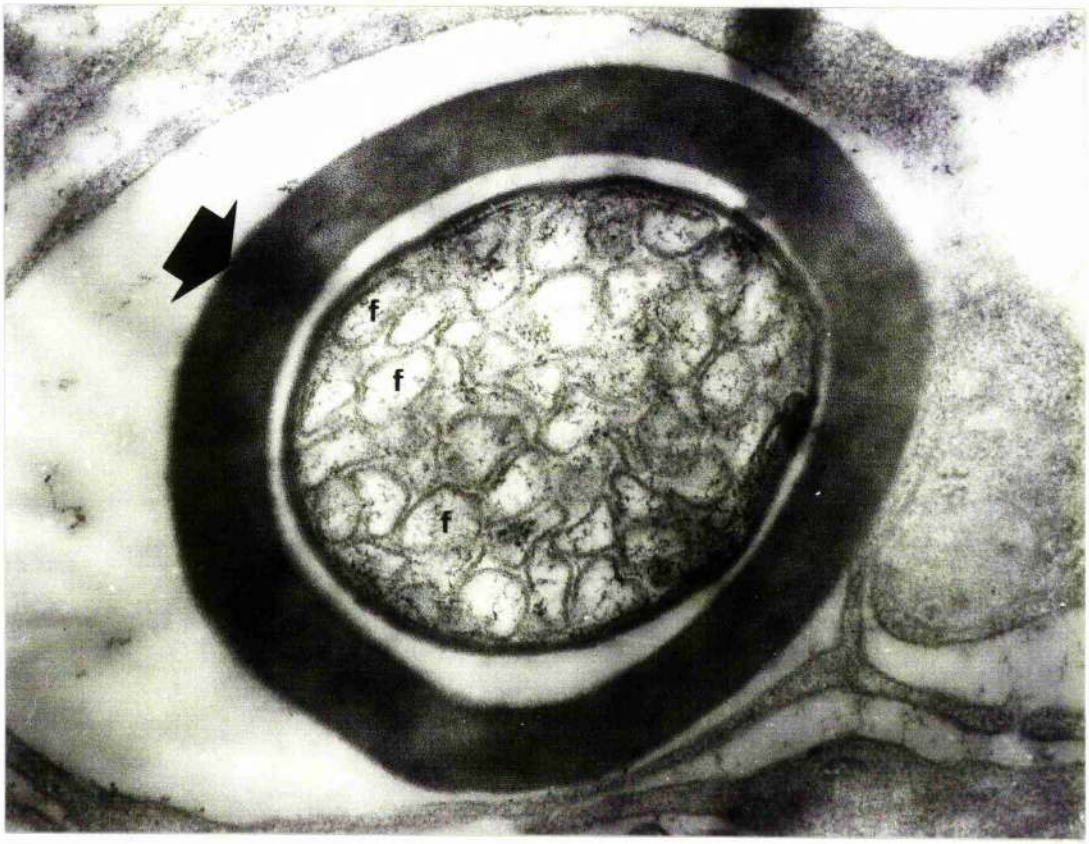
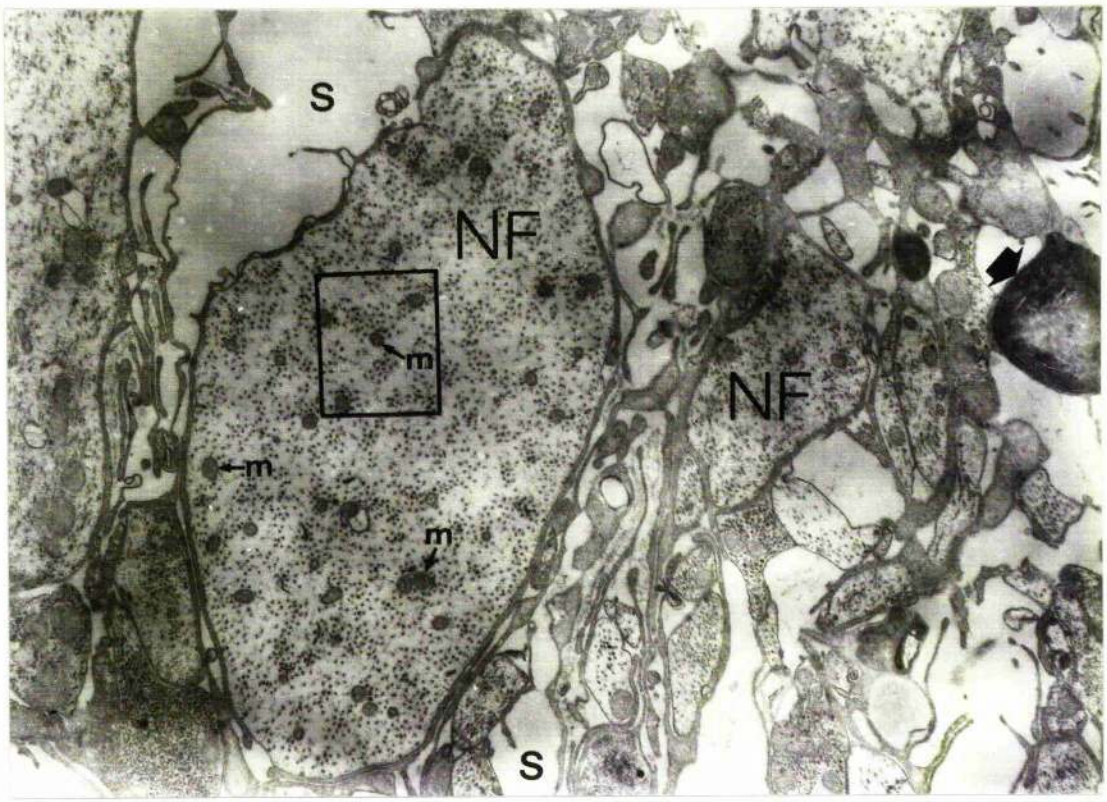

f
f
f

Fig. 1.34 Electron micrograph of part of the neuropile from a thoracic ganglion after 27 days in culture. Numerous clusters of microtubules can be seen within transversely sectioned nerve fibres (NF), and a number of mitochondria (m) are distributed among the microtubules. The largest nerve fibre in this picture is over 8 μm in diameter, and could be one of the smaller giant fibres which run rostro-caudally through the ventral nerve cord. A degenerative whorl (arrow) and some apparently empty spaces(s) can also be seen. Magnification : x 12,444.

Fig. 1.35 Electron micrograph of the boxed area in Fig. 1.34. Four mitochondria (m), and numerous microtubules (t) with characteristic star-like appearances can be seen. Magnification : x 88,578


s
NF
m
NF
m
m
s

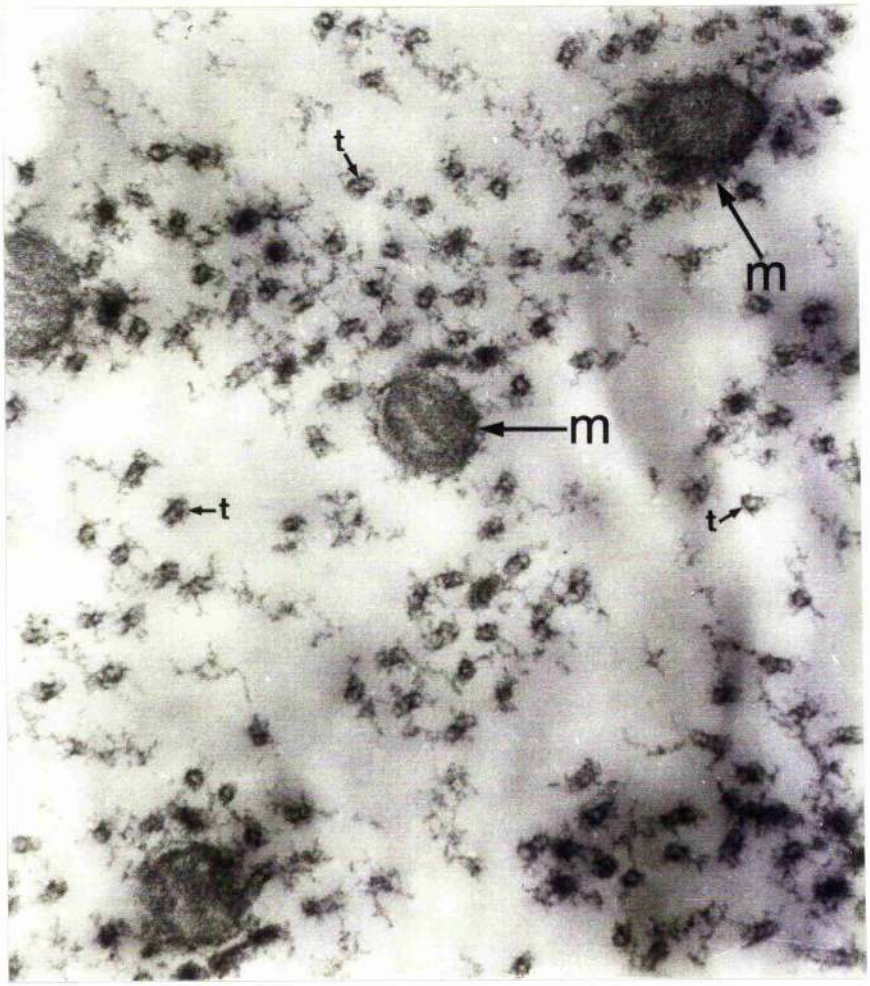
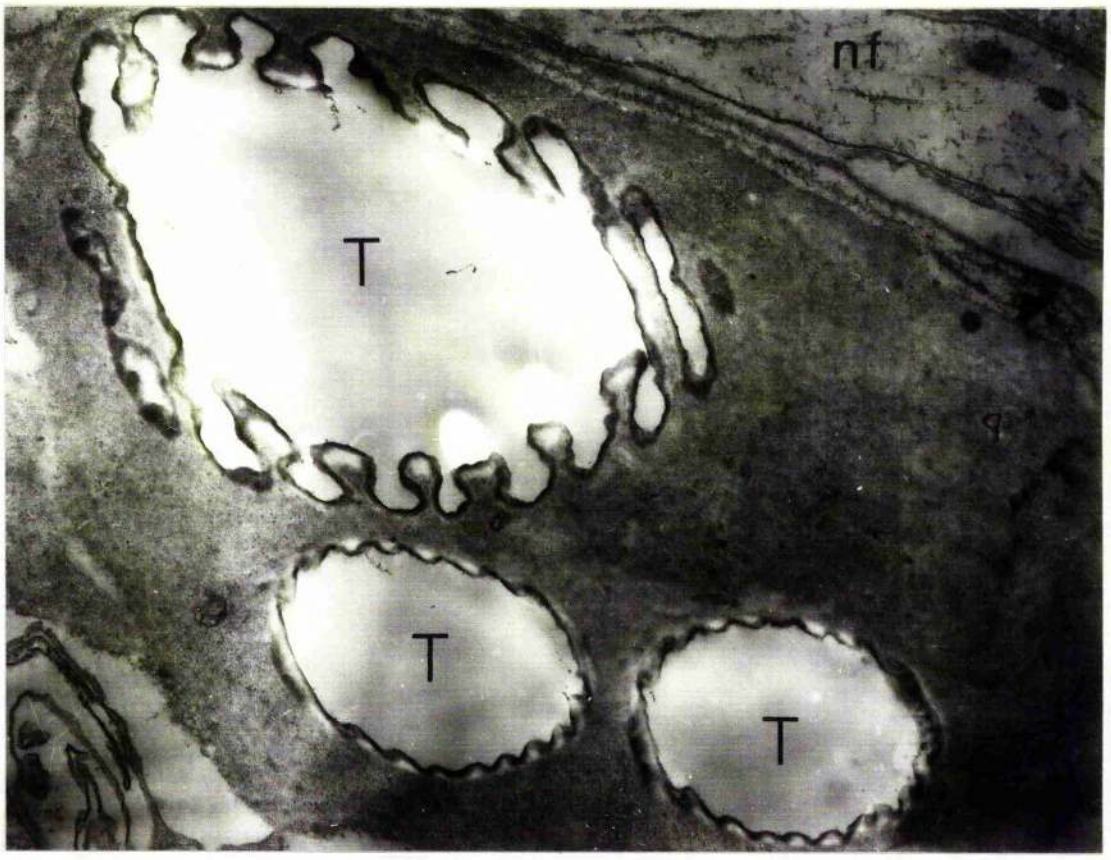

t
m
m
t
t

Fig. 1.36 Electron micrograph of transverse section through tracheal tubes (T) from a thoracic ganglion. Note the corrigations in the walls which reinforce the tracheal tubes. A longitudinally sectioned nerve fibre (nf) can be seen in the top right hand corner.
Magnification : x 30,360

Fig. 1.37 Electron micrograph showing the general appearance of the neuropile from an adult thoracic ganglion after 27 days in culture. The following structures can be seen: tracheal tubes (T), mitochondria (m), degenerative whorls (solid arrows), nerve fibres in transverse section (NF) and longitudinal section (nf) containing clusters of microtubules..
Magnification : x 8,440


nf
T
T
T


NF
T
m
nf
NF
T
m

bodies were present in fairly normal numbers in these cultured ganglia.

(2) SCANNING ELECTRON MICROSCOPE

Observations were made on ganglia from adult and small nymphs after either 4 weeks or 7 weeks in culture. The same basic features were seen in all preparations:

(i) General Shape of Ganglion

When viewed under the scanning electron microscope, some ganglia appeared to have shrunk, bearing large sunken areas, which gave them an appearance of a deflated ball (Fig. 1.38). This, however, was due to some effect of the preparation technique, since the ganglia did not usually have this appearance before fixation. Light microscope observations of transverse sections support this, as no concave dips of any appreciable size were ever seen in the profiles of the ganglia.

(ii) Fibre Outgrowth

Numerous fibres were seen growing out from the explant (Fig. 1.39 and 1.40). These formed a complex network, frequently branching and intermeshing with one another (Fig. 1.41). Many processes were varicose along their length; these varicosities were approximately 5 μm in diameter. Structures resembling growth cones were frequently found at the tips of fibres (Fig. 1.42). In some cases a fibre extended from one of these growth cones to give rise to a second growth cone, and occasionally a further fibre branching from the second gave rise to a third (Fig. 1.43 a). Growth cones varied in conformation, but all

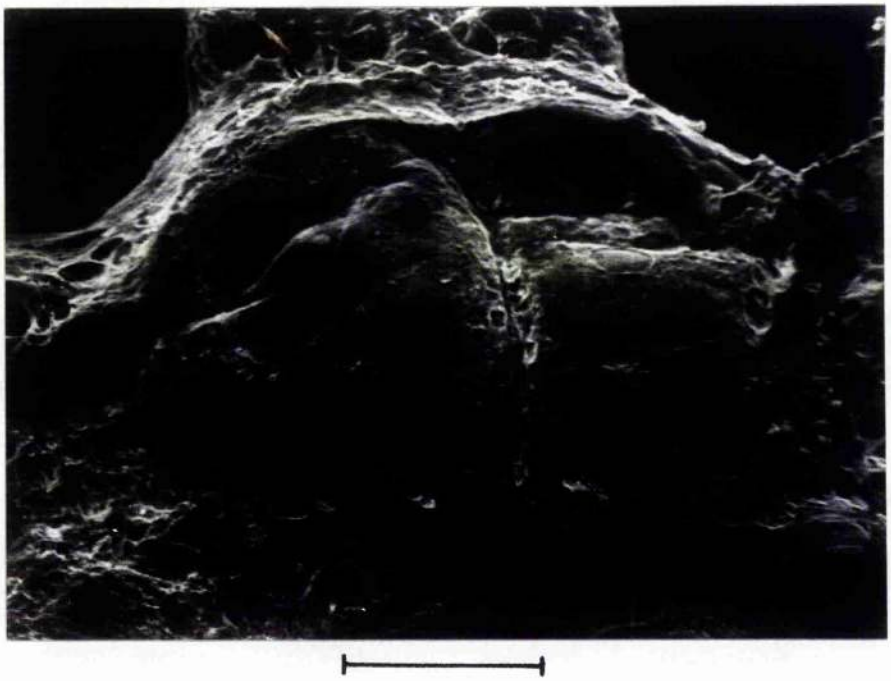
had characteristic microspikes extending from their margins.

(iii) Other Features

Some small isolated cells of unidentified type were seen in the area around the explant (Fig. 1.41). Certain areas around the outgrowth from the explant appeared to be covered with a thin blanket of cells (Fig. 1.41).

Fig. 1.38 Scanning electron micrograph of a thoracic ganglion from a young nymphal cockroach after 27 days in culture. The surface appears sunken in parts, but this is due to the technique used to prepare the tissue for scanning electron microscopy. Calibration : 100 µm

Fig. 1.39 Scanning electron micrograph of the same preparation as in Fig. 1.38, showing the general appearance of the ganglion and fibre outgrowth. Calibration : 1 mm



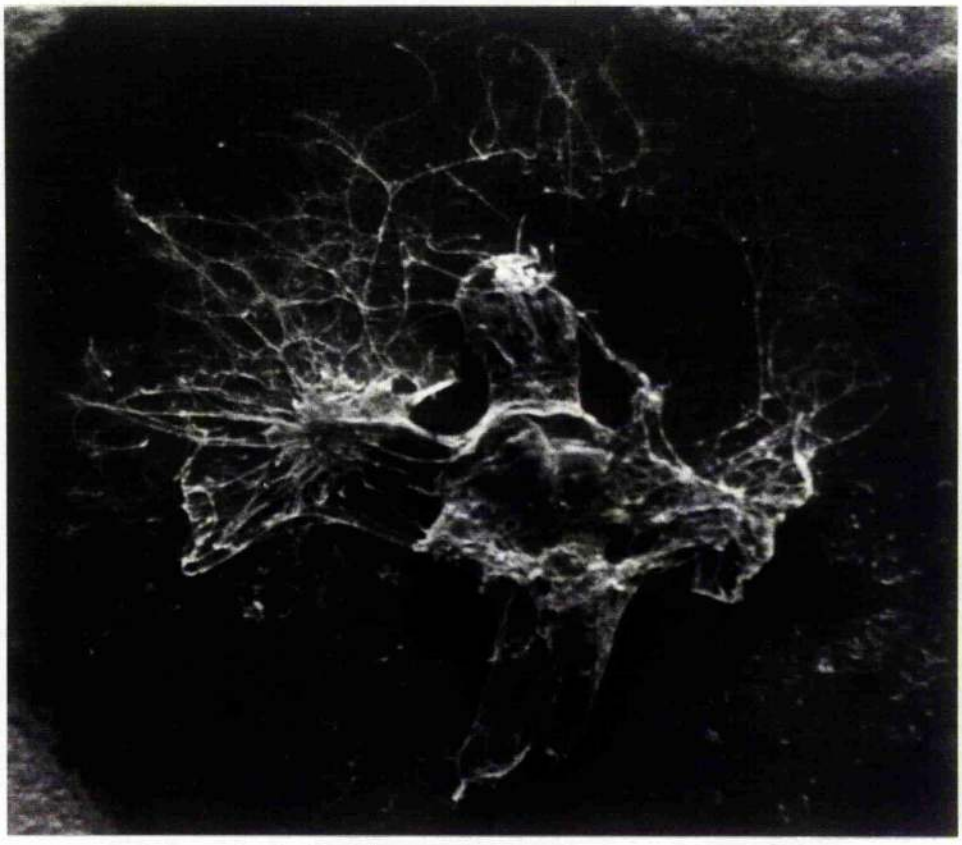
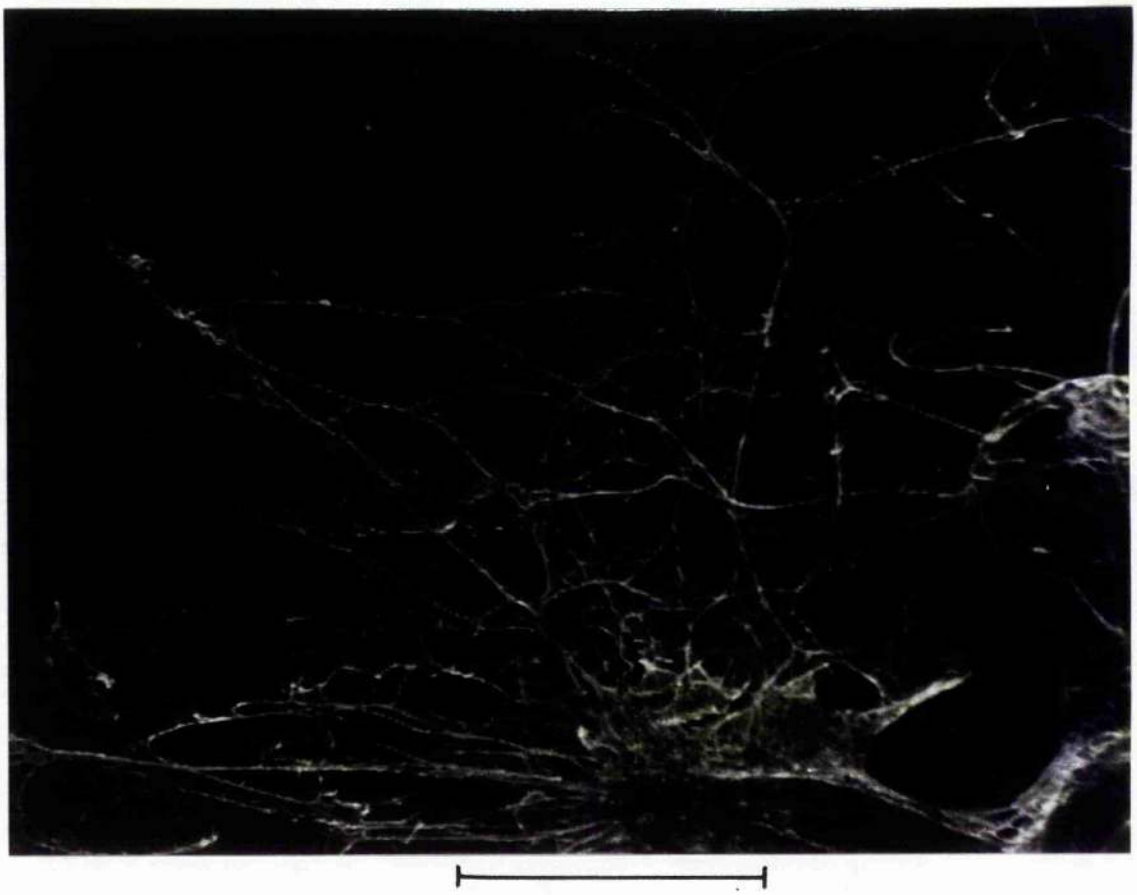


Fig. 1.40 Scanning electron micrograph of the same preparation as in Fig. 1.38, showing more detailed appearance of some of the fibre outgrowth. Calibration : 400 µm

Fig. 1.41 Scanning electron micrograph showing the complex branching and interconnections of fibres from a thoracic ganglion maintained in culture for 27 days. Some varicosities (V) can be seen in the fibres. A 'blanket' layer of unidentified cells, particularly evident in the top right hand corner of the picture, is present underneath the fibre network. Calibration : 20 µm



