SOME ELECTROPHYSIOLOGICAL AND BIOCHEMICAL EFFECTS OF DENERVATION ON THE CENTRAL NERVOUS SYSTEM OF THE COCKROACH, PERIPLANETA AMERICANA L.

Jonathan Anthony David

A Thesis Submitted for the Degree of PhD
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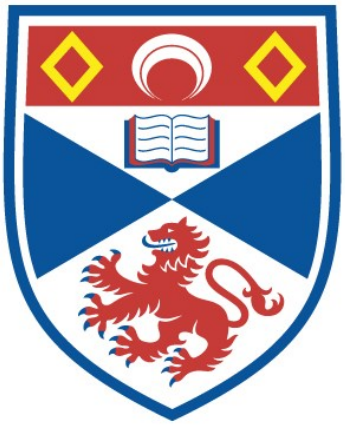

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ABSTRACT

FACULTY OF SCIENCE

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Some Electrophysiological and Biochemical Effects of Denervation on the Central Nervous System of the Cockroach, Periplaneta americana L.

by Jonathan Anthony David.

The effect of denervation on the excitability of cockroach central neurones has been investigated using both extracellular and intracellular recording techniques.

Stimulation of the anterior connectives produced a response in the flexor tibiae muscle 143b of the metathoracic segment. This muscle response could be correlated with action potentials in the anterior connective and fifth nerve trunk innervating the leg. The ganglionic delay for this pathway was 1.42 ± 0.07 ms (n=6) suggesting the presence of a single synapse. This was reduced to 0.86 ± 0.10 ms (n=10) 19-96 hours after cutting the contralateral fifth nerve trunk. It is suggested that this reduction in ganglionic delay could result from an increase in the excitability of the postsynatpic cell caused by deafferentation.

Intracellular recording were made from the cell body of the fast coxal depressor motoneurone (D_f) in the metathoracic ganglion. Acetylcholine (ACh) applied to the soma either topically or by iontophoresis caused depolarisation of the cell membrane and if repeated in large doses resulted in

rapid desensitisation and depression of the response. Some D_f motoneurones were axotomised by cutting the ipsilateral fifth nerve trunk 4-118 days before the experiment. The sensitivity to ACh increased approximately three fold following axotomy. However there was no change in the mean resting potential, input resistance or membrane time constant.

The change in sensitivity to ACh produced by axotomy was apparently not a result of a redistribution of cholinoceptors on the soma membrane since the rise time of the ACh responses were similar in control and axotomised preparations. Analysis also showed that their was no difference between control and axotomised cells in the number of ACh molecules needed to combine with individual cholinoceptors to produce a response. Similarly there was no significant difference in the mean values of the ACh reversal potential in control and axotomised neurones (as determined by extrapolation).

It is suggested that the supersensitivity to ACh is due to a decreased inactivation of ACh by acetylcholinesterase since (i) the anticholinesterases physostigmine and neostigmine produced a greater potentiation of the ACh response in normal than in axotomised neurones and (ii) there was no significant difference in the sensitivity of control and axotomised cells to carbamylcholine. This suggestion is supported by the results obtained from a biochemical study in which the cholinesterase activity of normal and denervated metathoracic ganglia was determined. Denervation was produced by transection of both fifth nerve trunks 5-8 days before the assay. Denervated ganglia showed an approximately 33% reduction in their capacity to hydrolyse AThCh.

SOME ELECTROPHYSIOLOGICAL AND BIOCHEMICAL EFFECTS OF DENERVATION ON THE CENTRAL NERVOUS SYSTEM OF THE COCKROACH (PERIPLANETA AMERICANA L).

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

by

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May, 1979.

CERTIFICATE

I hereby certify that Jonathan A. David has spent nine terms engaged in research work under my direction, and that he has fulfilled the conditions of General Ordinance No. 12 (Resolution of the University Court, No. 1, 1967) and that he 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 fullfillment of the requirements governing candidates for the degree of Doctor of Philosophy, in the University of St. Andrews, entititled 'Some Electrophysiological and Biochemical Effects of Denervation on the Central Nervous System of the Cockroach (*Periplaneta americana*, L)' 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 Dr. R.M. Pitman.

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Finally I would like to thank my parents for their continual interest and encouragement throughout my education.

ACADEMIC RECORD

I was educated at Earls Colne Grammar School and Portsmouth Polytechnic where I graduated in Physiology in June, 1975. The work described in this thesis was carried out between November, 1975 and July, 1978.in the Department of Physiology and Pharmacology, University of St. Andrews.

i do not see why men
should be so proud
insects have the more
ancient lineage
according to the scientists
insects were insects
when man was only
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'archy and mehitabel' by Don Marquis

CONTENTS

CHAPTER I

THE EFFECTS OF DENERVATION ON TWO NERVOUS PATHWAYS (A & B) THROUGH THE METATHORACIC GANGLION.

INTRODUCTION.

It is often assumed that once cells are laid down during development they remain stable throughout adult life. However cells possess an inherent plasticity, that is, they are capable of undergoing pronounced changes in their structur metabolism and connections when presented with a novel situation. This is particularly apparent in the cell response to either presynaptic denervation (deafferentation) or post-synaptic denervation in the form of axotomy. Both types of denervation often produce a change in the excitability of the cell, either to synaptic activation or to applied drugs. Generally if any such change is induced, deafferentation tends to produce a hyperexcitability and axotomy a reduction in excitability. This is discussed on pages 2 to 22. The changes which occur in axotomised neurones and the significanc of these changes on the cells excitability are discussed in more detail on pages 57 to 79 .

DEAFFERENTATION - CHANGES IN EXCITABILITY.

Over a century ago it was noticed that following denervation, peripheral structures become extremely sensitive to apparently normal stimulation. Philipeaux and Vulpian reported in 1863 that after section of the hypoglossal nerve, stimulation of the lingual nerve resulted in a prolonged contraction of the tongue. Rogowicz (1885) noted a similar increase in the sensitivity of the facial muscles. After secti of the facial nerve, sympathetic stimulation caused a contract of the muscles on the denervated side. In 1893 Sherrington

demonstrated that stimulation of a peripheral nerve following section of the ventral roots caused a slow contraction of the muscles of the extremities. Elliott in his much quoted paper on the sympathomimetic nature of adrenaline (1905) mentioned that the denervated iris and nictitating membrane of the cat became noticeably more sensitive to intravenous injections of adrenaline. Similar results were obtained with adrenal extract (Langley, 1905).

The subsequent discovery of similar phenomnea led to the formation of Canon's "Law of Denervation" in 1939: " when in a series of afferent neurones a unit is destroyed, an increased irritability to chemical agents develops in the isolated structure or structures, the effect being maximal in the part directly denervated". The term denervation supersensitivity has since been used to incorporate many phenomena where a reduction or removal of afferent information has induced an increase in the responsiveness of the denervated structure. Supersensitivity in one form or another may be involved in epilepsy (Echlin, 1959), muscular dystrophy(Howe, Telfer, Livett and Austin, 1977), some withdrawl symptoms following chronic intoxication by depressant drugs (Jaffe and Sharpless, 1965) and some effects of sensory deprivation (Doane, 1959).

Denervation supersensitivity in skeletal muscle.

The phenomenon of denervation supersensitivity in mammalian skeletal muscle is well documented. Early experiments consisted of measuring the mechanical response to acetylcholine (ACh) applied by injection of small doses into the

general circulation or the arterial supply (Frank et al, 1922; Dale and Gasser, 1926). Quantitative observations on the sensitivity of the muscle were not possible using this method of application however since the drug was significantly diluted in the circulatory system. Brown et al (1936) overcame this problem by using a more direct technique. Working on the gastrocnemius muscle of the cat, all blood vessels except those supplying or draining this muscle were tied off. Drugs were then injected directly into the main artery through a permanently implanted cannula and the mechanical response measured. 10-18 days following denervation the sensitivity and power of contraction of the muscle increased over a thousan fold. Using a similar technique, Brown and Harvey (1938) demonstrated an approximately ten fold increase in ACh sensitivity in denervated muscles of the frog and fowl.

1) Proliferation of receptors.

Kuffler (1943) demonstrated that ACh in low concentrations depolarised the muscle fibre when applied to the frog neuromuscular junction. Denervation supersensitivity was attributed to an increased chemical susceptibility confined to this area. However it was not until the advent of iontophoresis (Nastuk, 1953) that the localised application of drugs was possible. This technique revealed that the chemosensitive areas spread away from the endplate regions in skeletal muscles following denervation (Axelsson and Thesleff, 1959). In contrast to the supersensitivity observed with general bath application the sensitivity of the denervated endplate region to iontophoretically applied ACh does not

appear to be significantly different from that in normal muscle. In a normally innervated fibre of the frog gastrocnemius muscle the ACh sensitive area is restricted to a region approximately 1 mm in length surrounding the neuromuscular junction (Miledi, 1960a). Seven to eight weeks after denervation this area increased to 5-6 mm progressing to occupy the whole length of the muscle fibre within about ten weeks. After reinnervation the chemosensitive area contracts until it becomes confined to the region around the nerve termianls (Miledi, 1960b). This reaction resembles the situation in developing foetal muscle where th ACh sensitive areas are distributed along the entire length of the fibre. These areas shrink to around the endplate region after connection with the nerve (Diamond and Miledi, 1962). For this reason it has been suggested that denervated skeletal muscle undergoes a process of de-differentiation, reverting to the more primitive state.

Usherwood (1969) has demonstrated denervation supersensitivity in locust muscle. The distribution of chemosensitive areas on an insect muscle fibre appear to follow the same pattern as in vertebrate skeletal muscle. L-Glutamate rather than ACh is probably the excitatory transmitter substan at insect neuromuscular synapses (Usherwood and Machili, 1966; Kerkut, Shapira and Walker, 1965). By iontophoretically applying glutamate to localised areas on locust retractor unguis muscle fibres Usherwood was able to demonstrate the confinement of chemosensitive areas to within 10-35 μm of either side of a synapse. The extrajunctional areas were about 10,000 times less sensitive than the synaptic areas in

innervated muscle. The distribution of glutamate sensitive areas was studied from 1-32 days after denervation. In these muscles the whole of the fibre was sensitive to iontophoretically applied glutamate and in some preparations the sensitivity of the extrasynaptic membrane was indistinguishabl from that of the synaptic membrane.

Two types of extrajunctional glutamate receptor are present in locust muscle fibres (Cull-Candy, 1975). The D receptor responsible for a depolarising response is activate by L-glutamate but not by the glutamate analogue DL-ibotenate while the H receptor responsible for a hyperpolarising response is activated by both L-glutamate and DL-ibotenate. Utilising this difference in drug response, Cull-Candy (1975) showed that denervation of locust muscle fibres produces a selective proliferation of D receptors only.

Frank and Inoue (1966) investigated the sensitivity of a normal endplate after partial denervation of the muscle fibre. This was made possible by the unique innervation of the frog sartorius muscle. Each fibre is dually innervated, receiving an axon from each of two distinct nerve trunks. If one nerve trunk is cut the ACh sensitivity spreads away from the endplate although more slowly than in completely denervate mammalian muscle. Miniature endplate potentials (m.e.p.p.'s) at the innervated endplate were of a larger amplitude than in preparations with complete innervation.

2) Histochemical and Biochemical Evidence.

The mechanism underlying denervation supersensitivi has recently received much attention. Prominant amongst these

investigations has been the measurement of extrajunctional ACh receptors using saturation binding of ^{125}I-α-bungarotoxin. The specific and irreversible binding of this snake venom fraction to ACh receptors has allowed quantitative measurements of receptor density.

Diaphragm muscle of the rat has proved to be a convenient preparation for such investigations since the endplates are restricted to a narrow zone that can be easily removed. The muscle can then be bathed in a solution of the toxin for a given period of time and the number of binding sites measured either by autoradiography or scintillation spectrometry. In normal muscle the density of binding sites of presynaptic areas is about 10,000/μm^2 and 10/μm^2 at endplate free areas (Barnard, Wieckowski and Chiu, 1971; Berg, Kelly, Sargent, Williamson and Hall, 1972; Hartzell and Fambrough, 1972). 2-3 Days following denervation the number of binding sites in extrasynaptic regions starts to increase, reaching about 1000/μm^2 after 14 days. This subsequently falls to about 500/μm^2 after 45 days. Miledi and Potter (1971) using a similar technique showed that denervation of rat diaphragm muscle produced a 200 fold increase in α-bungarotoxin binding to endplate free regions.

The distribution of ACh receptors in dystrophic muscles of the mouse appears to resemble the situation found in denervated muscle. Howe et al have detected an increase in junctional receptor density in the dystrophic biceps femoris muscle similar to that produced by 16 days of denervation (Howe, Telfer, Livett and Austin, 1977). However whether or not dystrophic muscles are functionally innervated is at the

moment under some dispute (see McComas, Sica and Cambell, 1971 Harris and Marshall, 1973; Harris and Ribchester, 1978).

3) Mechanism for the Proliferation of Receptors.

The proliferation of receptors in denervated skeletal muscle has been attributed to a: (i) redistribution of existing receptors, (ii) activation of dormant receptors and (iii) synthesis of new receptors.

The redistribution of receptors usually refers to their spread from the endplate to the extrajunctional regions. This theory has now been almost completely abandoned. Katz and Miledi (1964a) showed that an increase in ACh sensitivity develops in cut segments of skeletal muscle devoid of endplate regions. In denervated rat diaphragm muscle the number of α-bungarotoxin binding sites in the endplate does not appear to decrease markedly (Hartzell and Fambrough, 1972) but the total number of binding sites per muscle shows a progressive increase (Miledi and Potter, 1971; Berg et al, 1972).

Indirect evidence has been produced in support of the synthetic theory. Fambrough (1970) and Grampp, Harris and Thesleff (1971) demonstrated that protein and RNA synthesis are necessary for the development of denervation supersensitivity. This was demonstrated more specifically by Lunt, Stephani and DeRobertis (1971) who measured the incorporation of tritiated leucine into a proteolipid fraction of muscle. This fraction was believed to be the ACh receptor since it bound ACh, methylhexamethonium and other cholinergic drugs. After denervation the rate of leucine incorporation into the proteolipid increased indicating a possible increase in receptor synthesis.

4) Other factors responsible for denervation supersensitivity in skeletal muscle.

By far the most popular explanation for denervation supersensitivity has been a proliferation of receptor sites. This would explain the rise in the sensitivity of denervated skeletal muscle to certain chemical stimuli. Even so, other changes have been reported which may increase the effect produced by occupation of the chemoreceptor. Isaacson and Sandow (1967) found that the increase in the movement of calcium ions across the membrane in response to caffeine was much greater in denervated than in normal muscle. Similarly Howell et al (1966) showed that denervated striated muscle could accumulate more calcium than innervated muscle. It has been suggested by Thorpe and Seeman (1971) that this would permit the release of more calcium ions from the plasma membrane and so produce a greater contractile response to caffeine.

Electrophysiological and pharmacological studies have revealed differences in the properties of junctional and extrajunctional receptors. In frog muscle normally innervated fibres show two kinds of ionic permeability. At the endplate the ACh reversal potential is about -15 mV but in the region immediately surrounding the endplate the reversal potential is about -44 mV (Feltz and Mallart, 1970). After denervation the entire fibre becomes sensitive to ACh. The reversal potential in the extrajunctional regions is about -41 mV, the same as in normal muscle. Beranek and Vyskocil (1967) have shown that junctional and extrajunctional receptors of rat diaphragm muscle differ in the relative potencies of curare

and atropine to block the ACh response. The results indicate a lower proportion of nicotinic receptors in the extrajunctional regions of the denervated muscle than in the region of the endplate.

Characteristic changes also occur in the electrical properties of denervated skeletal muscle. The membrane resistance increases two fold after denervation due to a decrease in the resting potassium conductance (Nicholls, 1956; Thesleff, 1963; Albuquerque et al, 1971). This effect however is far too small to account for the increase in sensitivity to ACh. Denervated skeletal muscle shows a significant fall in the level of acetylcholinesterase (AChE) in both junctional and extrajunctional regions. A decrease in this hydrolytic enzyme would increase the amount of ACh available to interact with the receptor and no doubt contribute partly to the supersensitivity of the muscle. Guth and Brown (1964) examined AChE histochemically in alternate sections of normal and denervated rat muscle. Following denervation the enzyme activi at both endplate and non-endplate regions fell dramatically reaching 50% of normal within 1 week. The reduction of AChE seemed to be fairly specific since after denervation the total protein content fell more gradually reaching a 50% level only after 3 weeks. Similar changes have been reported in the denervated diaphragm muscle of the rat (Kiauta et al, 1977; NcConnell, 1974). Reinnervation of the sternomastoid muscle restores the AChE level to normal (Guth and Brown, 1965).

Denervation supersensitivity in the autonomic nervous system.

Early investigations into denervation supersensitiv of autonomic ganglion cells were focused primarily on the

superior cervical ganglion of the cat. The activity of the nictitating membrane was used to determine the activity of the ganglionic neurones. The results of these investigations are open to criticism however since no allowance was made for the sensitisation of the decentralised nictitating membrane. This problem was overcome by Brown (1969) who quantified the amount of ACh, injected intra-arterially, which produced a contraction of the nictitating membrane equal to that elicited by a known quantity of noradrenaline. This technique revealed a 3 fold increase in the sensitivity of the denervated superior cervical ganglion to ACh but no significant change in the sensitivity to nicotine and carbamylcholine. Similar results were obtained by McKinstry and Koelle (1967). However conflicting evidence was produced by examining the amount of preganglionic stimulation required to mimic the effects of postganglionic stimulation of the nictitating membrane (Green, 1969). Green reported a reduction in the sensitivity of the denervated ganglion. The observations made by Brown and co-workers explain this discrepancy (Brown, 1965; Brown, Dearnaley and Geffen, 1967). The use of phenoxybenzamine to block the uptake of noradrenaline by adrenergic receptors allowed the quantity of liberated noradrenaline to be detected in the venous effluent. It was found that the amount of noradrenaline released during stimulation of the splenic nerves increased after preganglionic denervation. Thus more noradrenaline would be rquired to mimick the response to ganglionic stimulation after denervation.

Chein (1960) adopted a different approach. The effe of ACh on the superior cervical ganglion cells of the cat was

monitored by measuring the quantity of noradrenaline released from the postganglionic nerve endings. This was determined indirectly using the in vivo nictitating membrane as an indicator after callibrating its response to various known doses of noradrenaline. 13-15 Days after preganglionic denervation the control side of the ganglion required 4 times as much ACh as the denervated side in order to release the same quantity of noradrenaline.

Much information concerning the mechanism of the supersensitivity of autonomic ganglion cells has been achieved from studies on a number of recent preparations in which the surface of the neurones with their synapses can be seen. These preparations come from parasympathetic cardiac ganglia in amphibians. Kuffler and his associates examined the sensitivity to iontophoretically applied ACh of localised regions of parasympathetic ganglion cells from the interatrial septum in the heart of the frog. In normally innervated cells the synaptic regions alone are highly sensitive to ACh (Harris, Kuffler and Dennis, 1971). During the second day after the two vagus nerves to the heart were cut, the ACh sensitivity of extrasynaptic regions of the neurones began to increase (Kuffler, Dennis and Harris, 1971). Within 5 days the sensitivity of extrasynaptic areas was indistinguishable from that of synaptic regions. In addition the rise times of the ACh responses were similar wherever the drug was applied to the surface of the cell body. The authors concluded that denervation induced a proliferation of receptor sites over the cell membrane. This condition persisted for over 3 weeks. The time course of the response to denervation is more like that of denervated mammalian skelet

muscle (Axelsson and Thesleff, 1959) than of denervated frog skeletal muscle (Miledi, 1960).

Synapses can also be visualised on the multiply innervated parasympathetic cardiac ganglion cells of the mudpuppy (Roper, 1976; 1976a). As in frog cardiac ganglion cells identified synaptic regions of the cells were more sensitive to iontophoretically applied ACh than were extrasynaptic regions. Following partial denervation of the ganglion cells (accomplished by cutting the vagus nerve) the density of extrasynaptic ACh receptors appeared to increase. Within about 4 weeks the distribution of receptors appeared to be uniform over the whole of the cell membrane. A similar proliferation of receptors occured in the neurones of ganglia isolated in organ culture. However this response was more rapid than in denervated cells kept in vivo reaching a maximum within 4 days. This paralleled the increased rate of degeneration of the preganglionic nerve terminals.

Denervation supersensitivity in the central nervous system.

Unfortunately the evidence for denervation supersensitivity in the CNS is mostly indirect. This is largely a result of the technical difficulties encountered in the visual identification of individual central neurones and subsequent microapplication of test compounds.

1) The Spinal Cord.

The effect of semi-section of the spinal cord on the response to intra-aortal injections of ACh has been studied in the cat by monitoring the response of the quadricep

muscle (Cannon and Haimovici, 1939). The quadriceps on the side which was transected responded to smaller quantities of ACh than on the control side. It was shown that the sensitivity of the quadriceps muscle itself increased although the denervation produced by spinal transection was not direct but involved an intermediate or "penultimate" neurone. Later experiments carried out by Drake, Seguin and Stavraky (1956) showed that 4 days after semi-section of the spinal cord the frequency of nervous discharge from the anterior horn cells recorded in the peripheral nerves was greater in the operated side following intra-arterial injection of ACh.

Similarly disuse of a spinal pathway produces an increase in the excitability of ventral root motoneurones (Beranek and Hnik, 1959; Kozak and Westerman, 1961). These authors produced disuse by cutting the tendon of the gastrocnemius muscle in the cat. This operation would relieve tension on the stretch receptors and so reduce impulses in the Ia afferent fibres supplying the muscle spindle. After several weeks a significant increase in the frequency of monosynaptic action potentials recorded in the ventral root was observed.

2) The Brain Stem.

A number of workers have shown that cholinergic denervation of the hippocampus by transection of the medial septum causes a decrease in the hippocampal AChE, located mainly in the presynaptic termianls (Srebro et al, 1973; Storm-Mathisen, 1972). The sensitivity to ACh of hippocampal pyramidal cells in lesioned and unlesioned preparations has been investigated by Bird and Aghajanian (1975) using iontophoretic drug application. Denervation produced a six

fold increase in sensitivity after about 48 hours. However there was no change in the sensitivity to carbamylcholine or glutamate. In addition physostigmine produced no potentiation of the ACh response in lesioned preparations compared to a 50% potentiation in unlesioned preparations. The authors concluded that the supersensitivity to ACh was probably due to a decreased inactivation of ACh by AChE. The time course of the supersensitivity correlates well with the time course for AChE depletion. Receptor proliferation seems to be excluded since no increase in the binding of the potent muscari antagonist quinuclidinyl benzilate occurs in the lesioned hippocampus (Yamamura and Snyder, 1974).

3) The Cerebral Cortex.

It has been known for some time that slices of cerebral cortex which had been partially isolated show a supersensitivity to ACh (Rosenberg and Echlin, 1968). It has been suggested (Echlin, 1959; Sharpless and Halpern, 1962) that these effects are due to the denervation of cerebral neurones which produces a supersensitivity similar to that which occurs in denervated skeletal muscle. This supersensitivity is thought to underly the hyperexcitability observed during epileptic seizures (Brenner and Merritt, 1942; Tower, 1960).

Rosenburg and Echlin (1968) have shown a decrease in ChE activity in the isolated cortex of the monkey. This reaches a maximum at about the same time as the maximum susceptibility to denervation induced epileptic activity (abou 2 weeks). It is possible therefore that the increase in sensitivity of isolated cerebral cortical cells to ACh is partl due to a reduction in ChE. Chu et al (1971a; 1971b) have

produced evidence indicating a parallel increase in the number of ACh receptors: subcellular membrane fractions from chronically isolated cortex show an increase in the binding of ^{14}C-tubocurarine.

Injections of 6-hydroxydopamine (6-OHDA) into the lateral ventricle of the brain causes a specific degeneration of central catecholaminergic nerve terminals and a depletion of catecholamines (Uretsky and Iversen, 1970; Kostrzewa and Jacobowitz, 1974). In slices of rat cerebral cortex, activation of either α or β adrenergic receptors leads to an increased production of cyclic adenosine 3',5'- monophosphate = cyclic AMP (Schultz and Daly, 1973). After pretreatment with 6-OHDA the production of cyclic AMP in response to the activation of α and β receptors is increased (Palmer, 1972; Kalisker et al, 1973). The possibility that this is due to denervation supersensitivity has been investigated by Sporn et al (1976). These authors determined the density of β-adrenergic receptors in slices of rat cerebral cortex by measuring the degree of the binding of a ^{125}I-labelled specific β-adrenergic receptor agonist (hydroxybenzylpindolol - HYP). Within 9 days of beginning 6-OHDA administration the concentration of ^{125}I-HYP binding sites in the cortex had increased by about 30% indicating a similar increase in the number of β-adrenergic receptors.

Denervation supersensitivity in the invertebrate CNS.

Wine (1969; 1972) produced indirect evidence of denervation supersensitivity in neurones involved in the escape reflex of the crayfish. The reflex involves a pair of giant interneurones located in the lateral giant fibres

running the length of the nerve cord. These interneurones can be activated by mechanical stimuli applied to the side of the abdomen. This produces a vigorous flexion of the abdomen via a small number of motoneurones in each abdominal segment. The excitability of these local reflexes increased dramatically within 10 minutes of cutting the nerve cord between the last thoracic and the first abdominal segment. This was proceeded several days later by a further increase in excitability. Single threshold stimuli produced prolonged discharges from the motoneurones innervating the flexor muscles and previously ineffective stimuli became increasingly effective. The mechanism underlying this phenomenon has not been studied.

In the nervous system of the leech the central ganglia are linked by two large bundles of axons - the connectives. Jansen et al (1974) noticed changes in synaptic transmission between ganglia within 30 days of cutting one of the connectives. When a 'pressure' (P) or 'nociceptive' (N) sensory neurone was stimulated in one ganglion an abnormally large inhibitory potential was recorded in the longitudinal (L) motoneurone of the next most anterior ganglion. Weak and rather inconsistent traces of inhibition were detected in normal preparations. The authors suggested that these inhibitor pathways become exagerated and more potent after cutting one of the connectives. In addition a marked increase in the amplitude and duration of certain e.p.s.p.'s in annula erector (AE), L motoneurones and Retzius cells was observed in denervated ganglia. These phenomena could well be due to denervation supersensitivity. A cell such as the L motoneurone has over 1,000 synaptic connections (Purves and McMahan, 1972).

It is therefore unlikely that none of these afferents would be affected by cutting one of the ganglionic nerve trunks.

AXOTOMY - CHANGES IN EXCITABILITY.