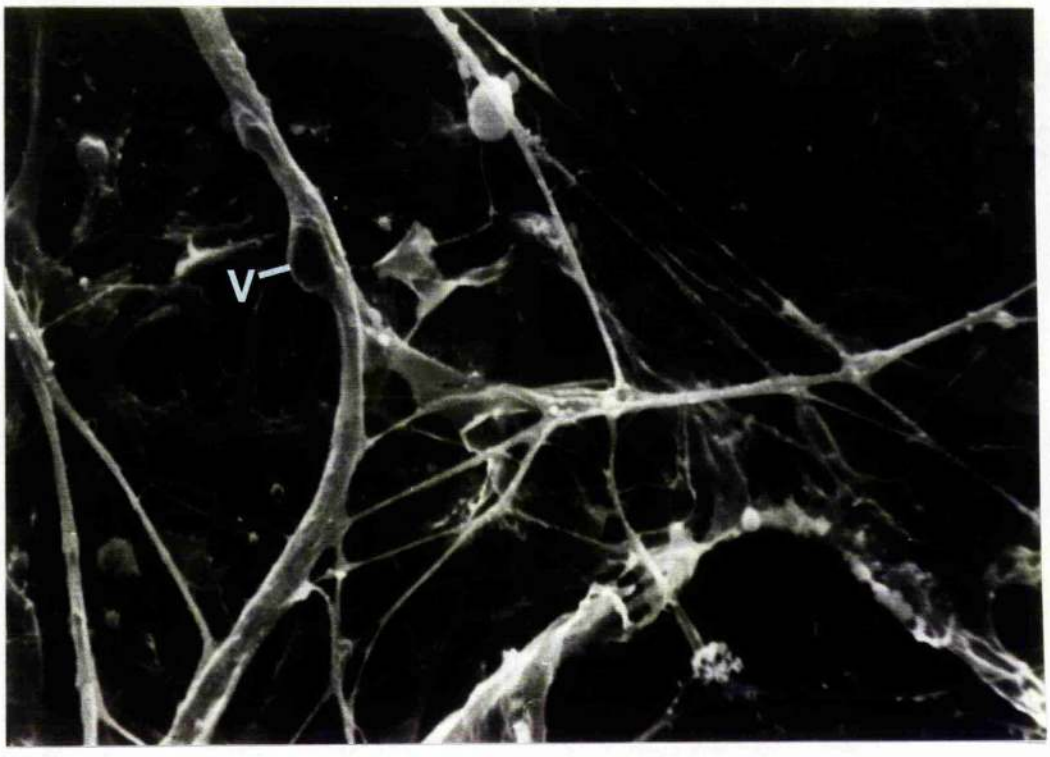
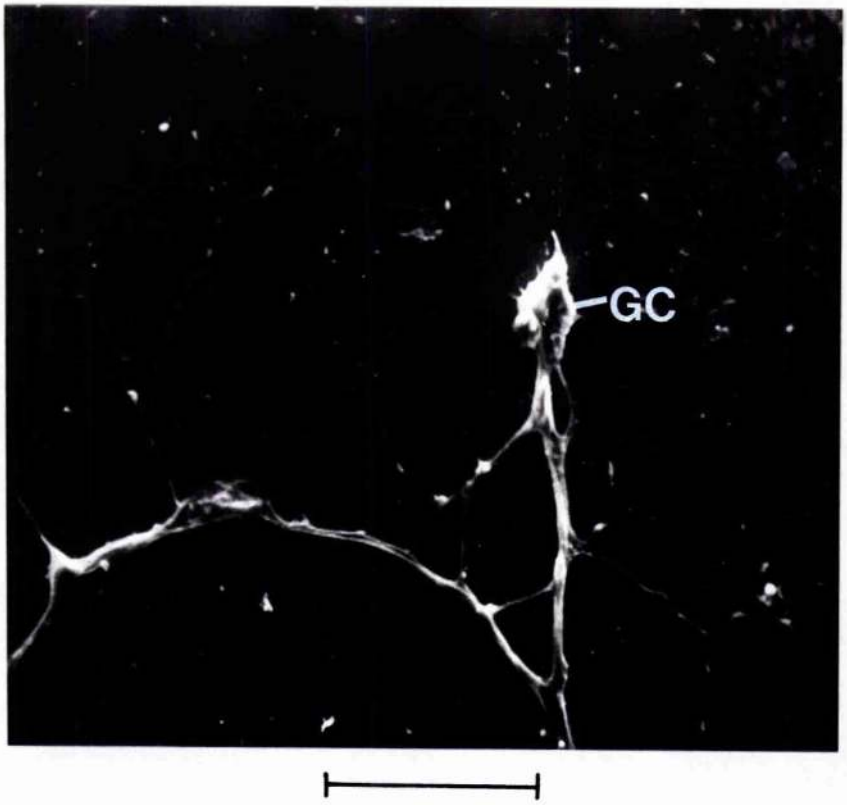
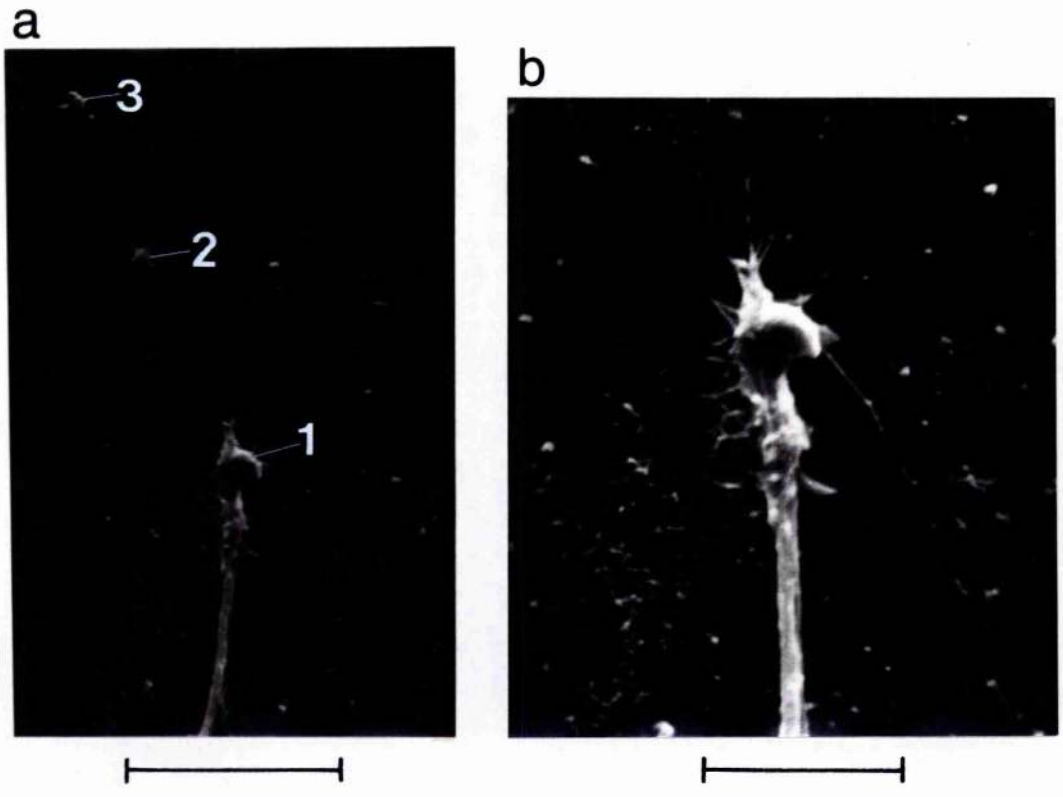

V

Fig. 1.42 Scanning electron micrograph of a branching fibre from a thoracic ganglion. One branch terminates in a growth cone (GC) from which extend a number of fine processes. Calibration : 20 μm

Fig. 1.43 (a) Scanning electron micrograph of a fibre supporting a series of three growth cones (1, 2 and 3) in culture. Calibration : 20 μm

(b) Detailed appearance of the primary growth cone (numbered 1) in (a). Calibration : 10 μm


GC


a
3
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DISCUSSION

As mentioned in the Introduction to this chapter, the technique of tissue culture is potentially a very useful one, especially for eliminating certain variables. However, tissue culture proved to be of limited use in answering the original question posed at the outset of this study. That is, 'how do regenerating insect neurones make specific functional connections with appropriate targets?'. The outgrowth produced by adult ganglia *in vitro* was so immensely complex, that it became difficult to identify the origin, or indeed the site of termination of the majority of nerve fibres. Although the internal structure of ganglia maintained in culture sometimes appeared poor (see later), the fibre outgrowth from these adult and nymphal ganglia was profuse, even when cultured alone. This contrasts with the results of Seshan et al. (1974) who were unable to obtain any substantial fibre outgrowth from non-neurosecretory explants from adult cockroaches. Further work on optimising the growth conditions for these cultured explants would be necessary to determine, for example, the composition of the optimal growth medium and substratum and the most successful combinations for co-culture.

A number of features of the explant cultures are worthy of discussion.

General Features of Fibre Outgrowth

One of the most striking features of explanted ganglia is the rapidity with which fibre outgrowth begins. A significant number of explants produced visible fibre sprouting after 2 or 3 days in culture, and by 6 days in culture, over half of the successful explants had begun to produce fibres. This rate is similar to that observed in adult molluscan (Limnaea) cells *in vitro*, where outgrowth may begin 20-50 hours after explantation (Geletyuk, 1977). This delay is considerably shorter than the delay of 13 days reported for cockroach

motoneurones regenerating *in vivo* by Denburg et al. (1977) and Denburg and Hood (1977). However, recent studies by Pitman (personal communication) using intracellular dye injection have shown that axonal sprouting *in vivo* may often begin within 4 days of axotomy. Thus the onset of nerve fibre growth in culture is probably similar to that of fibres regenerating *in vivo*. This rapid onset of regeneration would explain how a certain degree of functional recovery may occur in living animals within 2 weeks of thoracic fifth nerve section. This has been observed using behavioural criteria (personal observations) and electrophysiological criteria (Whitington, 1979). Denburg et al. (1977) used cobalt backfilling to study axonal regeneration. This method is probably inaccurate as it is dependent on the ability of cobalt to be taken up by and travel in freshly regenerated nerve fibres, which may have an extremely small diameter (Pitman and Rand, 1981b).

It was not possible to obtain conclusive evidence that the fibres growing from explants were actually neuronal in origin; many, however, had features which would be expected of nerve fibres; they were long, often branching structures, with no apparent cell bodies, frequently containing a number of varicosities along their length. Many processes terminated in growth cones. Fibre diameters (1-5 μm) were also comparable with those of molluscan neurones (1-3 μm) in culture (Geletyuk, 1977).

It was only possible to estimate the rate of axonal growth indirectly (see Methods), as growth of individual fibres could not be followed over sufficiently long periods of time without time lapse photography facilities. The method used would tend to give a low estimate of growth rate. The rate of growth estimated for the cockroach nerve fibres *in vitro* was 2-11 μm/h, and is comparable to that of 3 μm/h given by Geletyuk (1977) for molluscan neurones *in vitro*.

This is in fact considerably slower than that given by Denburg et al. (1977), who estimated the growth rate of cockroach motor axons *in vivo* at about 0.9 mm/day (37.5 μm/h). This value, however, is subject to the uncertainties discussed previously, and therefore may be unreliable.

Varicosities were particularly common features of fibres growing from cockroach ganglion explants. Similar varicosities have also been observed along nerve fibres from embryonic (Levi-Montalcini and Chen, 1969), and late nymphal instar (Marks et al, 1968) cockroach neurones, and from leech neurones (Wallace et al., 1977). Levi-Montalcini and Chen (1971) commented that "according to Wigglesworth, a mitochondrion is present in each of these dilatations." Wallace et al.(1977), however, claim that the varicosities which they saw in leech nerve fibres contained clear and dense-cored vesicles. Whatever the significance of these varicosities, they appear to be a common feature of regenerating neurones in a number of species. (Occurance of varicosities *in vivo* is discussed in Chapter 3).

Growth cones indicated the neuronal origin of outgrowing fibres; under optimal conditions of illumination and magnification these could be seen in living cultures, but were more readily observed under the scanning electron microscope. A number of fine microspikes extended from the perimeter of the growth cones, occasionally with veil-like expansions. Similar structures have been described by many authors, including Bray (1973), Letourneau (1975), Pfenninger and Rees (1976), Bunge (1977) and Wessells et al, (1978). Bunge (1977) found that growth cones of rat sympathetic nerve cells in culture differed from those seen *in vivo*, in that they contained many more organelles. The author suggests that this is perhaps because the fibres are regenerating rather than growing for the first time. Vesicles and granules are a common feature of growth cones, and it has been suggested that they result from active endocytosis at the filopodia, or

microspikes (Bray, 1973; Bunge, 1977). Occasionally, small flecks were visible in the growth cones of cockroach neurones observed with phase contrast microscopy. However, these could not be identified at the magnification available.

The fibres growing from cockroach ganglia exhibited contact guidance: once one fibre had made contact with a target, other fibres followed it closely, making it difficult in some cases to be sure whether there was actually one thick fibre, or several finer ones. However, as the bundles became thicker, it became clear that they consisted of a number of fibres running together. This suggests that in at least some cases, a pioneer fibre first establishes contact with a target, and is then followed by other nerve fibres which use it as a guide. Such pioneer neurones have been described in the nervous systems of other insects (Bate, 1976a; Edwards & Palka, 1976; Edwards & Chen, 1979; see also Anderson et al.,1980, for review). Contact guidance leading to bundle formation has also been clearly seen between isolated leech cells in culture (Ready & Nicholls, 1979).

Fibre bundles were observed as fasicles around explants, as well as between explants; such bundles can thus extend for considerable lengths without passing through ganglia, showing the importance of fibre-fibre interaction in their formation (Provine et al.,1973). Free-ended neurites of unknown function grew from such lateral fibre bundles. These may possibly act as 'anchors' for the complex network of fibre bundles, helping to hold the culture in position, or may stabilize the fibre network by producing tensions which oppose those produced by branches with other orientations (see later).

Once initial contact was established between fibres and a target, the fibres straightened and became detached from the surface of the culture dish for an appreciable length. The straightening was apparently accompanied by an increase in tension similar to that

observed by Provine et al.(1976) in fibres from embryonic cockroach ganglia *in vitro*. This often caused explants which were connected by fibres to become drawn closely together, sometimes to such an extent that they became fused. Similar fusion of ganglia has also been observed in embryonic cultures (Provine et al., 1976). Development of tension in regenerating nerve fibres which have made contact with their target could have an important function *in vivo*. The drawing together of nerve cells and target may reduce the distance which subsequent nerve fibres need cover before reaching the target, and also provide them with a firmer, more definite guide for growth. Such an effect may be important in small animals in which the total distance to be covered by regenerating fibres is relatively short. Fibres from cultured ganglia must, at times, produce considerable tension in order to produce physical movement of ganglia.

In cultures where explants became fused together, the problem of identifying sites of fibre origins or terminations could become extremely difficult. It was therefore an advantage to place explants reasonably well apart (around 1 mm) so that connections could be formed, but fusion was less likely. This made it possible to observe the general features of the fibre outgrowth between explants.

A further, and perhaps more interesting effect of tension in fibres has been demonstrated by Bray (1979). He discovered that growth cones at the tips of fibres were capable of pulling, and suggested that the force was exerted through the filopodia (Fig. 1.15). He found that a release in tension produced by cutting a fibre was followed by branching at the fibre tip in 87% of cases. A change in direction of force exerted on a growth cone could be produced by displacing the fibre using a microelectrode. Such manipulations caused the growth cone to change its direction of advance so as to once again directly

oppose the force. Removal of the microelectrode allowed the former direction of force to return, and the growth cone resumed its original direction of growth. Bray suggested that if the neurone were to be considered in equilibrium, then branching of the fibres could be explained by the need for opposing forces (in this case pulling by the growth cones) to cancel each other out. This may often result in symmetry if both branches have similar diameters. Asymmetrical branching may occur, however, if one branch is thicker than another, since tension exerted is related to fibre diameter. As Bray mentions, the direction taken by a growth cone has been shown in a number of experiments (e.g. Letourneau, 1975) to preferentially follow a path of high adhesivity, and it is feasible that it may 'test' how strong the adhesion is by contracting against the point of contact. If adhesion is good, the fibre will not pull away and the growth cone can then advance in that direction. The branching seen in fibres growing in culture is therefore likely to be a result of variations in tension (which may also occur when the culture dish is disturbed during observation) and adhesive properties of the different surfaces encountered. Such a mechanism as described by Bray could function *in vivo* to guide growing nerve fibres along paths of high adhesivity to their targets.

Connections formed between explants showed little or no selectivity: nervous tissue explants became connected to one another or to muscle explants, regardless of whether or not they would normally do so *in vivo*. Levi-Montalcini and Chen (1971) have reported that nerve fibres from embryonic thoracic ganglia make connections with leg explants in preference to other ganglia. This was not seen, however, in the adult ganglia used in the present study. No absolute specificity was seen between ganglia and muscle, although there may be a quantitative

difference between the relative density of connections. Adult abdominal ganglia could form connections with leg muscle explants. Such connections were, however, less robust, and seen more infrequently than those with other abdominal ganglia, or than those between thoracic ganglia and muscle explants. This could be, as suggested by Levi-Montalcini and Chen (1971), because abdominal ganglia contain fewer motoneurones than do thoracic ganglia. The present study also shows that adult ganglia may become connected in any sequence or orientation in vitro; similar results have been reported for embryonic ganglia in culture (Provine et al., 1976).

At this level of observation it was not possible to tell whether or not any specific connections were formed between individual cells. It is quite possible that within the mass of fibres, specific connections were formed between some nerve cells and their targets, but that these were masked. Isolation of specific cells would allow the connections made by them to be followed precisely. It is possible, in principle, to remove single, identified neurone cell bodies from cockroach ganglia and maintain them in culture; they could then be presented with a choice of appropriate and inappropriate targets, to see whether they showed any preference. This has been achieved with leech neurones; cells which are normally electrically coupled in vivo become preferentially coupled in vitro (Ready and Nicholls, 1979). Unfortunately, attempts to carry out similar experiments with identified cockroach neurones were unsuccessful. Very few cells produced any fibre outgrowth, regardless of whether they were placed in a fresh culture environment, or co-cultured with a well-established explant culture. Any early signs of fibre outgrowth aborted within a few days. It is possible, however, that improvements in the growth conditions for these cells might produce more successful results.

The apparent lack of specificity reported here indicates that neurones in cultures of adult ganglia either lack sufficient information to enable them to reach and recognize their appropriate targets, without some cues from the environment, or that they become de-differentiated under the culture conditions.

The Effect of Temperature on Fibre Outgrowth

These cultures will tolerate temperature fluctuations between 19°C and 37°C. This is perhaps understandable when one considers the lifestyle of the animal from which the explants have been derived. In its natural habitat, Periplaneta americana would probably be living in temperatures around 28°C, being in a fairly hot climate, but keeping largely to dark shady places. It is, therefore, reasonable that the nervous system should operate optimally in this temperature range. If, however, the animal moves temporarily into the direct sun and its body becomes heated, or if during the night or cooler months it becomes cooled, it is necessary for it to remain active at these temperature extremes. Since the animal has very limited non-behavioural means of regulating its body temperature, the nervous system would also experience these temperature changes, and must be able to function throughout. These animals are in fact able to survive at temperatures as low as 4°C, and although spontaneous movement almost ceases at this temperature, once they become warmed, they appear to function again perfectly normally.

The optimum temperature for fibre outgrowth was around 28°C. Although a temperature of 37°C produced less reliable and less profuse outgrowth, the time taken for outgrowth to begin in cultures which did show sprouting was not significantly different from the time at which outgrowth began at 28°C. The subsequent rate of outgrowth was only

slightly slower at 37°C than at 28°C, and taking into account the inaccuracy of the method used to estimate this, the difference may be insignificant. It therefore seems that temperature has a fairly marked effect on the proportion of cultures that produce outgrowth, but has little or no detectable effect on the subsequent progress of those cultures which do show outgrowth. This relative insensitivity means that the cultures will be little affected by minor temperature fluctuations which occur while they are under observation.

The effect of temperature on the cockroach nervous system, both *in vivo* and *in vitro*, is therefore very different from that on the nervous systems of homiothermic animals, where a temperature change of only a few degrees in either body temperature or culture environment may cause death.

Morphology of Cultured Ganglia

(a) Light Microscope Observations

i) Change in Shape

The flattening seen in ganglia after some days in culture also occurs in some dissociated cell cultures (Folkman and Moscona, 1978). It probably enhanced adhesion of the explant to the dish. Reduction in the dorsoventral thickness of the ganglion resulting from this flattening would also reduce the problem of diffusion to the centre of the explant.

ii) Changes in Cell Bodies

The cell bodies of some cockroach neurones have been reported to become swollen between 2 and 6 days after axotomy (Cohen and Jacklet, 1965), but subsequently become shrunken if reconnection with the muscle is not achieved (Jacklet and Cohen, 1967 b). Since motoneurones are axotomised during prepara tion of ganglion explants, it is not surprising that they become reduced in size. Motoneurone cell bodies are normally among the largest in the cockroach ventral nerve cord, and consequently this effect is particularly noticeable. In fact, several changes observed in these cultured cells show a remarkable similarity to those seen in axotomised motoneurones *in vivo*. Changes in RNA distribution within the cell bodies of axotomised neurones *in vivo* and *in vitro* appear similar and have comparable time courses (c.f. Cohen and Jacklet, 1965). Movement of the nucleus to an eccentric position within the soma is observed in culture, and is also characteristic of axotomised cells.

Cohen and Jacklet (1967 b) suggest that the characteristic changes in RNA distribution are a pre-requisite for regeneration. Since these changes have a similar time course *in vivo* and *in vitro*, it would be predicted that the time at which regeneration starts should also be similar in each case. As mentioned in Results, the onset of nerve sprouting occurs earlier *in vitro* than that reported *in vivo* by Denburg et al. (1977). These results suggest that, although the RNA changes occurring in the cell body are tied up with regeneration of the neurone, it is not necessary for the RNA aggregate to become dispersed before regeneration can begin, as has been suggested previously (Cohen, 1967; Jacklet and Cohen, 1967 b). More recent observations *in vivo* also suggest that this may be the case (Whitington, 1979; Brogan and Pitman, 1981).

iii) Changes in the Ganglionic Sheath

It is interesting that the nerve sheath of adult ganglia in organ culture became thickened, since sheaths of cultured embryonic ganglia disintegrate. The cells of the embryonic nerve sheaths have been reported to migrate from the ganglion, leaving the neurone cell bodies exposed (Hicks, 1981). It is likely that the sheath thickening seen in cultured adult ganglia was at least in part responsible for the poor preservation of tissue for morphological studies. Preliminary observations of sections made before removal of wax, revealed that wax impregnation into the centre of the tissue was extremely poor. The resulting absence of mechanical support to the central regions of the ganglia may have caused poor sectioning of the tissue.

In the sections examined, no breaks in continuity of the sheath were seen, suggesting that no fibres had actually grown through the sheath. However, Marks et al., (1968) have obtained histological evidence that the neurilemmal sheath of cultured ganglia from late instar nymphs did show breaks penetrated by nerve fibres. It is possible that the sections in the study presented here were not sufficiently thin to reveal such tiny breaks.

iv) Changes in the Neuropile

There are several alternative explanations for the changes observed in transverse sections of ganglia maintained in culture. It is possible that the appearance after varying periods in culture reflects different degrees of necrosis within the neuropile. However, neurones in culture can produce a profusion of varicose nerve sprouts which largely run longitudinally and parallel to one another through the tissue (see Chapter 3). These processes and varicosities would be circular in transverse section, and may be at least in part responsible for the increase in number of such profiles seen in the sections. If this were the case, then the relative decrease in number of circular profiles seen as the cultures aged could be due to a progressive growth of nerve fibres in more varied directions through the neuropile, possibly coupled with a regression of some longitudinally running fibres or varicosities. The apparent recovery of a fairly organised neuropile seen in long term cultures may not be identical to that of a normal ganglion in vivo, although a symmetrical pattern appears to develop.

If these results can be taken at face value, it would seem that the neuropile structure of ganglia in culture initially becomes disrupted, but subsequently becomes reorganised. However, the results should be interpreted with caution. It is possible that they only represent a selection process through which explants have passed: the older cultures, for example, would only have been maintained for long durations of time if they produced good fibre outgrowth. At early stages the potential of explants to produce outgrowth could not be assessed, and consequently the population studied could have included a number of non-viable as well as viable explants. It is possible, then, that the results merely represent the difference between healthy and less healthy cultures. If this is so, however, they do at least show that in healthy explants, the internal organisation of the ganglion can be maintained for several months.

Ganglia from Small Nymphs

Less radical changes were seen in these ganglia; this may be attributed to their small size which would present no problem for the diffusional exchange of substances within the central neuropile. This may also explain why nymphal ganglia became less flattened *in vitro* than did larger ones.

(b) Electron Microscope Observations

When sections through cultured ganglia were observed under the transmission electron microscope, a number of organelles were seen throughout the neuropile, such as micro-filaments and mitochondria, indicating that the cultures had been alive and active. There were, as would be expected

following the extensive damage caused by isolation of the ganglia, a number of degenerative whorls throughout the neuropile.

The observations made under the scanning electron microscope provided evidence to suggest that the outgrowing fibres were, in fact, nerve fibres. Fibres extending from ganglia possessed varicosities which were characteristic of insect axons *in vitro* (Chen and Levi-Montalcini, 1969; Seshan and Levi-Montalcini, 1971); growth cones were also observed at the ends of fibres.

Cells observed migrating from the explant could not be positively identified. It is likely that they are either glial cells, or tracheoblasts, as these cell types are thought to migrate from embryonic ganglia *in vitro* (Levi-Montalcini and Chen, 1969).