Axotomised neurones, in addition to showing classical symptoms of chromatolysis, often undergo a reduced responsiveness to synaptic activation. Chromatolysis or the axon reaction is discussed in Chapter II. Synaptic modifications were first described in ventral horn motoneurones of the cat and monkey by Campbell (1944). The motoneurone was axotomised by transectio of the ventral root. From 10 days to several weeks after nerve section there was a complete loss of the spinal reflex normally produced by stimulation of the dorsal root. Subsequent investigations have shown that the e.p.s.p. evoked in motoneurones by muscle afferent volleys is considerably reduced in amplitude after axotomy (Eccles, Libet and Young, 1958; Bradley and Brock, 1959). However at the same time the axotomise motoneurone can be excited by stimulation of fewer afferent fibres than for normal motoneurones.

This paradox has been resolved to some extent by information gained from intracellular recording techniques. Eccles et al (1958) suggested that there are abnormally excitable patches of membrane on the cell body and dendrites in order to explain the variety of 'humplike' depolarisations seen superimposed on the e.p.s.p.'s. Kuno and Llinas (1970; 1970b) arrived at the same conclusion since the peak of the monosynaptic e.p.s.p. was usually below the threshold level and would require amplification to produce the action potentials

observed. This reduction in e.p.s.p. amplitude occured in spite of the fact that the quantal release of transmitter from the presynaptic nerve terminals was normal.

The reduction in the amplitude and the slower rise times of the e.p.s.p.'s recorded in these central motoneurones is probably due to the detachment of the presynaptic terminals, accompanied by an invasion of glial processes into the synaptic clefts (Blinzinger and Kreutberg, 1968; Hillman, 1970). Blinzinger and co-workers observed that many motoneurone perikarya and proximal dendrites of the rat facial nucleus had lost about 80% of their normal synaptic contacts 4 days after axotomy. This appeared to be a result of a proliferation of microgliocytes which physically displaced the synaptic boutons from the neuronal membrane. The abnormality of e.p.s.p. in axotomised cells may be due to the selective loss of proximal synapses on the soma and dendrites since e.p.s.p.'s from synapses on more distal sites are usually smaller (Rall, 1967). In axotomised hypoglossal neurones of the rat, synaptic disruption also occurs but this is at least partly due to a 'retraction' of the dendritic tree. This process is reversible; a re-expansion of the dendritic field occurs between 5 and 10 weeks after nerve section.

This phenomenon is not confined to the somatic motor system. Brown and co-workers have described a complete failure of synaptic transmission through the stellate, inferior mesenteric and superior cervical ganglia of the rabbit after postganglionic axotomy (Brown and Pascoe, 1952; 1954; Brown, McLennan and Pascoe, 1952a). The conduction of impulses in both pre- and post-ganglionic nerve trunks

appeared to be normal. In addition the output of ACh (collected in the venous effluent) on stimulation of the preganglionic nerve fibres of the superior cervical ganglion was normal after postganglionic denervation. ACh injected into the arterial supply was ineffective in exciting the ganglion cells. It was therefore concluded that the failure of ganglionic transmission was due to a loss of sensitivity of the ganglion cells to ACh. McLennan (1954) attributed this loss of sensitivity to the reduction of AChE within the ganglion which accompanies postganglionic axotomy. Mathews and Nelson (1975) working on the superior cervical ganglion of the rat showed that section of the postganglionic nerve fibres led to a similar depression of synaptic transmission. However at the same time the number of synapses within the ganglion fell by about 75%. Electron microscopy showed normal presynaptic nerve endings but these were not apposed to postsynaptic membrane structures. The authors suggested that synapses are normally held in place partly by mechanical binding of "desmosome-like attachment plaques" which were found almost entirely between nervous profiles. These structures fell in number in parallel with the fall in intact synapses. The final separation seemed to be due to infiltration of the disrupted synapses by extensions of Schwann cells. Similar results were obtained by Purves (1975) working on the same ganglion in the guinea-pig.

Depression of synaptic transmission in the axotomised chick ciliary ganglion seems to be due to post-synaptic changes (Brenner and Johnson, 1976). Three to four days following postganglionic denervation the e.p.s.p.'s recorded in the postganglionic nerve trunk were reduced in

amplitude by a factor of 4 but this was accompanied by only a 17% reduction in the number of synapses. Brenner and Martin (1976) using intracellular recording techniques and iontophoretic drug application were able to demonstrate an 8 fold reduction in the ACh sensitivity of ciliary ganglion cells after axotomy. Thus it seems that the presynaptic terminals in the chick ciliary ganglion are more resistant to synaptic disruption than the presynaptic terminals of central and sympathetic ganglia. Although the mechanism appears to differ, the degree of synaptic depression is similar in both cases.

Invertebrate Central Neurones.

Horridge and Burrows made advantage of the relatively simple connections between the neurones in the insect CNS. Recording from the fast extensor tibiae motoneurone (FETi) of the locust metathoracic ganglion, a 1:1 relationship was observed between e.p.s.p.'s in this neurone and impulses in four large descending visual interneurones. The synapses of these interneurones seem to be distributed equally between the FETi motoneurones on either side of the ganglion. This symetrical arrangement provides a control for investigating the effects of axotomy on the synaptic input of one of these motoneurones. Axotomy was performed by amputation of the metathoracic leg. Changes in synaptic transmission were seen within 24 hours; these consisted of an increase in the amplitude of the e.p.s.p.'s recorded in the motoneurone on the operated side. After about 2 days synaptic transmission became very variable. This was followed by a progressive fall in the amplitude of the e.p.s.p

to a point barely visible above noise within 8 days. These changes appear to be a direct result of axotomy, since e.p.s.p.'s produced in the control FETi motoneurone were normal.

Light microscope study revealed no gross morphological changes in cobalt filled terminal branches of the descending interneurones or dendritic branches of the FETi motoneurones.. A similar stability of dendritic branching has been reported in axotomised cockroach central motoneurones (Pitman, Tweedle and Cohen, 1973). These results do not necessarily indicate that presynaptic terminals on locust and cockroach motoneurones remain intact after axotomy. In the cockroach at least (Pitman et al, 1973) synapses upon central motoneurones seem to occur mainly on the very fine branches of dendritic processes. Synaptic integrity after axotomy can only be determined from studies at the electron microscope level.

In conclusion it would seem that the axotomised neurone, whilst undergoing pronounced changes in its metabolism (discussed in Chapter II) is deprived of afferent input by disruption of its presynaptic terminals. It should be noted that in the insect CNS most of the ganglionic nerve trunks contain axons carrying afferent information to the ganglion as well as axons from motoneurones within the ganglion. Section of the nerve trunks supplying the leg for example may well cause direct deafferentation of some of the motoneurones which are axotomised.

PLAN OF EXPERIMENTS.

This study is concerned with the effects of denervation upon the motoneurone activity in the cockroach central nervous system. The motoneurone cell bodies are located within the ganglia of the central nervous system. Their processes pass through the neuropile where they make synaptic contacts before passing out through the nerve trunks. This arrangement has enabled a fairly extensive investigation into the location of the individual motoneurone cell bodies and the paths of their axons (e.g. Cohen and Jacklet, 1967). The recently developed Procion Yellow and cobalt chloride dye injection techniques (Stretton and Kravitz, 1968; Pitman, Tweedle and Cohen, 1972) have been particularly useful in these studies. Much information is also available about the innervation of the limb musculature by individual motoneurones (e.g. Pearson and Bergman, 1969; Pearson and Iles, 1970; Pearson and Fourtner, 1973).

Thus motoneurones are relatively accessible to stimulating and recording techniques and can be identified by recording from the muscles which they innervate. In addition motoneurones receive afferent inputs from a variety of sources (see Pearson and Iles, 1970; Wilson, 1965) and so transection of the various ganglionic nerve trunks is likely to succeed in producing partial deafferentation.

Extracellular stimulating and recording

techniques were used in order to examine pathways in the nervous system and the effect on these of section of some of the nerve trunks. Extracellular techniques have the distinct advantage over intracellular methods in that they may be used to simultaneously sample the activity of many units. Likewise extracellular stimulation of the nerve can excite many of its axons. Therefore if partial denervation produces a change in a certain pathway in the nervous system this is more likely to be detected using extracellular rather than intracellular techniques. An increase in the sensitivity of a postsynaptic neurone would be expected to be reflected in its response to presynaptic stimulation. For example, the synaptic delay may be reduced since less transmitter would be required to reach the postsynaptic membrane in order to excite the cell.

SECTION I

The effects of denervation on 'Pathway A'

SECTION I. PATHWAY 'A'

METHODS.

1) The Preparation.

Adult male cockroaches (Periplaneta americana, L.) obtained from a dealer were maintained under warm conditions with a constant supply of water and fed on 'Minced Morcels' dog food. Adult males were used to eliminate any possible variations resulting from sex differences and because they generally contain a smaller amount of fatty tissue than females This facilitates dissection and setting up of the preparation. The above applies to all animals studied in this and the proceeding chapters.

The dissection was as follows: the head and legs were removed to reduce muscular movements during the course of the experiment, The animal was then pinned down on a 'Sylgar resin block dorsal surface uppermost. Part of the tergum of the first abdominal segment was removed by cuts on either side of the mid-line which exposed the underlying nerve cord and allowed most of the gut to be pulled free with a coarse pair of forceps; the remainder was cut and allowed to retract into the abdominal cavity. The interior of the preparation was washed with saline to remove any gastric juices, dried with absorbent tissue and filled with vaseline through a 1 ml syringe to prevent dehydration. Care was taken to prevent the vaseline from coming into contact with the exposed part of the nerve cord so as not to impair electrical contact with the stimulating electrodes. Figure 1 illustrates a dissected preparation.

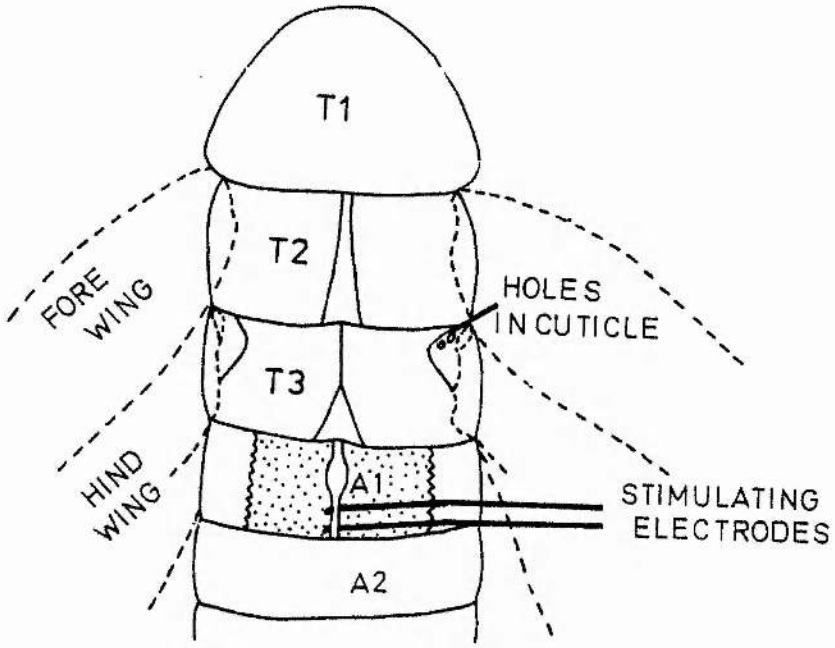


Figure 1. The dissected preparation, dorsal view. Both pairs of wings were removed before positioning the electrodes. The recording electrodes were placed in the holes made in the cuticle of the third thoracic segment. T1-T3, the thoracic ganglia; A1 and A2, the abdominal ganglia.

The arrangement of the stimulating and recording electrodes is shown schematically in Figure 2. The stimulating electrodes consisted of two hooked silver wires 0.28 mm in diameter secured at the end of a 1 ml syringe held at an angle of about 45° to the vertical in a 'Prior' micromanipulator. The electrodes were placed beneath the nerve cord just posterior to the first abdominal ganglion and raised to free the point of stimulation from the rest of the preparation. This area was then wiped with an absorbent tissue and covered with vaseline.

The recording electrodes consisted of two fine copper wires 0.1 mm in diameter insulated to the tip, and supported on a piece of 'Veroboard' which in turn was secured in a 'Prior' micromanipulator by means of a brass rod approximately 8 cm long. The muscle labelled 177a by Carbonell (1948) has its point of origin (the anterior tergal process) beneath a slight elevation of the cuticle close to the point of attachment of the hind wing (see Figures 1 and 3). This muscle is one of the more important branches of the main depressor of the coxa which inserts on a common apodemal tendon attached to the proximal end of the trochanter. Viewing from above with a binocular microscope, two small holes were made in the cuticle of the anterior tergal process using entomological pins. The recording electrodes were then inserted through the holes and the area covered with vaseline. It was important that the electrodes were not too far apart (no more than 1 mm) as being extracellular, they would be prone to record activity

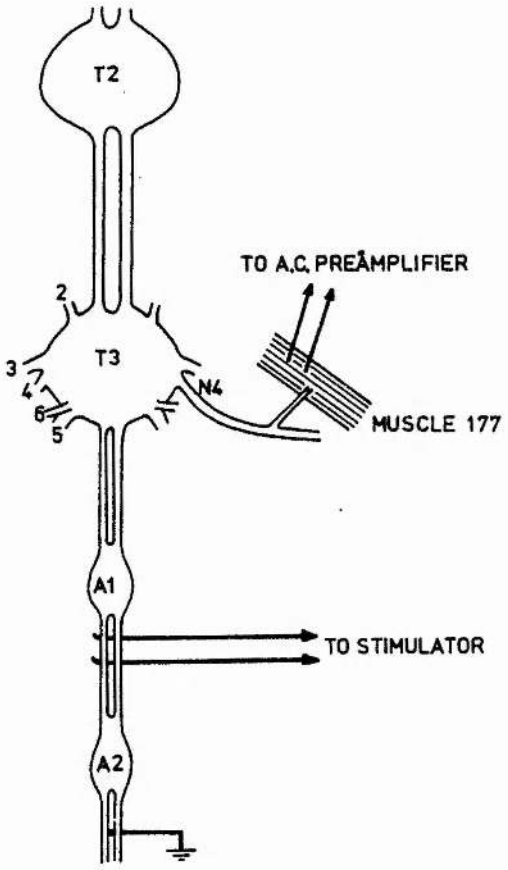


Figure 2. The experimental arrangement for stimulating and recording from the preparation (ventral view). T2 and T3, the second and third thoracic ganglia; A1 and A2, the first and second abdominal ganglia; 2-5, the ganglionic nerve trunks.

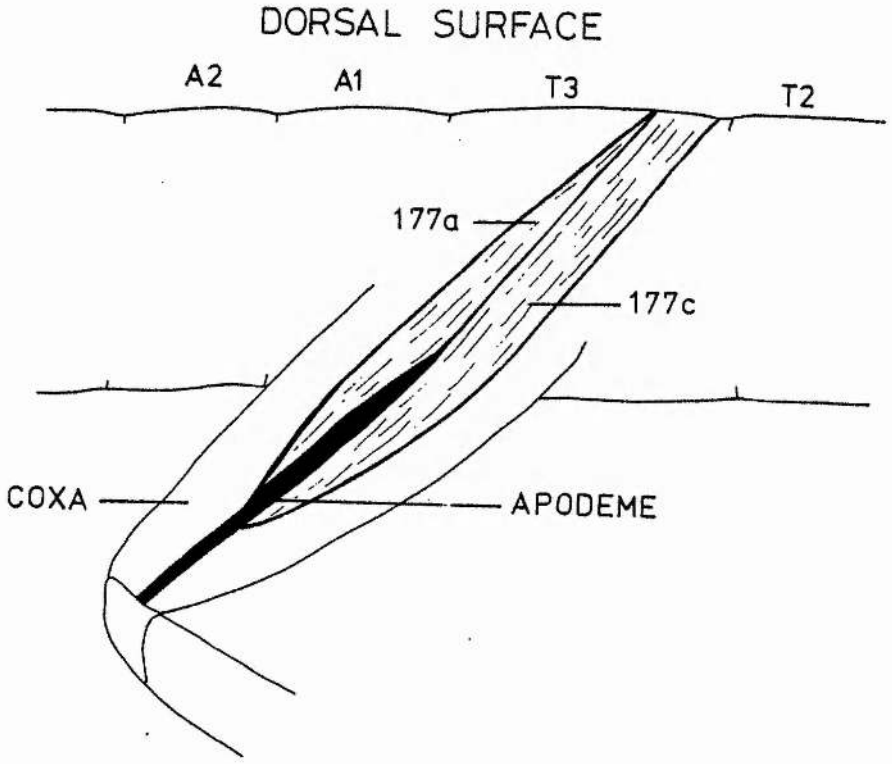


Figure 3. Lateral view of the coxal depressor muscles 177a and 177c. These muscles insert on a common apodemal tendon in the coxa and have their origin on the anterior portion of the metatergum.

from neighbouring muscles. The preparation was left to stabilise for 5-10 minutes.

2) Saline.

The saline used throughout these experiments was similar to that used by Kerkut, Pitman and Walker (1969) but in place of the 1 mM Tris-HCl buffer, 10 mM N-tris-(Hydroxymethyl) methyl-2-aminoethane Sulfonic Acid, (TES), was used. The composition was as follows:

Sodium Chloride	214 mM
Potassium Chloride	3.1 mM
Calcium Chloride	9.0 mM
TES	10 mM

The pH of the solution was adjusted to 7.2 with sodium hydroxide. The concentration of sodium and potassium ions was routinely checked on a flame photometer.

3) Stimulation, Recording and Display.

The preparation was stimulated by a 'Palmer' type 8128 stimulator, the output of which was isolated from earth and led directly to the stimulating electrodes. The output from the recording electrodes was fed into an 'Isleworth' type A102 differential A.C. preamplifier set to a gain of x100. The output was taken to a 'Tektronix' 5103N oscilloscope. The signals were also monitored through an audio amplifier and loudspeaker. Traces were photographed on 'Kodalith Ortho type 3' film directly from the screen of the oscilloscope.

4) Lesions to the Central Nervous System.

The following nerve trunks were severed: the left and right connectives between the second and third thoracic ganglia, and the left and right fifth nerve trunks of the third thoracic ganglion. Each of these can be seen under the binocular microscope as the cuticle is sufficiently transparent in these regions. The cuts were made with iris scissors previously wiped with ethanol. The animal was then put in a small sterilised aquarium. It was very important to provide a ready supply of water as loss of haemolymph during the operation caused significant dehydration. Complete section of the fifth nerve trunk (N5) was indicated by the leg becoming immobilised and raised to a more anterior and dorsal position (c.f. Jacklet and Cohen 1967).

Carbon dioxide anaesthesia was deliberately avoided as it has been shown to have relatively long-term effects on the electrical properties of cockroach motoneurones (Pitman, unpublished).

TABLE I

delay. time (ms) mean ± S.E.	number of animals	operation	P
4.4 ± 0.1	15	Normal	
4.4 ± 0.1	5	Ipsil.ant.conn.	> 0.5
4.3 ± 0.1	5	Cont.ant.conn	> 0.5
4.5 ± 0.1	7	Ipsil. N5	> 0.5
4.4 ± 0.1	5	Cont. N5	> 0.5

The effect of cutting various nerve trunks of the meta-thoracic ganglion on the delay time of the pathway illustrated in Figure 2.

Delay time - as described in text.

Ipsil.ant.conn. - ipsilateral anterior connective.

Cont.ant.conn. - contralateral anterior connective.

Ipsil. N5 - ipsilateral fifth nerve trunk.

Cont. N5 - contralateral fifth nerve trunk.

P - probability of results from normal and operated preparations being the same (as described on page 37).

RESULTS.

Spontaneous electrical activity was recorded from muscle 177a. This consisted of short bursts of action potentials every 5-30 seconds. Stimulating the preparation with 0.5-2.0 V, 0.5 ms pulses at 0.5 Hz gave rise to a single muscle response with consistent amplitude and delay time (Figure 4). The delay time was measured from the beginning of the stimulus (indicated by the stimulus artefact on the trace) to the beginning of the action potential response. In normal preparations this value was 4.38 ± 0.07 ms (n=15). Experimental animals were examined from 4-24 days after cutting one of the following metathoracic nerve trunks: (i) ipsilateral anterior connective, (ii) contralateral anterior connective, ipsilateral fifth nerve trunk (ipsil. N5) and (iv) contralateral fifth nerve trunk (cont. N5). None of these operations produced a significant change in the delay time (see Table 1).

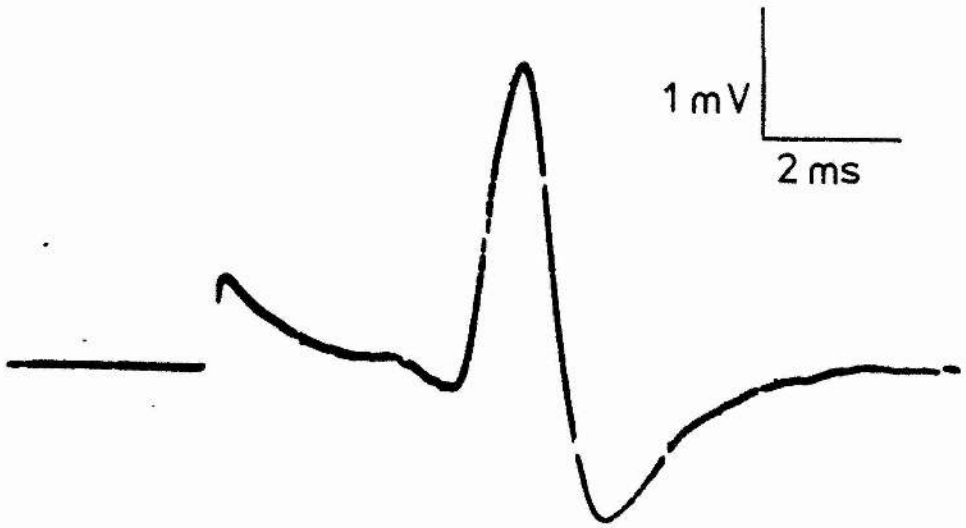


Figure 4. Extracellular record from muscle 177a showing a'fast' muscle potential evoked by stimulating the preparation between the first and second abdominal ganglia. Stimulus, 0.7 V, 0.5 ms. Initial deflection is a stimulus artefact.

DISCUSSION.

Stimulation of this preparation gave rise to a suprathreshold response in muscle 177a. This would indicate that the fibres of muscle 177a are innervated by the 'fast' type axons described by Usherwood (1961; 1967). Pitman (personal communication) has shown that this muscle is innervated by the motoneurone numbered 3 on the map of Cohen and Jacklet (1967). The axon of this cell leaves the ganglion through the fourth nerve trunk (N4). Dresden and Nijenhuis (1958) in their morphological study of the mesothoracic segment have shown that N4 contains one large axon (10-20 μ diam.) which innervates muscle 177a. This is apparently the only innervation received by this muscle.

Transection of the anterior connectives or the ipsilateral and contralateral fifth nerve trunks (N5's) produced no significant change in the delay time between the beginning of the stimulus and the onset of the muscle response. This could simply be because of an absence of afferent input via these nerve trunks, however this is unlikely. Pringle (1940) and Wilson (1965) have shown that sensory information from the hind leg influences the musculature of both the ipsilateral and the contralateral limb; the majority of this sensory information enters the ganglion via the fifth nerve trunks (Nijenhuis and Dresden, 1952). Transection of either the ipsilateral or the contralateral N5 would therefore be expected to reduce the afferent supply to the motoneurone innervating muscle 177a.

It is possible that deafferentation produced by section of these nerve trunks had no effect on this pathway through the ganglion. Alternatively a change in synaptic delay may have been induced but was not recognised because of the experimental technique. The delay time was taken as the time which elapsed between the onset of the stimulus and the beginning of the electrical response recorded from the muscle. Thus the delay time would be dependent upon a number of variables; the time taken for the stimulus to reach the presynaptic terminals, the synaptic delay, the time taken for the postsynaptic potential to reach the muscle and the neuromuscular synaptic delay. A change in synaptic delay may well have been masked by variation in these other events from one preparation to another. The experiments described in the next section were therefore designed in an attempt to overcome this problem.

SECTION II

The effects of denervation on 'Pathway B'

SECTION II. PATHWAY 'B'

METHODS.

A) MEASUREMENT OF GANGLIONIC DELAY.

1) Microelectrodes.

The microelectrodes were made from 'Pyrex' glass tubing 1.58 ± 0.025 mm O.D. and 1.47 mm I.D. The glass was pulled on a 'Narashige' type PE-2 vertical microelectrode puller. The microelectrodes were filled with 1M potassium acetate by the following method: the microelectrode was filled to within about 1 cm of the tip using a length of glass tubing attached to a 1 ml syringe. Holding the microelectrode upwards, a fine glass rod was inserted and passed towards the beginning of the taper where the liquid was drawn to the tip by capillarity. Care was taken to prevent the rod from penetrating the tip of the microelectrode, this being ensured by selecting a piece too large to enter the fine portion of the taper. The microelectrodes used had a resistance between 8 and 13 M ohms.

To reduce the damage caused by movement of the muscle, the electrodes were held on a floating mount (Figure 5). This consisted of a copper wire 2 cm long and 0.1 mm in diameter, soldered to a silver/silver chloride wire of 0.28 mm diameter bent into a coarse 'spring' at the end so as to form a tight fit inside the microelectrode. The whole unit was suspended from a 4 mm 'banana' plug which fitted into the input socket of the preamplifier.

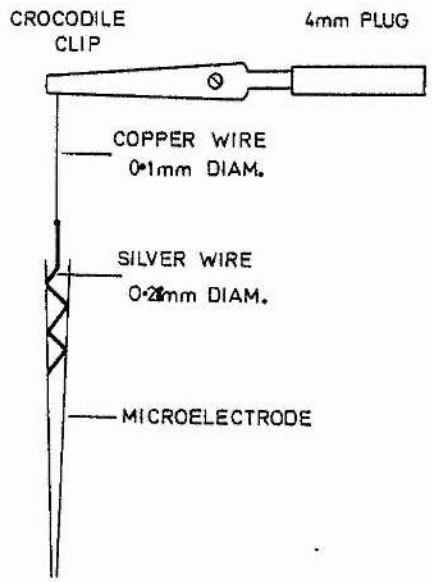


Figure 5. The 'floating mount' microelectrode holder. The silver wire was chloridised before inserting into the base of the microelectrode.

2) The Preparation.

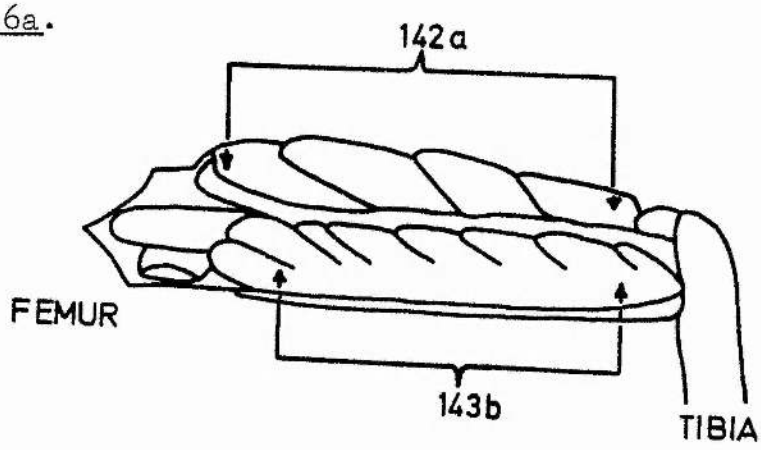
The head and all but the left metathoracic leg were removed and the animal pinned down on a 'Sylgard' resin block ventral surface uppermost. The leg was secured by carefully enclosing the sides in 'Plasticine' leaving the ventral surface of the femur lying horizontal. The first two abdominal ganglia were exposed by making a cut along the left side of the first and second sterna, gripping these with forceps and lifting them free of the underlying connective tissue by gentle separation with a seeker. The sterna were then cut loose. Each of the three thoracic ganglia were exposed as follows. The sternum was removed by first cutting the furca-sternum in two. The basisternum was then cut on either side and the sternum lifted free. The fifth metathoracic nerve trunk (N5) was exposed as far as the coxa by removing the cuticle and underlying tracheae. During the above operation the preparation was periodically bathed in saline.

The flexor tibiae muscles designated 143a, b and c (Dresden and Nijenhuis 1953) occupy the anterior and central regions of the femur, muscle 143b possessing by far the greater number of fibres (see Figure 6a). This muscle has points of origin along the whole length of the anterior femoral wall and inserts on the anterior side of the apodeme at the base of the femur. The microelectrode was inserted through a hole in the cuticle made with a fine entomological pin. A consistent recording site was maintained using the large spinal sensilla as landmarks. This is illustrated in Figure 6b. Care was taken not to disturb any of the underlying

Figure 6a. Ventral view of the left metathoracic leg, from Dresden and Nijenhuis (1953).

Figure 6b. Ventral view of the left metathoracic leg showing the position of the recording microelectrode. Same scale as above.

Figure 6a.



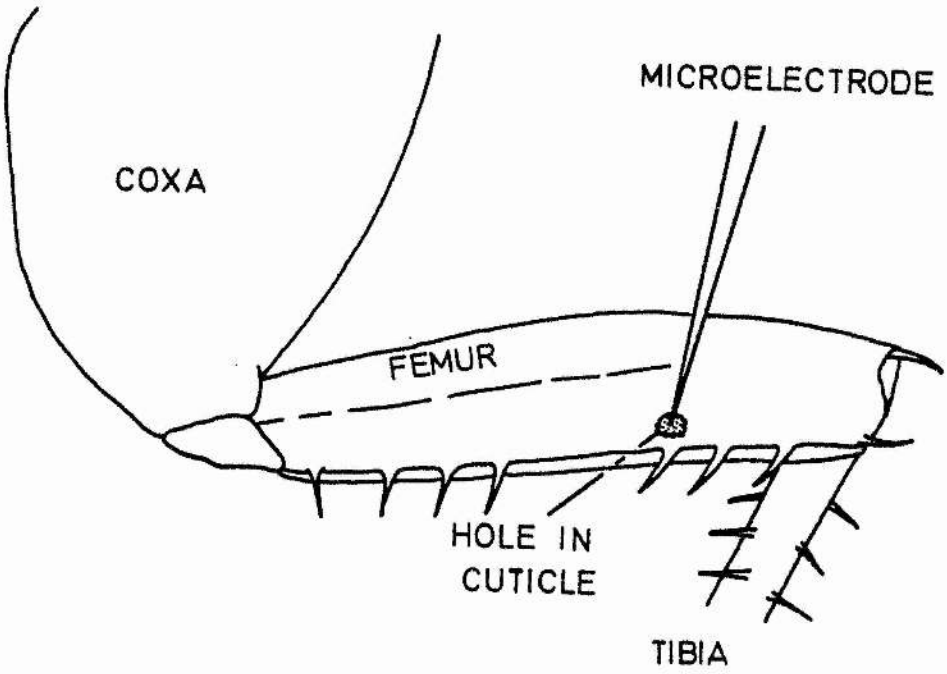


Figure 6b.

muscles when removing the cuticle. This was done by separating the tissues with a fine pin. The wound was immediately covered with a drop of saline.

The extracellular electrodes E1, E2 and E3 were the same as those described for Figure 2 except that only one hook of silver wire was used. The positions of these electrodes is illustrated in Figure 7. E1 was placed midway between the first and second thoracic ganglia by lifting the connectives with a seeker and sliding the electrode underneath using the micromanipulator. Similarly E2 was placed beneath the anterior connectives and E3 beneath N5, both as close to the ganglion without actually touching it. The electrodes were then raised to lift the nerve trunks clear of the body. The areas around the electrodes were dabbed with an absorbent tissue in order to prevent short-circuiting of the electrical impulse, and the entire preparation was covered with vaseline.

Observing through the binocular microscope, the microelectrode was lowered into the drop of saline above the hole in the cuticle of the femur. The electrode resistance was checked and the microelectrode lowered into the muscle until a fibre giving a stable resting potential was penetrated. The preparation was then stimulated using 0.05 ms pulses at a frequency of 2 pulses per minute. The stimulus strength was gradually increased from zero to a maximum of 5 volts until a response was produced in the muscle.

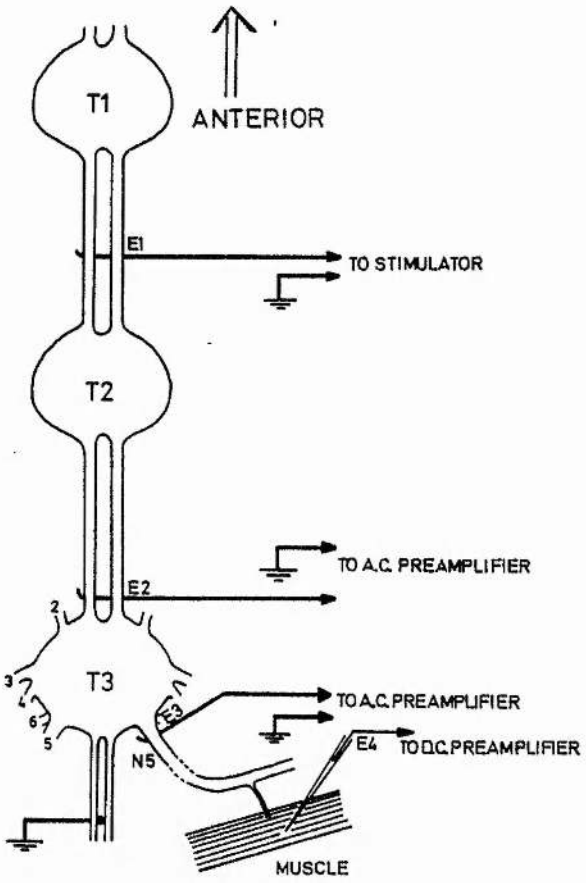


Figure 7. The experimental arrangement for stimulating and recording from the preparation (ventral view). T1-T3, the thoracic ganglia; N5, the fifth nerve trunk; E1-E3, silver wire hook electrodes; E4, glass microelectrode. The paired segmental ganglionic nerve trunks are numbered on the right side of the ganglion (2-6).

3) Lesions to the Central Nervous System.

The right N5 was severed in the experimental animals in the same manner as described in the previous section.

4) Recording and Display.

The preparation was stimulated in the same manner as described in the previous section with the negative output of the stimulator running to earth. The method of extracellular recordings was the same as that already described but one of the inputs to the differential amplifier was earthed. Intracellular muscle potentials were recorded using a unity gain high input impedance D.C. amplifier. This was mounted on a 'Prior' micromanipulator. The output from the amplifier was fed into a differential input on the oscilloscope.

5) Statistical Analysis.

The proabability of two populations of results being significantly different from each other was determined using a Student's two tailed t-test.

B) COBALT CHLORIDE STAINING.

Animals were pinned down ventral surface uppermost and the metathoracic ganglion exposed as described above. Working away from the ganglion, the left N5 and its ramifications were freed of connective tissue up to about 2 mm past the beginning of the femur and cut at this point. The

remaining nerve truks were severed and holding one of the anterior connectives, the ganglion and N5 were lifted free and transferred to the dye diffusion chamber (Figure 8). N5 was positioned so that about 1 mm of its distal end lay in the smaller compartment of the chamber, and using an electrically warmed piece of wire the bridge between the two chambers was sealed with vaseline. The end of N5 was cut obliquely to leave about 0.5 mm of the nerve in the smaller chamber which was then filled with 100 mM cobaltous chloride solution. The compartment containing the ganglion was filled with saline and the chamber placed in the refrigerator (4°C) in a humid environment for 18 hours. On removal from the chamber the ganglion was washed in saline and then immersed in saline containing 1% ammonium sulphide for 5-10 minutes where the cobalt was precipitated as black cobaltous sulphide. The tissue was dehydrated by passing it through a series of alcohols and finally immersed in creosote. The whole ganglion was observed under the microscope and photographed on 'Recordak Micro-File' film. Two photographs were taken: one with the preparation tilted from the horizontal 5° to the left; and the other with the preparation tilted 5° to the right. By mounting the prints of these photographs at a suitable distance apart, a stereo image of the ganglion could be obtained if viewed through standard stereo glasses.

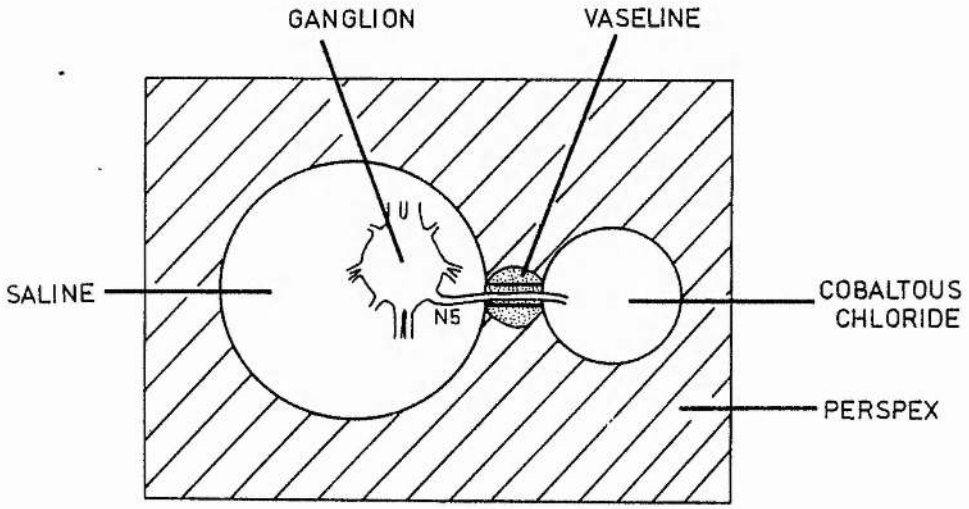


Figure 8. The dye diffusion chamber viewed from above.

C) INTRACELLULAR RECORDING AND STIMULATION.

1) Dissection.

The third thoracic ganglion was exposed and the leg prepared for intracellular recording from the flexor tibiae muscle 143b as described on page35. Entomological pins were used to secure the leg so that toxic components of the plasticine would not be released into the circulating saline. A pin was placed on either side of the coxa and another through the tibia. The nerve trunks N2, N3 and N4 on the right side of the animal were cut allowing a black piece of plastic to be slipped underneath the metathoracic ganglion. This enhanced illumination of the neurone cell bodies. The ganglion was then desheathed as follows: a small drop of 20% methylene blue was applied to the surface of the ganglion using the tip of a syringe needle, and immediately washed off. This procedure left the sheath lightly covered with the dye which was then grasped at the most anterior part of the ganglion close to the mid-line with two pairs of finely ground watchmakers forceps. The sheath was torn at this point by gently drawing apart the tips of the forceps, continuing until almost the entire ventral surface of the ganglion was desheathed. The whole preparation was then transferred to the recording chamber.

2) The Recording Chamber.

The recording chamber is shown in Figure 9. It consisted of a 'Perspex' chamber with a glass front of internal dimensions 5 x 6 x 7 cm. The walls were lined with

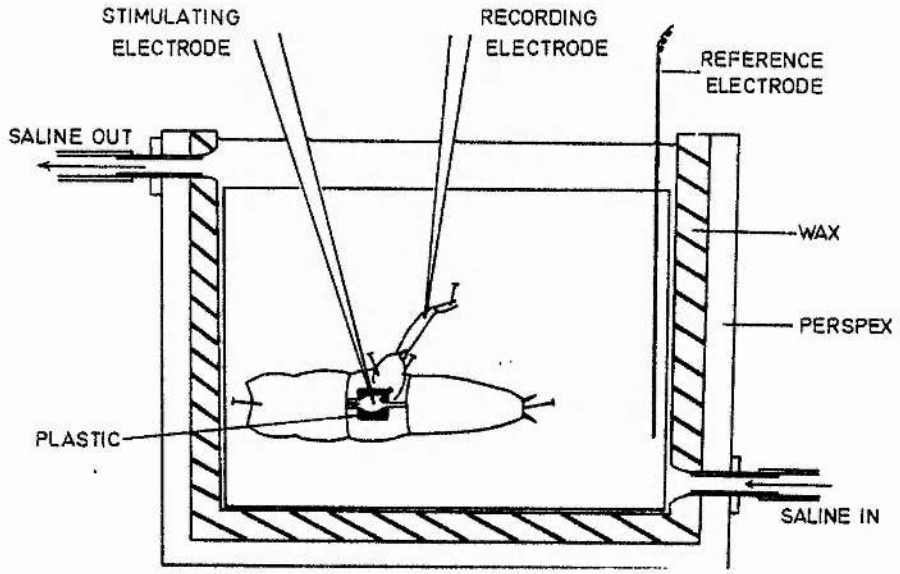


Figure 9. The recording chamber viewed from the front. A dark background for viewing the ganglion was provided by a thin sheet of black plastic; this was inserted after cutting the ganglionic nerve trunks on the animals right side.

paraffin wax so as to form a secure fit for the block on which the preparation was mounted. The block was held inclined at an angle of approximately 45° by filling the bath with wax. Saline solution was circulated by means of a vertical column of glass through which a 95% O_2, 5% CO_2 mixture was bubbled; this simultaneously circulated and oxygenated the saline. The reference electrode consisted of a silver/silver chloride wire 4 cm long and 0.28 mm in diameter suspended in the saline.

3) Observation and Illumination.

The preparation was observed through a x80 magnification 'Nikon' zoom binocular microscope held horizontally in front of the bath. The ganglion was illuminated from behind the bath by a beam of light projected at an angle of approximately 45°. The beam passed through a moveable lens and focused onto the preparation. By moving this lens the light path could be finely adjusted so as to illuminate any region of the ganglion and by adjusting the angle of incidence, cells previously unseen could be rendered visible. The system is essentially the same as that illustrated in Figure 16.

4) Recording and Display.

The system for recording the intracellular muscle potentials was identical to that described on page 37. The microelectrodes used to record from the motoneurones were of the same type as those used for the muscle but were filled with a 4% solution of procion navy blue and held in a 'Perspex'

electrode holder (see Figure20). The electrode holder was attached to a 4 mm plug which fitted into a socket forming the input of a unity gain high input impedance D.C. pre-amplifier. This was held in a 'Prior' micromanipulator as previously described (page34). The preamplifier was equipped with a bridge circuit to allow the cell membrane to be polarised during recording. Microelectrode resistance could also be monitored before and during the course of the experiment (see Figure19). A facility was provided for passing current directly through the recording microelectrode. This was operated by means of a relay within the preamplifier headstage which when activated connected the microelectrode directly to the stimulator. The output from the amplification system was fed into a differential input on the oscilloscope. The stimulating current was monitored by measuring the potential drop across a known resistance between the reference electrode and earth. The preparation was stimulated in the manner described on page28.

5) Structure of the Central Nervous System.

Wigglesworth (1960) classified the components of the more complex parts of the cockroach nervous system such as a ganglion as follows, proceeding from the periphery inwards: (i) a fibrous neural lamella - the sheath, (ii) a layer of cells - the perineurium, (iii) outer glial cells associated with a sinus system, and (iv) inner glial cells of the neuropile. The neuropile consists of axons and other neurone processes partially or entirely surrounded by folds

of glial membrane, the neurone cell bodies lying within the outer glial zone. The axons of these cell bodies branch within the neuropile where it appears that synaptic contacts with other cell processes are made. The general structure of the C.N.S. can be seen in Figure 10 which is a photomicrograph of a transverse section of the metathoracic ganglion.

The desheathing process, whilst to some extent disturbing the cells of the perineurium, did not remove the outer glial cells; it was therefore necessary to expose the neurone cell bodies. This was done by directing a jet of saline onto the ganglion surface through a broken off microelectrode attached to a 1 ml syringe. Care was taken to prevent the mainstream of the jet from hitting the neurones, and in this way, as far as could be seen under the binocular microscope, the outer surface of the cell bodies was cleared of any overlying tissue.

6) Penetration and Stimulation of the Motoneurone.

A fibre of muscle 143b was first penetrated in the manner described on page 36. The stimulating electrode was held in the recording mode and the electrode resistance checked by the same method used for the intracellular muscle microelectrodes.

The cell body of selected motoneurones were impaled with a microelectrode under visual control. Contact of the microelectrode tip with the motoneurone membrane was signalled by a small (approximately 2 mV) deflection in the potential

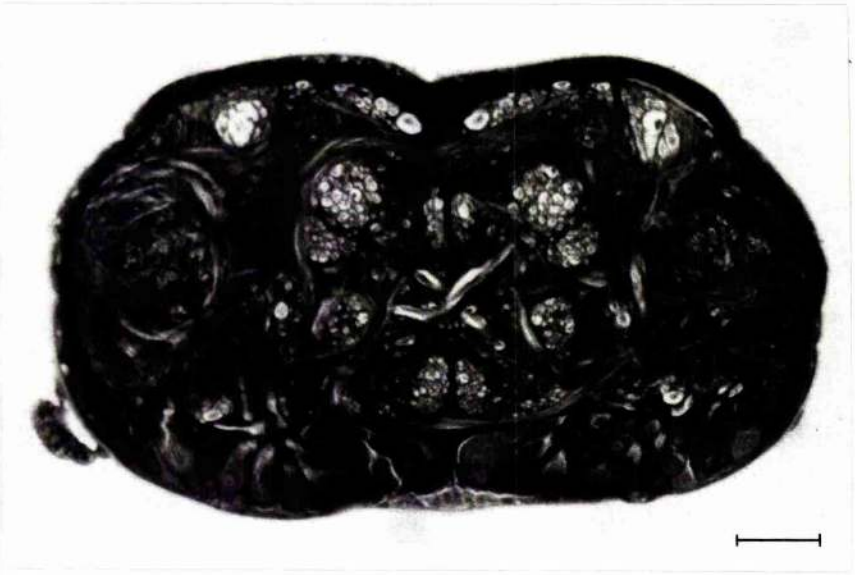


Figure 10a.

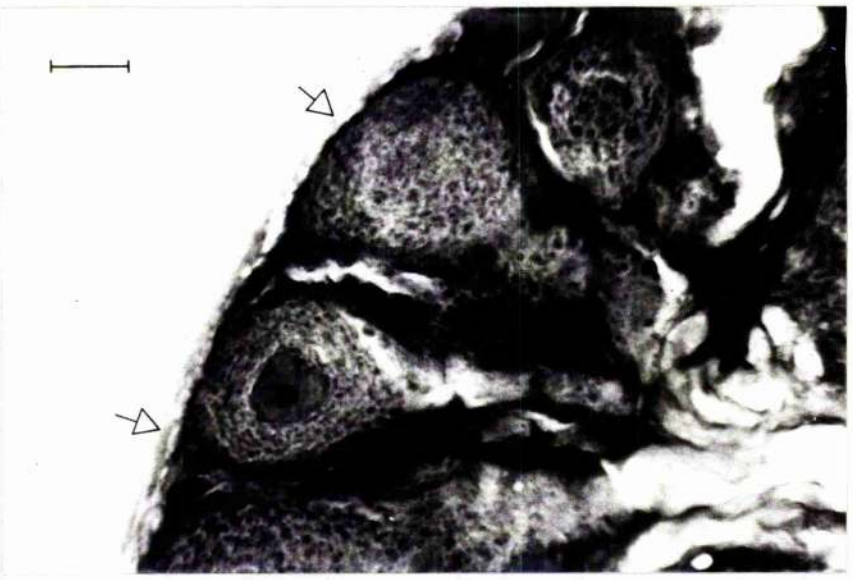


Figure 10b.

Figure 10. Cross section of a ganglion.

a) A transverse section through a metathoracic ganglion stained with 1% osmium tetroxide. This photograph shows clearly the bilateral symmetry of the ganglion and the sharp division between the neuropile area and the neurone cell bodies. These cell bodies are either motoneurones or interneurones, the majority being located near the ventral surface of the ganglion. Large bundles of fibres can be seen passing longitudinally through the ganglion in the neuropile. Horizontal bar, 100 μ.

b) A detail from a section similar to the above showing individual cell bodies. The axons of three neurones can be seen entering the neuropile (lower right). The arrows (⊳) show the neural lamella (sheath) which appears as a thin fibrous membrane at the surface of the ganglion. Horizontal bar, 10 μ.

recorded from the microelectrode. The microelectrode was then driven into the cell by passing 45 nA negative current pulses through the microelectrode tip. If the microelectrode tip did not penetrate the cell membrane it was lowered slightly and the procedure repeated.

When a stable resting potential (>50 mV) was obtained the relay in the preamplifier headstage was activated so that large amounts of current could be passed through the microelectrode to stimulate the motoneurone. 100 ms positive pulses provided by a 'Palmer' type 8128 stimulator were used for this purpose.

7) Procion Navy Blue Injection

The neurone was injected with procion navy blue by applying 100 ms negative pulses to the electrode at a frequency of 5 Hz. A 5 nA injecting current was usually used and the process continued for one hour (or less if the neurone became sufficiently stained). After completion of the experiment the ganglion was prepared and photographed as described on page 38.

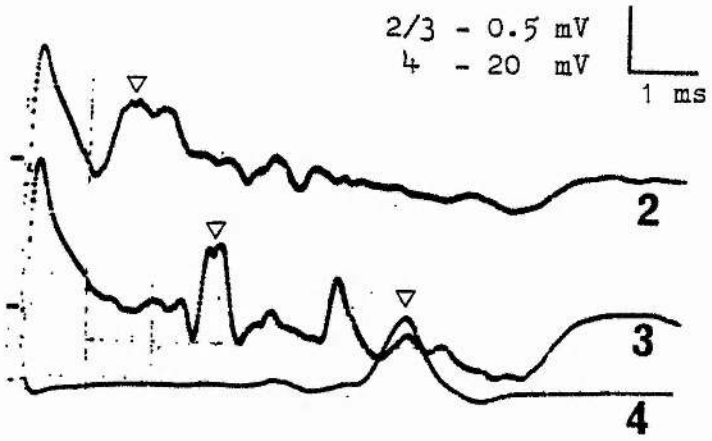
RESULTS.

Simultaneous recordings were made of the action potentials entering the ganglion (from the T2-T3 connectives) and leaving the ganglion (in the fifth nerve trunk) and of the activity of single units of muscle 143b (see Figure 7). Unfortunately a stable recording from the muscle fibre could only be maintained for a few minutes as the microelectrode tended to be dislodged by contraction. However this period was usually adequate if a fibre was penetrated about 30 seconds before stimulating the preparation. Stimulation usually produced 1 or 2 distinct depolarisations of the muscle fibre membrane with an amplitude of 20-30 mV and a duration of 1-3 ms; indicating that the muscle fibres are innervated by the 'fast' excitatory type axons described by Usherwood (1961;1967).

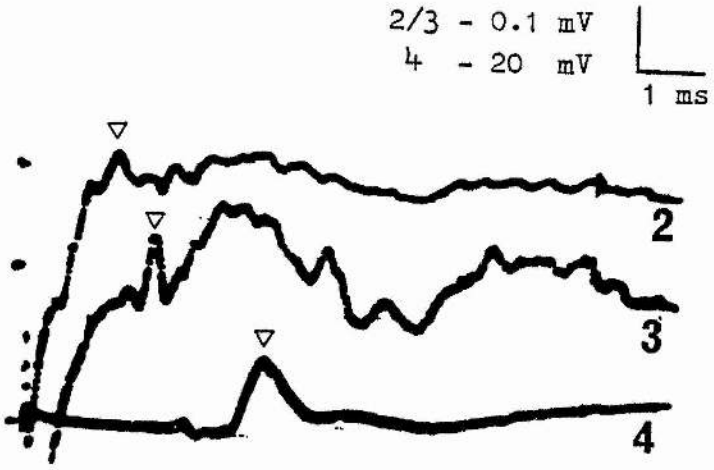
By reducing the strength of the stimulus a point was reached when the muscle response disappeared. This was usually accompanied by the disappearance of 1 or 2 action potentials recorded in the nerve trunks entering and leaving the ganglion. Only potentials which bore a 1:1 relationship in the anterior connective, N5 and the muscle fibre were chosen for analysis (Figure 11). No muscle response could be produced in 4 out of 10 control preparations and 9 out of 20 denervated preparations.

Figure 11. The response of a normal and a denervated preparation to stimulation of the anterior connectives between the first and second thoracic ganglia. 2,3 and 4, signals recorded by the electrodes E2, E3 and E4 respectively. 4, intracellular record from a fibre in muscle 143b showing the 'fast' muscle potential evoked by the action potential recorded in the fifth nerve trunk. The arrows (▽) indicate the signals which showed a 1:1 relationship. Initial deflection is a stimulus artefact.

Figure 11.



NORMAL PREPARATION



DENERVATED PREPARATION

1) Ganglionic delay.