CHAPTER 2

THE EFFECT OF CONDITIONING LESIONS ON NERVE FIBRE REGENERATION

INTRODUCTION

A number of reports have shown than one nerve lesion (known as the 'conditioning lesion') can affect the axonal growth from the nerve after a subsequent lesion (known as the 'testing lesion') to that nerve. Landreth and Agranoff (1976) crushed one optic nerve in the goldfish, and then 1-2 weeks later, explanted the retinae from both eyes into culture. They found that neuritic outgrowth from the 'prior-crush retina' began within a few hours after explantation, whereas the control retina showed no signs of outgrowth until 2-4 days after removal into culture. The extent of outgrowth from the 'prior-crush' explants was still greater than that from the controls after 2 weeks. Johns, Heacock and Agranoff (1978) have shown these neurites to arise from ganglion cells. Similar enhanced neuritic outgrowth following a conditioning lesion has been shown in the retina of Xenopus (Agranoff, Field and Gaze, 1976).

The effect of the conditioning lesion is apparent in goldfish optic axons with intervals as little as 2 days and as great as 28 days between conditioning and testing lesions (Forman, McQuarrie, Labore, Wood, Stone, Braddock and Fuchs, 1980). The effect on axonal growth was maximal when the delay between conditioning and test lesions was 14 days (Edwards, Alpert and Grafstein, 1981). Ultrastructural studies of this preparation revealed that the conditioning lesion accelerated the initial stages of regeneration (Lanners and Grafstein, 1980). A number of axon sprouts arranged in bundles were seen as soon as 2 days after the testing lesion (compared to 6 days in unconditioned nerves). A similar enhancement was observed in the rate of development of these

axonal sprouts: microtubule formation within the sprouts, and glial invagination, for example, occurred sooner in such axons.

The experiments described above were performed on cold blooded vertebrates. However, conditioning lesions can also affect axonal growth rates in mammalian nervous systems in vivo (Hall-Craggs and Brand, 1977; McQuarrie, Grafstein and Gershon, 1977; Scheff, Bernado and Cotman, 1977; Wells, 1977; Wells and Bernstein, 1978). McQuarrie and Grafstein (1973) reported that the axonal growth rate in mouse sciatic nerve following a test lesion was increased by 27% in nerves which had received a conditioning lesion. Similar results were obtained by McQuarrie et al.(1977) using rat sciatic nerve. They made a conditioning lesion in the sciatic nerve by transecting the tibial nerve at the ankle. The subsequent testing lesion was made 2 weeks later by crushing the sciatic nerve at the mid-thigh level. This ensured that the testing lesion was well away from the area of damage caused by the conditioning lesion. The axonal growth rate in nerves which received a conditioning lesion increased by 23% over that in nerves receiving a testing lesion only. The axonal growth rate was estimated by locating the leading sensory axons, by histological techniques in the experiments on mice, and by the 'pinch test' in the experiments on rats. The 'pinch test' involves pinching the exposed nerve, beginning distally and gradually moving proximally until a withdrawal reflex is elicited. It should be noted, however, that this test shows only an increase in growth rate of leading axons; the growth rate of other axons may not be enhanced.

In the experiments on mammalian nerves described above, a conditioning lesion resulted in an increase in axonal growth rate. In contrast, no appreciable change was found (only about 15% reduction) in the delay before the onset of axon regeneration. These results differ from those on goldfish and Xenopus retina, where the delay was greatly reduced.

McQuarrie, Grafstein, Dreyfus and Gershon (1978) have found yet other effects of conditioning lesions on the regeneration of adrenergic axons in rat sciatic nerve; regeneration was initiated after a shorter interval, but subsequently progressed more slowly. The initial delay, estimated by extrapolation to zero outgrowth distance, showed a 50% decrease in conditioned nerves. Direct histological examination of axonal growth in the mouse sciatic nerve with time (McQuarrie, 1979) also showed a marked decrease in nerves that had received a conditioning lesion. The growth rate of both sensory and adrenergic axons in the sciatic nerve is similar following test lesions only. This highlights the differential effect of conditioning lesions, since the growth rate of sensory axons is increased, while that of adrenergic axons is decreased following such lesions.

In summary, conditioning lesions seem to affect axonal outgrowth in two ways. Firstly, in some neurones they may cause a reduction in the delay before axonal sprouting begins. However, in other neurones, such a change apparently does not occur.

The second effect is on the rate of subsequent axonal growth. This may be either increased or decreased, depending on the type of neurone involved. The results from the experiments discussed above, therefore, indicate that the initiation of axonal outgrowth following a second lesion is controlled by a different mechanism from that regulating the subsequent rate of growth, since these two parameters of growth appear to be affected independently. It has been suggested on the basis of indirect evidence (Grafstein and McQuarrie, 1978) that the above alterations in nerve regeneration may result from changes in protein synthesis and transport produced by conditioning lesions.

Some cases of enhanced reinnervation or regeneration <u>in vivo</u> are consistent with the above results. Hall-Craggs and Brand (1977)

found that prior injury to a nerve which was destined to innervate a transplanted muscle graft resulted in superior formation of tension in the graft. They attributed this to the more rapid, and therefore more successful, reinnervation of the muscle by previously damaged nerves.

Kao, Chang and Bloodworth (1977) transected the dog spinal cord, and one week later grafted a segment of nerve into the region of the lesion. They reported that axons traversed the graft into the distal portion of the spinal cord. They suggested that this was because after a week, the glial scar did not reform, and hence nerve fibre growth was not impeded as after the first transection. However, it is possible that this enhancement of growth could be yet another instance illustrating the effect of conditioning lesions.

The effects of conditioning lesions upon invertebrate nervous systems have not been documented in the literature; nor have the experiments described above included any long term observations on the final state of recovery of the animal. The experiments described in this chapter were therefore designed to analyse the effects of conditioning lesions in an invertebrate, taking three different aspects into consideration. These were:

a) the delay before axonal outgrowth begins (in vitro)

b) the rate of subsequent axonal growth (in vitro)

c) the rate and extent of functional recovery (in vivo)

Whatever the mechanisms, conditioning lesions appear to have an influence on nerve regeneration in many animals. Since the effects are not peculiar to one species, or even one class of animal, it seems possible that observations made on the growth of neurites under these circumstances may shed further light on the different mechanisms involved in regeneration.

METHODS

(a) Conditioning Lesions for the in vitro Preparation

Young adult male cockroaches (Periplaneta americana) were anaesthetized with humidified 100% carbon dioxide, and secured, ventral surface uppermost, to a dissection board. The largest leg nerve (nerve 5) was cut on the right side of the animal in both the mesothoracic and metathoracic segments. This is a relatively simple operation to perform, since this nerve runs in a superficial position, and can be easily seen through the transparent articular membrane on the ventral surface of the animal, where the coxa joins the thorax (Fig.2.1). Fine iridectomy scissors were cleaned in 95% ethanol and then dipped in insect saline (Table 2.1) containing 0.8 mg/ml of gentamycin sulphate (taken from commercial stock of 40 mg/ml obtained from Roussel Laboratories Ltd., London) immediately before use. A small slit was made in the cuticle over nerve 5, which was then carefully cut, avoiding damage to nearby muscle or tracheal tubes. Once severed, the proximal and distal ends of the nerve parted and disappeared into the thorax and coxa respectively. It was possible to check that the nerve had been successfully sectioned by observing the limb movement of the animal on recovery from anaesthetic. If the nerve had been completely transected, the leg was held in an elevated position, slightly extended away from the body (Jacklet and Cohen, 1967a). This was due to the denervation of both the coxal depressor muscles, and the flexor muscles of the femur (Dresden and Nijenhuis, 1958). Little haemolymph was lost through the small cut in the cuticle during this operation, if the animal was well anaesthetized. Since the cut healed over quickly, it was not necessary to seal the wound.

Fig. 2.1 Diagram of the cockroach metathoracic region, as seen from the ventral surface. Part of the coxal cuticle has been removed to reveal the route taken by nerve 5. This nerve can be conveniently transected at the position indicated by the broken line, where it runs directly beneath a thin, transparent articular membrane.

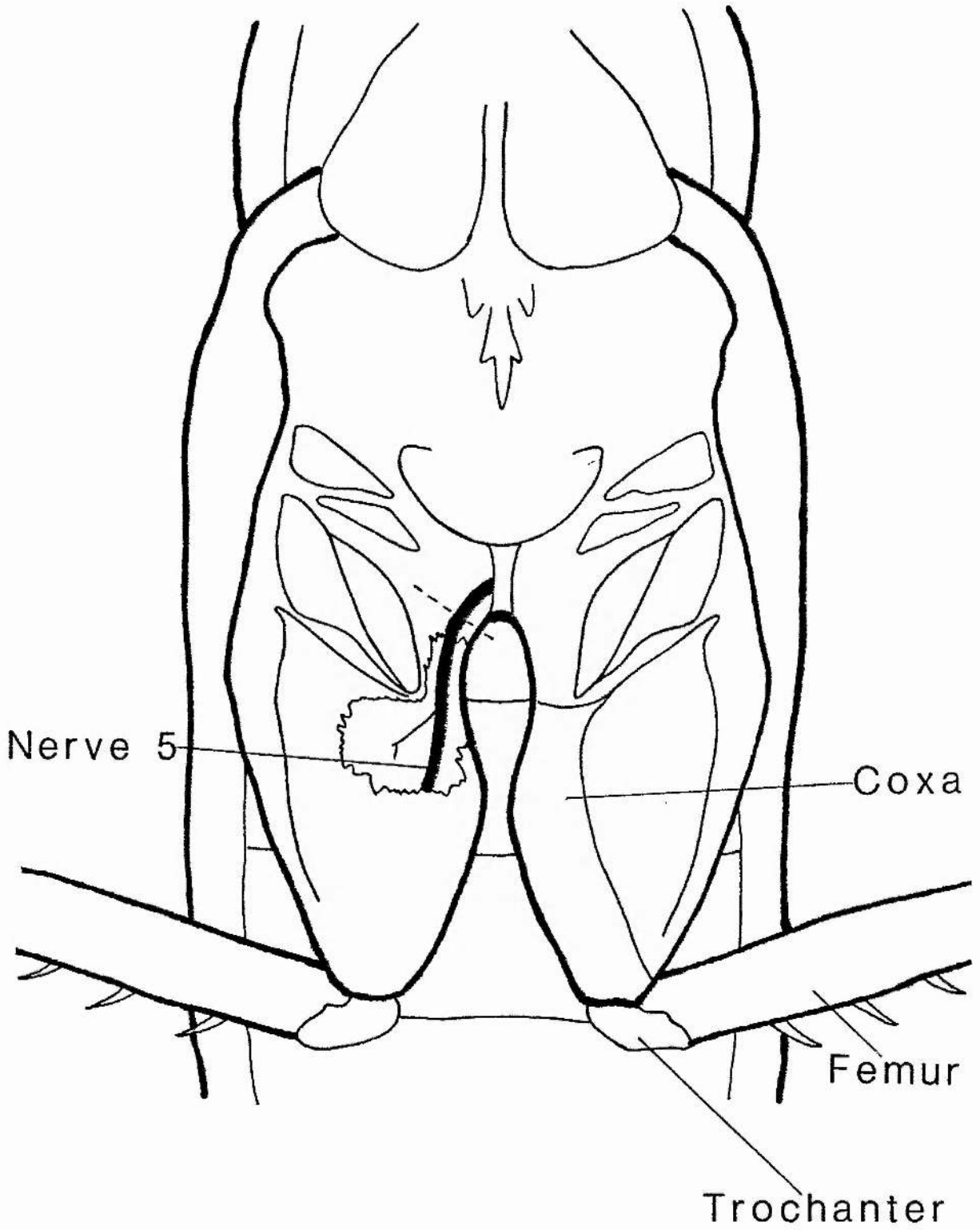

Nerve 5
Coxa
Femur
Trochanter

TABLE 2.1

Composition of Insect Saline

214 mM NaCl

3.1 mM KCl

9 mM $CaCl_2$

10 mM TES

The pH was adjusted to 7.2 with NaOH. Sodium and potassium concentrations were checked by flame photometry.

Animals were allowed to recover and were housed individually in small plastic boxes. They were supplied with a constant source of water and Minced Morsels Moist Dog Food. After 3 weeks, the animals were sacrificed and their thoracic ganglia explanted into separate Petri dishes for culture, as described in Methods, Chapter 1. The explants were observed at 6 hourly intervals, and fibre outgrowth from the pre-sectioned nerve 5 was compared with that from the homologous contralateral nerve.

(b) Conditioning Lesions for the in vivo Preparation

The preliminary operation was very similar to that described above, but differed slightly in 2 ways:

(i) The nerve was crushed using a fine pair of forceps, instead of being cut. If this was done efficiently, then all the axons within the nerve should have been severed, leaving only the nerve sheath intact. The crush was considered to have been successful if a clear gap was visible in the nerve at the point of crush, and if, on recovery from anaesthetic, limb movement showed similar abnormalities to those observed after the nerve had been cut. The nerve sheath was left intact in these experiments to aid relocation of the nerve for a subsequent operation.

(ii) The right nerve 5 was crushed only in the metathoracic segment. This was a less traumatic operation than crushing nerves in two adjacent segments, and therefore animals were more likely to survive. This was important, since long-term survival was crucial to such experiments.

The animals were allowed to recover from anaesthetic, and kept for 3 weeks before performing a second operation. They, and a group of previously unoperated animals, were divided into 4 groups:

First Operation	Second Operation
Crush right N5	Cut right N5
Crush right N5	Cut right and left N5
None	Cut right N5
None	Cut right and left N5

The animals were again allowed to recover, and were observed over a period of 7 months for restoration of normal leg functioning. If function was restored, the leg was no longer elevated or extended abnormally during movements, and the animal was once again able to grip with the tarsus. During walking and running, the previously denervated leg was used in the normal sequence, resulting in co-ordinated movement of the animal as judged by direct visual observations.

RESULTS

(a) The Effects of Conditioning Lesions Observed In Vitro

In the Introduction to this chapter, evidence was presented that a conditioning lesion may affect two aspects of neurite growth following a test lesion. These aspects were studied in the experiments presented here using the cockroach nervous system, and will be considered separately.

(1) Delay Before Initiation of Neurite Growth from Lesioned Nerves

Out of 22 preparations examined, 86.4% (19 preparations) showed fibre outgrowth from the nerve 5 which had been previously cut in the living animal (referred to as the conditioned nerve) before the non-pre-cut (or non-conditioned) contralateral homologous nerve (Fig. 2.2). In the remaining 3 preparations, outgrowth from both the conditioned and non-conditioned nerve 5 began after approximately the same delay. In no instance was outgrowth observed from the non-conditioned nerve before the conditioned nerve.

(2) Subsequent Growth Rate of Nerve Fibres

The rate of fibre growth (estimated as described in Methods, Chapter 1) varied considerably between preparations, and also with time after explantation. In some cases, rates of more than 22 μm/h were estimated, whereas in others, the maximum rate was as little as 8.7 μm/h. Fig. 2.3 shows some examples of fibre growth rate against time in culture. After an initial phase of several days during which growth rate increased, the rate of extension normally declined (Fig.2.3, A - C), and occasionally there was even some regression (Fig. 2.3, A). In many cases this was followed by a secondary

Fig. 2.2 The effect of conditioning lesions on fibre outgrowth.

(a) Adult mesothoracic ganglion maintained in culture for 7 days. The animal's right nerve 5 was sectioned 16 days prior to explantation. Fibre outgrowth from this 'conditioned' nerve (open arrowhead) is more extensive than that from the 'non-conditioned' homologous nerve (solid arrowhead), or from any other nerve trunk. Calibration : 1 mm

(b) The same preparation as in (a), one day later. Outgrowth from nerves other than the 'conditioned' nerve is becoming extensive, but there is still little, if any, outgrowth from the 'non-conditioned' nerve 5. Calibration : 1 mm

(c) Adult metathoracic ganglion maintained in culture for 5 days. The right nerve 5 was sectioned 16 days prior to explantation. Once again, fibre outgrowth from the 'conditioned' nerve (open arrowhead) is more extensive than from the 'non-conditioned' nerve 5 (solid arrowhead). Calibration: 1 mm

(d) The same preparation as in (c), two days later. There seems to have been some fibre regression from the 'conditioned' nerve, but outgrowth from this nerve is still more extensive than from any others. Calibration : 1 mm

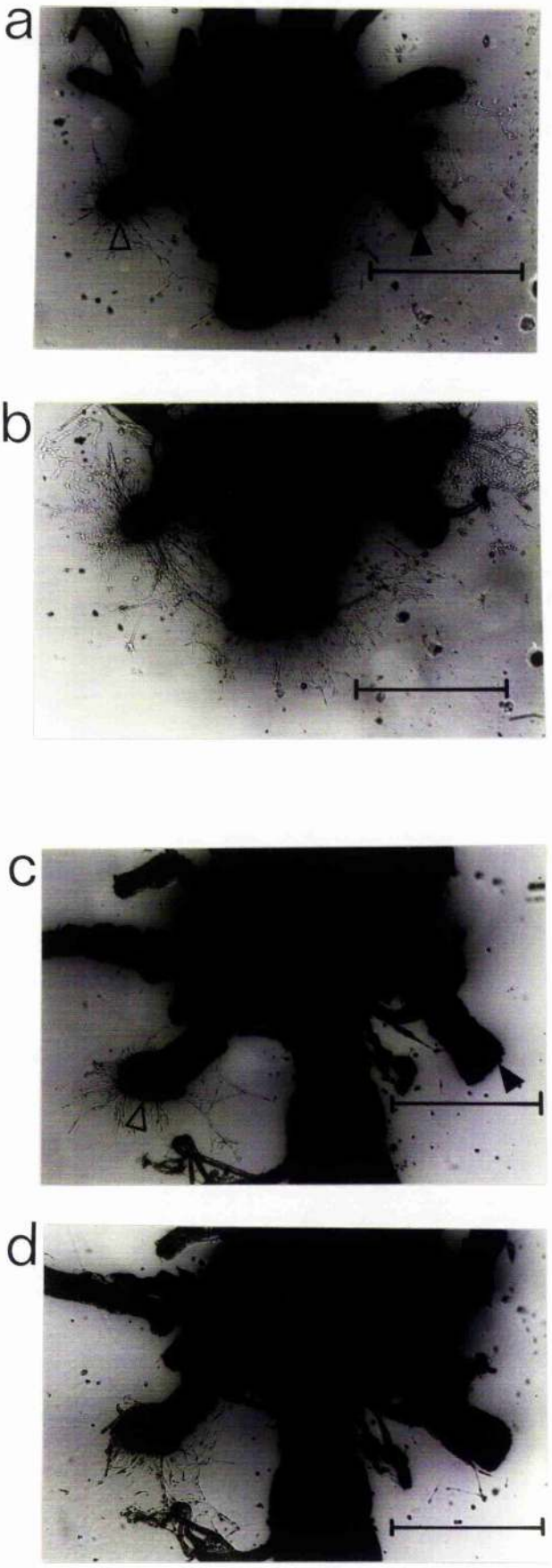
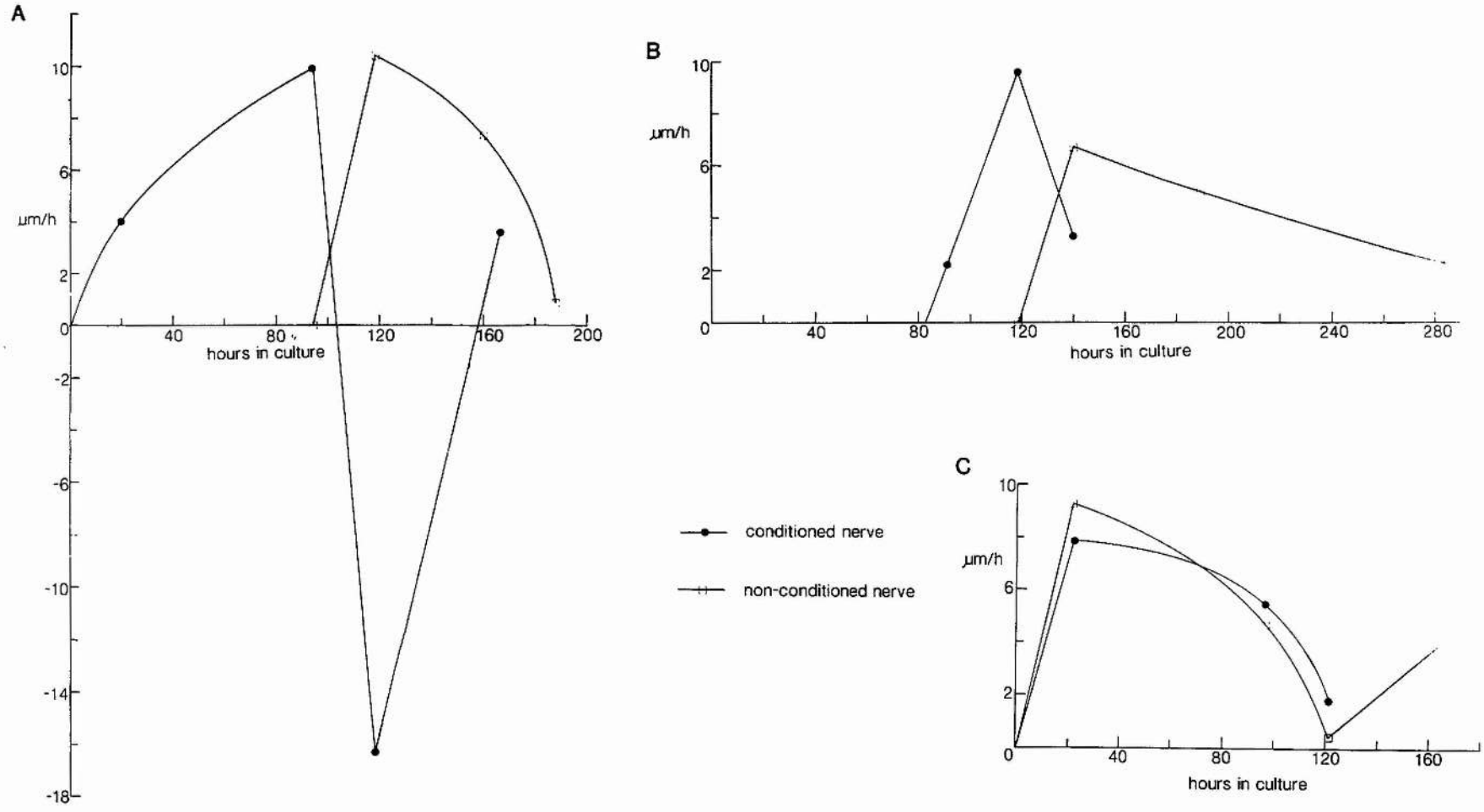

a
b
c
d

Fig. 2.3 Comparisons of fibre growth rates in vitro from 'conditioned' and 'non-conditioned' nerve 5. In A and B, outgrowth from the 'conditioned' nerve began well in advance of the 'non-conditioned' nerve; in C, outgrowth began almost simultaneously from both nerves. The maximum growth rate reached by nerve fibres from either of the nerves was usually about 8-10 μm/h. Growth rates from the two nerves frequently followed a similar pattern within a given preparation, as in B and C.


A
10
6
µm/h
2
0
-2
-6
-10
-14
-18
40
80
120
160
200
hours in culture
B
10
µm/h
6
2
0
40
80
120
160
200
240
280
hours in culture
conditioned nerve
non-conditioned nerve
C
10
µm/h
6
2
0
40
80
120
160
hours in culture

phase of accelerated growth (Fig. 2.3, A and B). Fibre growth rates from conditioned and non-conditioned nerves were frequently similar within a single preparation; when dissimilar rates were seen, growth could be faster from either the conditioned or non-conditioned nerve.

Outgrowth from the non-conditioned nerve soon became difficult to follow as there was almost invariably a delay of several days before this began. By this stage fibre growth from other nerves had begun, and soon became sufficiently prolific to obscure growth from the non-conditioned nerve. The number of comparisons of fibre growth rates which could be made was, therefore, limited, as was the time-span over which individual preparations could be followed.

(b) The Effect of Conditioning Lesions Observed In Vivo

Fig. 2.4 A shows the leg positions of a normal animal. After section of nerve 5, the denervated leg became elevated and extended (Fig. 2.4, B and C), and the tarsus no longer gripped the substrate (Fig. 2.4 C).