The time taken for a signal to pass through the ganglion (ganglionic delay) was taken as the time which elapsed between the arrival of the signal at E2 and the recording of the postsynaptic response at E3 (Figure 11). The ganglionic delay in 6 normal preparations was 1.42 ± 0.07 ms indicating that a single synapse was probably present in the pathway (Roeder, 1967; Iles, 1972; Horridge and Burrows, 1974). The effects on delay of cutting the contralateral (right) N5 from 19-96 hours prior to the experiment are shown in Table II. No change in ganglionic delay was observed up to 10 minutes after cutting the right N5 in situ. Nerve section reduced the ganglionic delay by 14-57% within 22 hours. This modification was observed up to 96 hours after the operation, however no animals were examined after longer time periods. The mean ganglionic delay for all 10 experimental animals was 0.86 ± 0.10 ms. The difference observed between the control and the denervated preparations was statistically significant ($P < 0.005$).

2) Cobalt chloride staining.

In order to determine whether or not this change in ganglionic delay was accompanied by changes in the postsynaptic membrane an attempt was made to identify the cell body(ies) of the neurone(s) innervating muscle 143b. Cobalt chloride was passed along the fifth nerve trunk towards the ganglion; the cell bodies

TABLE II

Results obtained from stimulating the preparation illustrated in Figure 7 . The delay times were taken from the beginning of the stimulus artefact on the trace (Figure 11).

E2 (ms) = the time delay (in ms) between the stimulus and the beginning of the response recorded at the anterior connective.

E3 (ms) = the time delay (in ms) between the stimulus and the beginning of the response recorded at the fifth nerve leaving the ganglion.

Muscle (ms) = the time delay (in ms) between the stimulus and the beginning of the response recorded from a fibre of muscle 1$\frac{1}{2}$3b.

Gangl. delay (ms) = ganglionic delay = E3 - E2 ms.

TABLE II

	time after operation (hrs)	E2 (ms)	E3 (ms)	muscle (ms)	gangl. delay (ms)
CONTROL		1.2	2.6	4.6	1.4
		1.3	2.7	6.2	1.4
		1.0	2.3	4.5	1.3
		1.6	2.9	5.8	1.3
		2.0	3.7/4.2	4.6/6.6	1.4/1.4
EXPERIM.	48	1.2/1.6	1.8/2.2	5.0/6.8	0.6/0.6
	96	1.1	2.0	3.8	0.9
	24	1.3/1.5	1.9/2.2	3.7/6.2	0.6/0.7
	22	1.2	1.8/2.0	3.8	0.6/0.8
	46	1.5	2.4/2.6	4.2/5.2	0.9/1.1
	19	0.9	2.4	4.0	1.5
	24	1.3	2.1	4.9	0.8
	26	1.2	2.0/2.3	4.2	0.8/1.1
	24	0.9	2.1	4.2	1.2
	30	1.0/1.3	1.4/2.0	3.6	0.7/0.7

of the neurones whose axons pass through the nerve should then be filled with the dye.

Figure 12 shows the result of passing cobalt chloride through the fifth metathoracic nerve trunk on the right side of the animal. The dye entered the nerve trunk at a point corresponding approximately to the position of the trochanter of the leg. Most of the neurone cell bodies stained should therefore have axons entering or passing through the femur. This was not always the case since some of the inhibitory neurones of the leg muscles were invariably filled with the dye (see: Pearson and Bergman, 1969; Pearson and Iles, 1971; Pearson and Fourtner, 1973). However with these exceptions the cell bodies containing cobalt occupied an anterior and ventral position on the ipsilateral side of the ganglion. It is in this region that the motoneurone cell body(ies) innervating the flexor tibiae muscle would be expected to lie.

3) Electrophysiological identification of motoneurones innervating muscle 143b.

The resting potential of the cell bodies recorded through Procion Navy Blue (P.N.B.) filled electrodes was usually slightly lower than the value obtained through conventional potassium acetate filled electrodes. These varied between 45-60 mV. As in most cockroach motoneurones (Pitman, Tweedle and Cohen, 1972) the cell bodies occupying the anterior-ventral region

Figure 12.

Figure 13.

Figure 12. Photograph of a metathoracic ganglion seen from the ventral surface. Cobalt was introduced into the neurones through the left fifth nerve trunk which was cut between the 16th and 17th rami (designation of Nijenhuis and Dresden, 1954). All of the cell bodies stained are on the side ipsilateral to the infused nerve trunk and lie close to the ventral surface of the ganglion. The majority of these cell bodies occupy an anterior-lateral position. The two cells located close to the posterior mid-line are probably common inhibitory motoneurones identified by Pearson and co-workers (Pearson and Bergman, 1969; Pearson and Iles, 1971; Pearson and Fourtner, 1973).

Figure 13. Photograph of a desheathed metathoracic ganglion seen from the ventral surface. Procion Navy Blue was injected into the cell body of a neurone which when stimulated gave rise to synaptic potentials in muscle 143b (Figure 14). The cell body occupies an anterior-lateral position on the ventral surface of the ganglion. The position of the neurone is slightly distorted by the desheathing process.

of the metathoracic ganglion were not excitable. For this reason relatively large transmembrane currents were required to produce a response in the muscle. These currents varied between 50-500 nA when applied through the bridge circuit.

Five cells were penetrated which when sufficiently depolarised produced a response in muscle 143b. All of these cells were from different preparations and occupied approximately the same position in the ganglion (Figure 13). The muscle responses were equally consistent with amplitudes and durations characteristic of fibres innervated by 'slow' excitatory axons (Figure 14). Unfortunately no cell could be found which produced signs of fast excitatory innervation in the muscle.

At least one other motoneurone would be expected to innervate this section of the muscle. Dresden and Nijenhuis (1957) observed that taking the individual sections of muscle 143b as a whole, innervation was supplied by some 6 or 7 axons. These were classified as either 'medium' (5-10 μm diameter) or 'large' (10-20 μm diameter). It was concluded that each section received at least two axons. The two most likely reasons for this apparent failure to locate another motoneurone innervating this section of the muscle are: (i) other motoneurones innervating the muscle but with cell bodies in another region of the ganglion may have escaped the staining process, and (ii) the maximum

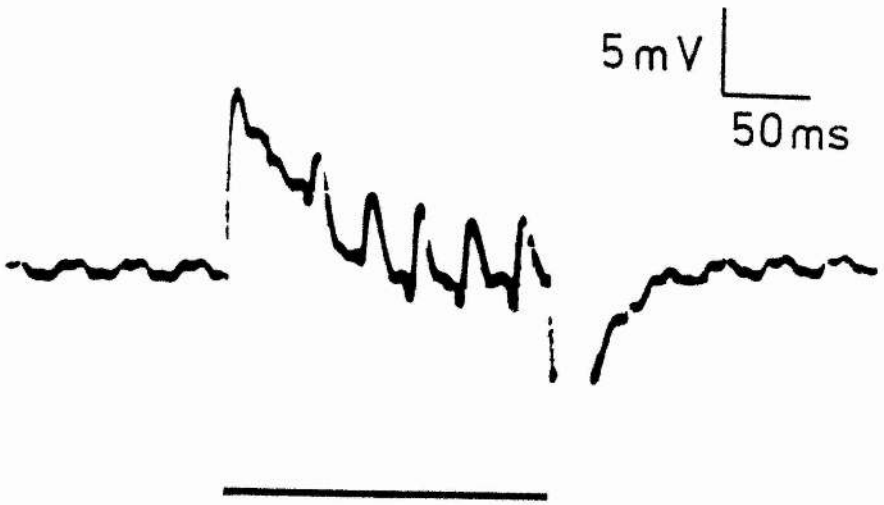


Figure 14. Intracellular record from a fibre in muscle 143b showing the 'slow' muscle potentials evoked by depolarisation of a motoneurone cell body in the meta-thoracic ganglion. Horizontal bar denotes duration of the stimulus.

current injected into the cell body could have been insufficient to excite its axon.

DISCUSSION.

In this cockroach preparation, stimulation of the anterior connectives produced a response in the flexor tibiae muscle 143b. The muscle response could be correlated with action potentials in the anterior connective and the fifth nerve trunk innervating the leg. The ganglionic delay for this pathway was approximately 1.42 ms suggesting the presence of a single synapse. Roeder (1967) estimated the synaptic delay of the cercal nerve-giant fibre preparation in the cockroach terminal ganglion to be about 1.3 ms. Iles (1972), after examining excitatory inputs to the fast coxal depressor motoneurone in the metathoracic ganglion estimated a mean synaptic delay of 1.5 ms between abdominal sensory interneurones and the motoneurone.

It is interesting to compare these results with those obtained by Horridge and Burrows (1974) who recorded from the soma of the fast extensor tibiae motoneurone (FETi) in the locust metathoracic ganglion. Impulses recorded from visual interneurones in the anterior connective showed a 1:1 relation with potentials recorded in the FETi cell body. The delay between the arrival of the presynaptic impulse at its entry to the metathoracic ganglion and the onset of the e.p.s.p. was about 0.9 ms. These results were taken to indicate that the pathway was monosynaptic. This locust preparation resembles the preparation examined in this study in that both pathways through the ganglion appear to be monosynaptic and both

are initiated by stimulation of the anterior connectives. Disparities in the estimated synaptic transmission times are to be expected between these two preparations since the postsynaptic potentials were recorded from entirely different locations.

Pearson, Stein and Malhotra (1970) demonstrated that the amplitude of action potentials recorded extracellularly in insect nerves are proportional to the square of the axon's diameter:

$$d = 7 \times \sqrt{V}$$

where d is the axon diameter in μm and V is the amplitude of the action potential in mV. Using this relationship the diameter of the axon innervating muscle 143b can be estimated. The mean amplitude of the action potential recorded in N5 which coincided with the electrical response recorded in muscle 143b was 0.6 mV. Therefore the size of the axon producing this action potential was approximately $7 \times \sqrt{0.6} = 5.4$ μ. In some preparations two such action potentials were recorded, both with similar amplitudes. In other preparations two muscle responses were recorded, each associated with separate action potentials of similar amplitudes. The time interval between these action potentials varied from 0.2-0.6 ms indicating that the impulses were produced by two separate axons. These results are compatible with the morphological study of Dresden and Nijenhuis (1957) who found that muscle 143b is composed of a number of separate sections

each section receiving at least two axons. In addition it was concluded that the muscle as a whole receives 9 axons, 4 of which were between 10-20 μ in diameter and 5 of which were between 5-10 μ in diameter.

The Effect of Cutting the Contralateral Fifth Nerve Trunk.

Following transection of the contralateral N5 it is assumed that recordings were made from the same axons as in the normal preparation. The action potentials recorded entering the ganglion through the anterior connective and leaving the ganglion through N5 appear to be produced by the same axons since (i) the response produced in muscle 143b bore a 1:1 relation to the responses recorded from these nerve trunks and (ii) the proportion of preparations in which two action potentials recorded in the nerve trunks gave rise to a single response in the muscle were about the same for both control and experimental animals. In addition the amplitude of these potentials were similar in normal and denervated preparations indicating that the axons carrying the action potentials were of about the same diameter. The reduction in ganglionic delay following denervation of the ganglion is therefore taken to represent a synaptic modification of the pathway.

Unfortunately it was not possible to identify the motoneurone involved in the postsynaptic events of the ganglionic pathway; the cell may have escaped the staining process or perhaps the maximum current injected into the cell body was insufficient to excite its axon. For this

reason the mechanism underlying the observed reduction in ganglionic (and probably synaptic) delay were not determined. Since this synaptic modification does not occur immediately after cutting the contralateral N5 the removal of some inhibitory control over the pathway would seem to be excluded.

Afferent input from the metathoracic leg of the cockroach has been shown to influence the muscluature of the contralateral limb. Pringle (1940) demonstrated an increase in the frequency of electrical activity recorded in the extensor tibiae muscle following stimulation of the contralateral N5. Similarly forced flexion and extension of the leg produced modifications in the frequency of the discharge recorded from the contralateral coxal extensor trochanteris muscle. Similar results have been obtained by Wilson (1965). Considering the co-ordination necessary for locomotion between the hind legs of the cockroach and the fact that the majority of axons enter or leave the leg through N5, it is likely that the afferent input reaching the flexor tibiae motoneurone would be affected by transection of the contralateral N5.

Thus denervation supersensitivity could well account for the observed reduction in ganglionic delay. The ganglionic delay was reduced within about 24 hours of nerve section; denervation supersensitivity in some vertebrate preparations follows a similar time course. The time course would obviously be dependant upon the mechanism, for example a proliferation of receptor sites

would be limited by the rate of protein production while a reduction in cholinesterase activity would be determined partly by the rate of turnover of the enzyme. Denervation of parasympathetic ganglion cells in the frog (Kuffler, Dennis and Harris, 1971) and the mudpuppy (Roper, 1976) causes a proliferation of receptor sites on the cell membrane within 48 hours. On the other hand Bird and Aghajanian (1975) noted an increase in the sensitivity of rat hippocampal pyramidal cells within 24 hours of denervation and concluded that this supersensitivity was the result of a reduction in cholinesterase activity.

Whether these changes are induced by a change in electrical activity or by withdrawl of some trophic factor has not been investigated here; such studies have so far been confined to supersensitivity in vertebrate skeletal muscle. In this preparation supersensitivity due to nerve degeneration is certainly a possibility; the orthograde degenerative process in insects can be extremely rapid. The first histological changes occur only a few hours after nerve section in the cricket (Edwards and Palka, 1971) and within 24 hours in Calliphora (Boeckh, Sandri and Akert, 1970). Hess (1960) observed changes in synaptic morphology in the cockroach CNS from 2-30 days after nerve section. Whether or not such changes occured at an earlier stage however was not investigated. Biochemical changes could of course take place before any morphological signs become visible.

CONCLUSION.

A pathway (probably monosynaptic) through the metathoracic ganglion incorporating one of the flexor tibiae motoneurones can be modified by transection of the contra-lateral fifth nerve trunk. This operation is believed to remove afferent input to the motoneuorone and produce a subsequent reduction in the synaptic delay time. It is suggested that this reduction in delay time is due to an increase in the excitability of the postsynaptic membrane to synaptic activation. In contrast the ganglionic pathway incorporating cell number 3 on the map of Cohen and Jacklet (1967) - a motoneurone to muscle 177a, showed no apparent change after transection ov various nerve trunks to the ganglion. It is possible that a small change in synaptic transmission may have occured but escaped detection because of the experimental technique; a relatively large number of events were involved between stimulation and response. However synaptic transmission in this pathway was very stable in that stimulation always produced a response in muscle 177a (n=37). In comparison, synaptic transmission in the pathway involving the flexor tibiae motoneurone was relatively labile; no muscle response could be produced in 4 out of 10 normal preparations and 9 out of 20 denervated preparations. It is therefore possible that the former more stable pathway is more resistant to modifications that might be produced by partial de-afferentation.

SUMMARY - CHAPTER I.

The effects of denervation on two nervous pathways through the metathoracic ganglion of the cockroach, Periplaneta americana, have been examined.

1) A single action potential was produced in muscle 177a in the metathoracic segment by a 0.5 ms stimulating pulse applied between the first and second abdominal ganglia. There was no apparent change in this pathway up to 24 days after cutting the left and right anterior connectives or fifth nerve trunks. The possible reasons for this stability are discussed.

2) Stimulation of the anterior connectives produced a response in the flexor tibiae muscle 143b. This muscle response could be correlated with action potentials in the anterior connective and fifth nerve trunk innervating the leg.

3) The ganglionic delay for this pathway (2) was 1.42 ± 0.07 ms (n=6) indicating the presence of a single synapse. This was reduced to 0.86 ± 0.10 ms (n=10) 19-96 hours after cutting the contralateral fifth nerve trunk. It is suggested that this reduction in ganglionic delay could result from an increase in the excitability of the postsynaptic cell caused by deafferentation.

4) The cell body of the flexor tibiae motoneurone involved in this pathway is probably located in the anterior-lateral region on the ipsilateral and ventral side of the ganglion.

CHAPTER II

ELECTROPHYSIOLOGICAL AND BIOCHEMICAL CHANGES IN DENERVATED METATHORACIC GANGLIA

INTRODUCTION.

Neurones whose axons have been cut either atrophy, degenerate or regenerate. Although generally more pronounced in the invertebrates than in the vertebrates, neurones from both groups show a remarkable capacity for regeneration of their axons. Before regeneration occurs the neurone cell body undergoes a series of marked morphological and chemical changes some of which are continued through the process of axonal growth. In the vertebrate neurone the entire morphological response to axonal injury is known as chromatolysis although the term was originally used to describe the enhanced staining which accompanied the initial breakdown of Nissl bodies in the perikaryon (Marinesco, 1897). However not all axotomised vertebrate neurones show this staining reaction. For this reason and in order to include the other cellular changes associated with axotomy the response to axonal injury in both vertebrate and invertebrate neurones will be referred to as the 'axon reaction' (Lieberman, 1971).

Separation of the axon from its cell body in the vertebrates results in a degeneration of the portion distal to the lesion (Wallerian degeneration). Changes in the cell body occur quite soon (about 24 hours) after injury in most neurones and are well developed within 2-3 days. The maximum response may take several weeks to develop after which the cell

body begins to revert back to its normal state. This final stage of the axon reaction may take up to several months.

The responses of cockroach central neurones to axonal injury have received extensive study, largely from Cohen and co-workers. For this reason and because of parallels between the axon reaction in cockroach and vertebrate neurones, most of the invertebrate material for this review will be drawn from work on the two cockroach species Periplaneta americana and Diploptera punctata.

1) THE AXON REACTION.

a) Basophil Material.

The early light microscope studies on axotomised vertebrate neurones revealed a dispersal of Nissl substance within the perikaryon. This phenomenon was first described by Nissl in rabbit facial motoneurones (1892) and later in spinal motoneurones (1894). These bodies of cytoplasmic bsophil material (Nissl substance) began to disintegrate 24 hours after injury. Within 2-3 days they were converted to fine dust-like granules. Subsequent studies have demonstrated a similar disintegration of Nissl bodies in many classes of vertebrate central and peripheral neurones.

There is general agreement that this process

starts centrally particularly in the region of the axon hillock (Nandy, 1968; Gersh and Bodian, 1971) and spreads to the periphery of the cell body (Nissl, 1892; Cerf and Chacko, 1958). Some authors (e.g. Nicholson, 1924) have reported a complete disapearance of basophil material after axon section.

In the cockroach some authors (Cohen and Jacklet, 1965; Cohen, 1967) have reported that central neurone cell bodies normally lack Nissl bodies. The perikaryon was described as showing a uniform basophilia due to the scarcity of endoplasmic reticulum and uniform distribution of ribosomes. However this is probably a result of the thick paraffin sections used which would tend to obscure fine profiles. Byers (1970) found clearly visible basophilic aggregates in thin sections of neurone cell bodies from a different cockroach species, Diploptera. Both species (Periplaneta americana and Diploptera punctata) show a marked redistribution of basophilia within the perikaryon after axotomy. An aggregate of basophilic material forms around the nucleus within 12 hours in Periplaneta and 24 hours in Diploptera. This increases in density to reach a maximum by about 3 days after axon section. Cohen and Jacklet (1965) refer to this basophil aggregate as a perinuclear ring. Although not strictly correct since the aggregate forms a three dimensional shell, it is intended to adopt their term here to describe

this phenomenon. The fine structure of the Nissl bodies in this perinuclear ring does not appear to differ from the normal situation; the bodies remain associated with mitochondria and other organelles (Byers, 1970). The same redistribution of Nissl substance occurs in injured neurones of other invertebrates e.g. cephalopod central neurones (Young, 1932). Although in most vertebrate neurones the situation is reversed i.e. the Nissl bodies aggregate around the periphery of the cell, the pattern in axotomised Purkinje cells appears to be the same as that found in cockroach neurones (Llinas, Precht and Kitai, 1967).

b) Protein and RNA Metabolism.

In the cockroach the first morphological changes are observed 12-24 hours after injury. These do not seem to be connected with regeneration as axonal growth occurs much later. Jacklet and Cohen (1967) suggest that the reorganisation is a constructive preparation for increased protein synthesis which occurs approximately 3 days later (Byers, 1970). Cohen (1967) obtained autoradiographical evidence that RNA synthesis is a major factor in the production of the basophilic aggregate. The perinuclear ring was shown to contain a concentrate of RNA; in sections of axotomised cells previously treated with ribonuclease the ring was absent. Using tritiated uridine it was shown that in axotomised cells a high concentration

of labelled uridine appeared in the perinuclear ring. This was taken to indicate that the RNA was newly synthesised. However using a similar technique in Diploptera, Byers (1970) concluded that Nissl bodies around the nucleus were not synthesised but moved from the periphery towards the nucleus. If animals were given tritiated uridine postoperatively there was no increase in the labelling of the perinuclear region but this was produced by preoperative administration (1-6 weeks). Therefore the perinuclear ring was formed from RNA present in the cell before its axon was cut. A similar migration of Nissl substance has been observed in axotomised vertebrate neurones (Smith, 1961; Porter and Bowers, 1963; Holtzman, Novikoff and Villaverde, 1967).

The dispersal of the RNA aggregate coincides with the emergence of axons from the cut central nerve stump 10-14 days after injury (Bodenstein, 1957; Guthrie, 1962). Protein production therefore seems to fall after axotomy and returns after the breakdown of the RNA aggregate.

In the vertebrate neurone, RNA synthesis also increases after axotomy. The first conclusive evidence of this was produced by Brattgard, Edstrom and Hyden, 1957). RNA was extracted from isolated neurones of the rabbit hypoglossal nucleus. Employing photometric techniques these authors were able to demonstrate a 50% increase in RNA content 15 days

after axotomy. This reached an increase of 100% after about 30 days and returned to normal by 80 days. Before the period of increased RNA production began the distribution of cytoplasmic RNA changed from large aggregates characteristic of Nissl bodies to a finely dispersed state. Watson (1965; 1968) measured the uptake of tritiated nucleosides in axotomised hypoglossal neurones of the mouse. The RNA content of the nucleolus was found to increase 2 days after axotomy coinciding with an increased rate of transfer of RNA from the nucleolus to the cytoplasm. The maximum increase in nucleolar RNA content occured during the period of maximal increase in nucleolar RNA synthesis.

Thus an interesting difference exists in RNA synthesis during the axon reaction of vertebrate and cockroach neurones. In the normal vertebrate neurone the cytoplasmic RNA is organised into Nissl bodies. Increased protein synthesis after axotomy is preceded by the dispersal of these aggregates. The cockroach on the other hand normally has its cytoplasmic RNA in a dispersed state. Before the neurone can begin protein synthesis for regeneration the RNA must first be attached to cisternae of the endoplasmic reticulum and once again disperse. Cohen (1967) considers this breakdown of the RNA aggregate in the cockroach to be analogous to the dispersal of RNA aggregates of chromatolysed vertebrate neurones.

In both cases this is followed by increased protein synthesis.

c) The Nucleus.

The majority of studies on the axon reaction in vertebrate neurones have shown changes in the position of the nucleus which becomes shifted to the periphery of the cell body within about 24 hours after axon section (e.g. Harkonen, 1964). In mammalian motoneurones the nuclei seem to migrate to the pole opposite to the axon hillock (Barr and Hamilton, 1948) whereas in ganglion sensory cells the nuclei tend to be displaced towards the axon hillock (Lieberman, 1971). The nuclei of cockroach central neurones are displaced from their central position towards the axon hillock. This differs from the vertebrate situation in that nuclear eccentricity does not appear until about 4 weeks after axon section (Jacklet and Cohen, 1967; Byers, 1970). However in both the vertebrates and the cockroach, the movement of the nucleus towards the cell periphery corresponds to the period of maximum axon growth and would seem to be associated with the increase in protein synthesis.

d) The Golgi Apparatus and Mitochondria.

Changes in the Golgi apparatus have been shown using metal impregnation techniques. After section of the axon in vertebrate neurones the Golgi

apparatus becomes shifted to the periphery of the cell and often completely disappears. This applies to both motor and sensory neurones (Penfield, 1920). Byers (1970) reported a reduction in the size of the Golgi bodies in cockroach central neurones but observed no change in their position.

Light and electron microscope studies have demonstrated an increase in the number and volume of mitochondria in axotomised vertebrate neurones (Hartmann, 1954; Hudson, Lazarow and Hartmann, 1961). The mitochondria of regenerating cockroach motoneurones in addition to becoming enlarged and less tightly packed together, gain a denser matrix under the electron microscope (Byers, 1970).

e) Enzyme Activity.

Vertebrate neurones undergo pronounced metabolic changes after axon section. This is illustrated by the numerous histochemical and biochemical studies on enzyme activity and distribution during the course of the axon reaction. Both the activity and distribution of various enzymes are usually reduced after axotomy, for example; enzymes of the tricarboxylic acid cycle (Watson, 1966a; Barlow, 1969; Lieberman, 1971b); acid phosphatase (Kawai, 1963; Soderholm, 1965); tyrosine hydroxylase and dopamine-B-hydroxylase (Kopin and Silberstein, 1972; Cheah and Geffen, 1973; Ross, Joh and Reis, 1975) and choline

acetylase (Hebb and Silver, 1963). This change in enzyme metabolism is usually reversible, lasts for several weeks and is apparently due to a fall in the rate of enzyme synthesis.

Numerous studies have been made on the acetylcholine hydrolysing enzyme acetylcholinesterase (AChE) in normal and axotomised vertebrate neurones. Investigation has been focused on this enzyme largely for two reasons: (i) it is present in large amounts in cholinergic and cholinoceptive neurones and (ii) there have existed for some time sensitive and specific biochemical and histochemical techniques for its detection. Because of its importance in this study AChE will be dealt with in a separate section.

2) CHOLINESTERASES.

a) A General Introduction.

Carboxylic esterases hydrolyse the carboxylic acid esters of alcohols, phenols and napthols. These esterases differ from each other in their preference for particular substrates and in their reaction to specific inhibitors (Augustinsson, 1948; Harkonen, 1964). Esterases have been divided into two major groups; non specific esterases (ns.Es) and cholinesterases (ChEs). Substances such as eserine and neostigmine specifically inhibit ChE activity but have no affect on the activity of ns.Es (Easson and Stedman, 1937; Richter and Croft, 1942). Cholinesterases are subdivided into two groups, each originally characterised by their substrate specificity: (i) non specific or pseudo cholinesterases (ns.ChEs) and (ii) acetylcholinesterase (AChE). Non specific cholinesterases hydrolyse long chain choline esters, such as butyrylcholine, more rapidly than does AChE (Ord and Thompson, 1952). Various biochemical studies have demonstrated acetyl-B-methylcholine to be a specific substrate for AChE and benzoylcholine for ns.ChE (e.g. Mendel and Rudney, 1943; Mendel, Mundell and Rudney, 1943). Alternatively, the two groups of cholinesterases can be distinguished by their reaction to specific inhibitors, such as B.W.284C51 dibromide, which inhibits AChE activity and ethopropazine hydrochloride which inhibits

ns.ChE activity (see: Klingman, Klingman and Poliszczuk, 1968).

b) Acetylcholinesterase.

A high activity of AChE is associated with almost all neurones receiving a cholinergic input. Sites of AChE activity have been found in association with the perikaryon membrane of central neurones in the cockroach (Iyatomi and Kanehisa, 1958; Smith and Treherne, 1965). The active site of this enzyme consists of an anionic (N^+ attracting) subsite and an esteratic (ester binding) subsite. Hydrolysis of ACh involves the formation of a reversible enzyme-substrate complex followed by acetylation of the esteratic subsite and release of choline into solution:

$$E + ACh \longrightarrow EACh + EA$$

where: E = enzyme; ACh = acetylcholine; EACh = enzyme-substrate complex; EA = acetylated cholinesterase. The acetylated cholinesterase is very unstable and reacts with water to give free enzyme and acetic acid. Choline reacts with the receptor site in a similar manner to ACh but with a much lower affinity. Hydrolysis of ACh is therefore equivalent to its inactivation. The competitive inhibitors such as eserine (physostigmine) and neostigmine bind to the esteratic site and thus render it inactive.

ACh receptors and the sites of AChE activity seem to be structurally linked together (reviewed by Silver, 1974). Little direct information is available concerning the localisation of functional AChE; whether it is present inside the neurone or on the surface membrane. The evidence at present suggests that 'external' AChE is responsible for the hydrolysis of ACh released during synaptic transmission and that 'internal' AChE forms a reserve store. Accumulating evidence favours the idea that the greater part of the enzyme associated with the axon and its terminals originates in the cell.

The distribution of cytoplasmic AChE demonstrated by histochemical techniques corresponds to that of the Nissl substance (see: Fukuda and Koelle, 1959) which in turn probably represents the rough endoplasmic reticulum where AChE may be synthesised and then transported to the surface of the cell and its' processes (Fukuda and Koelle, 1959). Biochemical studies show that AChE is associated with the intracellular membranes of the soma and nerve endings (Aldridge and Johnson, 1959; Holmstedt and Taschi, 1959). AChE activity seems to be localised in the endoplasmic reticulum in the pre- and postsynaptic membranes (Torack and Barrnett, 1962; Lewis and Shute, 1964). In the cockroach, sites of ChE activity seem to be concentrated on the cellular glial sheaths surrounding the neurone cell bodies and the axon membranes

within the neuropile (Smith and Treherne, 1965). However, Burt and co-workers (Burt, Gregory and Molloy, 1966), using the thiocholine technique of Gomori (1952), observed that 'certain' neurone cell bodies stained for ChE activity as heavily as the neuropile. Iyatomi and Kanehisa (1958) obtained similar results to Smith and Treherne (1965); ChE activity was detected in the cytoplasm but was strongest on the nerve sheaths and perikaryal membranes. The question of whether AChE is synthesised locally in the axon or transported from the cell body will be considered later.

3) CHANGES IN CHOLINESTERASE ACTIVITY AFTER AXOTOMY.

a) The Autonomic Nervous System

Sympathetic Ganglia

The first comprehensive investigation into the AChE activity of axotomised neurones was carried out by Sawyer and co-workers (Sawyer and Hollinshead, 1945; Sawyer, 1946). Using manometric techniques these authors reported a total loss of AChE from the superior cervical ganglion of the cat after postganglionic nerve section. Correlated with this was a reduction in the activity of ns.ChE. Qualitatively similar changes were observed in axotomised rat superior cervical ganglia (Brown, McLennan and Pascoe, 1952a; 1952b; McLennan, 1954). These authors demonstrated (manometrically) a 50% reduction in total cholinesterase activity 3 weeks after postganglionic nerve section. This was accompanied by a failure of ganglionic transmission while the ACh output to the blood remained normal (Brown, McLennan and Pascoe, 1952a; Brown and Pascoe, 1954). McLennan (1954) did not observe any hydrolysis of benzoylcholine by normal ganglia and so attributed this fall in hydrolytic activity entirely to a reduction in AChE. These results are at variance with those produced by Dhar (1958) and Klingman and co-workers (Klingman and Klingman, 1969) working on the same ganglion. Using AChE and ns.ChE specific

substrates, Dhar produced evidence of significant ns.ChE activity in this tissue. Postganglionic nerve section produced a reduction in both enzymes: 3 weeks after axotomy AChE activity was reduced by about 50% and ns.ChE activity by about 15%. Almost identical results were obtained by Klingman and Klingman (1969) using AChE and ns.ChE specific inhibitors.

These observations were confirmed histochemically by Brown (1958) and later in more detail by Harkonen (1964). In normal ganglia almost all of the cells showed a positive cholinesterase reaction. The AChE activity seemed to be concentrated along the intracellular membranes and fibres of tissue surrounding the cells. Non specific ChE was localised in the intercellular spaces and only a few cells showed positive activity in the cytoplasm. Postganglionic denervation produced an almost total disappearance of AChE from the chromatolysed ganglion cells, dendrites and preganglionic fibres. A corresponding fall in ns.ChE was also detected. The time course of the ChE response closely parallels the chromatolytic response. Concommitant with the gradual dispersal of the Nissl substance beginning some 12 hours after axotomy, the AChE and ns.ChE activity disappeared from the neurones within 2 days. Non specific esterase activity decreased somewhat during this period, becoming concentrated in the central area of the ganglion cells. 2-30 days after axotomy the Nissl substance could be seen only

in the periphery of the cell bodies. At the same time AChE and ns.ChE reactions remained negative in the perikarya and their intensity diminished in the interstitial tissue. After approximately 100 days the distribution of AChE and ns.ChE had returned to normal. However, the activity of AChE was significantly higher than in control cells whilst the ns.ChE activity appeared normal.

Parasympathetic Ganglia.

The ciliary ganglion has proved to be a convenient preparation for investigating the ChE response of cholinergic neurones to nerve section. Both histochemical and biochemical techniques have shown that mammalian ciliary ganglion cells are almost exclusively cholinergic, that is: ACh is the transmitter substance in the synapses of this ganglion (Giacobini, 1959; Koelle and Koelle, 1959). A high level of AChE activity has been demonstrated in the ciliary ganglion of mammals (Koelle and Koelle, 1959; Huikuri, 1966) and of birds (Szentagothai, 1957). Axotomy produces a reduction in the AChE activity of ciliary ganglion cells of both these groups (Taxi, 1961; Huikuri, 1966). Huikuri, working on this ganglion in the rat demonstrated histochemically a strong AChE activity in all synapses and postganglionic nerve fibres while only a few of the preganglionic nerve fibres, gave a positive reaction. AChE had almost totally disappeared within 3 days after

axotomy. Recovery began some 30 days later and appeared normal by the 60th day. In normal ganglia, ns.ChE seemed to be distributed randomly throughout the intercellular tissue and appeared in some of the cells. Axotomy produced a reduction of the enzyme in the interstitial spaces, with no activity apparent in any of the perikarya. The onset and recovery times of these changes in ns.ChE were similar to those observed for AChE. As in the superior cervical ganglion of the rat (Harkonen, 1964), most of the preganglionic synaptic terminals lost their AChE acitivity (however c.f. McLennan, 1954).

b) The Central Nervous System

Spinal Motoneurones.

The motoneurones of the spinal cord have been the subject of several histological and biochemical studies. Sites of AChE activity have been located in the cell bodies and dendritic processes of mammalian spinal motoneurones (Koelle, 1951; 1955a). In contrast ns.ChE seems to be localised in 'non-neural' structures such as the myelin sheath (Gerebtzoff, 1959) and glial cells (Cavanagh, Thompson and Webster, 1954).

In motoneurones of the spinal cord of the guinea-pig, Kumamoto and Bourne (1963) observed that division of the sciatic nerve produced a decrease in ns.ChE activity in a number of cells from 4-12 days after axotomy. The reduction in enzyme activity was

particularly strong around the cell periphery. Chacko and Cerf (1960), using specific inhibitors were able to demonstrate a reduction in AChE activity in the motoneurone cell bodies of the spinal cord of the bullfrog and common toad. The authors were unable to detect any sign of ns.ChE activity in this tissue either before or after axotomy. AChE was located mainly in the perikarya of the motoneurones and to a lesser degree in the region of the neuropile. The perikaryal AChE activity fell dramatically from 4 days after axotomy, however no attempt was made to study the intracellular distribution of the enzyme.

In apparent contrast to amphibian spinal motoneurones, normal motoneurones of the spinal cord of the rat show only a moderate perikaryal AChE activity while the enzyme activity of the synapses and nerve fibres is intense (Soderholm, 1965). Axotomy produced a decrease in AChE activity in the cell bodies after 3-70 days and a decrease in the synaptic activity after 5-130 days. Ns.ChE in the anterior horn of the spinal cord was confined to the capillaries and showed no change after axotomy. It is interesting that the reduction in AChE activity of these motoneurones was restricted to the peripheral regions of the cell body, bearing a close resemblance to the reaction of ns.ChE observed in axotomised spinal motoneurones of the guinea-pig (Kumamoto and Bourne, 1963).

The Brain Stem.

Investigations into the response of motoneurones of the brain stem to axon section have been confined almost exclusively to the region of the hypoglossal nucleus. This is understandable since these motoneurones can be axotomised by a relatively simple operation - section of the hypoglossal nerve in the neck region. Under the light microscope the hypoglossal nucleus is seen to contain many large motoneurones. Many of the changes characteristic of chromatolysis in spinal motoneurone cell bodies are not seen in hypoglossal neurones of the rat. For example changes in the position of the nucleus have not been observed. However dispersal of cytoplasmic basophilia does seem to occur during the early stages of the axon reaction (Cammermeyer, 1963).

Schwarzacher (1958) demonstrated, histochemically, a signifificant AChE staining reaction in the cell body and processes of hypoglossal neurones. Within 4 days of cutting the hypoglossal nerve AChE was reduced reching its lowest level after about 16 days. This change in the enzyme paralleled the chromatolytic response and returned to normal after about 5 weeks. Lewis and co-workers reinvestigated this phenomenon using the electron microscope (Lewis and Shute, 1965; Flumerfelt and Lewis, 1969). Most of the cytoplasmic AChE activity was found to be associated with the rough endoplasmic reticulum localised in scattered areas

within the lamellae. Ns.ChE was also found distributed within the endoplasmic reticulum of some motoneurones. Extracellular ChE consisted of AChE only and appeared to be associated with synaptic structures and axons. Within 2 weeks after axotomy much of the AChE and all of the ns.ChE activity had dissapeared. This seemed to be associated with fragmentation of the endoplasmic reticulum.

4) AXOPLASMIC TRANSPORT OF CHOLINESTERASES.

The changes which follow axotomy seem to be due largely to the failure of some substance(s) reaching the cell body by axoplasmic transport (reviewed by Cragg, 1969). It is now widely accepted that proteins and low molecular weight compounds are carried from the soma to the axon (orthograde transport) and from the axon to the soma (retrograde transport).

Autoradiographid studies have shown that labelled protein originating in the cell body moves down through the axoplasm as a discrete band (Droz and Lebland, 1963; Taylor and Weiss, 1965). This protein may be associated with neurotubules and neurofilaments (Droz, 1966) or with soluble protein (Ochs, Johnson and NG, M-H., 1966) There seem to be several rates of orthograde axoplasmic transport but these can be placed into one of two categories: fast axonal transport and slow axonal transport. The bulk of the protein transported down the axon from the soma is carried by slow axonal transport - 1-5 mm/day (Koenig, 1958; Taylor and Weiss, 1965; Bray and Austin, 1968). Some protein is carried to the nerve endings and across the synaptic gap to the postsynaptic cell by fast axonal transport - 100-500 mm per day (Miani, 1963; Lasek, 1968).

Various studies have shown that AChE accumulates proximal to a ligature. This lead to the proposal that the enzyme is synthesised in the cell bodies of neurones and transported along the axon towards the nerve

ending (Hebb and Waites, 1956). This has since been confirmed by experiments on axoplasmic transport of AChE. Lubinska, Niemierko and Oberfield (1961), using biochemical techniques, were able to demonstrate a gradient of AChE activity along the sciatic nerve of the dog. After nerve section the accumulation of the enzyme at the proximal nerve stump was accompanied by a depletion of enzyme in the more proximal parts of the nerve. Similar accumulations of AChE activity have been observed in transected sciatic nerves of the rabbit (Zelena and Lubinska, 1962), cat and rat (Lubinska, Niemierko, Oderfeld, Szwarc and Zelena, 1963) and frog (Klodos and Niemierko, 1968). By examining the AChE activity in isolated segments of the sciatic nerve of the dog Lubinska and Niemierko(1971) were able to demonstrate a movement of the enzyme in both directions along the nerve. The intensity and velocity of the migration was greater in the descending (towards the peripheral stump) than the ascending stream (away from the peripheral stump). Ochs and Ranish (1969; 1970) observed the movement of tritiated leucine in cat sciatic nerves after injection of the lumbar seventh ganglia. The velocity of movement did not change when the nerve was transected just distal to the ganglion.

In conclusion, the evidence at present suggests that following axon section: (i) AChE transport along the axon towards the periphery continues and (ii)

AChE synthesis in the cell body is reduced or eliminated. It is interesting that the reduction in the catecholamine biosynthetic enzyme dopamine-β-hydroxylase, which occurs in axotomised central noradrenergic neurones, has been shown to be due entirely to a diminished rate of synthesis (Ross, Joh and Reis, 1977). The reduction in ChE activity seems to occur during the period of increased protein production. During this recovery phase protein synthesis associated with regeneration apparently takes priority over protein synthesis related to neurotransmission.

SECTION I

The effect of axotomy on the electrical characteristics and drug responses of the cell body of the fast coxal depressor motoneurone (D_f).

PLAN OF EXPERIMENTS. - SECTION I.

A fall in the ChE activity associated with the cell bodies and dendrites of neurones in the cockroach CNS after axotomy would be expected to result in an increase in the ACh sensitivity of the neurone. This possibility has been investigated in the cell body of an identified neurone - the fast coxal depressor motoneurone numbered 28 by Cohen and Jacklet (1967) located in the third thoracic ganglion (Figure 15). This is identical to the neurone designated D_f by Pearson and Iles (1970). The cell body of this neurone is particularly large (about 50 µm in diameter) and can be located visually in the whole ganglion preparation.

Cell 28 innervates the coxal depressor muscles 177d, 177e, 178 and 179 through the fifth ganglionic nerve trunk (Carbonell, 1947; Pearson and Iles, 1970). This motoneurone can therefore be axotomised by transection of the fifth nerve trunk at the base of the leg.

TRANSMITTER SUBSTANCES IN THE COCKROACH CNS.

The most probable excitatory transmitter in the cockroach CNS is ACh (reviewed by Sakharov, 1970; Pitman, 1971). Colhoun (1958) found an increase in the ACh content of cockroach central ganglia following electrical stimulation. Treherne and co-workers have demonstrated high levels of ChE in the same tissue (Treherne and Smith, 1965; Smith and Treherne, 1965).

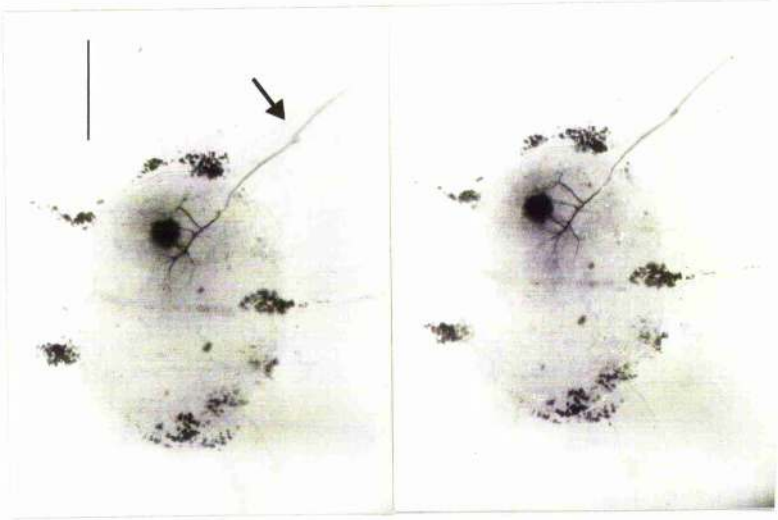


Figure 15. Stereo photographs of the metathoracic ganglion showing cell 28 stained by injecting cobalt chloride into the cell body. A three dimensional image can be obtained by observing these pictures through standard stereoscopic viewing glasses. The entire ganglion was mounted ventral surface uppermost on a depression slide and immersed in creosote. The cell body is located near the ventral surface of the ganglion with the initial process running towards the dorsal surface. The axon leaves through the fifth nerve trunk (arrow) on the animals' left side. The anterior edge of the ganglion is towards the left of the page. Vertical bar, 0.5 mm.

Pharmacological studies on the cercal nerve-giant fibre interneurone synapse have produced compelling evidence for cholinergic transmission at this synapse. Hemicholinium-3 (HC-3) produces breakdown of cholinergic transmission either by preventing uptake of choline (MacIntosh, 1959) or by disrupting ACh binding sites (MacIntosh, 1961). HC-3 in 10^{-3} M concentrations applied to the terminal ganglion produces synaptic blockade with repeated stimulation within 1 hour. The radiolabelled snake neurotoxin ^{125}I-α-bungarotoxin seems to bind specifically and with high affinity to ACh receptors (Lee, Tseng and Chiu, 1967; Potter, 1974). A correspondence has been shown between the ability of various cholinergic ligands to inhibit ^{125}I-α-bungarotoxin binding in the abdominal nerve cord with their competence to modify transmission at the cercal nerve-giant fibre synapse (Gepner, Hall and Sattelle, 1978; Sattelle, Gepner and Hall, 1979).

Pitman and co-workers applied ACh to the soma of cockroach dorsal unpaired medial (DUM) cells in the terminal ganglion by iontophoresis (Kerkut, Pitman and Walker, 1969; Pitman and Kerkut, 1970). The threshold sensitivity was shown to be about 10^{-13} M; the same order as for snail central neurones and the crustacean neuromuscular junction. The estimated reversal potential for excitatory postsynaptic potentials (epsp's) recorded in the soma was similar to that of the response to iontophoretically applied ACh. In addition

both the ACh response and the epsp were potentiated by physostigmine and antagonised by gallamine.

γ-amino butyric acid (GABA) is a possible inhibitory transmitter in the cockroach CNS. The nerve cord has been shown to contain GABA in concentrations of the same order as those found in mammalian brain (Ray, 1964). Gahery and Boistel (1965) showed that GABA in 10^{-2} M concentrations blocked synaptic transmission in the sixth abdominal ganglion. When applied iontophoretically to the soma membrane of central neurones, GABA hyperpolarises the cell in 10^{-13} M concentrations (Kerkut, Pitman and Walker, 1969; Pitman and Kerkut, 1970). The estimated reversal potentials and ionic permeability changes for inhibitory postsynaptic potentials (ipsp's) and responses to iontophoretically applied GABA were similar.

METHODS.

1) The Bath.

The experimental bath is illustrated in Figures 16a and 16b. The sides were made from 6 mm 'Perspex' and the front of glass cut from an ordinary microscope slide. The internal dimensions of the bath measured 2.8 x.2.2 x 1.8 cm, the volume being adjusted to 6 ml with wax which formed an inclined surface on which the block holding the preparation rested. Solutions were added by means of a glass tube (1.6 mm external diameter) opening at the base of the preparation holder, and removed through similar tubing held directly above the inlet so as to give a constant volume of 2.5 ml. The bath was completely filled in this way with the colouring produced by methylene blue in 20 seconds, and completely cleared by washing with saline in 1.5 minutes. The preparation was held dirctly above the point of entry of the solutions on a block of black 'Perspex' measuring 4.0 x 2.0 x 0.2 cm. The saline was circulated and oxygenated by a jet of 95% O_2/ 5% CO_2 directed obliquely at its surface.

2) Observation and Illumination.

The preparation was observed and illuminated as described on page 40 (see Figures 16a and 16b) but the fine control of the lighting was made by moving the whole bath with respect to the beam of light.

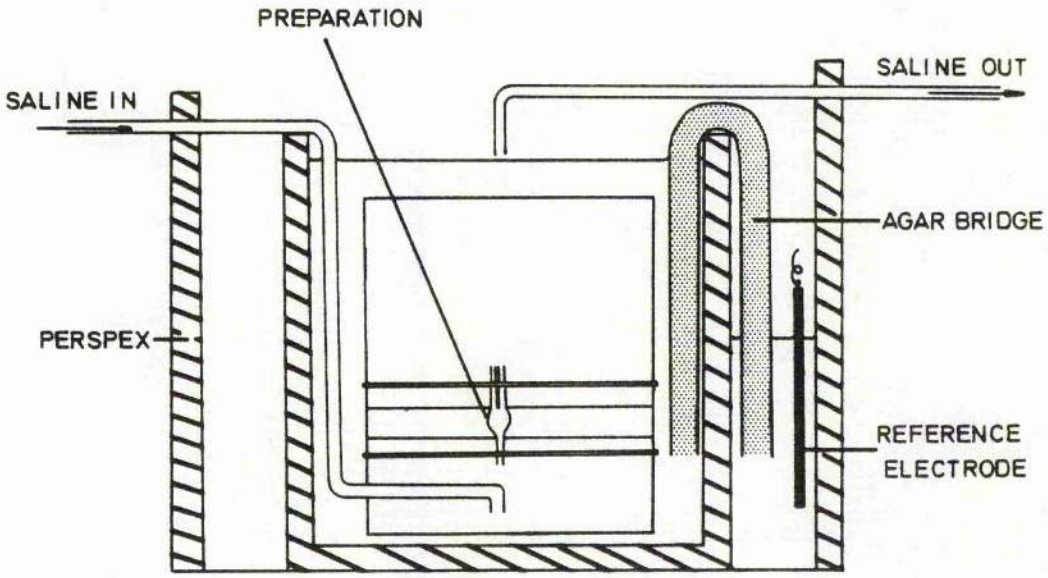


Figure 16a. The experimental bath viewed from the front.

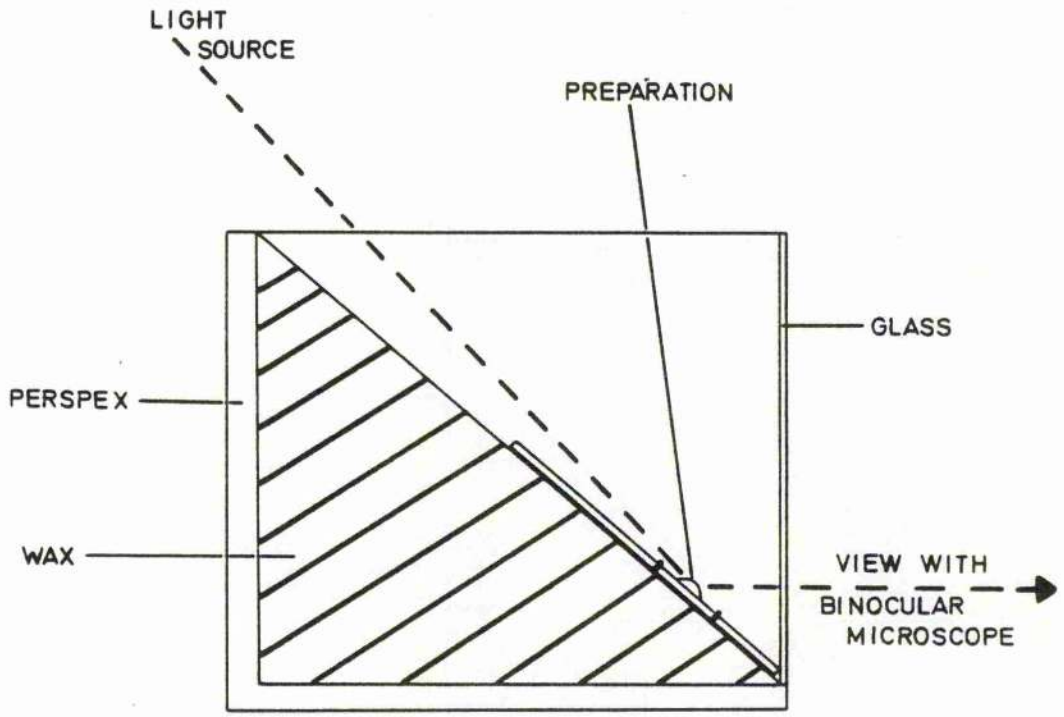


Figure 16b. The experimental bath viewed from the side.

3) The Preparation.

The CNS was exposed from the prothoracic to the second abdominal segment as described on page 39. The connective lying just posterior to the second abdominal ganglion was held in a pair of forceps, and the lateral nerve trunks cut free as far as the prothoracic ganglion. This left the exposed portion of the CNS free to be removed and placed on the preparation block where it was secured by two elastic bands; one across the connectives joining the meso- and metathoracic ganglia and the other between the first and second abdominal ganglia. The metathoracic ganglion was positioned ventral surface uppermost across a strip of 'Araldite' approximately 1 cm from the bottom of the block.

The metathoracic ganglion was desheathed as quickly as possible, in the manner described on page 39, and the preparation block transferred to the bath. The oxygenating system was turned on and cell 28 located using the following two morphological landmarks. (i) cell 28 usually lies close to and lateral to cell 27, another large motoneurone. The orientation of these two cells to one another, together with a slight separation from the general mass of neurone cell bodies in this area, makes them quite distinct. (ii) The ventral side of the metathoracic ganglion possesses a bilaterally symetrical tracheal system in the shape of two V's; one on either side, with the taper of the V pointing away from the centre. Cells 27 and 28 usually lie within the

last posterior bifurcation of these tracheae. The glial and other unwanted tissues covering the surface of cell 28 were removed as described on page 42. A prepared ganglion is shown in Figure 17.

4) Microelectrodes.

The recording microelectrodes were prepared in the manner described on page 34. They were filled with 3M potassium acetate since chloride ions diffusing from microelectrodes filled with 3M potassium chloride can cause an increase in the internal chloride ion concentration and a consequent alteration in inhibitory synaptic potentials (Kerkut, Pitman and Walker, 1969).

The iontophoretic microelectrodes were made in a similar manner to the recording electrodes and filled with 0.1 M acetylcholine chloride. The electrical resistance of these microelectrodes was checked for every experiment and varied between 60-100 M ohms.