The functional recovery observed in living animals following conditioning and/or testing lesions are summarised in Table 2.2b, i and ii. A number of tentative conclusions can be drawn from these results, but it must be emphasised that the number of animals used was small (only four in each of the groups described under Methods), and evaluations of the extent of recovery were only based on behavioural observations. The results indicate that the following may be true:

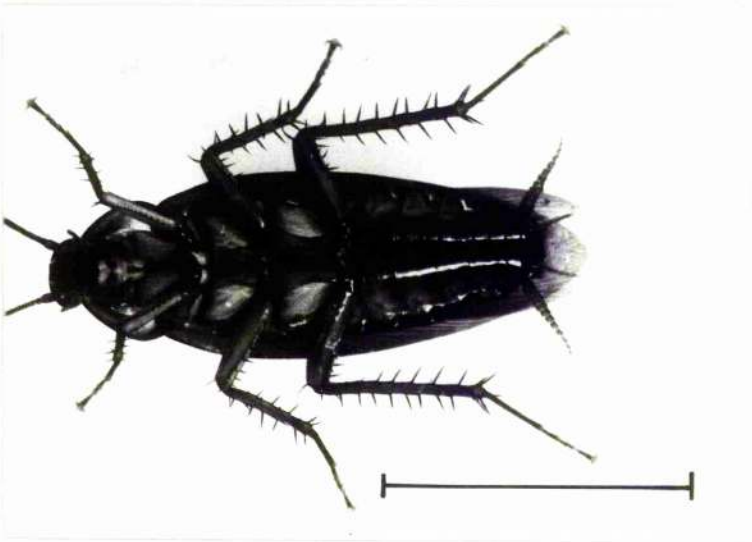
Fig. 2.4 The effect of nerve 5 lesion.

(a) Normal adult male cockroach viewed from the ventral surface. Note that the tarsi firmly grip the surface to which the animal is clinging. Calibration : 2 cm.

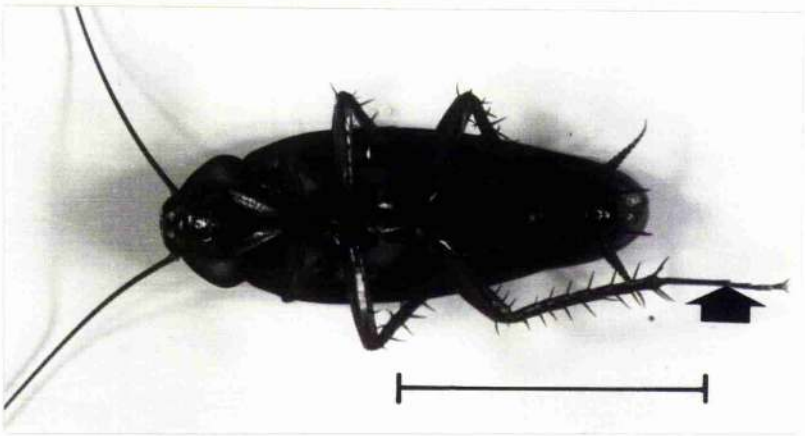
(b) Lightly anaesthetised adult male cockroach after section of the animal's right metathoracic nerve 5. Note the extension of the right metathoracic leg (arrow). Calibration : 2 cm.

(c) Adult male cockroach, fully recovered from anaesthesia after section of the right metathoracic nerve 5. The right metathoracic leg is held in an elevated position due to denervation of the coxal depressor muscles; the upturned tarsus no longer grips the surface (arrowhead) and is consequently seen here out of focus. Calibration : 2 cm.

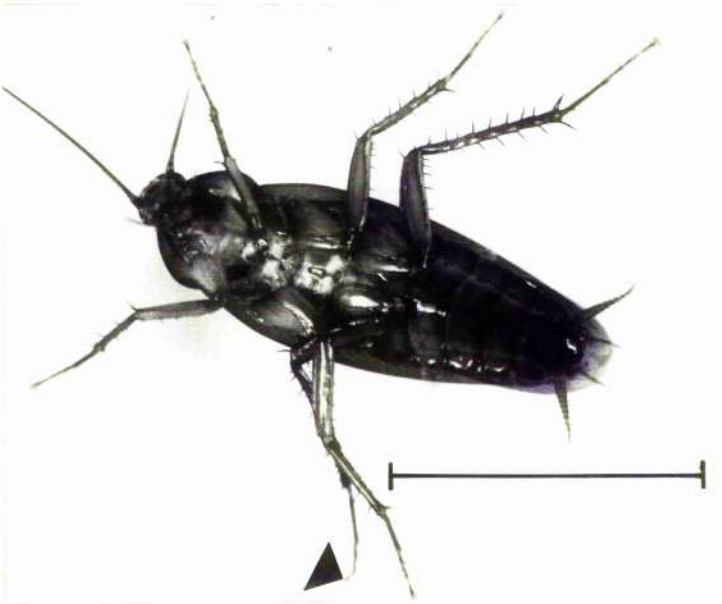
a



b



c



(A) Nerves receiving conditioning lesions show more rapid functional recovery rates than do nerves receiving only one lesion:

(1) A comparison of recovery in a number of experimental animals showed that functioning of the leg returned more quickly if the nerve concerned had received a conditioning lesion than if it had not. However, by about 9 weeks after the testing lesion, the recovery in the two groups was similar: the non-conditioned nerves seemed to have 'caught up' with the conditioned nerves, and presumably had established functional contact with their targets (see Table 2.2b, columns (a) and (b)). In long term animals (31 weeks after test lesion), the recovery of non-conditioned nerves was, if anything, slightly superior to that of conditioned nerves.

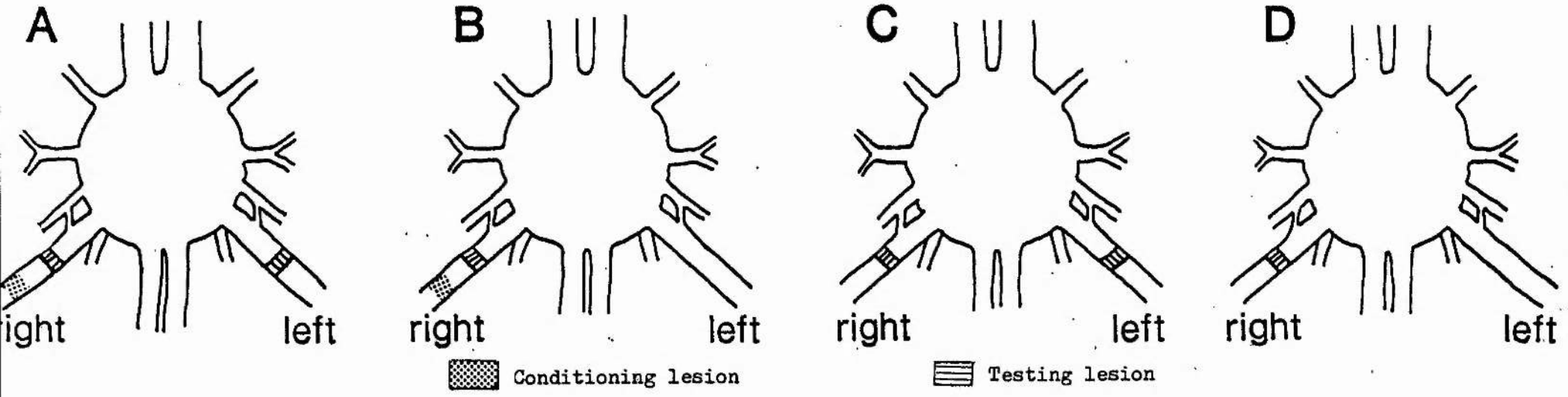
(2) Comparisons of recovery of conditioned and non-conditioned nerves in different animals (Table 2.2b, columns (a) and (b)), or within individual animals (Table 2.2b, columns (c) to (e)) produced similar results.

(B) An effect of a lesion is transferred to the contralateral side of the ganglion:

Recovery of function seemed to be superior if homologous nerves were sectioned simultaneously on both sides of the animal than if there was a spatial or temporal asymmetry in lesions (Table 2.2b, columns (f), (g) and (h), (i)).

Table 2.2a

Animals were divided into four groups with different combinations of non-lesioned, non-conditioned and conditioned nerves.



Column in Table 2.2b	Lesion programme observed
a	A(right); B(right)
b	A(left); C(right); C(left); D(right)
c,d,e	A(right) compared with A(left)
f	A(left)
g	C(right); C(left)
h	A(right); A(left); C(right); C(left)
i	B(right); D(right)

	a	b	c	d	e
No. of weeks After Testing Lesions(s)	% CN with good recovery	% NCN with good recovery	% CN with better recovery than NCN	% NCN with better recovery than CN	% NCN and CN equally well recovered
Up to 5	0 (8)	0 (16)	0 (4)	0 (4)	0 (4)
7	62.5 (8)	37.5 (16)	50 (4)	0 (4)	50 (4)
9	62.5 (8)	62.5 (16)	50 (4)	0 (4)	50 (4)
11	75 (8)	71 (14)	25 (4)	50 (4)	25 (4)
12	83 (6)	82 (11)	25 (4)	25 (4)	50 (4)
31	100 (5) (20% appeared completely normal)	100 (10) (60% appeared completely normal)	33 (3)	67 (3)	0 (3)

TABLE 2.2b(i) Comparison of recovery of nerve function in conditioned and non-conditioned nerves.

CN - CONDITIONED NERVE
NCN - NON-CONDITIONED NERVE

(Columns (c), (d) and (e) show comparisons between nerve recoveries in individual animals)

Numbers in brackets show total number of observations made in each case.

	f	g	h	i
No. of weeks After Testing Lesions(s)	% NCN with good recovery opposite CN	% NCN with good recovery opposite NCN	% Nerves with good recovery in animals with bilateral lesions	% Nerves with good recovery in animals with unilateral lesions
Up to 5	0 (4)	0 (8)	0 (16)	0 (8)
7	25 (4)	25 (8)	50 (16)	37.5 (8)
9	75 (4)	87.5 (8)	81 (16)	50 (8)
11	75 (4)	100 (6)	86 (14)	62.5 (8)
12	50 (4)	100 (4)	83 (12)	80 (5)
31	100 (3)	100 (4)	100 (10)	80 (5)

TABLE 2.2b(ii) The effect of asymmetries in lesions to pairs of nerves on the recovery of nerve function.

CN - CONDITIONED NERVE
NCN - NON-CONDITIONED NERVE

Number in brackets show total number of observations made in each case.

DISCUSSION

The bilateral symmetry of the cockroach nervous system provides a suitable preparation for making comparisons of fibre growth from 'conditioned' and control nerves. Since many experiments involved comparison of nerves from the same animal, differences in such growth could be attributed to the effect of a conditioning lesion rather than to differences between individuals.

(a) The Effect of Conditioning Lesions Observed In Vitro

The results leave little doubt that a conditioning lesion causes a reduction in the time taken for the initiation of fibre growth. Even if the culture system had an effect on the delay before growth initiation or upon the rate of subsequent fibre growth, it is unlikely that it would have opposite effects upon conditioned and non-conditioned nerves. It is possible that the reduced delay for nerve fibre outgrowth occurs because 'conditioned' neurones are already primed for regeneration when subjected to a second injury. A number of authors have suggested that aggregation and subsequent dispersal of RNA is a necessary pre-requisite for regeneration of insect neurones (Cohen and Jacklet , 1965; Cohen, 1967; Guthrie, 1967; Denburg, Seecof and Horridge, 1977). The aggregation is first seen after about 3 to 5 days. If it is assumed that neurones have undergone this process following the first lesion, then they should be already primed for regeneration when subjected to the testing lesion. The effect of a conditioning lesion on growth initiation would perhaps be more apparent in insects where the normal delay is relatively long compared to that in mammalian nervous systems. The delay before growth initiation in non-conditioned rat sciatic

nerve, for example, is about 1.6 days (McQuarrie et al., 1977); that for cockroach neurones is probably about 4 days (see Chapter 1).

Conditioning lesions did not appear to have any effects on the growth of nerve fibres in vitro subsequent to the onset of growth. More extensive experiments are necessary to confirm this, but the results obtained suggest that growth initiation and subsequent fibre growth are affected independently. The effect of temperature on fibre growth also supports this view; maintenance temperature may affect growth initiation but have no effect on subsequent growth rates of regenerating nerve fibres (see Chapter1).

(b) The Effect of Conditioning Lesions Observed In Vivo

The results from the experiments on living animals to some extent support those from the experiments in vitro, in that recovery of function occurred more rapidly if nerves had received conditioning lesions. However, the decrease before nerve fibre growth begins does not fully account for the increased recovery rate of conditioned nerves. Limbs re-innervated by conditioned nerves could become functional up to two weeks earlier than those re-innervated by non-conditioned nerves. The difference in time for initiation of growth would only account for about 3 to 4 days difference in time to recovery (Brogan and Pitman, 1981). It therefore seems likely that the rate of axonal elongation in vivo is also increased by a conditioning lesion. To confirm this, experiments to measure fibre outgrowth directly are necessary.

Conditioning lesions caused more rapid recovery of function by the nerve, but by 9 weeks after nerve section, limb function appeared equally good regardless of whether the re-innervating nerve had or had not been conditioned. (See Table 2.2b,columns (a) and (b)).

This indicates that the ultimate state of recovery in the animal was not enhanced by such lesions. In fact in some of the long term experiments, recovery on the control side was superior to that on the conditioned side (see Table 2.2b,columns (c) to (e); also columns (a) and (b)). This may be due either to the formation of weaker connections by the conditioned nerve, poorer long term viability of nerve fibres from this nerve, or perhaps even to the formation of some inappropriate connections.

The results also seem to suggest that if there is an asymmetry in lesions, a transneuronal or systemic effect may be transmitted across the ganglion from one set of cells to their contralateral homologues. Injury to nerve 5 on both sides of the animal tended to result in more successful functional recovery on both sides than did injury of one nerve 5 only (see Table 2.2b, columns (h) and (i)). This was found whether or not the nerves had received conditioning lesions, and may be explained if greater overall damage to the nervous system produced a greater stimulus for regeneration. However, functional recovery of non-conditioned nerves was more rapid if the contralateral nerve was also non-conditioned; nerves in which the contralateral nerve was conditioned showed slower recovery of function (see Table 2.2b,columns (f) and (g)). It would therefore seem that any asymmetry in lesions, whether spatial or temporal, causes poorer nerve recovery. It is possible that intact cells may respond to damage of their contralateral counterpart, so becoming less responsive to a later injury.

In summary, this series of experiments indicates that, as with vertebrate nerves, regeneration of invertebrate nerves is affected by conditioning lesions. The delay before growth begins

is reduced. Intracellular dye injection into conditioned neurones *in vivo* is necessary to demonstrate conclusively the effects of such lesions on nerve fibre growth rate.

Enhancement of regeneration could be of clinical value; in man, recovery after peripheral nerve lesions is improved if nerve suture is postponed for several weeks after injury (Nicholson and Seddon, 1957). Since nervous systems in many animals show similar responses to conditioning lesions, the relatively simple invertebrate nervous system appears to provide a suitable model.

CHAPTER 3

THE EFFECT OF CENTRAL NERVOUS SYSTEM LESIONS UPON THE STRUCTURE OF AN IDENTIFIED MOTONEURONE

INTRODUCTION

The connections made by a neurone can be affected either indirectly, by damage to or removal of surrounding cells, or directly, by damage to the neurone itself. An understanding of the way in which neurones respond to nerve damage may give us a deeper insight into the events which lead to successful regeneration.

There are several ways in which a neurone may be affected by, and react to, damage of its neighbouring cells. Partial denervation of a vertebrate muscle caused by damage to some of the nerves may cause the remaining intact nerves to sprout and take over the functional innervation of the denervated area. This phenomenon, known as collateral sprouting, has been discussed in the General Introduction. When native nerves regenerate and return to the muscle, these collateral sprouts may begin to retract (Brown and Ironton, 1976). The exact stimulus for collateral sprouting is not clear, as it can occur under a variety of different conditions. The activity of the muscle plays an important part: inactivation of a fully innervated muscle can induce collateral sprouting, (Brown and Ironton, 1977a), and direct stimulation of a denervated muscle can largely suppress it (Brown and Ironton, 1977b). However, a block in axonal transport in the nerves of a fully innervated, active muscle can also induce collateral sprouting (Cooper, Diamond and Turner, 1977).

Collateral sprouting has also been observed in invertebrates. Destruction of nociceptive neurones (or "N" cells) in the leech central nervous system causes the remaining N cells to increase their receptive field, spreading into the denervated area (Blackshaw, Nicholls and Parnas, 1981).

Deafferentation of mammalian neurones can cause a reduction in the branching and the diameter of the dendrites. (Jones and Thomas, 1962; Matthews and Powell, 1962; Cowan, 1970). This dependence of the

dendrites upon synaptic input is also evident during the development of the vertebrate nervous system, and has been discussed by Berry, Bradley and Borges, (1978).

Axotomy of vertebrate neurones can produce dendritic changes similar to those seen after deafferentation; there are several reports of a reduction in branching and dendrite diameter (Cerf and Chacko, 1958; Sumner and Watson, 1971; Sumner and Sutherland, 1973; Cohen, 1976). The proximal region of the severed axon produces sprouts which may, in some cases, regenerate sufficiently well to establish functional synapses (Bjorklund and Lindvall, 1979; Wood and Cohen, 1979). Regenerative sprouting of central neurones has been reviewed by Reis, Ros, Gilad and Joh, (1978).

The structure and connections of neurones from some immature invertebrates may change considerably during normal development. Radical changes were found during the development of identified neurones in a nematode. Motoneurones initially make contact with muscles on the dorsal side of the animal, but subsequently lose these connections and innervate muscles on the ventral side (White. Albertson and Anness, 1978). Neuroblasts of identified neurones in the grasshopper have been shown to form supernumerary central branches which subsequently disappear later in development (Goodman and Spitzer, 1979). Similarly, dendritic regression, followed by extensive sprouting has been observed in identified motoneurones during metamorphosis of a moth (Truman and Reiss, 1976).

Removal of a synaptic input has been shown to cause changes in the dendrites of invertebrate neurones similar to those seen in vertebrates, provided the denervation occurs early in postembryonic development. Murphey, Mendenhall, Palka and Edwards (1975) caused deafferentation of the giant interneurones of the cricket by removing

a cercus immediately after hatching. They found the dendrites of the giant interneurone which would normally receive synaptic input from sensory cells in the cercus were reduced in length. The effect was more marked early in development when dendritic growth is rapid (Murphey, Matsumoto and Mendenhall, 1976), and the length could be restored to normal if reinnervation was allowed to take place before adulthood was reached. Hoy, Cassady and Rollins (1978) (see Anderson, Edwards and Palka, 1980) have shown that denervation of postembryonic cricket auditory interneurones causes marked changes in their dendritic growth. Not only is the growth of one dendrite reduced, but another dendrite, which is normally confined to the ipsilateral neuropile, grows and branches into the neuropile on the contralateral side of the ganglion.

In contrast, the traditional view of the adult invertebrate nervous system has been one of structural stability following either deafferentation or axotomy. Tweedle, Pitman and Cohen (1973) observed no dendritic changes in adult cockroach giant interneurones up to 7 weeks after deafferentation, while denervation of a crayfish motoneurone produced no observable dendritic changes in the cell for over a year after the destruction of the presynaptic cell (Wine, 1973). Axotomy of adult cockroach motoneurones produced no observable dendritic changes in the cells (Tweedle, et al. 1973). This apparent rigidity in structure was used to explain the stereotyped behaviour pattern of these animals (Cohen, 1976).

There has, however, recently been some evidence to suggest that the adult invertebrate nervous system is not as rigid as was initially supposed. Restoration of apparently normal swimming movements in a leech have been observed, even when regeneration of the appropriate damaged neurones had not taken place (Baylor and Nicholls, 1971).

This indicates that the nervous system is perhaps capable of making central modifications. Murphy and Kater (1978; 1980) have shown that axotomy of an identified snail neurone can produce marked dendritic sprouting and this is particularly extensive near the site of damage. Some cricket neurones have even been shown to be capable of producing supernumerary dendritic branches when isolated from their cell body (Clark, 1976). In this thesis, further evidence is produced to indicate that some neurones from adult invertebrates are capable of making extensive changes in dendritic branching following nerve lesions affecting their neural environment. Some of these results have already been reported (Pitman and Rand, 1981b; 1982).

The method of intracellular injection of cobalt ions into cells (Pitman, Tweedle and Cohen, 1972a) has proved extremely useful in the study of neuronal structure. The visualisation of identified cells has made it possible to tentatively correlate structure with function, (Pitman, Tweedle and Cohen, 1973; Goodman and Williams, 1976). Additional techniques of silver intensification (Tyrer and Bell, 1974; Bacon and Altman, 1977) and destaining (Pitman, 1979) have greatly increased the resolution of the cobalt method, so that it is now possible to see the finer details of the filled cells.

METHODS

Preparation for the Study of Neuronal Structure in Vivo

Healthy adult male cockroaches were anaesthetized with 100% carbon dioxide in a humid atmosphere and secured, ventral surface uppermost, to a dissection board. Carbon dioxide was passed over the animal throughout the operation. This abolished movement of the animal and thus minimized haemolymph loss. Before use, all dissection instruments were dipped in insect saline containing 0.8 mg/ml of gentamycin sulphate. The cuticle overlying the metathoracic ganglion was cut to expose the ganglion, but so that it remained attached to the animal along one edge. The resulting flap of cuticle was held back and the necessary nerve lesions performed (see Results for details of lesions). The flap of cuticle was replaced over the ganglion and the cut edges sealed with low melting-point wax ($49^{\circ}C$) using an electrically heated wire probe. This held the cuticle in place until the wound healed, and prevented loss of haemolymph.

Animals were allowed to recover and were given a constant supply of water and Minced Morsels dog food (Quaker Oats Ltd., Southall, Middlesex). They were sacrificed at various times after operation (between 12 and 175 days). The cuticle on the ventral surface of the animal overlying the nerve cord was removed. Segmental nerves from the ganglia were severed, and a section of the nerve cord extending from the first thoracic to the third abdominal ganglion was removed. Cobalt ions were injected into the soma of the fast coxal depressor motoneurone, D_f (Pearson and Iles, 1970), designated cell 28 by Cohen and Jacklet, (1967).

Micro- Injection of Cobalt Chloride

Thoracic ganglia previously maintained in culture (see Methods, Chapter 1) or freshly dissected from experimental animals were mounted onto a black, plastic slide with a strip of solidified Araldite across

the width. The nerve cord was positioned, ventral surface uppermost, so that the ganglion containing the cell to be injected was secured over the Araldite strip with rubber bands around the anterior and posterior connectives (see Fig. 3.1). The nerve cord was kept moist with insect saline.

The cell bodies are not easily visible through the mechanically tough perineural sheath which envelopes the ganglion. Removal of this sheath, therefore, assists penetration of neurone somata with microelectrodes. The de-sheathing must be done carefully to avoid damage to the underlying cell bodies. A small drop of methylene blue solution was placed on the ganglion, making the sheath easily distinguishable from underlying tissue. Two pairs of fine tipped forceps were used to remove the sheath which was carefully gripped with one pair of forceps at a position posterior to D_f, and raised slightly. The second pair of forceps was used to tear the sheath away from the ventral surface of the ganglion over the area containing D_f.

The preparation on the slide was then quickly transferred into a glass fronted bath containing insect saline (Fig. 3.1). A constant flow of 95% oxygen, 5% carbon dioxide was passed through the liquid throughout the experiment to oxygenate and circulate the fluid. The preparation slide was supported in the bath at an angle of approximately 45°, and viewed through the front of the bath using a X80 binocular microscope. The angle of the light shining on the preparation was adjusted so that the relevant area of ganglion was illuminated, and motoneurone D_f was visible. Sometimes, it was necessary to remove some of the surrounding glial cells before the soma was visible. This was done by gently blasting a small jet of saline around the cell body.

When the cell was clearly visible, a microelectrode was lowered into the saline and its resistance measured. Microelectrodes were

pulled from glass tubing with an external diameter of 1.5 - 1.7 mm, using a Narishige electrode puller, and were subsequently filled with a 100 mM solution of cobalt chloride dissolved in 100 mM potassium chloride. Their resistance was usually 10-20 MΩ

Figures 3.1 and 3.2 show the set up used for recording and micro-injection. The microelectrode, mounted vertically in a micromanipulator (Prior, England), was carefully positioned directly over the cell body. It was gently lowered until it just touched the surface of the cell. (This was indicated on the oscilloscope screen by a slight change in potential recorded at the microelectrode tip). Negative pulses of current were applied until the microelectrode penetrated the cell. The resting potential of the cell was noted. 10^{-7} A positive current pulses of 0.5 s duration and frequency of 1 Hz were passed through the micro-electrode. This expelled the positively charged cobalt ions from the microelectrode into the cell. After about one hour, the microelectrode was withdrawn from the cell and the slide taken out of the bath. The nerve cord was then removed and placed in a vial containing about 5 ml of insect saline and one small drop of ammonium sulphide solution. The sulphide was added to the saline immediately before use, since prolonged exposure to air caused a precipitate to form.

The preparation was left in ammonium sulphide solution for about 10 minutes to allow the precipitation of cobalt sulphide. After this time the cell body was visible as a black dot to the naked eye on the ventral surface of the ganglion. The tissue was then washed in saline for 5 minutes, transferred to a 1:1 mixture of 70% ethanol and saline and finally placed in a solution of 70% ethanol in saline. The preparation could be stored for several days in the refridgerator at this stage if necessary. The cobalt filled cell was then silver intensified, as described below.