5) Recording and Display.

A block diagram of the apparatus is given in Figures 18 and 19. The recording system is the same as that described on page 40, but the input to the oscilloscope of one of the channels was taken, in addition, to a 'Brush' mark 220 D.C. pen recorder set to a gain of 10 mV/division. The neuronal membrane was polarised by passing current through one of the microelectrodes set in the 'stimulating' mode, and connected to a 'Grass' S44 stimulator through a 100 M ohm resistor. The retaining current to the iontophoretic electrode was supplied

Figure 17. Photograph of a desheathed metathoracic ganglion mounted ventral surface uppermost in the experimental bath. The cell bodies of both the left and right cell 28s have been filled by injecting Procion yellow through a micro-electrode (shown impaling the cell 28 on the animals' right side). Note that the stained cells lie within the last posterior bifurcations of the tracheal system (see text). The anterior connectives can be seen in the upper half of the photograph.

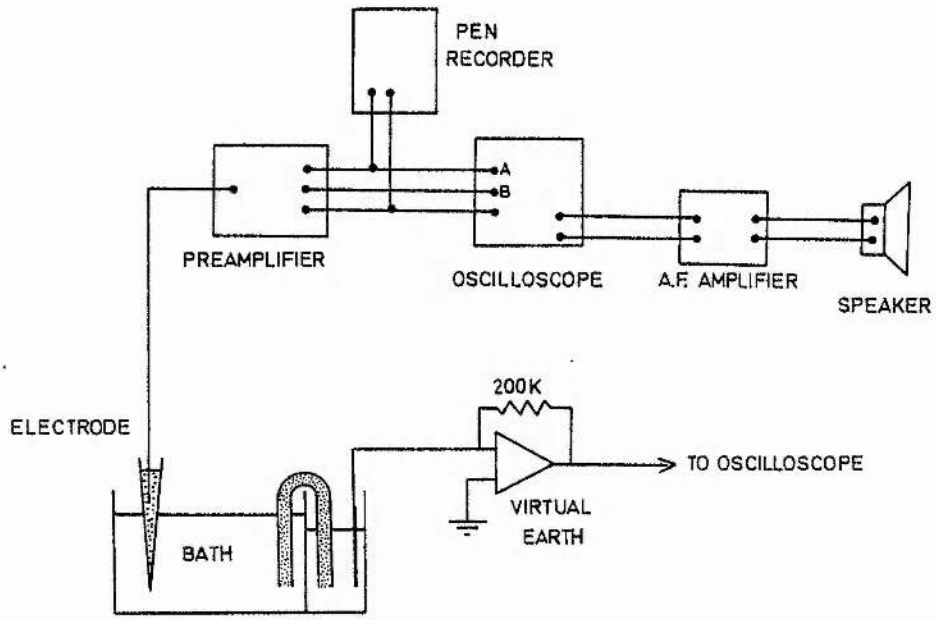


Figure 18. Block diagram of the apparatus used for intracellular recordings. Currents passing through the bath were monitored by measuring the potential difference across the 200 kohm resistance incorporated into the 'virtual earth' circuit.

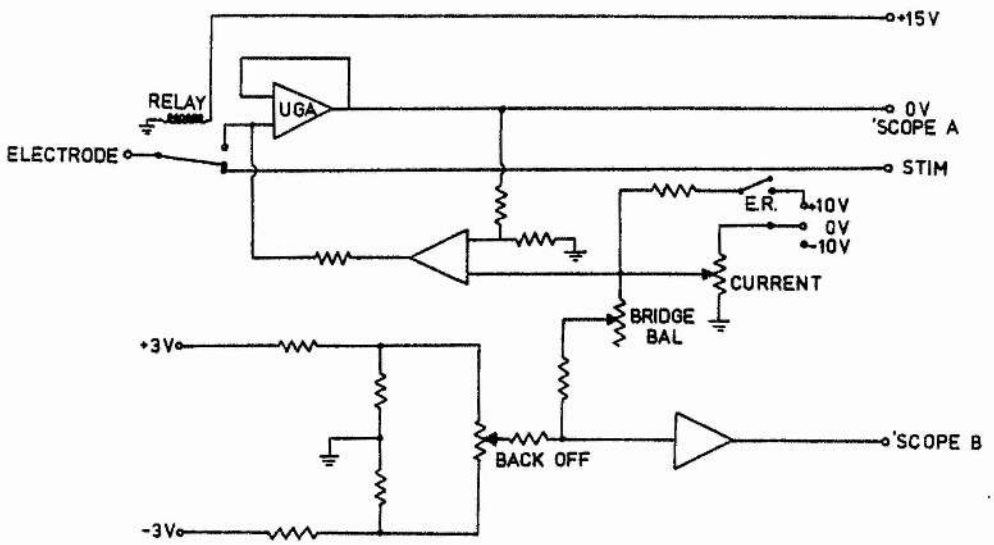


Figure 19. Simplified circuit diagram of the apparatus used for intracellular recording and stimulation. The apparatus is shown in the stimulating mode with the microelectrode connected directly to the stimulator via a 100 M ohm resistance (not shown). The relay and unity-gain amplifier (UGA) were mounted inside the preamplifier probe. E.R., electrode resistance check; bridge bal., bridge balance; 'scope A and 'scope B, preamplifier outputs leading to a differential input on the oscilloscope.

by a 9 volt battery connected to a 10k ohm resistor in series with a 10M ohm isolating resistance. Iontophoretic ejecting currents were provided by a 'Digitimer' DS9 stimulator connected to the microelectrode through a 100 M ohm resistor. The reference electrode consisted of a 1 mm diameter silver/silver chloride wire connected to the bath by an agar bridge and held at earth potential by a virtual earth circuit. Currents passing through the bath were monitored by measuring the potential difference across a 200k ohm resistance incorporated in the virtual earth (see Figure 19).

6) Penetration with the Recording and Stimulating Electrodes.

The microelectrodes were held at approximately 30° to the vertical, behind and to either side of the bath (Figure 20). The left cell 28 was impaled with two recording microelectrodes as described on page 42 . Currents could be passed through either of these microelectrodes by holding the preamplifier probe in the stimulating mode.

7) Positioning the Iontophoretic Electrode.

A 15 nA retaining current (just sufficient to prevent diffusion of the drug from the electrode, and consequent desensitisation of the neurone) was used (see Bradshaw et al, 1973). The iontophoretic electrode was placed above the surface of the neurone and lowered using the fine control of the micromanipulator until there was a slight fall in the retaining current (about 2 nA)

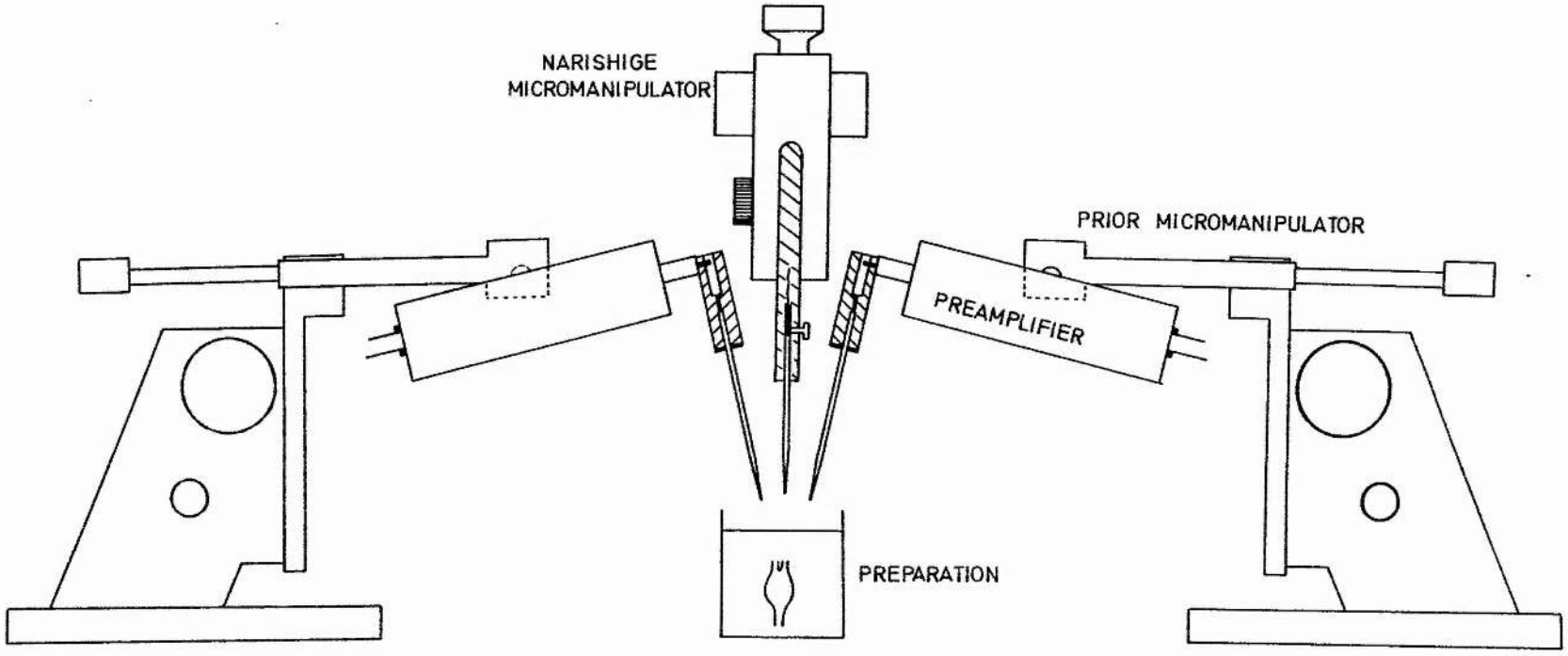


Figure 20. The arrangement for positioning the microelectrodes. The recording microelectrodes are held in 'Perspex' blocks which plug into the preamplifier probe. The iontophoretic microelectrode is held at approximately 30° to the vertical in a 'Narishige' micromanipulator.

indicating contact with the membrane surface. The micro-electrode was then raised slightly until the retaining current returned to normal.

8) Bath Application of Drugs.

Drugs were added to the bath by the method described on page 84 . Known concentrations of agonists were perfused through the bath until a maximum response was obtained, i.e. until the amplitude of the polarisation of the neuronal membrane reached a plateau. The bath was then washed out with saline. The point of entry for the solutions was as already described, directly below the centre of the ganglion in an attempt to use the response of the contralateral cell 28 as a control. The concentration of the agonists given is that which was actually applied to the bath and not that reaching the preparation; this presumably being appreciably lower.

Potentiating drugs (in this case anticholinesterases), since they do not have a direct action on the neurone, were perfused through the bath in 20 ml aliquots. This process lasted for 30-40 seconds, which from the dye perfusion experiments (page 84) was sufficient for the concentration of the drug reaching the preparation in the bath to approximate to the concentration of the drug in the sample. The preparation was then left for 2 minutes before iontophoretically applying ACh.

9) Drugs.

Drugs were made up from the solid, in saline for bath application and in distilled water for iontophoretic application. These were diluted to the appropriate concentration just prior to the experiment.

Acetylcholine chloride and physostigmine sulphate were stored in a dessicator at 0°C. GABA, neostigmine bromide and carbamylcholine chloride were stored in the dark at room temperature. Fresh stock solutions were made up each day.

RESULTS.

Throughout this chapter the terms experimental or axotomised are used to refer to preparations in which the left N5 had been cut 4-118 days prior to the experiment. The majority of these were examined from 4-10 days after the operation. All recordings were made from the soma of cell 28 on the left side of the animal.

1) Electrical Properties of Cell 28 in Normal and experimental animals.

The cell bodies had resting potentials of 64.9 ± 1.4 mV (n = 39) and 66.7 ± 1.9 mV (n = 30) for normal and experimental animals respectively. The two values were not significantly different ($P > 0.9$). These measurements are somewhat uncertain because of variations in the potentials recorded by the electrode caused by penetration of the membrane. When a cell was penetrated by two electrodes the resting potential recorded differed by as much as 5 mV. This discrepancy has also been noted in recordings from chick ciliary ganglion cells (Martin and Pilar, 1964; Brenner and Martin, 1976).

The membrane (input) resistance was determined for each preparation by passing current in 500 ms pulses through a second electrode and recording the change in membrane potential. The current required to produce 10 mV positive and negative polarisations was used for calculating the resistance (Figure 21). The values were

Figure 21. Current-voltage relation with two electrodes in the cell.

a). Record of voltage change (lower trace) produced by positive and negative current pulses (upper trace) passed through the second electrode.

b). The current-voltage relation for a normal (•) and an axotomised (o) cell 28. Membrane response was nearly linear with input resistance of approximately 5 M ohms.

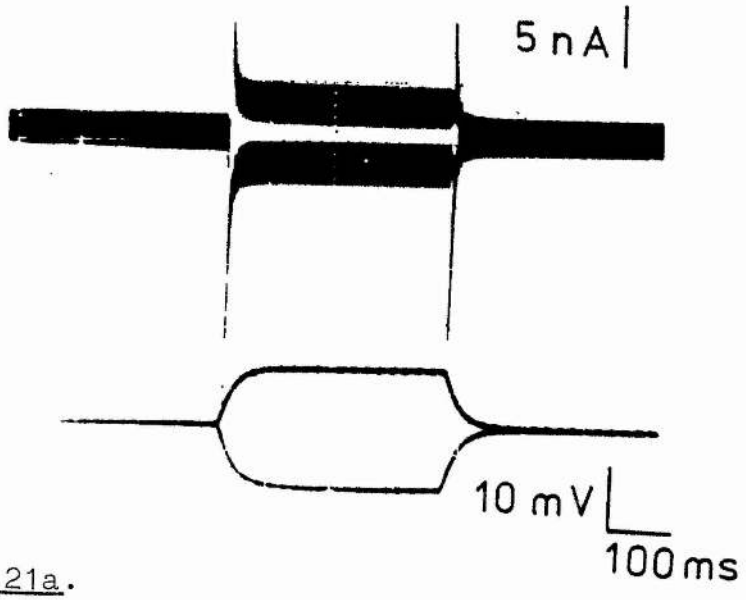


Figure 21a.

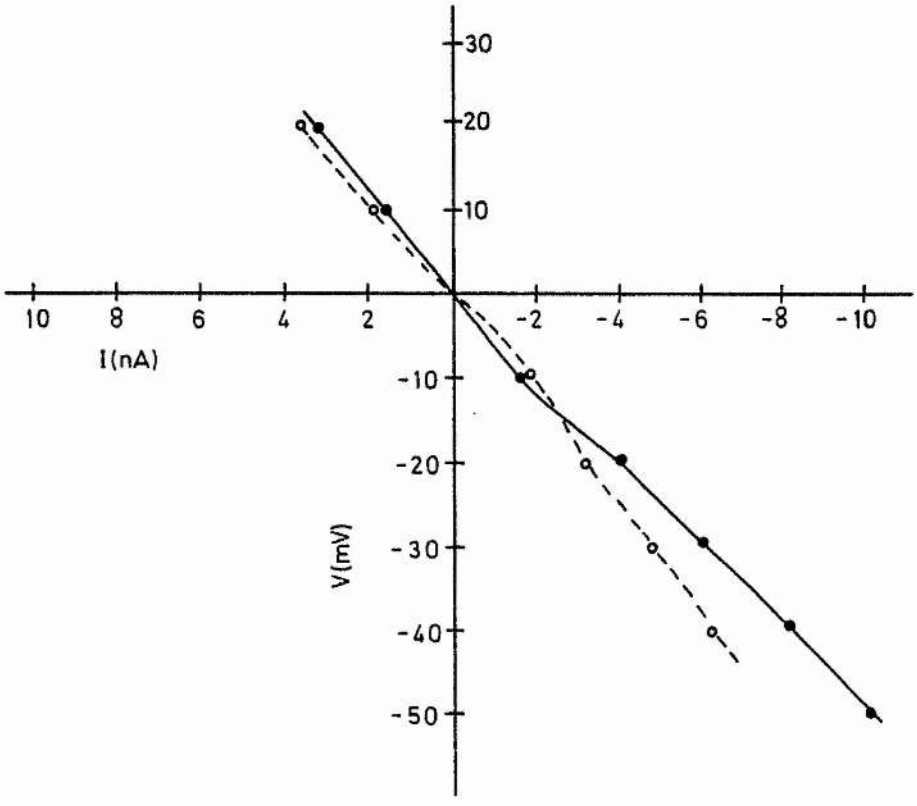


Figure 21b.

4.7 ± 0.5 M ohms (n = 33) for normal cells and 4.9 ± 0.5 M ohms (n = 30) for axotomised cells. There was no significant difference between the two values ($P > 0.8$). The current/voltage relationship was approximately linear from -50 to +20 mV with respect to the resting potential (Figure 21). Depolarising the cell above 20 mV normally produced damped membrane oscillations as shown in Figure 22). After nerve section many cells became electrically excitable producing all or none overshooting action potentials in response to a small depolarising current (Figure 22 ; see Pitman, Tweedle and Cohen 1972). The threshold depolarisation for these action potentials was in the region of 10-15 mV.

The time constant of the membrane was calculated from the exponential decay of a 10 mV 500 ms hyperpolarising pulse. The mean for the normal preparations was 13.1 ± 1.2 ms (n = 14) and 15.6 ± 1.2 ms (n = 14) for the experimental. The two values were not significantly different ($P > 0.2$).

2) Bath Application of Drugs.

The perfusion of saline through the bath produced a slight hyperpolarisation of the membrane potential in the region of 1-5 mV. Therefore in order to determine the effect of an applied drug, saline was continuously passed through the bath until a maximum level of hyperpolarisation was reached. The perfusion of saline was then replaced by the saline containing

Figure 22a. Damped membrane oscillations produced by a 6 nA depolarising pulse through the second electrode. Horizontal bar denotes duration of pulse. Cell resting potential, 64 mV.

Figure 22b. Overshooting action potentials evoked by passing a 2 nA depolarising pulse through the second electrode. The cell had been axotomised for 6 days. Horizontal bar denotes duration of pulse. Cell resting potential, 58 mV.

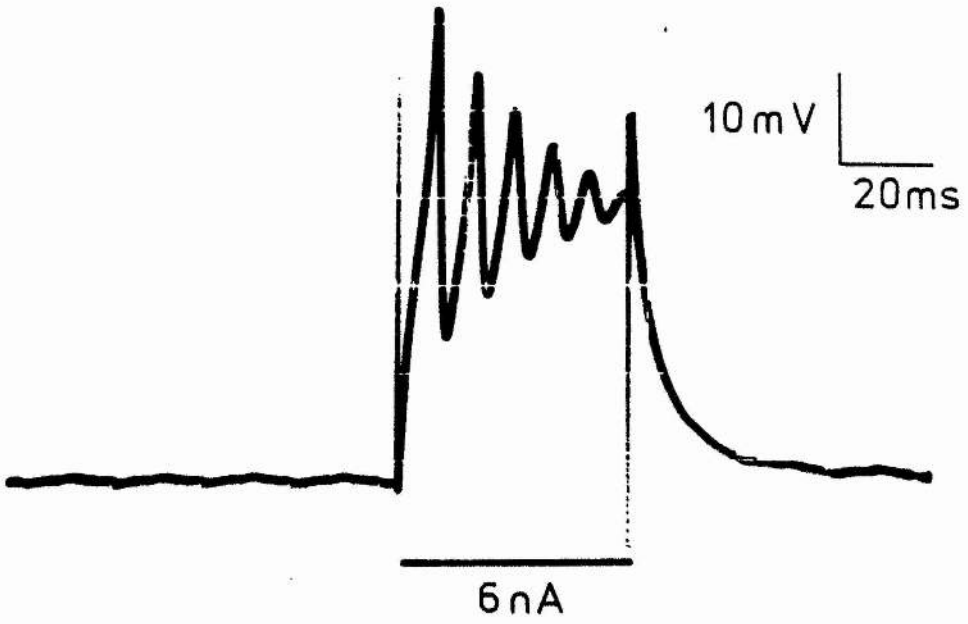


Figure 22a.

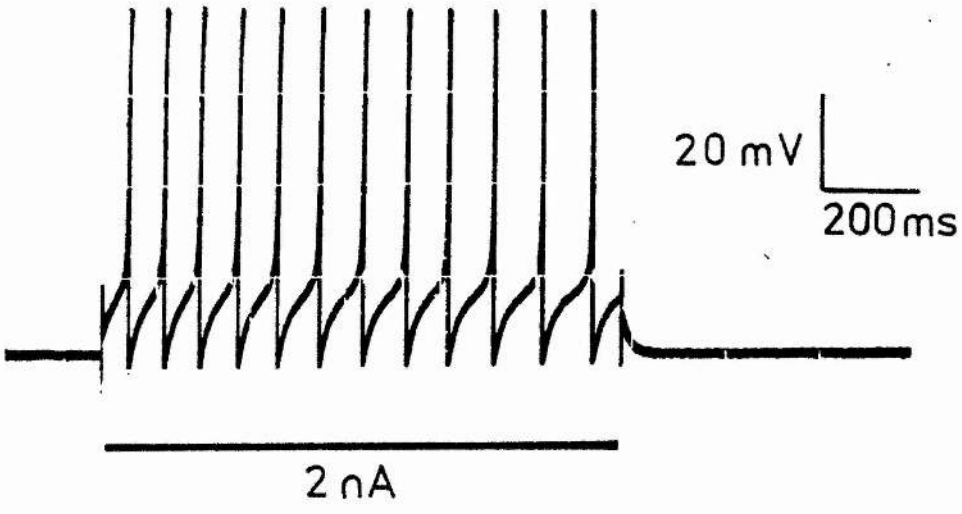


Figure 22b.

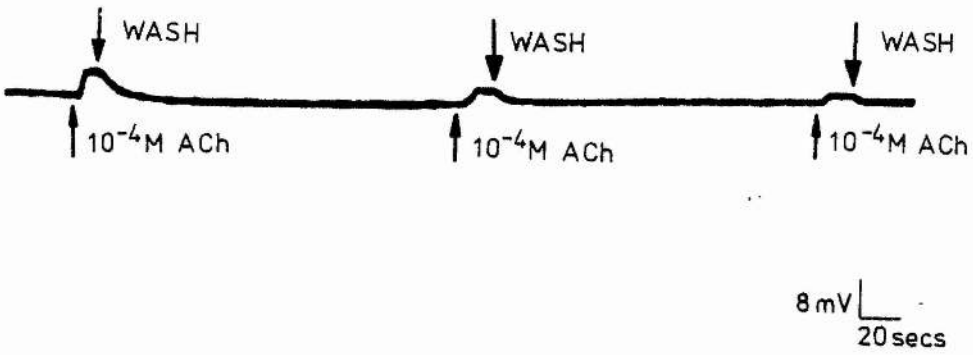


Figure 23. Membrane depolarisations produced by repeatedly challenging the preparation with 10^{-4} M acetylcholine (ACh). The preparation was washed continuously between drug applications. This attenuation of the response by desensitisation could be avoided by challenging each preparation with only one test solution (Figure 24).

the drug. It should be noted that the final drug concentration in the bath was not known; the concentrations quoted are those of the drug added to the bath.

Bath applied acetylcholine (ACh) produced a depolarisation of the cell membrane. The lowest concentration of ACh to have an effect was about 10^{-5} M in normal preparations. Unfortunately the receptors desensitised after only one application of the drug (Figure 23). The preparation required at least 10 minutes of washing before normal sensitivity was restored and so it was not possible to determine an accurate relationship between dose and response. In order to quantify the sensitivity of the cell to ACh a standard concentration of 10^{-4} M was applied to the bath and the cell response was taken as the peak amplitude of the first depolarisation. Similar problems were encountered by Sattelle and co-workers using the cercal nerve:giant interneurone synapse in the sixth abdominal ganglion of the cockroach. Quantitative results could be achieved only by a single application of the drug (Sattelle, McClay, Dowson and Callec, 1976; Callec, Dowson and Sattelle, 1975).

Figure 24 shows an example of the response to 10^{-4} M ACh in a normal and an axotomised cell. The mean response for the 22 control cells was 6.0 ± 0.9 mV. The sensitivity of the cells which were axotomised was appreciably higher; the same concentration of ACh in 19 preparations produced an average response of 13.9

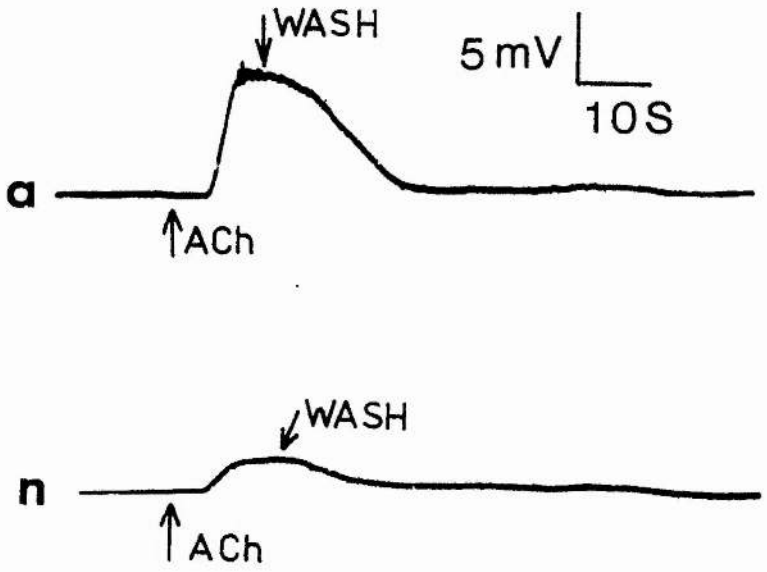


Figure 24. Membrane depolarisations produced by challenging a normal (n) and an axotomised (a) cell 28 with 10^{-4} M acetylcholine (ACh). The two cells were from two different preparations.

± 2.2 mV. The two values were significantly different ($P < 0.005$).

The threshold concentration of GABA applied to the bath was about 10^{-5} M. The preparation was again subject to desensitisation and so a single dose of 10^{-4} M GABA was applied. The peak of the hyperpolarisation was taken as the cell response (Figure 25). The mean response was 4.6 ± 1.1 mV (n = 11) for normal cells and 5.4 ± 1.7 mV (n = 4) for experimental cells. There was no signif icant difference between the two values recorded ($P > 0.6$).

The cholinomimetic carbamylcholine (CCh) has both nicotinic and muscarinic actions in the vertebrates (Jenkinson, 1960; Thron and Waud, 1968). In addition it has been shown to mimic the effect of ACh on the receptor mediating slow ipsp's (inhibitory postsynaptic potentials) in Aplysia neurones. Since CCh is not broken down by cholinesterases, one would expect the effect of a given dose to be the same in both the normal and the experimental cells if the increase in sensitivity following axotomy is due to a reduction in the amount of available cholinesterase.