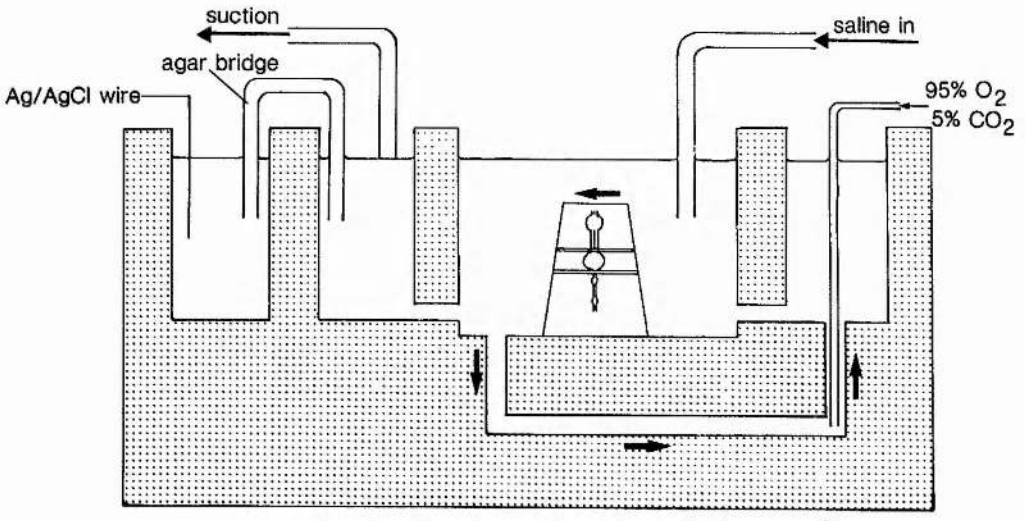
Fig. 3.1 The experimental bath used during intracellular recording and injection of D_f cell bodies. The bath is shown viewed from the front (a) and from the side (b). Major features of the set up include :

(1) Preparation on slide is mounted at about 45° to the vertical,

(2) Suction maintains saline solution at a constant level,

(3) Supply of 95% O_2/5% CO_2 gas mixture is used to induce constant re-circulation of the saline (direction indicated by arrows) and oxygenate the tissue.

a


suction
agar bridge
Ag/AgCl wire
saline in
95% O_2
5% CO_2

b

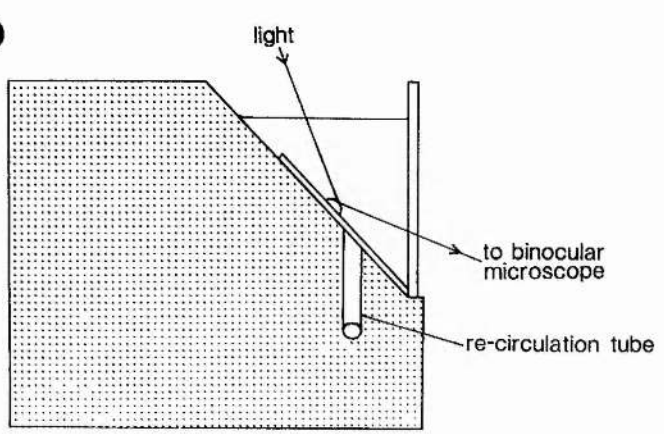
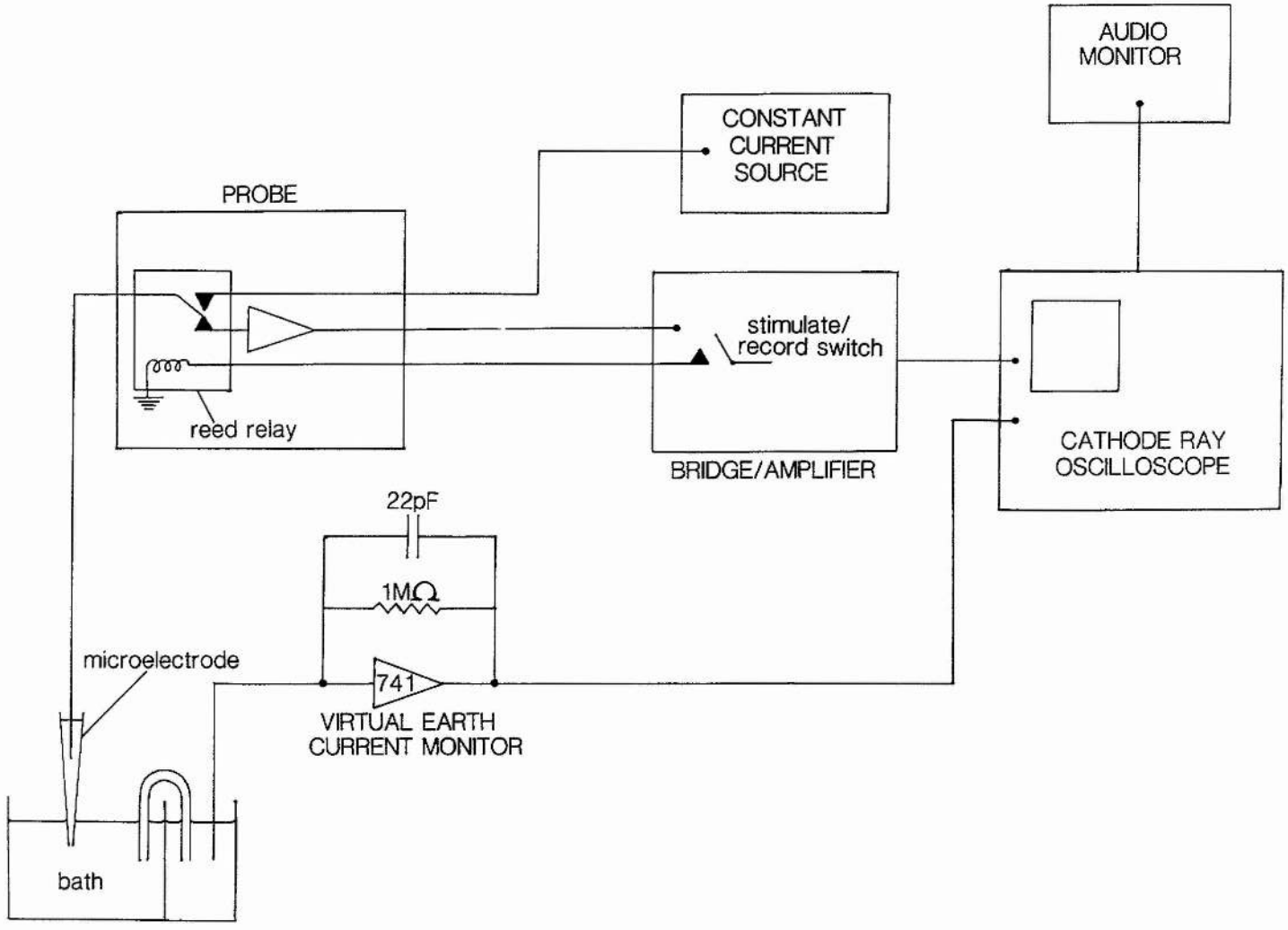

light
to binocular microscope
re-circulation tube

Fig. 3.2 Block diagram of the apparatus used for intracellular recording and injection of D_f cell bodies. The microelectrode was filled with 100 mM cobalt chloride. Major features of the system include :

(1) Current maintained by virtual earth circuit.

(2) Reed relay in probe allows remote switching between recording amplifier and a constant current source.

(3) Microelectrode filled with 100 mM cobalt chloride.


AUDIO
MONITOR
CONSTANT
CURRENT
SOURCE
PROBE
stimulate/
record switch
reed relay
BRIDGE/AMPLIFIER
CATHODE RAY
OSCILLOSCOPE
22pF
1MΩ
microelectrode
741
VIRTUAL EARTH
CURRENT MONITOR
bath

Wholemount Silver Intensification of Cobalt-Filled Neurones

A number of methods have been used for the wholemount intensification of cells (Strausfeld and Obermayer, 1976; Bacon and Altman, 1977; Stirling, 1978). The method used in these experiments differed in some respects from all of these, and incorporated a step based on the destaining procedure of Pitman (1979). Once in 70% ethanol, preparations were passed through the solutions given in Table 3.1. Stages (1) and (2) (destaining) were introduced to remove from the surface of the ganglion any contaminants which may have been present in the bath. Silver from electrodes was one source of such contamination. However, this was not the sole contaminant, as the destaining procedure was found necessary even when an agar bridge was introduced into the system. The bath used during injection of cells was also periodically washed out with destaining solution (potassium ferricyanide and sodium thiosulphate) to reduce contamination of the ganglion. These destaining precautions greatly reduced the non-specific darkening of the ganglion during intensification, thus enhancing the contrast between the stained cell and the surrounding tissue.

While in solution (8), each preparation was briefly observed with minimum lighting under a binocular microscope every 10 minutes and then returned to the dark. As the intensification neared completion (i.e. when the axon and dendrites of the cobalt filled cell were becoming black and obvious) the preparation was observed more frequently. When the preparation had reached its optimum state, the intensification reaction was arrested by transferring the nerve cord into solution (9). The citric acid in this solution lowers the pH from about 5 to 2-2.5. Below pH 2.3, intensification proceeds extremely slowly (Bacon and Altman, 1977) and therefore this solution arrests the reaction quickly.

Any leakage of cobalt from the filled cell into the surrounding

TABLE 3.1

SOLUTION	TEMPERATURE	TIME
*1. 1% potassium ferricyanide + 1.25% sodium thiosulphate in 50% ethanol (potassium ferricyanide dissolved in sodium thiosulphate solution immediately before use	Room Temperature	5 min.
*2. Wash in 1.25% sodium thiosulphate in 50% ethanol.	Room Temperature	10 min.
3. 50% ethanol	Room Temperature	10 min.
4. 1.25% gum acacia in 30% ethanol	45°C	15 min.
5. 2.5% gum acacia in 30% ethanol	45°C	15 min.
6. 5% gum acacia in 30% ethanol	45°C	15 min.
7. 1.6% citric acid + 2% hydroquinone in solution (6)	45°C	1 hour
8. 0.1% silver nitrate in solution (7) (in dark)	Room Temperature	$\frac{1}{2}$-1$\frac{1}{2}$ hours
9. 1.6% citric acid in solution (6)	4°C	10 min.
10. 1.6% citric acid in solution (6)	Room Temperature	10 min.
11. 2.5% gum acacia in 30% ethanol	45°C	15 min.
12. 30% ethanol	45°C	15 min.
13. 50% ethanol	Room Temperature	15 min.

Note. Metal forceps must not be used to transfer ganglia at stages marked with *.

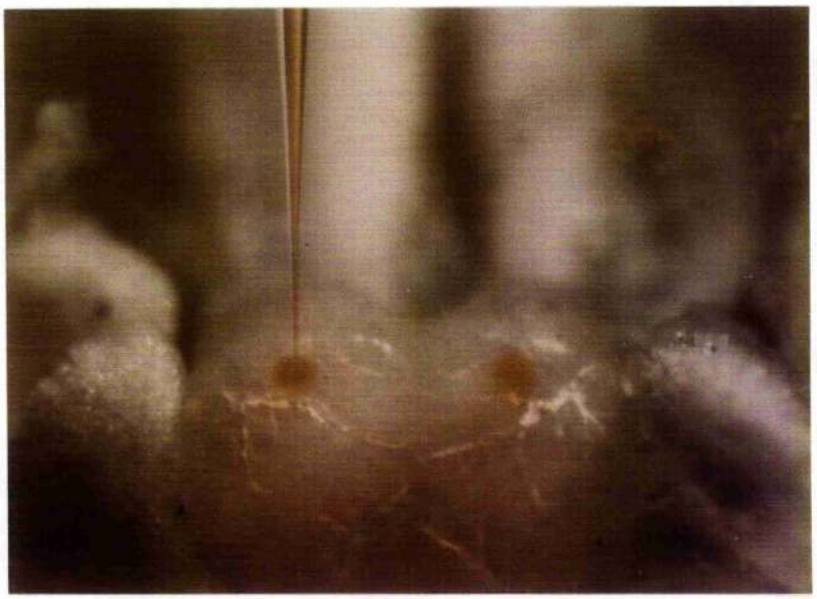
tissue can cause non-specific staining. After intensification, the resulting brown areas could obscure parts of the cell. If this occurred, ganglia were briefly passed through destaining solution (solution (1) in Table 3.1) before proceeding with dehydration (i.e. before step (13) in Table 3.1). The destaining was observed carefully, and when the majority of the non-specific staining had been removed, ganglia were quickly transferred to a 1.25% solution of sodium thiosulphate in 50% ethanol, using perspex forceps. They were washed in this solution for about 5 minutes. (Care had to be taken not to leave the preparations in destaining solution for too long, as this could remove stain from the filled cell.) Once washed, preparations were transferred to 50% ethanol.

When step (13) (Table 3.1) had been reached, preparations were dehydrated through an ascending series of ethanols (20 min in each solution), and cleared in creosote. The ganglia became sufficiently clear to see the general cell structure within about 10-15 minutes in creosote, but clearing was not complete until after about one hour, when more detail could be observed. Once cleared, ganglia were stored in creosote at -20°C. Preparations can be stored at this low temperature for many months before an appreciable deterioration of the ganglia occurs. However, if stored at room temperature or at 4°C, a general browning of the preparation is seen within a few days.

The Effect of Fixatives

In the method described above, preparations are immersed in a number of solutions between micro-injection of the cell and fixation in 70% ethanol. It is possible that this may cause some minor changes in the structure before fixation, and therefore attempts were made to preserve the preparations before precipitation of cobalt sulphide. Fixation in either 4% formaldehyde or 70% ethanol before precipitation

Fig. 3.3 The position of the fast coxal depressor motoneurone (D_f) in the cockroach metathoracic ganglion. The ganglion was desheathed and mounted ventral surface uppermost. The cell bodies of both left and right motoneurone D_f have been filled by injecting Procion Yellow through a microelectrode (shown impaling the cell body at the left hand side of the photograph). The cell bodies are situated in a characteristic location with respect to the tracheal tubes, which appear branched and silvery-white. The anterior connectives can be seen, out of focus, in the upper half of the photograph. Calibration : 0.5 mm



of the cobalt was unsuccessful. Pre-treatment with either of these fixatives tended to prevent precipitation of cobalt sulphide, and 70% ethanol caused an unacceptable leakage of cobalt from the cell if precipitation had not been performed first.

Recording and Analysis of Neuronal Structure

The soma of the metathoracic fast coxal depressor motoneurone, D_f (or cell 28), lies on the ventral surface of the ganglion. It usually has a characteristic position with respect to the tracheal system (Fig. 3.3) and often another large cell (cell 27, Cohen and Jacklet, 1967) can be seen just medial and posterior to cell 28. These two cells are usually the largest in this area of the ganglion. It was therefore frequently possible to identify D_f with reasonable accuracy on grounds of its cell body size and position alone.

Once stained, motoneurone D_f could be identified with more certainty. The axon leaves the ganglion in the fifth segmental nerve (or nerve 5), and a slight kink in the axon is often seen at the point where it enters the nerve. It then takes a very characteristic route, passing into the first ramification of the nerve (5 r 1) to innervate the coxal depressor muscles 177d', 177e', 178 and 179 (Pearson and Iles, 1971; Pearson and Bradley, 1972). The basic structure of D_f has been given in the literature (Pitman, Tweedle, and Cohen, 1972a; Tweedle, Pitman and Cohen, 1973), and is shown here under "Results" (Fig. 3.4). The majority of the dendrites from this cell are normally found towards the dorsal surface of the ipsilateral ganglionic neuropile. The three dimensional arrangement of the neurone poses great problems when attempting to make visual records. Low magnification micrographs of the cell show the general structure and the relative positions of the main branches, but details of the finer processes are lost. At higher magnification, limited areas of the cell can be recorded in detail, but information on the position

of the region relative to the rest of the cell is no longer available. It is sometimes difficult to record detailed information on areas deep within the ganglion.

Since no means of recording cell structure has been found which is ideal, various features of each preparation were recorded in three different ways. The preparations were placed in a depression slide containing creosote and covered with a glass coverslip ready for observation.

1. Stereoscopically Paired Photographs

The depression slide was secured to a microscope stage which could be tilted to two different positions. This allowed the preparation to be viewed from two angles, differing from each other by about 15^{o}. Preparations were photographed in the two positions using a Vickers J 35 Automatic Exposure Unit and an Autowind 35 mm camera attached to a Leitz microscope. By placing paired photographs side by side and viewing through stereoscopic spectacles, the two images could be superimposed. This provided valuable information on the three-dimensional appearance of the stained neurone at a low maginification.

2. High Magnification Photographs

Various regions of stained cells showing features of particular interest were photographed in greater detail under bright field illumination using a Reichert Kam ES - Electronic Camera System for photomicrography. It was not possible to photograph structures deep within the ganglion at these higher magnifications because of the short working distance of the objective lenses.

Details of photographic material used for low and high power micrographs are given in Table 3.2.

TABLE 3.2

DETAILS OF PHOTOGRAPHIC MATERIAL

FILM	DEVELOPER (DILUTION)	TIME FOR DEVELOPING	FIXER	TIME FOR FIXING
KODAK MICRO FILE 5669	KODAK D-76 (1:2)	6 min.	ILFORD HYPAM RAPID FIXER	2 min.
KODAK AHU MICROFILM 5460	KODAK D-76 (1:2)	12 min.	ILFORD HYPAM RAPID FIXER	2 min.

3. Drawings of Preparation

An image of the preparation was projected onto paper using a Gillet and Sibert microscope fitted with a projecting prism attachment. By gradually focussing on different planes through the ganglion and drawing the parts of image in focus, it was possible to produce an accurate picture of the cell. Usually the preparation was drawn from both dorsal and ventral surfaces to obtain the maximum amount of information. Although this provided a detailed picture of the branching pattern of the entire cell, three dimensional information was lost.

The strong light which was required to project the image caused the preparation to turn brown with time. Finer details of the cell gradually became indistinguishable from the background, and it was therefore important to draw the preparations as quickly as possible. A copper sulphate heat filter was introduced into the system between the light source and the preparation. Although this reduced browning of the preparation, it by no means eliminated the problem. This deterioration of the preparation was irreversible, and therefore drawings of the neurones were always made after all photographic records had been completed.

Histological Examination of Lesioned Ganglia

Metathoracic nerve lesions in adult male cockroaches were performed as described above. All segmental nerves except one nerve 5 were sectioned, and the anterior connective ipsilateral to the intact nerve 5 was crushed. Animals were divided into two groups; one group were sacrificed 4 days postoperatively, and the other group after 23 days. The metathoracic ganglia were removed for sectioning. Transverse 10 µm sections were stained with Pyronine-Malachite Green, as described in 'Methods', Chapter 1.

RESULTS

The fast coxal depressor motoneurone (D_f) was studied throughout these experiments. When impaling the cell for microinjection of cobalt chloride, it was tentatively identified by its size and position as described in Methods of this chapter (see also Fig. 3.3). More positive identification of this motoneurone after staining relied on observations of the characteristics of the dendritic tree, and of the course followed by the axon (see Figs. 3.4 and 3.5). The latter criterion was only of limited use when nerve lesions involved axotomy of D_f.

Figure 3.6 shows the structure of D_f from a control animal in which the metathoracic ganglion had been exposed and tracheal system around the ganglion disrupted. It does not appear noticeably different in structure to D_f from unoperated animals (Figs. 3.4 and 3.5). Various lesions to nerves of the metathoracic ganglion caused dendritic sprouting of this neurone, which developed a number of characteristic features in its dendritic tree. It was rare to encounter all of these changes in any one preparation, but all were seen on several occasions, and some were seen regularly. Figures 3.7 to 3.16 show the effects of a number of different lesions to nerves of the metathoracic ganglion upon the structure of motoneurone D_f. The dendritic changes which have been observed may be summarised as follows:

(1) Origin of outgrowth of sprouts

New sprouts usually originated at or near the ends of the normal branches of the dendritic tree, and were frequently found near the midline of the ganglion, since this is where the majority of the normal branches terminate. However, sprouts were also seen originating from the ends of the branches numbered 1 and 2 by Tweedle et al., 1973 (see Fig. 3.4), and occasionally from the process connecting the cell body to the axon (the 'initial segment').

(2) General path taken by sprouts

Although sprouts did branch, this was less intricate and frequent than the branching of normal dendrites. The course followed was of two basic types:

(a) Tortuous processes: a number of sprouts followed extremely winding paths near their origin (Fig. 3.17), often doubling back upon themselves, or forming complete loops (Fig. 3.11). Similar loops have been observed in the regenerating neurones of the tadpole tail fin (Speidel, 1935).

(b) Straight processes: on a number of occasions sprouts which had originally travelled in a tortuous, seemingly undirected manner, suddenly began to grow in a straight line (Fig. 3.17). This was almost invariably in a rostro-caudal direction, frequently close to the midline of the ganglion. It was not uncommon in these instances to see a number of fibres all travelling along the same basic route, thus forming a cluster of parallel fibres. Some of these fibres produced branches which also followed the same general path.

(3) Unusual sprouts within normal territory

In a number of preparations, unusual dendritic sprouts could be seen, but these remained within the normal dendritic field. This type of sprouting was the most difficult to distinguish from normal dendrites, and positive identification of such branches was not always possible.

(4) Unusual sprouts outside normal territory

These could be far more easily and positively identified. sprouts were seen to enter:

(a) ipsilateral foreign segmental nerves (Figures 3.11, 3.13, 3.14, 3.16 and 3.18). The majority of these nerves had

themselves been sectioned; a preference was shown for nerve 3 followed by nerve 4. However, in two preparations, sprouts entered an intact foreign segmental nerve.

(b) ipsilateral anterior and posterior connectives (Figures 3.9, 3.12, 3.16 and 3.17). Sprouts entered the anterior connective more frequently than they did the posterior, and almost as frequently as they did nerve 3. They did not, however, enter the connectives unless the anterior or both anterior and posterior connectives had been sectioned.

(c) contralateral neuropile (Figures 3.14 and 3.15). Sprouts occasionally crossed the midline to the contralateral side of the ganglion. These sprouts sometimes extended a considerable distance into the contralateral neuropile.

(5) Several sprouts entering any one nerve trunk

It was not uncommon to see a number of sprouts entering a single nerve trunk. This was frequently the normal nerve by which the axon of D_f would leave the ganglion (nerve 5). Foreign nerves(and connectives) receiving processes from D_f also often contained several rather than single sprouts (Figures 3.12, 3.13, 3.16, 3.18 and 3.19).

(6) Varicosities

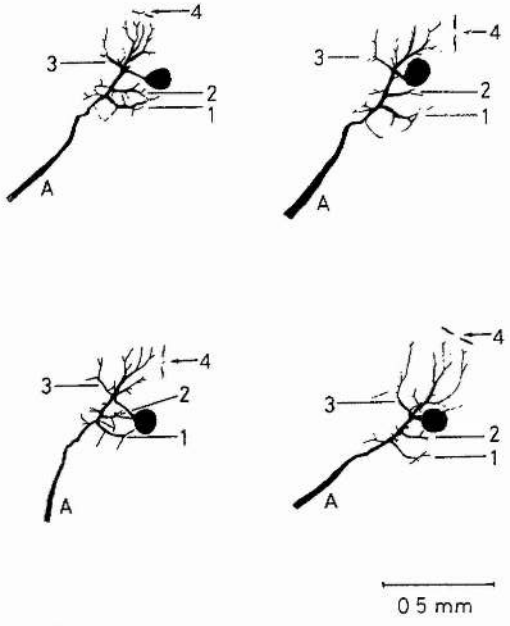
These were by far the most common and easily induced feature. Two types of varicosities were found in the dendrites:

(a) small varicosities (Fig. 3.19). There were usually several of these along a supernumerary dendrite giving it a beaded appearance. This type of varicosity only appeared in new sprouts, and was frequently found in straight, parallel running fibres mentioned in (2b).

Fig. 3.4 The basic dendritic structure of the fast coxal depressor motoneurone D_f (or cell 28). The figure is taken from Tweedle et al. (1973); anterior is at the right and posterior at the left of the figure.

Fig. 3.5 Camera lucida drawing of a cobalt stained motoneurone D_f in the metathoracic ganglion of a normal, unoperated animal. As with all following drawings, the neurone was silver intensified in wholemount, and drawn as viewed from the dorsal surface, with the soma outlined by a broken line so that overlying dendrites are not obscured. Calibration : 200 μm

Fig. 3-4



F 1. Tracings of the right motor neuron 28 made from photographs of whole-mount preparations o .he 3rd thoracic ganglion taken from 4 separate animals. The cells were stained by intracellular coalt injection. Ganglia were photographed from the dorsal surface with all cells in approximately similar orientation. The cell body is ventrally located with the initial process and dendritic tree e rging toward the viewer to ramify near the dorsal surface. The axon, A, leaves ganglion in a erior-lateral direction through right nerve 5. Numbers 1 3 indicate similar identified dendritic iches in the 4 preparations. A dendritic complex with somewhat variable branching pattern is cated at 4.

Fig. 3-5

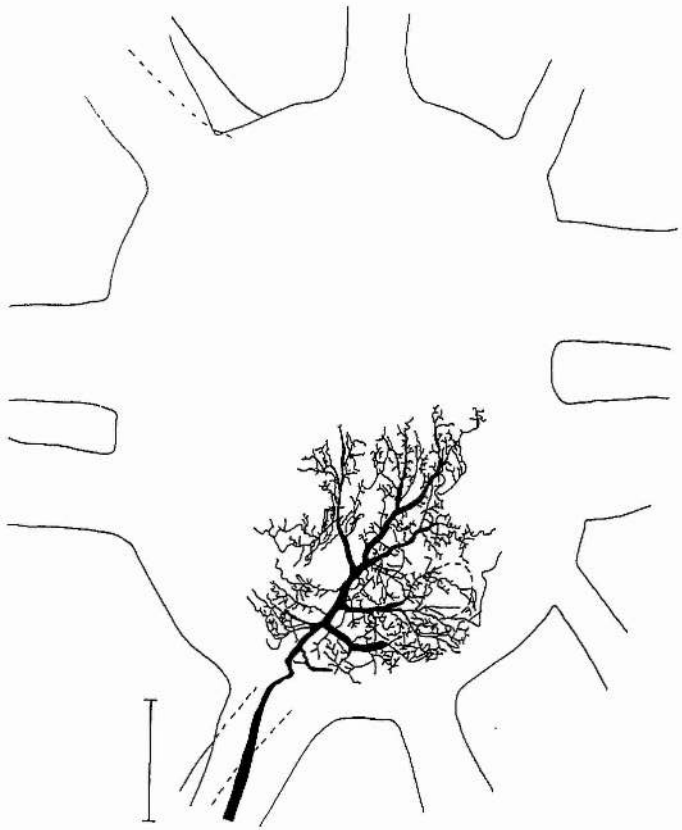
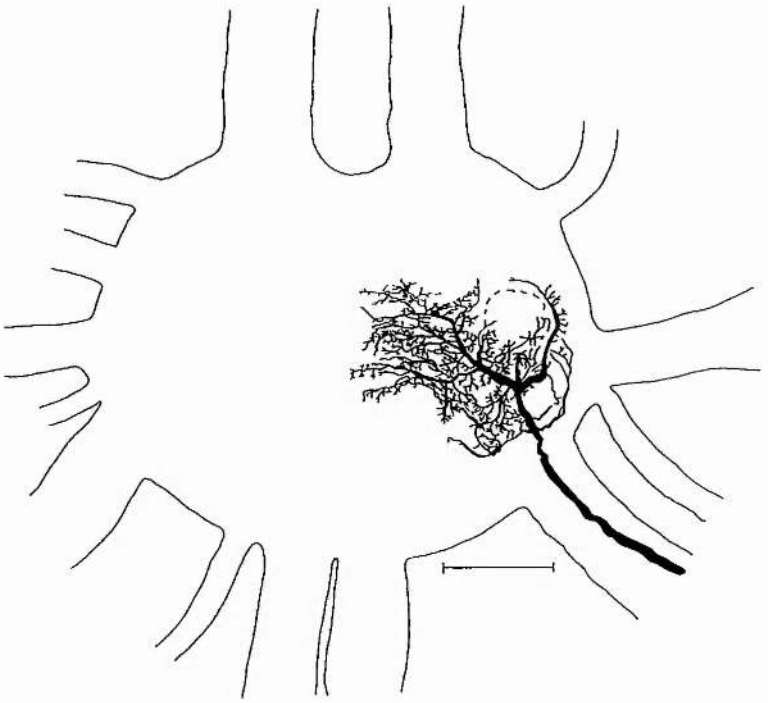


Fig. 3.6 Camera lucida drawing of cobalt stained D_f, 35 days after disruption of the tracheal system of the metathoracic ganglion. The dendritic branching pattern does not appear to be different from that of a normal neurone. Calibration : 200 µm

Fig. 3.7 Camera lucida drawing of cobalt stained D_f, 36 days after section of the ipsilateral nerve 5. The dendritic branching pattern does not appear to have altered, but several varicosities (V) can be seen at some of the dendrite terminals. Calibration : 200 µm



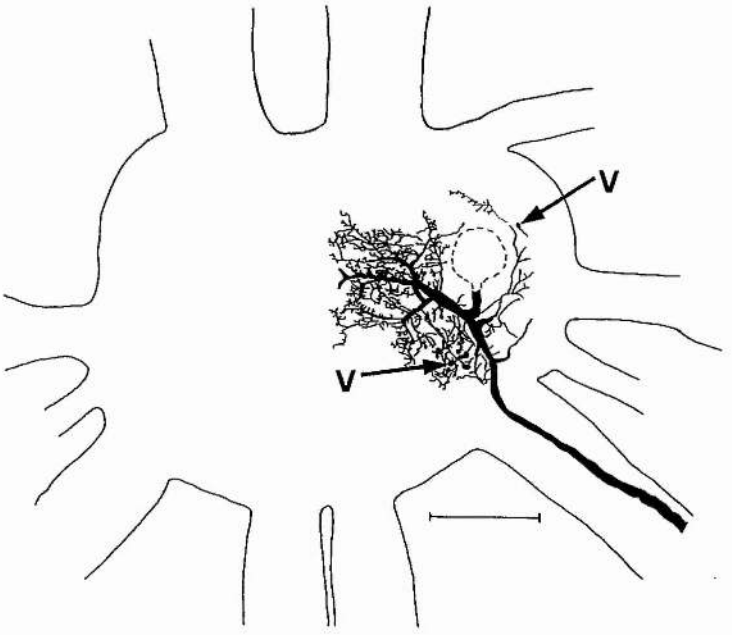
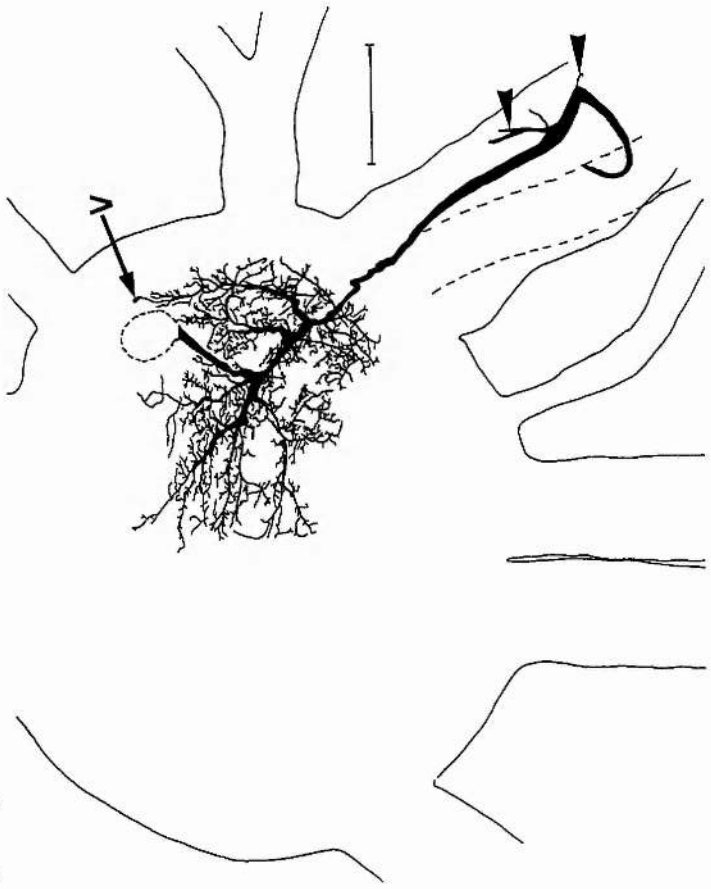

V
V

Fig. 3.8 Camera lucida drawing of cobalt stained D_f, 148 days after section of the ipsilateral nerve 5. The dendritic branching pattern still appears similar to that of a normal neurone. One varicosity (v) is present at the terminal of a dendrite near the soma. Sprouting from the end of the sectioned axon can be seen within nerve 5 (arrowheads).
Calibration : 200 µm

Fig. 3.9 Camera lucida drawing of cobalt stained D_f, 41 days after the ipsilateral anterior and posterior connectives. (As with all following camera lucida figures, sectioned nerves are indicated in the accompanying diagram by shading). The neuronal structure does not appear to differ from that of a normal neurone.
Calibration : 200 µm



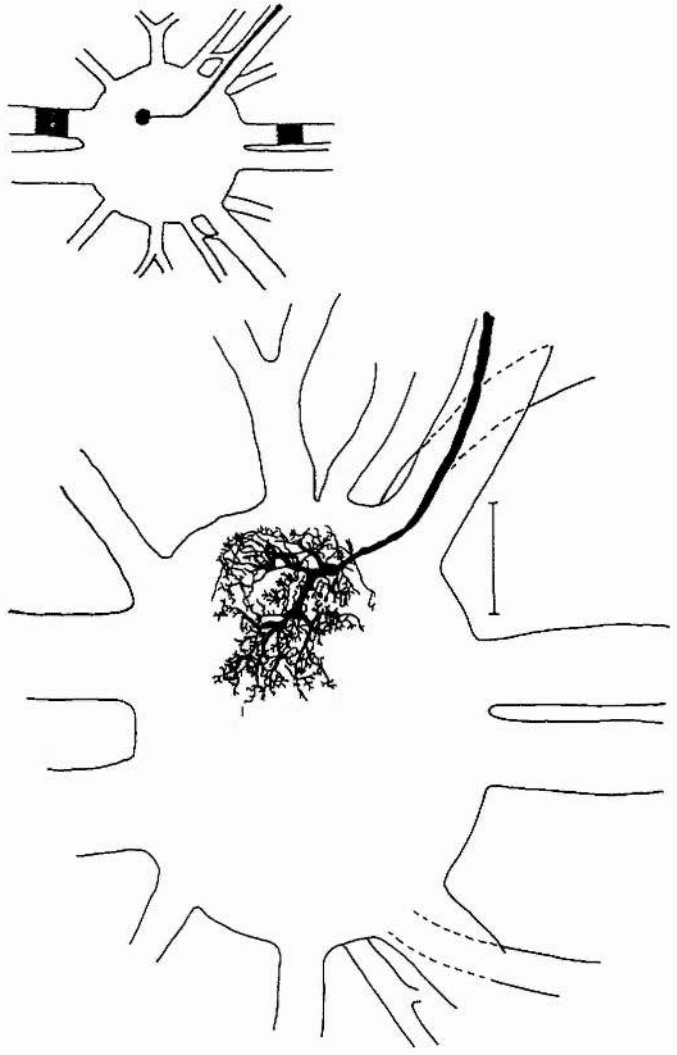
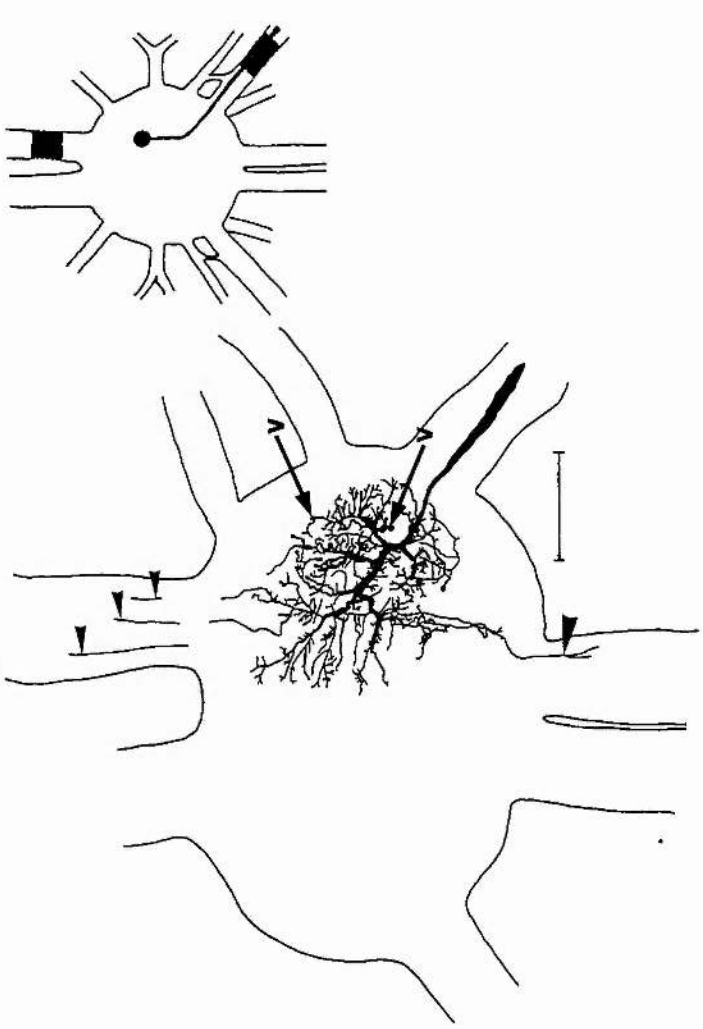


Fig. 3.10 Camera lucida drawing of a cobalt stained D_f, 23 days after the indicated nerve lesions. Sprouts have entered the ipsilateral anterior* (small arrowheads) and posterior (large arrowhead) connectives. Denritic varicosities (V) are also present. (* Processes entering the anterior connective are discontinuous in this drawing as the preparation had become non-specifically darkened, making it impossible to follow their entire course. Calibration : 200 μm

Fig. 3.11 Camera lucida drawing of a cobalt stained D_f, 46 days after the indicated nerve lesions. Abnormal sprouts can be seen both within the area of the normal dendritic tree (arrowheads) and entering nerve 3 (small arrow). One sprout has formed a complete loop (small arrowhead). Note also sprouts from the distal end of the axon in nerve 5 (large arrow) and varicosities (V). Calibration : 200 μm



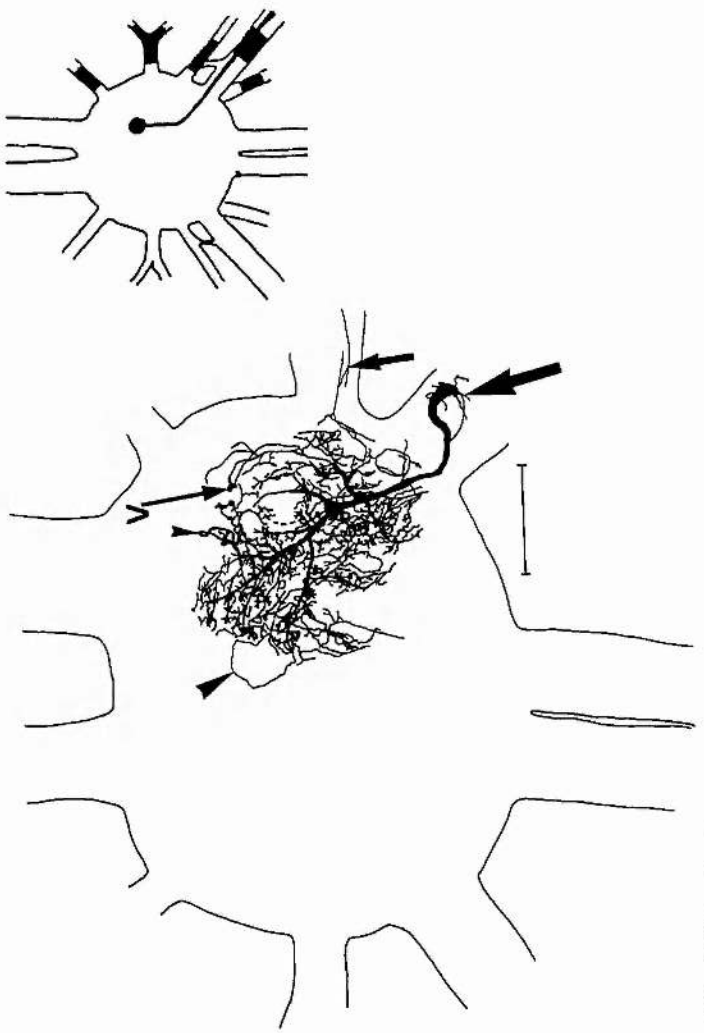
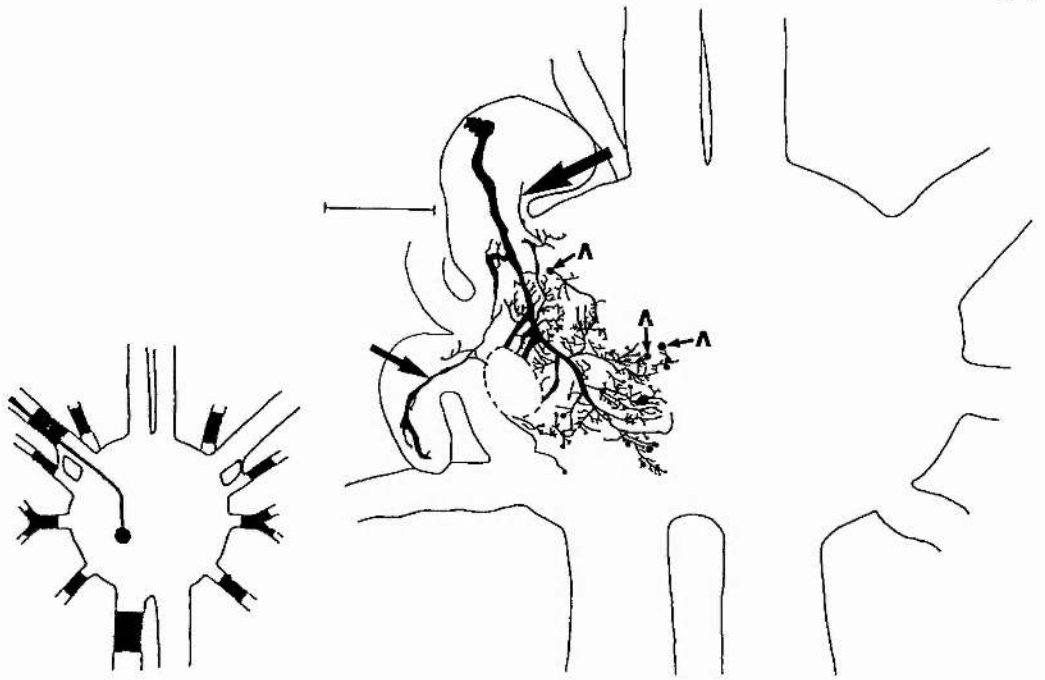


Fig. 3.12 Camera lucida drawing of a cobalt stained D_f, 21 days after the indicated nerve lesions. Although the neurone was not damaged by these lesions, it has sprouted extensively into the anterior connective (small arrowheads); some of these processes branch within the connective. Sprouts also extend close to the posterior connective (large arrowhead). (A photograph of this neurone can be seen in Fig. 3.17). Calibration : 200 μm

Fig. 3.13 Camera lucida drawing of a cobalt stained D_f, 22 days after the indicated nerve lesions. Numerous large varicosities (V) can be seen at the ends of dendrites. Sprouts enter both nerve 3 (small arrow) and nerve 5 (large arrow). Calibration : 200 μm



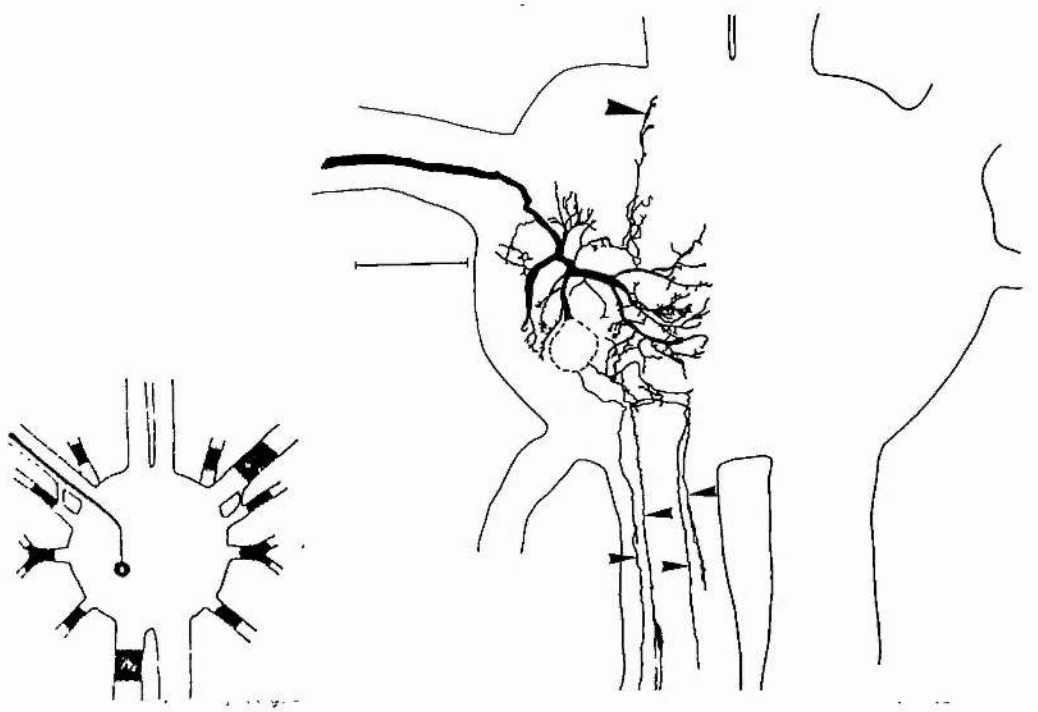
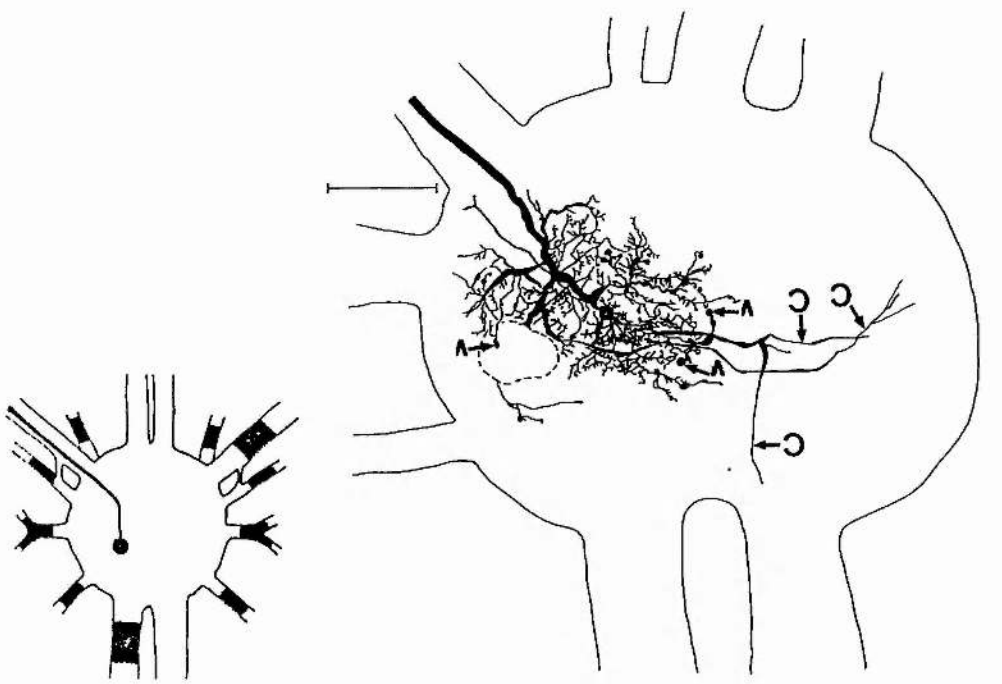


Fig. 3.14 Camera lucida drawing of a cobalt stained D_f, 49 days after the indicated nerve lesions. Sprouts can be seen within the area of the normal dendritic field (arrowhead), crossing into the contralateral neuropile (C), and entering nerve 3, where they branch (arrow). Calibration : 200 μm

Fig. 3.15 Camera lucida drawing of a cobalt stained D_f, 20 days after the indicated nerve lesions. Although the neurone itself was not damaged, extensive sprouting can be seen crossing the midline (C). Varicosities (V) are also present. Calibration : 200 μm