The response to 10^{-4} M CCh was 9.7 ± 1.2 mV (n = 7) for normal cells and 11.6 ± 3.3 mV (n =5) for experimental cells (see Figure 26); there was no significant difference between these two values ($P > 0.6$).

Figure 25. Membrane hyperpolarisation of a normal cell 28 produced by passing 10^{-4} M GABA through the bath.
A, beginning of perfusion of GABA; W, beginning of wash.

Figure 26. Membrane depolarisations produced by challenging a normal (N) and an axotomised (A) cell 28 with 10^{-4} M carbamylcholine (CCh).

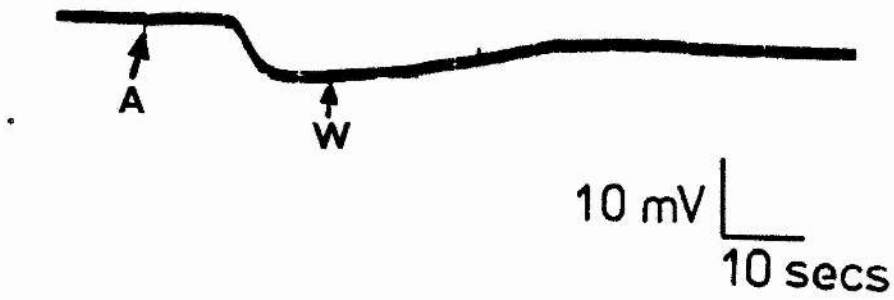


Figure 25.

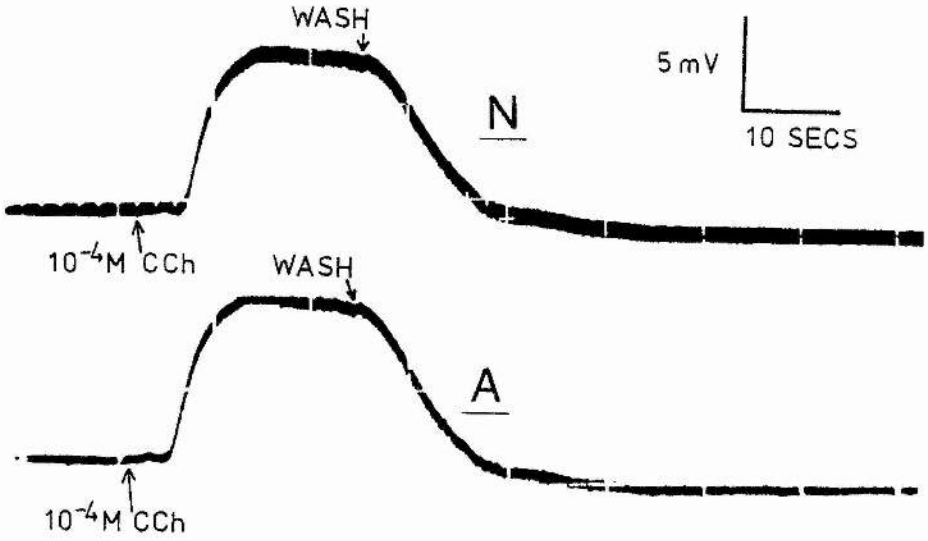


Figure 26.

3) Iontophoretic Application of Drugs.

When drugs are added to the experimental bath all of the cells in the preparation which are affected by the drug will respond. However appreciable time is required for equilibrium of the agonist concentration at these sites. This was undoubtedly the cause of the tremendous desensitisation observed. Moreover, the prolonged action of ACh can cause some ionic changes inside the cell (Karlin, 1967; Jenkinson and Terrar, 1973). In order to overcome these problems ACh and CCh were applied locally to the soma membrane by iontophoresis.

In most experiments 100 ms ejecting current pulses were used; for less sensitive cells the pulse duration was increased to 500 ms. Recovery times of up to 45 seconds were required and so a standard two minute interval between doses was adopted.

a) Acetylcholine.

In both normal and experimental cells the maximum depolarisation produced by iontophoretically applied ACh was graded with increasing iontophoretic charge (Figure 27). For a given charge this response was independent of the pulse duration over the range 0.1-1.0 seconds (Figure 29).

The charge necessary to produce a given response was considerably smaller in the axotomised cells. Figure 30 shows the relationship between log. iontophoretic charge and response for a normal and

Figure 27. Iontophoretic application of acetylcholine onto the nerve cell body.

a). Upper trace, membrane depolarisation.
Lower trace, iontophoretic current.

b). As the iontophoretic current was increased, so the maximum depolarisation increased. The iontophoretic charge was increased every two minutes; doses were 20, 50, 85 and 100 nC.

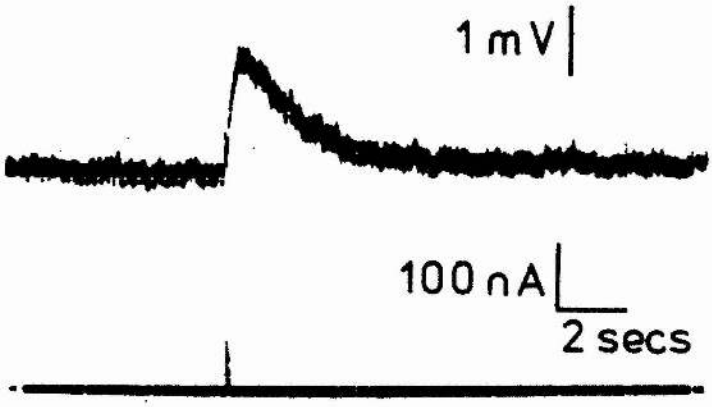


Figure 27a.

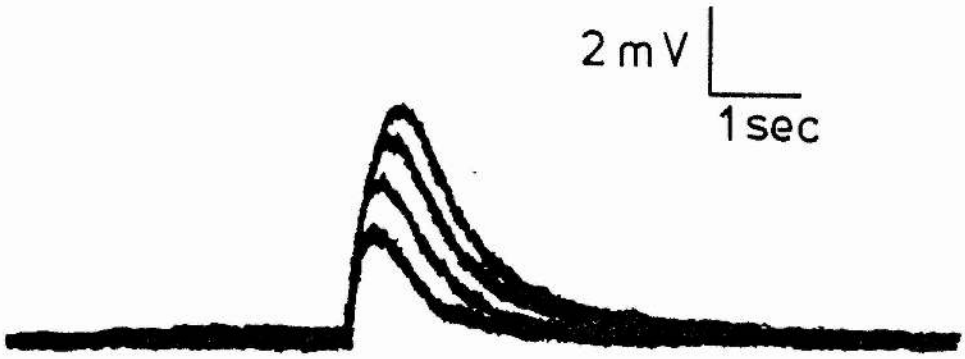


Figure 27b.

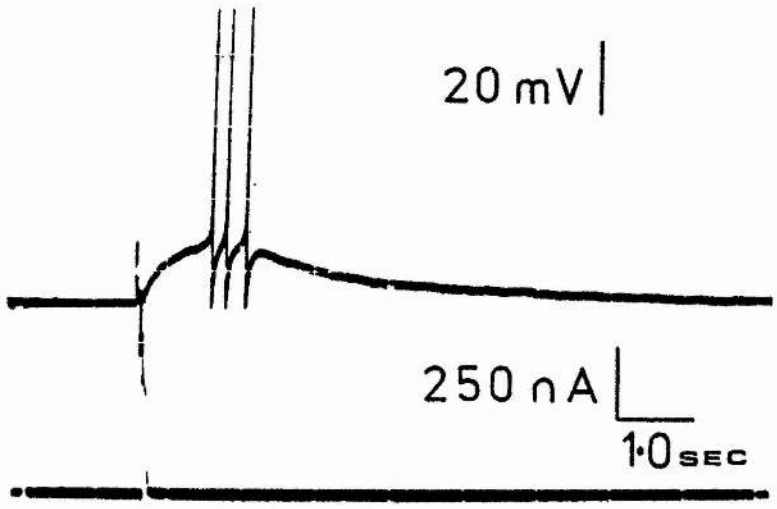


Figure 28. Overshooting action potentials (upper trace) recorded from an axotomised cell produced by a supra-threshold ACh response. Lower trace, iontophoretic current. Cell resting potential, 68 mV.

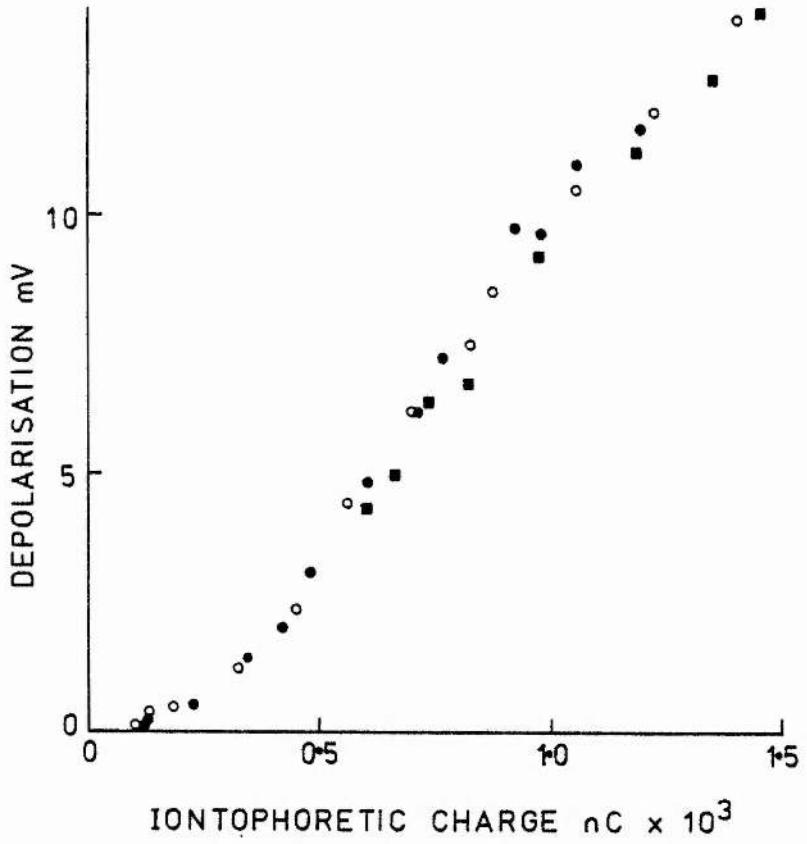


Figure 29. Relation between peak ACh response (depolarisation, mV) and total iontophoretic charge passed from micropipette for various pulse durations (seconds) ■, 1.0; o, 0.5; ●, 0.1. Response was independent of pulse duration for durations between 0.1 and 1.0 secs.

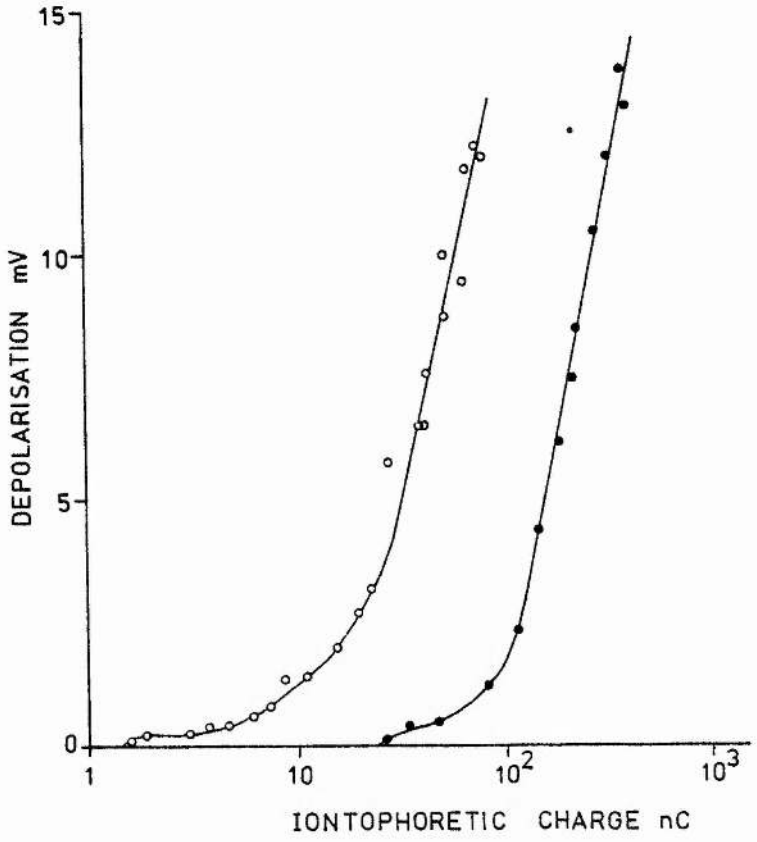


Figure 30. ACh dose-response curve for a normal (•) and axotomised (o) cell 28. ACh sensitivity was taken from the maximum slope of relation. Retaining current, -15 nA.

axotomised cell. The maximum slopes of such dose response curves were taken as measures of the ACh sensitivity of the cells. The mean sensitivity of 33 normal cells was 0.077 ± 0.020 mV/nC compared with a mean of 0.246 ± 0.039 for 20 experimental cells. The difference between the sensitivities of control and axotomised cells was statistically significant ($P < 0.001$).

b) Carbamylcholine.

Like ACh, iontophoretically applied CCh produced a depolarisation of the cell membrane (Figure 31). However, plotting the relationship between log. iontophoretic charge and maximum response (Figure 31) revealed two distinct features: (i) the threshold dose was much lower than for ACh applied to normal cells and (ii) the maximum slope of the graph was less steep than that of the ACh dose response curve. The relatively low threshold is most likely due to the resistance of CCh to hydrolysis by cholinesterase enabling a greater proportion of the molecules to reach the receptor sites. The reduction in the slope of the curve could be a result of desensitisation caused by prolonged exposure of the receptors to the agonist - the greater the dose, the greater the desensitisation. Alternativaly CCh may have a lower affinity for the receptors than ACh (Eldefrawi, Eldefrawi, Seifert and O'Brien, 1972; Sattelle, McClay, Dowson and Callec, 1976).

CCh dose response curves were similar for

Figure 31a. Iontophoretic application of carbamylcholine onto the nerve cell body. Upper trace, membrane depolarisation; lower trace, iontophoretic current.

Figure 31b. Carbamylcholine dose-response curve for a normal (•) and axotomised (o) cell. Retaining current, -15 nA.

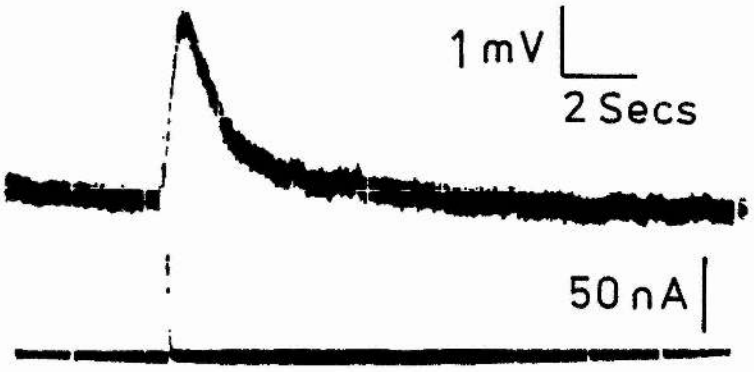


Figure 31a.

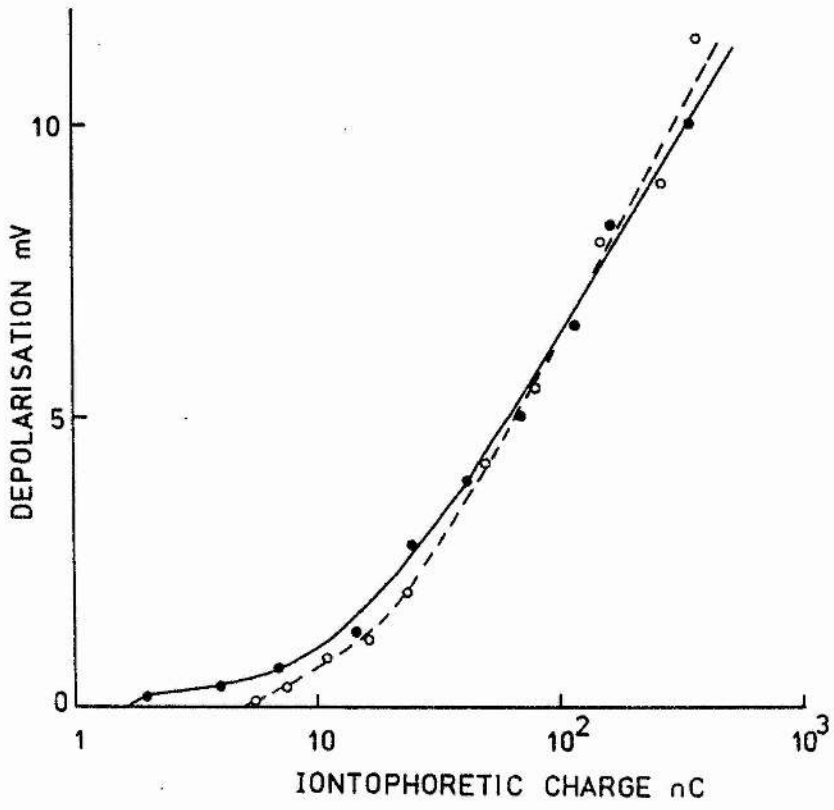


Figure 31b.

normal and axotomised cells. Sensitivities calculated from the maximum slopes were 0.068 ± 0.021 mV/nC for 5 control cells and 0.075 ± 0.035 mV/nC for 5 axotomised cells. The two values were not significantly different ($P > 0.4$).

It is interesting that following the application of CCh during a routine dose response determination, the sensitivity to ACh iontophoretically applied to approximately the same area on the cell membrane was increased in the region of 10-20 fold. Following bath application of 10^{-6} M physostigmine the sensitivity of the cell was further increased. These observations are consistent with the behaviour of CCh in vertebrate preparations, where it has been shown that it combines with the cholinesterase and acts as a weak enzyme inhibitor (Goldstein, 1951; Changeux, Meunier and Huchet, 1971).

4) Anticholinesterases.

The experiments with CCh suggest that the supersensitivity produced by axotomy results from a reduction in the ChE activity on or near the neuronal membrane which consequently slows the rate of inactivation of ACh. This possibility was investigated by examining the affect of anticholinesterases on the response to a given dose of ACh.

Both physostigmine and neostigmine blocked the ACh response when applied in sufficiently high concentrations.

TABLE III

	mean sensitivity before antiChE (mV/nC)		mean sensitivity after antiChE (mV/nC)		potentiation (%)		
PHYS.10^{-6} M							
N	0.044 ± 0.034	(P<0.05)	0.349 ± 0.181	(P>0.5)	1034 ± 305	(P<0.01)	(n=7)
E	0.185 ± 0.059		0.446 ± 0.206		199 ± 27		(n=7)
NEOST.10^{-6} M							
N	0.092 ± 0.039	(P<0.05)	0.688 ± 0.316	(P>0.9)	687 ± 150	(P<0.05)	(n=5)
E	0.219 ± 0.043		0.661 ± 0.222		349 ± 53		(n=7)
NEOST.10^{-7} M							
N	0.092 ± 0.030	(P<0.05)	0.386 ± 0.138	(P>0.5)	408 ± 25	(P<0.001)	(n=7)
E	0.185 ± 0.048		0.435 ± 0.175		214 ± 32		(n=6)

N = Normal cell 28s

E = Experimental/Axotomised cell 28s

The lowest concentration which produced a block was 10^{-5} M for both these anticholinesterases, although in 2 out of 7 preparations this occured with 10^{-6} M neostigmine. Similar results have been reported in other preparations. These include the cat superior cervical ganglion (Paton and Perry, 1953) and leech central neurones (Sargent, King-Wai Yau and Nicholls, 1977). High concentrations also inhibit ACh binding to isolated receptors from Torpedo (Eldefrawi, Eldefrawi, Seifert and O'Brien, 1972).

The strength of iontophoretic pulses was adjusted to give a depolarisation of approximately 1 mV before perfusing 30 mls of saline containing the anticholinesterase. The preparation was then left for two minutes before applying the same concentration of ACh. Only a brief pre-treatment with the anticholinesterase was possible since prolonged (5-10 minutes) exposure to 10^{-6} M physostigmine or neostigmine could alter the membrane properties so that the resting potential would fall. In addition the membranes of some cells became excitable and gave 10 mV to overshooting action potentials in response to small depolarising current pulses (see Figure 33).

Figure 32 shows the response to iontophoretically applied ACh before and after adding 10^{-6} M neostigmine. The degree of potentiation was taken as the percentage increase in the sensitivity to ACh:

$$= \frac{\text{sensitivity to ACh before antiChE}}{\text{sensitivity to ACh in presence of antiChE}} \times 100$$

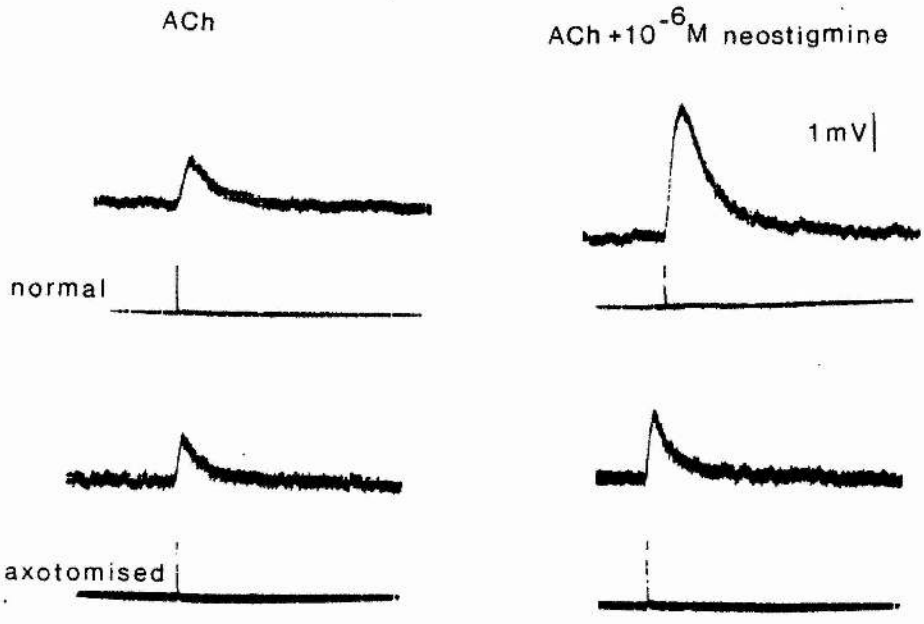


Figure 32. Potentiation of the ACh response by physostigmine. Left, the response of a normal and an axotomised cell to iontophoretically applied ACh. Upper trace, membrane depolarisation; lower trace, iontophoretic current (axotomised cell, 45 nA; normal cell, 160 nA). Right, the ACh response of the same cells after bathing the preparations in 10^{-6} M physostigmine for 2 minutes.

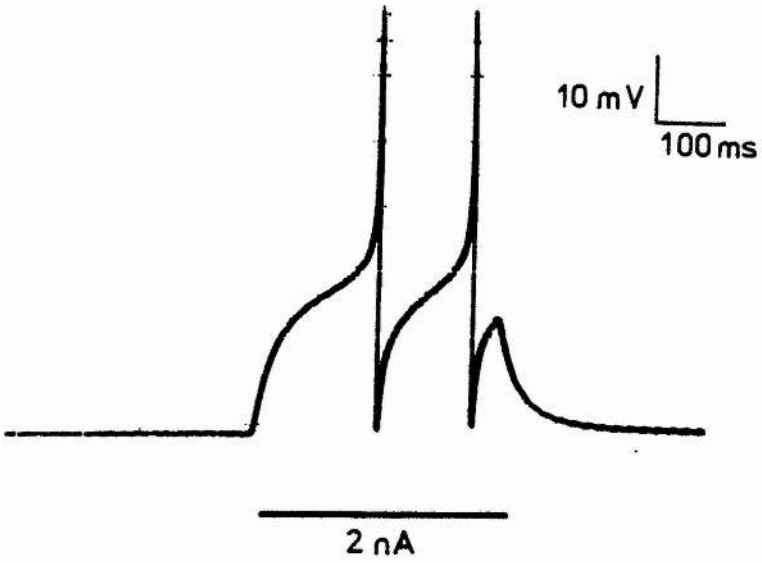


Figure 33. Excitability of cell membrane caused by bathing the preparation in 10^{-6} M physostigmine for 6 minutes. Horizontal bar denotes duration of depolarising current passed through a second electrode. Cell resting potential, 53 mV.

The results of these experiments are illustrated in Table III. The relatively large potentiation by physostigmine observed for normal cells is probably due to the relatively low initial sensitivities of these cells, assuming that this is governed to a significant extent by the cell ChE content. It is worth noting that in the dorsal muscle of the leech, physostigmine is about six times more potent than neostigmine in potentiating the response to iontophoretically applied ACh (Flack and Yeoh, 1968).

The degree of potentiation by both physostigmine and neostigmine was greater in normal cells than in axotomised preparations. This supports the conclusion drawn from the CCh experiments: that ChE loss is involved in the development of supersensitivity following nerve section.

5) Rise Times and Receptor Distribution.

The increase in sensitivity to ACh could have been due to the removal of a diffusion barrier between the point of drug application and the receptor sites on the membrane surface. This was investigated by examining the rise times of the ACh responses and the regional distribution of sensitive areas. Ejecting pulses of 100 ms duration were used in all of these experiments.

The rise times were relatively slow when compared to ACh responses of junctional membranes (c.f. Harris, Kuffler and Dennis, 1971; Feltz and Mallart, 1971).

The mean value for an approximately 1 mV depolarisation was 0.65 ± 0.12 secs (n = 23). In addition the rise times appeared to increase linearly with the dose (Figure 34). The slow rise times and their dependance on dose is reminiscent of the interaction between ACh and extra-junctional receptors on frog muscle fibre (Feltz and Mallart, 1971). Both of these phenomena can be explained by supposing that the density of extrajunctional receptors is lower than junctional receptors. At such relatively insensitive areas the nearest receptor site would become quickly saturated and the response would develop by stimulation of progressively more distant receptors. Larger doses would saturate larger areas of the membrane and so the time to reach the maximum response would increase.