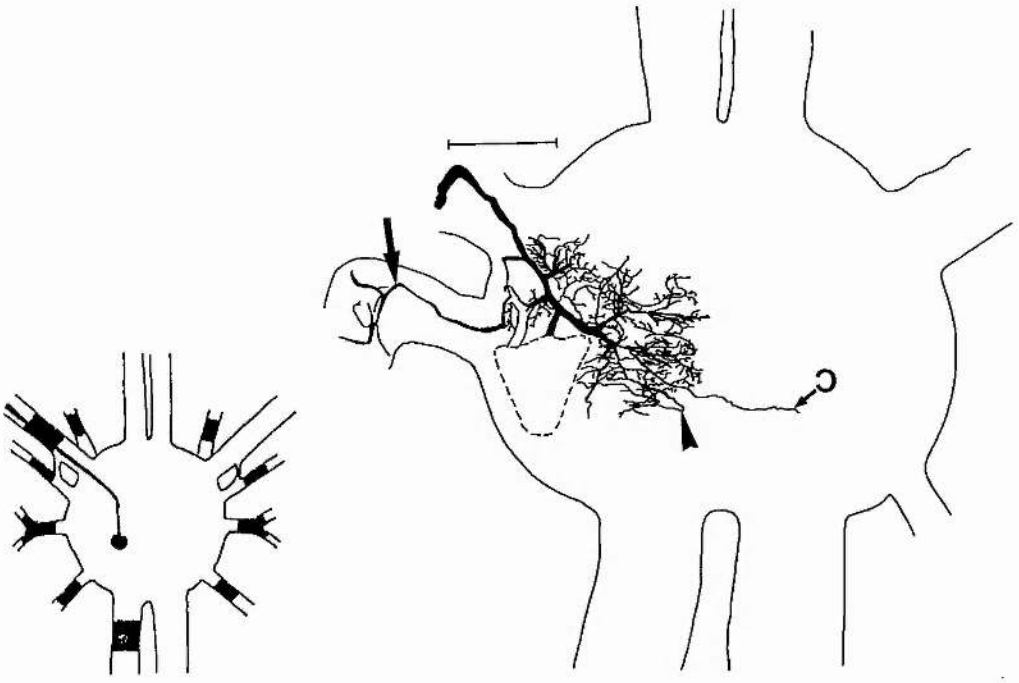


Fig. 3.16 Camera lucida drawing of a cobalt stained D_f after the metathoracic ganglion had been maintained in culture for 13 days. Extensive sprouting can be seen entering the anterior connective, forming a bundle of relatively straight, parallel processes (small arrowheads). Sprouts also extend towards the posterior connective (large arrowheads) and into nerve 2 (arrow). Calibration : 200 μm

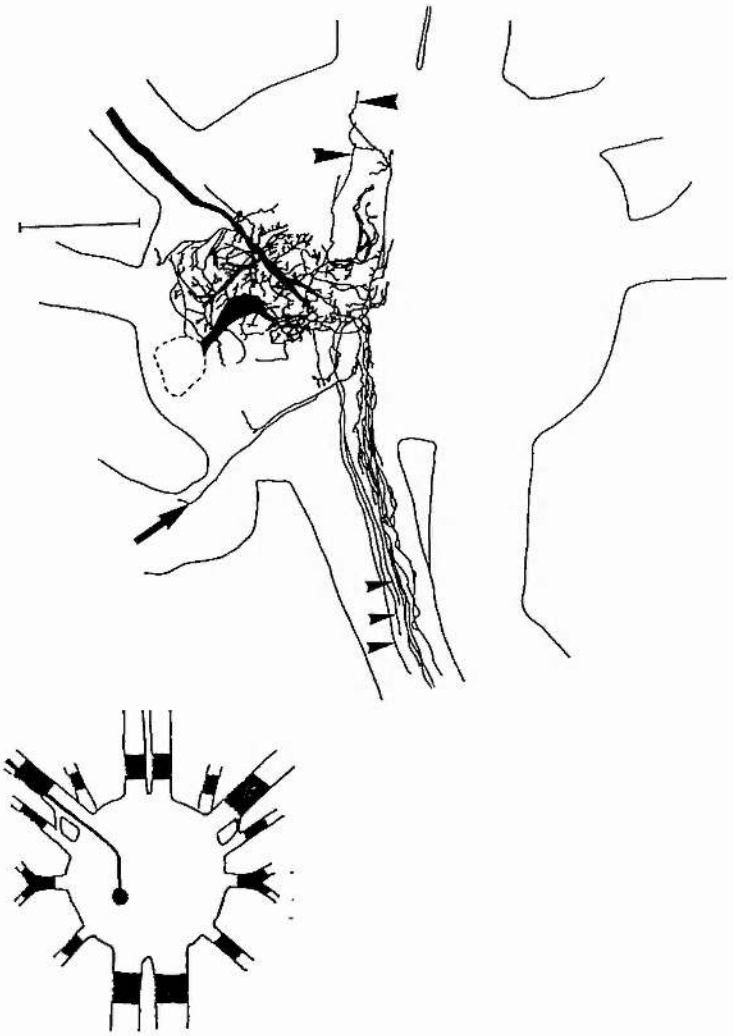


Fig. 3.17 Micrograph of cobalt stained D_f shown in Fig. 3.12. Some sprouts follow a tortuous route (arrow), and then begin to travel in a fairly straight manner (arrowhead) into the anterior connective. Calibration : 100 μm

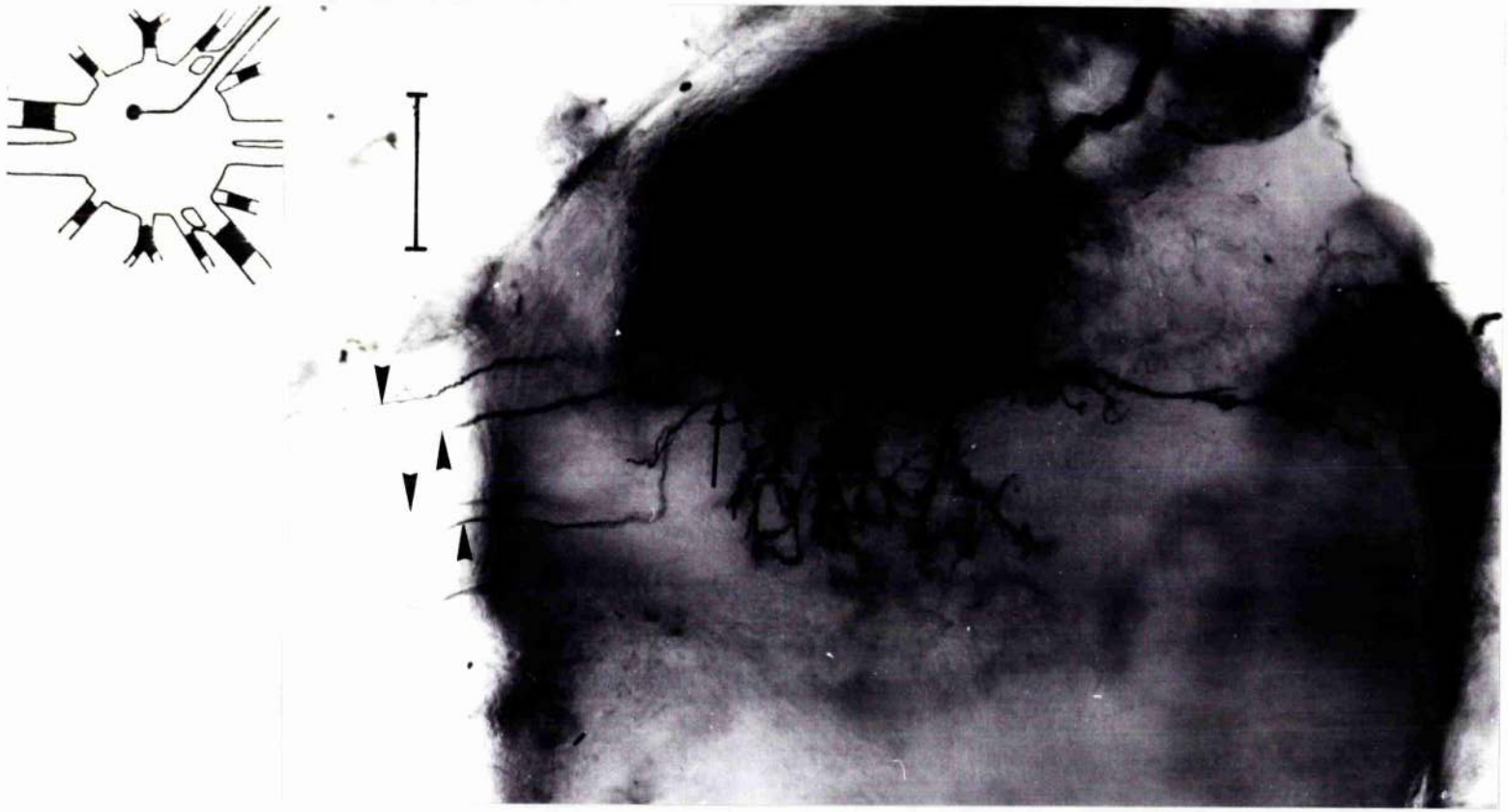
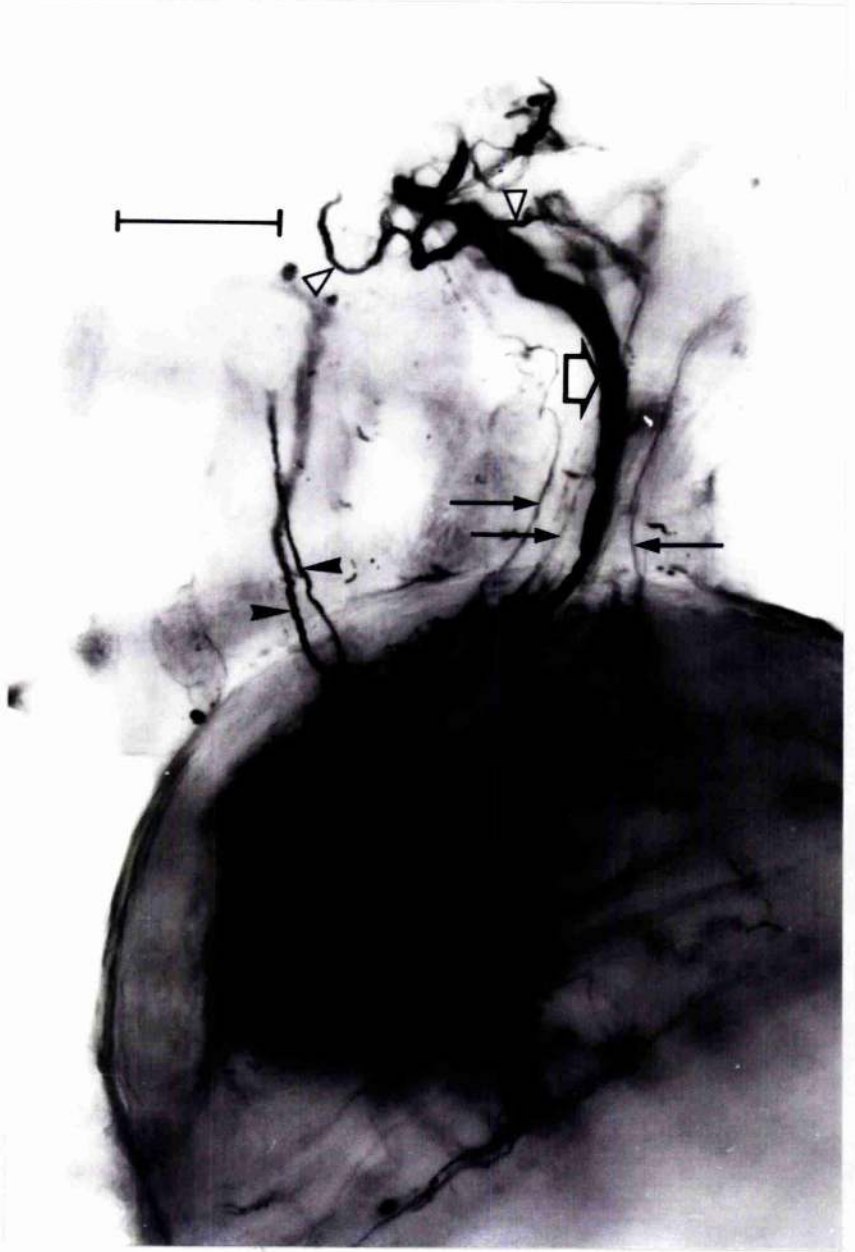


Fig. 3.18 Micrograph of a cobalt stained D_f, 35 days after the indicated nerve lesions. Sprouts can be seen entering nerve 3 (solid arrowheads) and nerve 5 (solid arrows). A number of sprouts (open arrowheads) extend from the distal stump of the original axon (open arrow). Calibration : 100 µm



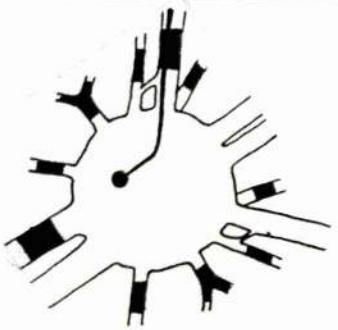
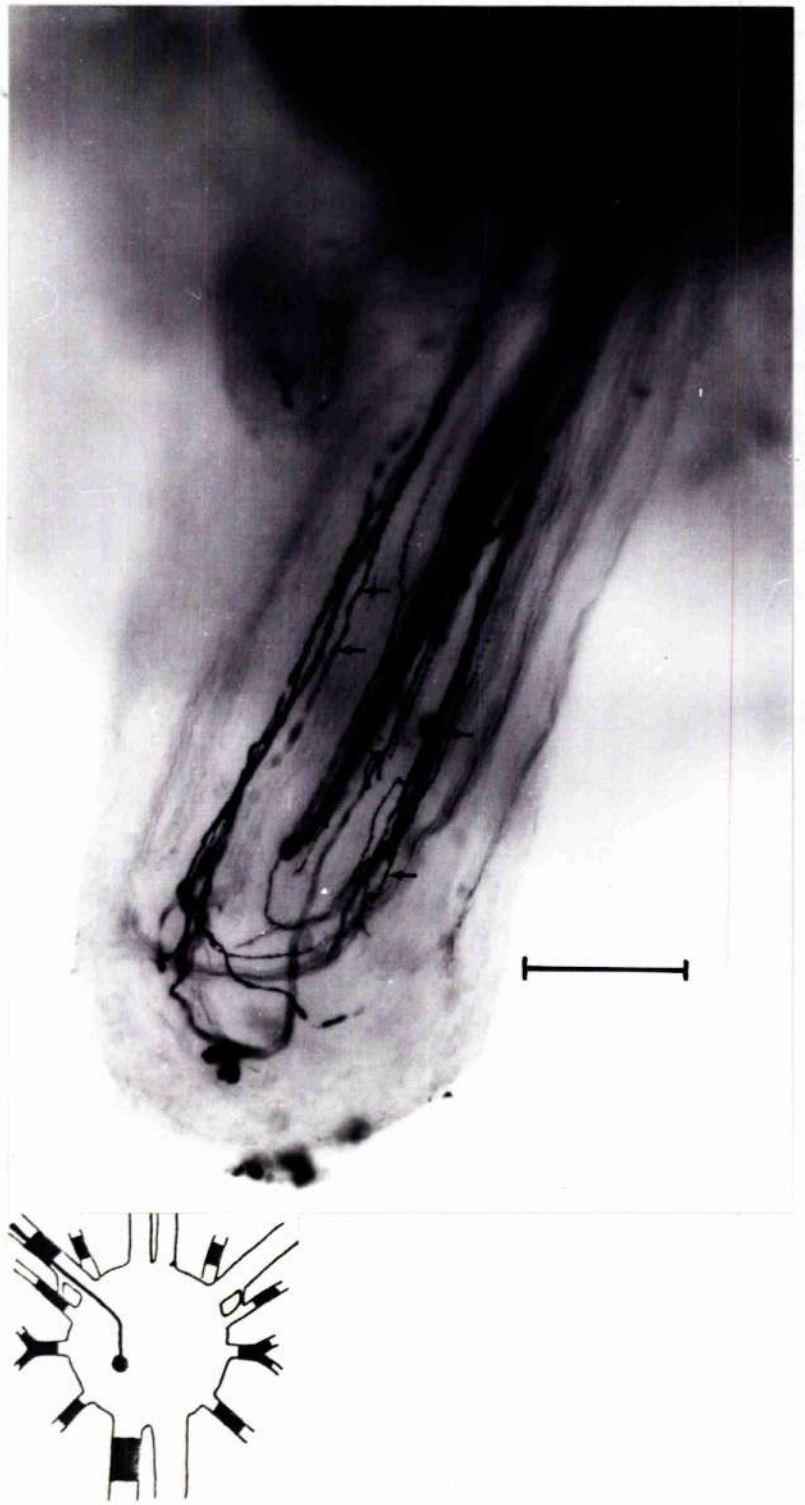
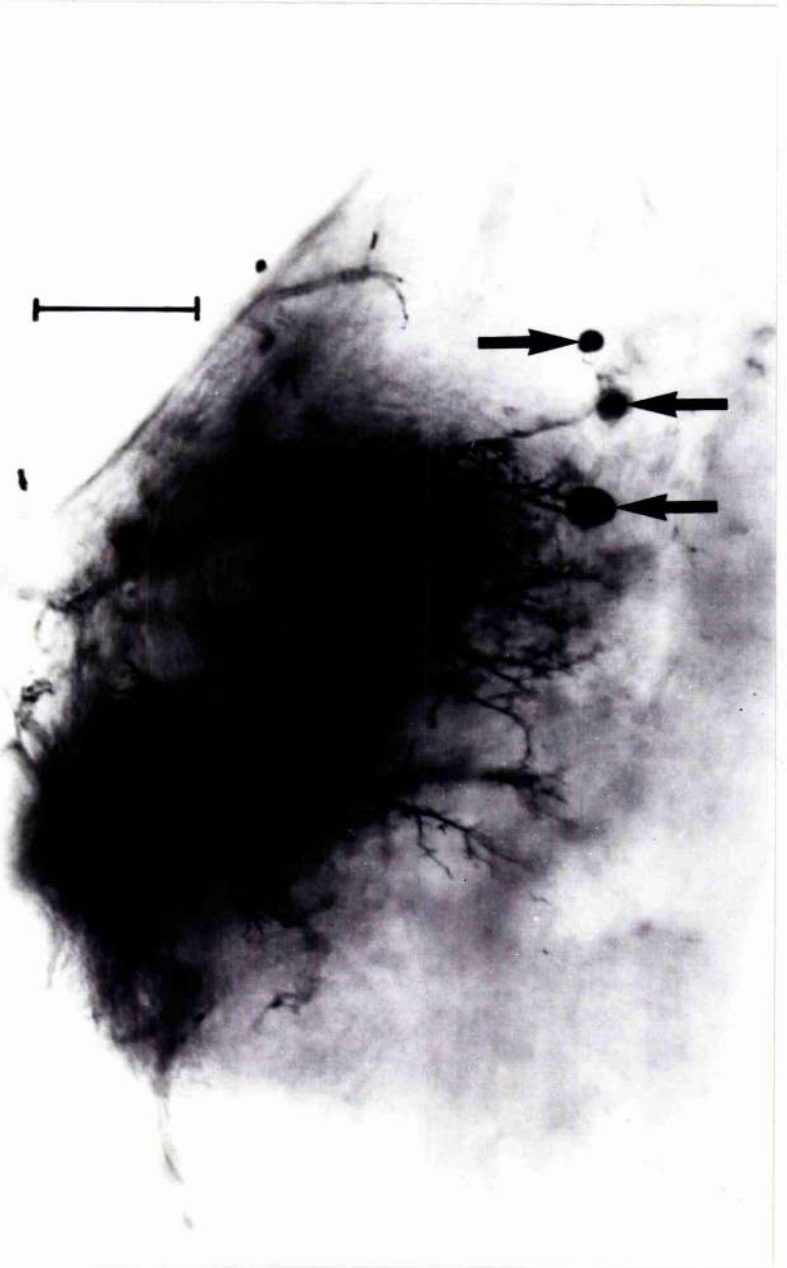


Fig. 3.19 Micrograph of extensive sprouting into an anterior connective from a cobalt stained D_f, 22 days after the indicated nerve lesions. The processes run straight and parallel to each other. Numerous small varicosities (arrows) are present along their length, giving these processes a beaded appearance. Calibration : 100 μm



3.20 Micrograph of cobalt stained D_f, 42 days after section of the ipsilateral nerve 5. Several large varicosities (arrows) can be seen at the ends of dendritic processes. Calibration : 100 µm



(b) large swellings (Figures 3.7, 3.9, 3.11, 3.12, 3.13, 3.15 and 3.20). These were usually found at or near the ends of the dendrites. Some were in newly formed sprouts, but others appeared to have developed at the end of original dendritic branches. They varied in size, but sometimes approached about 30 µm. Unlike the small varicosities, they were usually found singly, or occasionally in pairs.

The significance of the varicosities is unknown, but is considered in the Discussion.

(7) Axonal sprouts

In preparations where D_f had been axotomized, a number of sprouts were frequently seen growing from the axon, either at the end (Figures 3.8, 3.11 and 3.18) or occasionally from along its length. These sprouts sometimes followed winding paths, and occasionally doubled back upon themselves, growing proximally towards the ganglion (Figures 3.8 and 3.11).

The frequency with which dendritic sprouting occurred after different lesions is summarised in Table 3.3a. The table is arranged so that, on descending, the lesions performed become more extensive. The extent of neurone sprouting seemed to be related to the general extent of overall damage, rather than to any specific type of of injury. Quite extensive sprouting of D_f was observed in experiments where the cell itself had not been damaged, but where a number of segmental nerves other than nerve 5 had been sectioned.

Extensive sprouting could be observed after as little as 21 postoperative days, and was still seen in preparations as old as one year postoperative. The frequency with which many of the unusual

TABLE 3.3a. The Effect of Different Types of Nerve Lesion upon the Proportion of D_f Motoneurones Showing Sprouting.

The table does not indicate the extent of sprouting. Lesions as in line (6), for example, were very effective in causing limited sprouting, but this was less extensive than neurones showing sprouting after lesions as in lines (7) and (8).

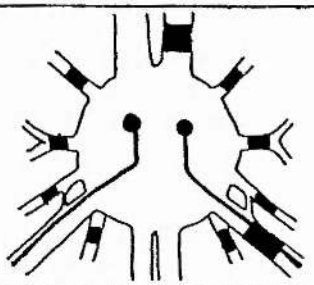
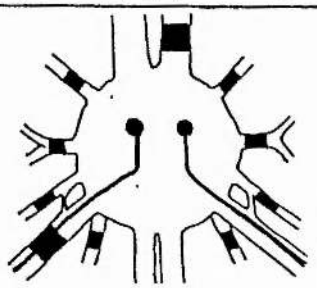
	LESION	NEURONE (left or right)	NO OF NON-SPROUTING NEURONES	NO OF SPROUTING NEURONES	% OF SPROUTING NEURONES
1	Control (tracheal system damaged	Either	5	0	0
2	Right nerve 5	Right	26	2	7
3	Right leg amputated	Right	5	1	16
4	Right anterior and posterior connectives	Right	4	0	0
5	Right nerve 5 and one or both of right anterior and posterior connectives	Right	5	2	29
6	Right nerve 2,3,4,5, and 6	Right	0	4	100
7		Left	8	5	38
		Right	1	5	83
8		Left	3	9	75
		Right	3	2	40
9	Cultured Preparations	Either	0	3	100

TABLE 3.3b Frequency with which Characteristics were seen in Sprouting Neurones.

This indicates the relative ease with which different features could be induced, taking results collectively from all the lesion types in Table 3.2. Some of these lesions produced few of the characteristics mentioned, whereas other lesions were effective in inducing many changes. (Preparations where nerve 5 only was sectioned were excluded from line (2), since such lesions produced no sectioned foreign nerve. Preparations maintained in culture were excluded from line (3), since no intact foreign nerves were present).

	FEATURE	AGE(Post-operative days	NO. NEURONES WITHOUT FEATURE	NO. NEURONES WITH FEATURE	% NEURONES WITH FEATURE	
1	Unusual dendritic Sprouts	< 40	34	19	36	38
		> 40	21	14	40	
2	Sprouts entering sectioned foreign nerve	< 40	23	8	26	24
		> 40	18	5	22	
3	Sprouts entering intact foreign nerve	< 40	32	2	6	4
		> 40	25	0	0	
4	Sprouts crossing midline to contra-lateral neuropile	< 40	51	2	4	8
		> 40	30	5	14	
5	Groups of Sprouts running parallel	< 40	47	6	11	7
		> 40	35	0	0	
6	Several Sprouts entering a single nerve	< 40	33	20	38	36
		> 40	23	12	34	
7	Varicosities	< 40	21	32	60	58
		> 40	16	19	54	

features were seen in neurones from animals which had been allowed to survive 6 weeks or more postoperatively was, however, lower than the frequency seen in younger preparations. The most notable exception to this is the development of sprouts crossing to the contralateral neuropile. The effect of age upon frequency of characteristics is summarised in Table 3.3b. This table only indicates the relative ease with which each feature can be induced in the total population of all lesion types. It does not indicate the frequency with respect to individual lesions, and as such gives an impression that some features (such as 3,4 and 5 in Table 3.3b) occurred very infrequently. This is because certain types of lesion were apparently inadequate to induce particular features, however, given an appropriate lesion, some features occurred far more frequently. For example, taking only experiments in which the types of lesion most effective in inducing sprouting to the contralateral side of the ganglion were performed, (lesions in line 7, right cell and line 8, left cell in Table 3.3a) this feature occurred in 28% of the preparations (c.f. 8% when considering all lesion types).

Histological Examinations

As already discussed in the General Introduction, the formation of a perinuclear shell of RNA and its subsequent dispersal has been considered necessary for formation of a new axon during regeneration in the cockroach (Cohen and Jacklet, 1965). If this is true, we might also expect the same to be required for a neurone to form new dendrites. The lesions were designed such that nerves to the metathoracic ganglion were extensively damaged while sparing the axon of D_f on one side (lesion in line 7, left cell in Table 3.3a). It had already been demonstrated that such lesions induce sprouting of the non-axotomized D_f in approximately 38% of animals.

(a) Cells stained for RNA after 4 postoperative days

The ring of RNA surrounding the nucleus in these neurones is normally at its peak around 4 days after axotomy. In the five preparations studied, all of the axotomized D_f cell bodies showed a brightly stained perinuclear ring. However, of the non-axotomized cells, only two showed a perinuclear ring of RNA (Fig. 3.21 b and d) which, although more heavily stained than that of a normal D_f motoneurone (Fig. 3.21 a), was less marked than that of the homologous axotomized cell (Fig. 3.21 c and e).

(b) Cells stained for RNA after 23 postoperative days

Since it is not known whether a perinuclear RNA accumulation occurs with a fixed delay after a neural lesion, or at the time during which maximal fibre growth is taking place, a further set of animals were allowed to live sufficiently long after extensive lesions for dendritic sprouting to have begun. Extensive sprouting in such cells had been observed by 21 days, and therefore 23 days should allow time for sprouting to be underway.

In 4 preparations studied, none of the axotomized nor non-axotomized D_f cells showed a stained RNA ring.

Fig. 3.21 Staining for RNA in cell bodies of regenerating D_f motoneurones.

(a) Transverse section (10 μm thick) through the motoneurone D_f from a normal, unoperated animal. The section was stained with Pyronine - Malachite Green, which stains RNA pink. A green filter was used during photography to enhance contrast. RNA is evenly dispersed through the cytoplasm, with only a slightly more dense aggregate (arrow) surrounding the nucleus (N).

(b) Transverse section through a D_f motoneurone within a ganglion where many of the segmental nerves were sectioned 4 days prior to fixation. The ipsilateral nerve 5 was spared, and therefore this neurone was not axotomised. The section was prepared and photographed as in (a). A shell of RNA (arrow) can be seen aggregated around the nucleus (N). This is considerably more dense than that of a normal neurone (a), but less dense than that in the contralateral, axotomised cell (c).

(c) Transverse section through the contralateral D_f motoneurone in the same preparation as (b). This neurone was axotomised by the nerve lesions to the ganglion. A dense aggregate of RNA (arrow) is seen surrounding the nucleus (N).

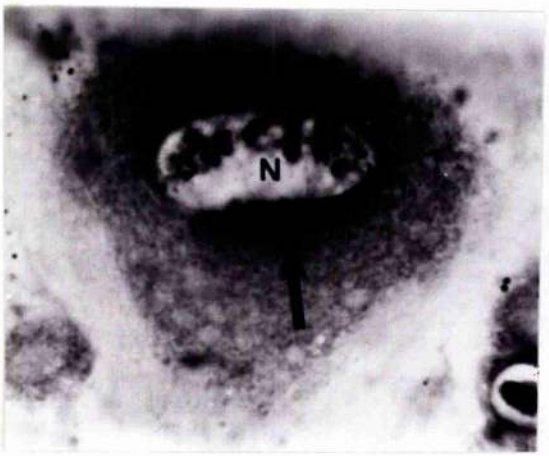
(d) Transverse section through a D_f motoneurone in another ganglion which received similar treatment to that described in (b). The neurone was not axotomised. A slight aggregation of RNA (arrow) is visible, but this is only marginally more intense than that seen in a normal cell body (a).

(e) Transverse section through the contralateral D_f motoneurone in the same preparation as (d). This neurone was axotomised by the nerve lesions. A dense perinuclear aggregate of RNA (arrow) can be seen.

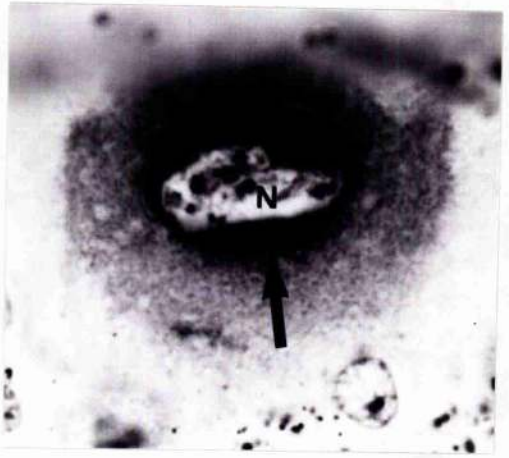
Calibration : 50 μm

a

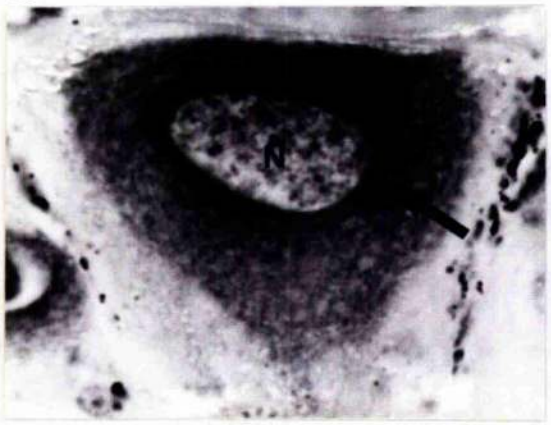
b



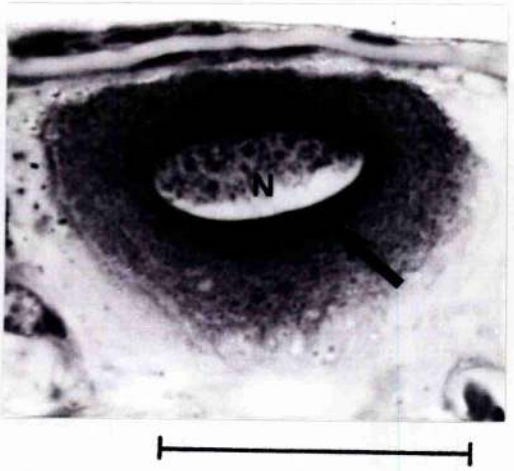
c



d



e



DISCUSSION

It is clear from the results reported here that some neurones within the adult insect nervous system can undergo radical structural changes as a result of nerve lesions affecting their environment. The structure is by no means as rigid as was initially supposed, indicating that these nervous systems have some potential for plasticity.

It is extremely unlikely that the staining method used here would produce such radical changes in the dendritic tree as were observed. However, it is important to remember that it may cause minor distortions to the structure of the cells. It could not, for example, be relied upon to make absolute measurements of the size of varicosities, or of fibre diameters; processes of D_f appear considerably thinner when stained with cobalt than when stained with Procion yellow or Lucifer yellow (Pitman, personal communication).

The possibility that cobalt has not been restricted to the primarily filled cell must also be borne in mind when making observations on the structure: under some circumstances, cobalt may cross chemical synapses (Strausfeld and Obermayer, 1976) or electrotonic synapses (Politoff, Pappas and Bennett, 1974), or even intact neuronal membranes (Fredman and Jahan-Pawar, 1980). No single piece of evidence proves that the observed 'sprouts' are actually part of D_f. However, collectively, a number of features of these sprouts indicate that it is extremely unlikely that they are part of a second neurone into which cobalt has passed from D_f. The evidence that supports this view is as follows:

(a) The general appearance of sprouts: they never showed the intricate and extensive branching of normal dendrites. Varicosities were also frequently seen along their length; such features have never been observed in normal adult cockroach motoneurones.

(b) Groups of parallel fibres frequently ran into the connectives, sometimes branching within the connective. Normally a neurone sends one process only into a segmental nerve or connective, and branching is absent unless a fork in the nerve is reached.

(c) The terminations of many fibres entering intact segmental nerves or connectives were abrupt, and frequently could be clearly seen.

(d) A second cell body never became stained in the ganglion.

The new growth produced by the motoneurones studied here has been referred to as 'sprouting'. This term is used since it describes the type of growth, and because it was not always possible to identify processes as being axonal or dendritic in nature.

The stimulus, or stimuli, for dendritic sprouting in these cells is still unknown, but the effect is increased with more extensive damage to the neuronal environment; neurones of ganglia maintained in culture showed such extensive dendritic changes(Pitman and Rand, 1981 a) that the stained cells were frequently unidentifiable, whereas sprouting was seldom observed in neurones after axotomy alone. This is consistent with the results of Tweedle et al. (1973), who reported that transection of the peripheral nerve containing the axon of the motoneurone D_f produced no changes in this cell. However, it is possible that the less refined staining techniques available at the time would not allow them to see minor changes such as those observed in the experiments presented here. Although sprouting was observed in cells where multi-nerve lesions had spared the axon, the frequency with which sprouting occurred was still greater with lesions of similar extent if D_f had been axotomized. This indicates that although axotomy is not essential to allow dendritic sprouting, it does perhaps enhance the stimulus.

The results presented here contrast with the results obtained with an identified leech neurone which did not sprout after removal of its target unless it had been directly damaged itself (Muller and Scott, 1980).

It seems likely that any combination of the following factors could be involved in the stimulus for dendritic sprouting in motoneurone D_f:

(a) Loss of presynaptic or postsynaptic contacts.
This seems a likely candidate, as a number of other neurones are known to respond to such changes.

(b) Materials released into the local environment by damaged neurones.

(c) Glial changes associated with neural damage.
Such a mechanism is possible, since glial changes may accompany neuronal damage in some animals.

(d) Damage to the cell itself.
Results indicate that this is not the sole factor, although it could play a contributory part.

The normal structure of D_f is basically very similar from one animal to another (See Results). In contrast, sprouting neurones often produce processes that penetrate areas which would normally be totally foreign to them. If this sprouting was sufficiently extensive it could mask the normal branching pattern of the neurone so that it became unidentifiable. This occurred less frequently than in the cultured neurones, but when it did, it was necessary to exclude data on these cells. It was thus inevitable that the sample in the results would be biased against those neurones showing the most radical changes.

Many sprouts entered foreign segmental nerves or connectives, and sometimes these sprouts branched within the foreign nerves.

The straight route taken by many of these processes suggested that they were following either a chemical trail laid down by degenerating fibres, or tracks of minimum mechanical resistance in the nerve. The majority of foreign nerves entered had themselves been sectioned, but some neurones sprouted processes into intact foreign nerves. The relatively small number of intact foreign nerves which were invaded suggests that the sprouts do not grow in an entirely random fashion, but follow some sort of guide. However, an alternative reason for this small number could be a function of the experimental design: lesions which caused extensive dendritic sprouting also left few intact foreign nerves. The two cases where sprouts entered a truly intact foreign nerve were produced in one case by amputation of the distal region of the leg, and in the other by sectioning the ipsilateral nerve 5 and anterior connective. On both occasions, sprouts entered nerve 3, probably because it is, with the exception of nerve 5, the largest of all segmental nerves leaving the ganglion, and also because it is relatively close to dendrites of D_f. Four other neurones sent processes into an intact posterior connective. However, in all cases the ipsilateral anterior connective had been crushed, and therefore, the posterior connective would contain a number of degenerating 'through fibres', i.e. nerve fibres passing through the metathoracic ganglion from more anterior ganglia.

Occasionally sprouts crossed the midline to the contralateral side of the ganglion, but never entered contralateral nerves or connectives. This crossing into the contralateral neuropile occurred whether or not the contralateral nerve 5 had been sectioned, and therefore could not have resulted from the neurone 'taking over' the function of the contralateral homologue. It also occurred irrespective of whether or not the cell itself had been axotomized. No cells, however, sprouted

into the contralateral neuropile if there had been no damage to the nerves on that side of the ganglion. Extensive lesions restricted to the same side of the ganglion as the nerve cell body provided an adequate stimulus for extensive sprouting. In such cases, however, sprouting was confined to the ipsilateral side of the ganglion. This restriction cannot, therefore, be attributed to an inadequate sprouting stimulus. A possible explanation is that degenerating fibres from damaged contralateral motoneurones or from interneurones with bilateral dendritic fields, attract sprouts to cross the midline.

One of the most notable features of these sprouting processes were swellings or varicosities along their length and at their ends. Swellings were also frequently encountered at the ends of dendrites which constituted part of the normal dendritic tree. The functional significance of these varicosities is unclear; they could be associated in some way with the dynamic state of the neurone, since they have been observed in different types of neurones undergoing physiological changes. Leech neurones have been shown to normally exhibit some varicosities, and it has been suggested, as a result of electron microscopical studies, that synapses are present at these points (Muller and McMahan, 1976). The number of varicosities greatly increases when the cells are damaged (Miyazaki, Nicholls and Wallace, 1976), again indicating that formation may be associated with a dynamic state of the neurone. Fibres growing from leech neurones in culture also develop numerous varicosities (Wallace, Adal, Nicholls, 1977). Similarly, varicosities develop in insect (Clark, 1976; Truman and Reiss, 1976) and molluscan (Murphy and Kater, 1978) neurones during regeneration or other dynamic changes such as metamorphosis. Several decades ago, Speidel (1933; 1935) described varicosities in the growing neurones which he observed in the tail fin of living tadpoles. As with the observations made here on cockroach

neurones, he found that a number of small varicosities developed along the lengths of neurites, giving them a beaded appearance, and that larger, more discrete swellings formed at the ends. He suggested that the smaller varicosities were found at the sites of slight temporary obstructions to the growth cones (Speidel, 1933), and that the larger swellings were degenerating balls which would later disappear, followed by retraction of the dendrite (Speidel, 1935). If these observations are true for the cockroach, this might explain why the smaller varicosities were always found in obviously extensively sprouting dendrites, whereas the larger swellings were found more within the normal territory of the cell and probably partly on original as well as newly formed dendrites.

Dendritic swellings have been observed after axotomy of mammalian central neurones (Purves, 1975b; Bernstein, Wells and Bernstein, 1978). Purves (1975b) suggested that they correspond to profiles observed under the electron microscope containing tubular organelles, vesicles and mitochondria. Bernstein et al (1978) believe the varicosities to be a characteristic of deafferented, and possibly also injured, neurones. The cockroach neurones described here would conform to this proposal; almost all dendritic sprouts, no matter how induced, bear varicosities.

Normally the dendrites of D_f form a complex pattern, branching frequently and repeatedly along their length. However, a number of supernumerary fibres produced by sprouting neurones may travel in relatively straight lines in groups, forming bunches of parallel fibres. This was particularly common in sprouts growing in an anterio-posterial direction. Many 'through fibres' (see earlier) normally taking this path would have been severed when the connectives were crushed. It is therefore likely that regenerating sprouts may follow the course of these degenerating fibres.

Long term experiments are necessary to answer the question of whether or not inappropriate branches regress once normal function is restored. Muller and Scott (1979, 1980) found that in the leech there was little regression of fibres deprived of their target cell for up to 5 months; the situation may be different for inappropriate branches when function is restored. For technical reasons, this question is difficult to answer in the system used here. The extent of damage to the nervous system required to produce an acceptably reliable degree of structural change in D_f is fairly extensive. In turn, this results in poor recovery of function in the experimental animals. The neurone under study is responsible for sudden, quick movements of the leg, and a recovery of functional contact by this cell is difficult to assess in the living animal. It should be possible to use another experimental cell, such as the slow depressor motoneurone (Ds), whose functional recovery could be more easily assessed as it is used more frequently for normal walking movements. However, the soma of this cell is less easily accessible without causing damage to the dendrites of the cell, and so is a less attractive choice for study.

Whatever the ultimate fate of inappropriate branches, it is clear that, given the right stimulus, these adult insect neurones can sprout profusely. Although some of the supernumerary branches appear to be directed randomly, a number appear to follow tracks, perhaps left by degenerating fibres. At present it is not possible to assign a role to these dendritic sprouts. If they play a positive part in the restoration of function, then any inappropriate branches may eventually be expected to retract or become inactive. However, it is possible that these sprouts may form no synaptic connections and serve no useful purpose in functional recovery; they may even be detrimental to recovery if they formed inappropriate synapses. Recent evidence suggests that

such branches may be induced under circumstances where regenerating fibres are prevented from making contact with their target (Pitman and Rand, unpublished observations). Long-term experiments on animals in which function has been restored are necessary to begin answering these questions.

Dendritic sprouting has been reported in another adult insect neurone (Clark, 1976). Supernumerary branches temporarily developed on the dendritic tree when it was isolated from the soma by hemisection of the ganglion, and it was suggested that perhaps the soma normally has a repressing influence on dendritic sprouting from which the neurone was freed. However, in the light of the experiments presented here, it seems more likely that the sprouting was induced by the extensive neuronal damage caused by the operation.

The response of the axon to section is also of interest. The point in the axon at which it was transected became slightly swollen in the majority of cases, and in many preparations, a number of fine processes grew from the proximal axon stump. These processes tended to fan out from their point of origin, and grew either in winding paths back toward the ganglion, or extended distally along nerve 5. Such sprouting was observed in some cases even when the target for D_f was removed by amputating the distal part of the leg. In the lamprey (Wood and Cohen, 1979, 1981) and in the leech (Van Essen and Jansen, 1976; Wallace, et al. 1977; Muller and Carbonetto, 1979; Muller and Scott, 1979), many fine fibres have been seen growing from the proximal axon stump. Therefore, after regeneration, a single thick axon is replaced by several fine processes. Brogan and Pitman (1981) have recently found that extensive axonal sprouting occurs in D_f when the main trunk of nerve 5 is crushed. Some of these sprouts entered branch 5r1, andothers proceeded past the branch point along

the main trunk of nerve 5. As with the dendritic sprouts, there appears to be a profuse production of processes which grow in directions both appropriate and inappropriate to restoration of normal function. The functional significance of replacing one thick axon with several fine processes is not clear; it would superficially appear to be disadvantageous to restoration of function, since impulse conduction would be both slower and more likely to fail in these fine fibres. It would be interesting to know whether some of these branches regress when functional connections are restored with the target muscles. Such a selection process has been described in regenerating retinal axons in the newt (Fujisawa, Tani, Watanabe and Ibata, 1982).

If the sprouting observed in D_f is a phenomenon common to other similar neurones, then the traditional view of the adult insect CNS must be re-thought to include properties which allow it to show morphological plasticity. Such plasticity may be necessary to allow some insects to regain function after nerve damage.

Histological Examinations of Sprouting Neurones

The non-axotomized D_f neurones in which sprouting had presumably been induced did not show any significant aggregation of RNA at the time when such sprouting would be expected (23 days postoperative). However, if the time course is similar to that seen after axotomy (maximal at 4 to 14 days), the response would have disappeared by this time.

At 4 days there was some indication of a little RNA aggregation. The small number of cell bodies showing this response may be explained if the nervous system lesions had not induced sprouting in all of these particular non-axotomized D_f neurones. This is a possibility: almost one third of the D_f neurones stained with cobalt after similar lesions

showed dendritic sprouting. In this case, we would expect at least one, or possibly two, of the five cells studied to have been induced to sprout. In fact two cells did show some RNA aggregation 4 days postoperatively, but the intensity was not as great as in the axotomized homologous cell on the contralateral side. RNA aggregation may have appeared less dense in these non-axotomized neurones for a number of reasons:

(1) The time course for RNA redistribution could be somewhat slower in non-axotomized cells where perhaps the stimulus is not so great, or so rapid to take effect. In order to ascertain this it would be necessary to sacrifice animals at intervals between 4 and 23 days postoperative.

(2) It is possible that all or most of these cells had been induced to sprout, but that sprouting is not in fact dependent upon RNA aggregation and dispersal. The minor degrees of aggregation observed may lie within the outer limits of the normal range of RNA distribution.

This technique of assessing RNA distribution is very much dependent upon subjective evaluation of the observer; borderline cases are sometimes difficult to assess. However, the fact that there did seem to be some RNA aggregation in at least one case, suggests that a degree of RNA redistribution may accompany dendritic sprouting alone.

SUMMARY

A (1) Thoracic and abdominal ganglia were explanted from adult (and occasionally young nymphal) male cockroaches, Periplaneta americana, and maintained in culture at 28°C for up to 6 months.

(2) Fibre outgrowth could be seen from the majority of these explants by about 5 days in culture. By 3-4 weeks, this outgrowth formed a complex, branching network of interconnecting fibres, some of which were seen to terminate in growth cones. Direction of fibre growth appeared to be largely random, although there was some formation of fibre bundles, presumably due to contact guidance.

(3) The delay before onset of fibre outgrowth was reduced by prior section of nerves in situ 2-3 weeks before explantation of ganglia into culture. Outgrowth from these nerves which had received a 'conditioning lesion' was observed as soon as 12-24 hours after explantation into culture. Similarly, functional recovery occurred sooner in vivo after equivalent operations: normal function returned to animals more quickly after a second lesion to the same nerve, than after one lesion only.

(4) Although cultures were relatively resistant to different temperatures, and could survive anywhere between 19°C and 37°C, the rate and extent of fibre outgrowth was optimal at 28°C. This relative tolerance may be because the nervous system of these poikilothermic animals is adapted to withstand temperature changes.

(5) Explanted ganglia co-cultured in a dish, but separated by distances of more than 1 mm, frequently became physically interconnected by the fibre outgrowth. There was no apparent selectivity in the formation of connections: ganglia became interconnected irrespective of whether or not they would be neighbours in vivo. Similar connections were made between ganglia and pieces of explanted coxal depressor muscle, again, with no apparent selectivity. No evidence was obtained to suggest that any of these interneural or neuromuscular connections were functional.

(6) Histological examinations of sections from ganglia which had been maintained in culture revealed a number of structural features that differed from those of normal ganglia. The sheath surrounding the ganglion appeared to thicken; many of the larger neuronal cell bodies were reduced in size; the structure of the neuropile underwent a series of changes, losing its highly organised, symmetrical appearance, and then, in longer term cultures, apparently regaining some organisation.

(7) Neurones from ganglia maintained in culture for several weeks were studied by intracellular injection of cobalt followed by wholemount silver intensification. Such neurones showed extensive sprouting which could extend into regions of the ganglion which would normally be foreign to the neurones.

B (1) A number of different types of lesion have been performed *in vivo*. The structure of an identified motoneurone was studied by intracellular cobalt injection at postoperative time intervals ranging from 2 weeks to several months.

(2) Lesions to the CNS caused changes in the branching pattern of this identified neurone (the fast coxal depressor motoneurone D_f). The extent of change was determined largely by the overall extent of damage to the CNS, rather than a specific type of lesion. Direct damage to the cell was not a pre-requisite for structural changes to occur.

(3) The structural changes observed included:

(a) sprouting of supernumerary branches into foreign territory;

(b) swellings or varicosities along the length of new sprouts, or at the ends of new or pre-existing branches;

(c) a tortuous route taken by sprouting processes, often followed by an abrupt change in growth to follow a straight course, along with other sprouts, forming groups of parallel fibres. It is suggested that this straight growth may be caused by new processes following the paths of degenerating fibres.

C It is concluded that at least some adult insect neurones are capable of extensive regenerative growth, and of undergoing a high degree of structural modification, both *in vivo* and *in vitro*. This indicates that some plasticity is a feature of the adult insect nervous system.

LIST OF ABBREVIATIONS

CNS	Central Nervous System
D_f	Fast coxal depressor motoneurone
NGF	Nerve Growth Factor
RNA	Ribonucleic Acid

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