In order to compare the rise times of ACh reponses in both types of cell the effect of ChE was eliminated by bathing the preparation in a 10^{-6} M solution of neostigmine bromide. As a result of this pre-treatment a constant dose of ACh would produce a similar depolarisation in the control and experimental cells (see Table 3). It was important to maintain the same dose from cell to cell because of the dose dependancy of the rise time described above. The average times to peak for 14 normal and 10 axotomised cells were 0.85 ± 0.13 secs and 1.09 ± 0.23 secs respectively using a 10 nC iontophoretic pulse. These values were not significantly different ($P > 0.9$).

Figure 34. Relation between ACh response amplitude and rise time for a normal cell 28. The time to reach the maximum response increases approximately linearly with the amplitude of the response.

Figure 35. Sample records of the sensitivity to iontophoretically applied ACh (mV/nC) of various spots on the surface of a normal and an axotomised cell 28. The sensitivity is fairly uniform over the exposed surface of the cell body.

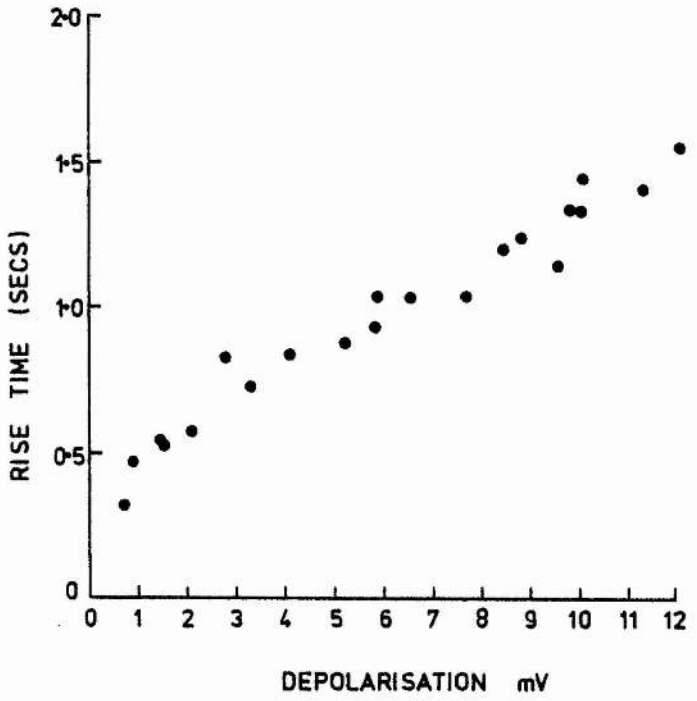


Figure 34.

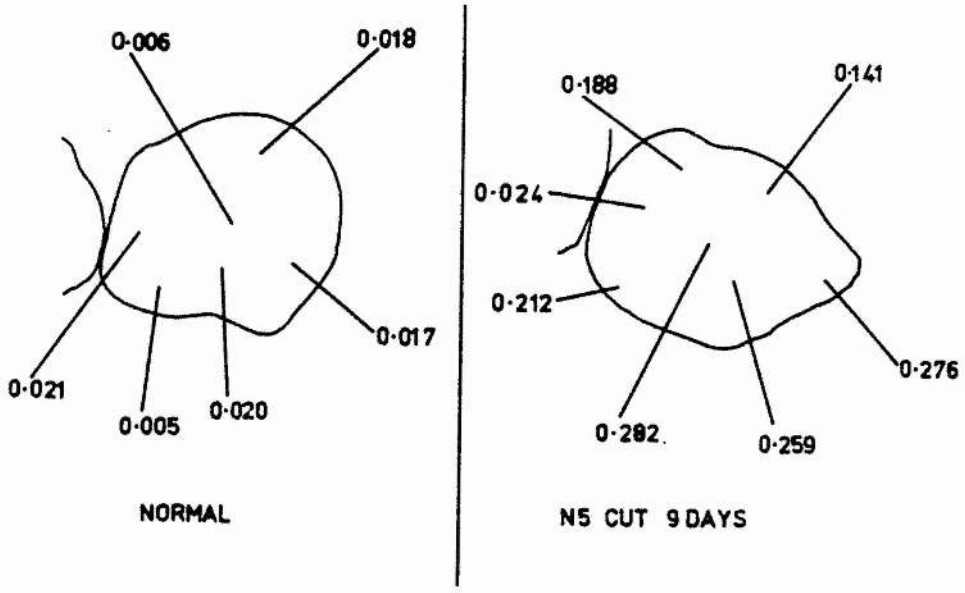


Figure 35.

The distribution of ACh sensitive areas was investigated by iontophoretically applying ACh at random points on the membrane surface. An ejecting pulse was selected to give a depolarisation of approximately 1 mV in order to standardise the response:dose ratio. Wherever the iontophoretic electrode was placed the rise time and the sensitivity were approximately the same. Figure 35 illustrates the sensitivity of different areas of the membrane in a normal and an axotomised cell. The sensitivity to ACh appeared uniform over the cell surface in all of the 6 normal and 6 axotomised preparations examined.

It would seem that any barrier to the diffusion of ACh from the tip of the micropipette to the receptive areas is equal in both cell types and uniformally distributed over the whole of the soma membrane. Similarly the distribution of receptors seems to remain unchanged after axotomy.

6) The Acetylcholine Reversal Potential.

The ACh response was greatly reduced in sodium free saline (Figure 36). Similar results were obtained from neurones of the sixth abdominal ganglion of the cockroach (Kerkut, Pitman and Walker, 1969). Since the ACh response has such a large sodium component, the increase in sensitivity seen after axotomy could result from a shift in the sodium reversal potential to a more positive value.

Figure 36. The effect of reducing the external sodium ion concentration on the response to ACh. Upper traces, membrane depolarisation; lower traces, iontophoretic current. Removal of external sodium reduced the effect of ACh (B). The small depolarisation seen in sodium free saline could be due to the presence of a small number of sodium ions still present around the preparation (Chamberlain and Kerkut, 1969). The ACh response was restored after washing with normal saline for 15 minutes (C).

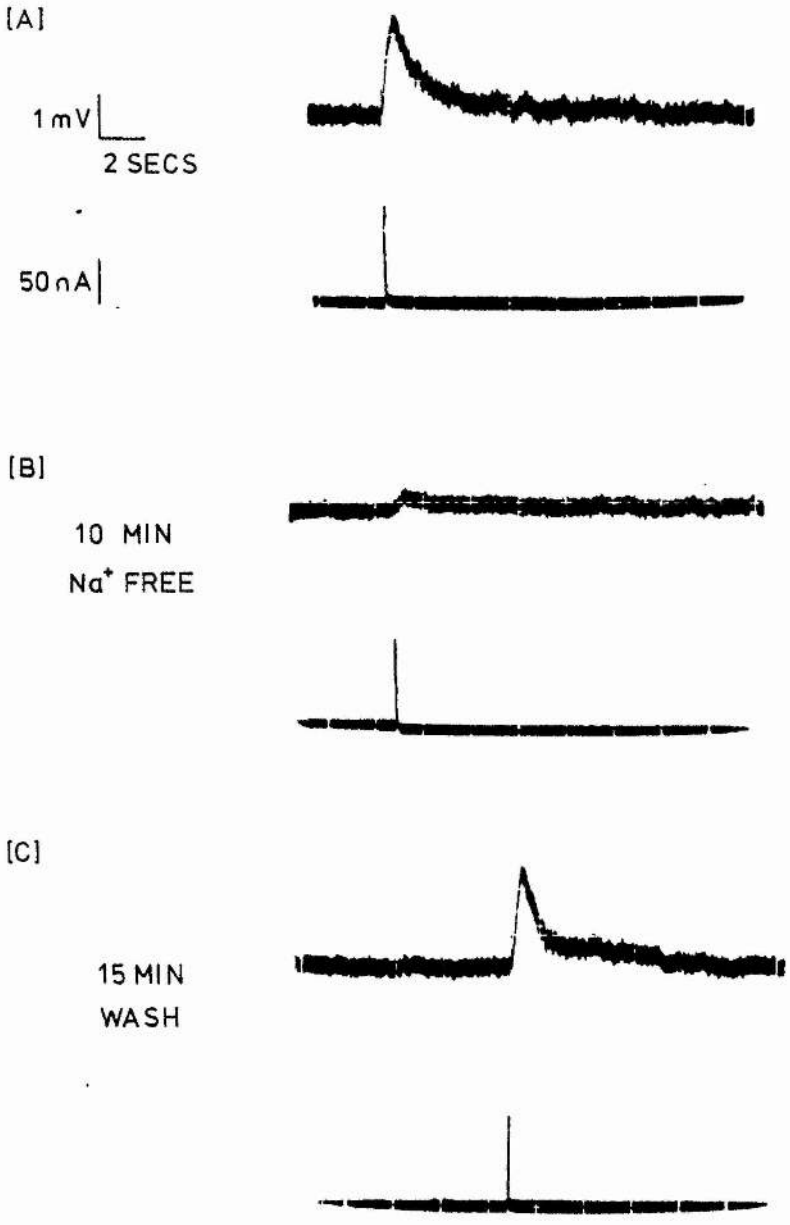


Figure 36.

To investigate this, the reversal potential was determined in 5 normal and 5 axotomised cells. Unfortunately it was not possible to measure this directly as sustained depolarisations of more than 20 mV produced a significant fall in the membrane resistance. The reversal potential was estimated by observing the response to a standard iontophoretic dose of ACh at different membrane potentials. The amplitude of the ACh response increased linearly with membrane potential as shown in Figure 37. Plotting this relationship gives a straight line graph (Figure 37). A linear regression through the points is justified since it has already been shown that the membrane resistance does not change significantly over this range of membrane potential. Extrapolation of this line should intersect the ordinate at the ACh reversal potential. Although this method of determination is subject to some error (Mallart, Dreyer and Peper, 1976) it should reveal any significant difference between the true values for normal and axotomised cells. The reversal potential obtained in this way for the five normal and five axotomised cells were -35 ± 2.4 and -33 ± 2.9 mV respectively ($P > 0.5$). These results indicate that the increase in sensitivity after axotomy is not due to a change in the ACh reversal potential.

7) The Hill Coefficient.

A reduction in the average number of ACh molecules

Figure 37a. The effect upon the iontophoretic ACh response of hyperpolarising the neuronal membrane. Normal resting potential, 65 mV.

Figure 37b. The magnitude of the ACh response as a function of membrane potential. (•) Normal cell; (o) axotomised cell. Linear regression line extrapolates to intersect abscissa at an estimated reversal potential of -36 mV (normal cell) and -32 mV (axotomised cell). Resting potentials: normal cell, -70 mV; axotomised cell, -65 mV.

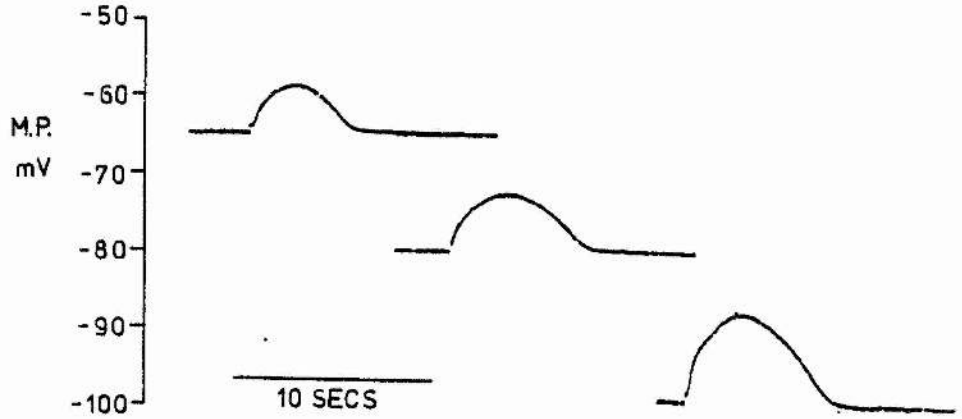


Figure 37a.

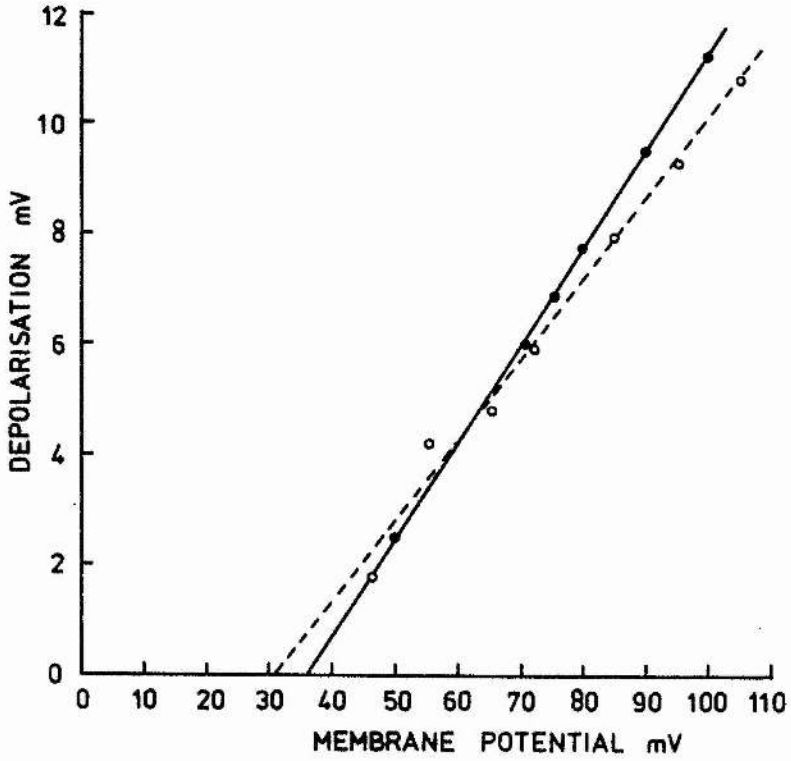


Figure 37b.

required to activate a receptor (Hill coefficient) would result in an increase in the response to a given dose of ACh; the converse would also be true. In order to calculate the Hill coefficient (n) the effect of iontophoretically applied ACh was expressed in terms of the change in membrane conductance in order to give values independent of resting potential and membrane resistance. The peak conductance change was calculated from the equation:

$$g = \frac{\Delta V}{R(E - \Delta V)}$$ from Brenner and Martin, 1976

Where ΔV was the peak depolarisation, R the membrane resistance and E the difference between the resting potential and the ACh reversal potential. The reversal potential was taken as -34 mV.

It is generally assumed that the reaction between an agonist and a receptor rapidly reaches equilibrium:

$$\text{Agonist} + \text{Receptor} \underset{\text{(rapid)}}{\rightleftharpoons} \text{Complex} \overset{\text{(slower)}}{\cdots\cdots>} \text{Response}$$

If a single receptor binds to a single agonist molecule then the fraction of receptors occupied at equilibrium (Pe) should be proportional to $\frac{\Delta g}{\Delta g_{max} - \Delta g}$ if one assumes that the effect due to ACh is directly proportional to Pe. From the law of mass action, Pe is described by the Langmuir equation:

$$Pe = \frac{A}{A + K_A} \qquad I$$

Where A is the concentration of ACh reaching the receptors

and K_A is the dissociation equilibrium constant. If equation I is obeyed, plotting log. $\frac{\Delta g}{\Delta g \text{ max} - \Delta g}$ against log. ACh concentration should give a linear graph of unit slope (Rang, 1973).

Unfortunately it was not possible to measure Δg max directly for the following reasons. (i) Maximal doses of ACh usually caused depolarisations of 20 mV or more; depolarisations of this magnitude normally produce a reduction in membrane resistance accompanied by membrane oscillations. (ii) Large responses have relatively long times to peak, for example 20 mV depolarisations took from 10-30 secs to reach maximum. The desensitisation produced by such prolonged doses required recovery times ranging from 1.5 to 5 minutes. The cell membrane was therefore desensitised significantly before its' maximum level of depolarisation was reached. (iii) Many of the experimental cells produced action potentials when depolarised more than about 10 mV.

However it was possible to determine whether the increase in sensitivity was accompanied by a change in (n) by using the method adopted by Brenner and Martin (1976). If Δg is small, Δg max - Δg can be treated as a constant in the calculations (Dreyer, Peper and Stertz, 1978; Kahn and Yaouanc, 1971). Therefore with low doses of ACh, plotting log. Δg against log. dose should give a straight line of slope proportional to (n) (Figure 38). The mean value of the slope was 1.16 ± 0.15 for 11 normal

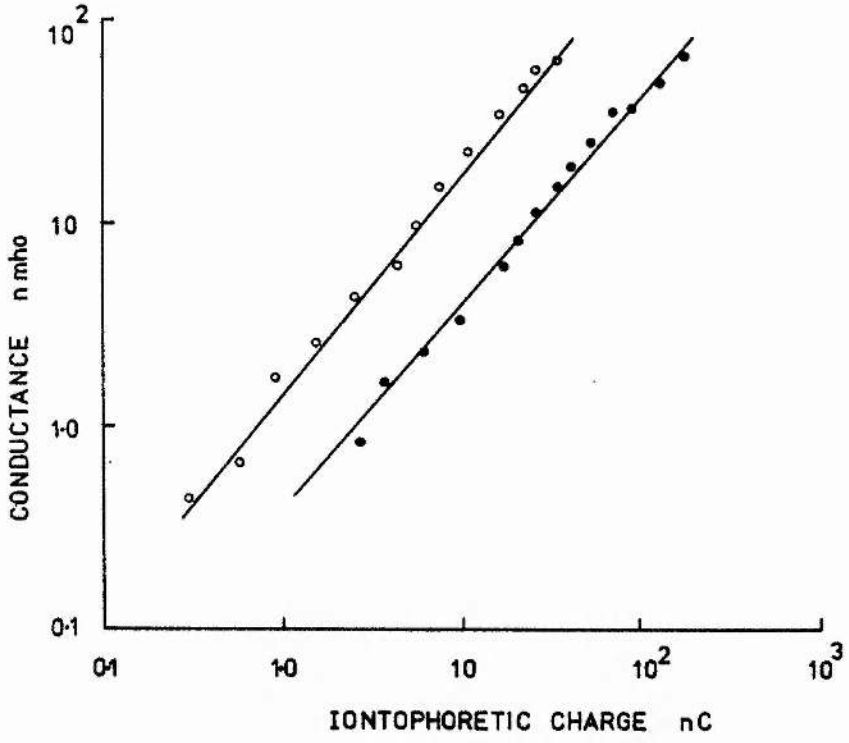


Figure 38. Dose-response relationship for a normal (•) and axotomised (o) cell, double logarithmic plot. Ordinate, peak conductance change calculated from the equation on page 101; abscissa, charge passed from iontophoretic electrode.

cells and 1.13 ± 0.19 for 11 axotomised cells. These values were not significantly different ($P > 0.5$).

8) The Time Course.

It would be of interest to know (i) the time course with which supersensitivity develops after axotomy and (ii) the effect of reformation of nerve connections. Jacklet and Cohen (1967) reported a partial return of movement in approximately 1 out of 3 limbs denervated by section of N5; this occured within 30-70 days of nerve section. Using behavioural and electrophysiological criteria many of these cases later developed a full functional recovery.

Unfortunately it was not possible to establish whether reinnervation had or had not occured using electrophysiological techniques since these experiments were performed on isolated portions of the CNS. However, some idea of functional reinnervation can be obtained by studying the movement of the affected leg during running and walking and by observing the ability of the leg to grasp at a pair of forceps; normally if a small object is brushed against the leg the animal will grasp it firmly in its pretarsal claws. This reaction was always absent in legs at relatively short times after the fifth nerve trunk had been cut. However two animals were able to clutch at objects brushed against the tarsus 112 and 118 days after nerve section. These animals also showed normal movement of

the leg during running and walking. Upon subsequent dissection the proximal and distal segments of the fifth nerves appeared to have re-united. The points at which they had been cut was marked by a mass of scar tissue. Some fifth nerve trumks were revealed upon dissection to have rejoined but the legs had shown no signs of a grasping reflex or of normal walking.

The failure of a nerve to regenerate does not appear to be simply a matter of time; some animals failed to re-develop normal leg function for more than a year after section of the fifth nerve.

The change in ACh sensitivity of cell 28 with time after section of nerve five is represented graphically in Figure 39. There seems to be no correlation between sensitivity and the post-operative period up to about 30 days following axotomy. After this period 4 out of 6 cells showed a marked reduction in sensitivity. The sensitivity of 2 of this group of 4 cells lay within normal values; these were examined 32 and 39 days following nerve section. The other 2 cells examined 112 and 118 days after nerve section had exceptionally low sensitivities, well below the values for normal cells (as already mentioned, the legs which these cells would normally innervate showed signs of functional re-innervation).

Cell 28 innervates, amongst other muscles of the coxa, muscle 177e (Pearson and Iles, 1971; Pitman,

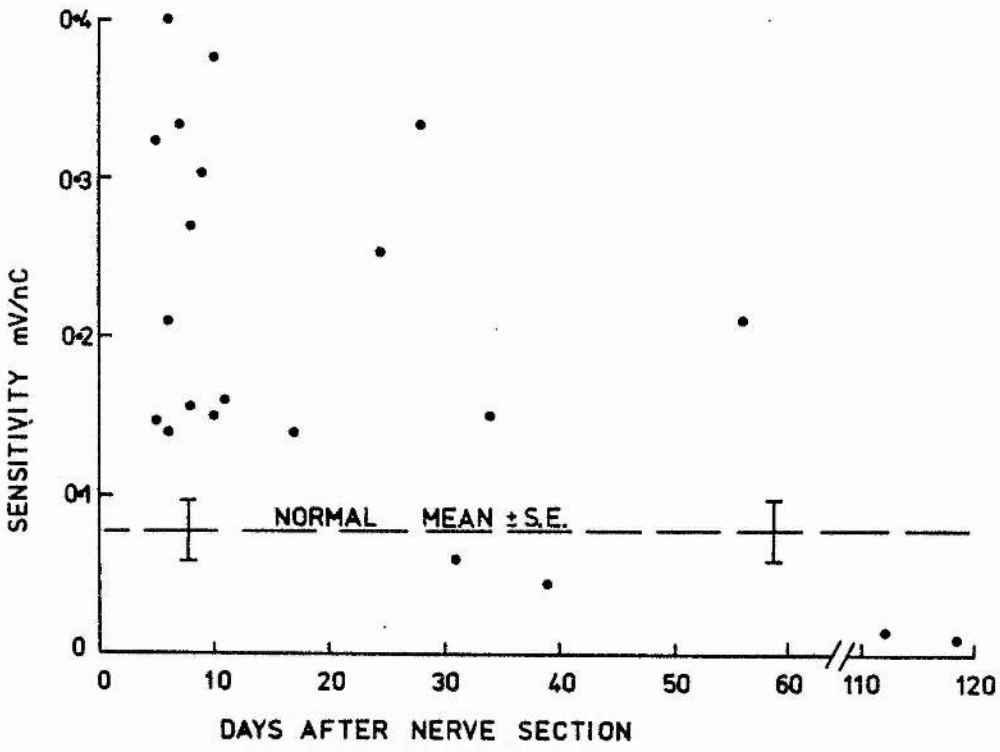


Figure 39. The sensitivity of individual cell 28s plotted against the number of days after section of their axons. Horizontal dashed line, mean sensitivity of 33 normal cells ± standard error of mean. The two cells studied 112 and 118 days after axotomy were from preparations in which the left metathoracic leg showed behavioural signs of functional reinnervation.

Tweedle and Cohen, 1972). Jacklet and Cohen (1967) observed that after cutting the fifth nerve, reinnervation of this muscle (if any) occured within about 45-100 days. Bearing in mind that most of the nerves showed no signs of reinnervation, it is probable that of the cells examined only those studied 112 and 118 days after axotomy could have reinnervated muscle 177e. It is of course possible that the regenerating axon of cell 28 could have reinnervated a foreign muscle, although in the cockroach regenerating motoneurones seem to connect exclusively with their correct muscles (Westin and Camhi, 1875; Pearson and Bradley, 1972).

DISCUSSION.

1) General Properties of the Cell Bodies.

Axotomy produced no significant change in the cell resting potential; the average resting potential recorded from the somata of cell 28s was 64.9 mV for normal cells and 66.7 mV for axotomised cells. These values are in aggreement with those obtained by Pitman, Tweedle and Cohen (1972) working on the same preparation. However the limitation of intracellular microelectrode techniques in determining absolute values for resting potentials should be acknowledged; potentials recorded by two separate microelectrodes inserted into the same cell were rairly identical. Despite such uncertainty this variation was never more than about 5 mV and so it seems safe to conclude that the resting potentials of axotomised cells lie within the normal range. Comparable values have been obtained for the resting potential of dorsal unpaired median (DUM) neurone cell bodies in the cockroach terminal ganglion (Kerkut, Pitman and Walker, 1969) and metathoracic ganglion (Crossman, Kerkut, Pitman and Walker, 1971).

The perikaryal membrane of cell 28 is normally electrically inexcitable; membrane depolarisations of 30 mV or more fail to produce action potentials. Increasing the magnitude of the injected current usually gives rise to a series of damped membrane oscillations with a maximum amplitude of about 15 mV (Figure 22).

Similar membrane responses have been observed in crustacean muscle fibres (Fatt and Katz, 1953; Atwood, 1967). Currents in the order of 10^{-6} amps are required to excite the axon of cell 28 and produce a response in the innervated muscles (Pitman, Tweedle and Cohen, 1972). This inexcitability is found in the majority of motoneurone cell bodies in the cockroach CNS, exceptions being the DUM cells located on the dorsal surface of the metathoracic and sixth abdominal ganglion (Pitman and Kerkut, 1969; Crossman, Kerkut, Pitman and Walker, 1971) and phallic neurones of the sixth abdominal ganglion (Crossman and Parnas, 1973). Early reports of excitable cell bodies may be suspect since the recording sites were not visible and may well have been from structures within the neuropile (Hagiwari and Watanabe, 1956; Rowe, 1964).

Within about 10 days after cutting the axon of cell 28 relatively small membrane depolarisations (10-15 mV) give rise to overshooting action potentials in the cell body (Pitman, Tweedle and Cohen, 1972). Pitman (1975; unpublished observations) has examined the ionic mechanism of the action potentials which can be evoked in axotomised or cochicine treated cells. In both of these situations the action potential is sodium dependent; the response is abolished in the presence of sodium free saline or saline containing tetradotoxin.

Action potentials could be evoked in

approximately 1 out of 3 axotomised cells in this study probably because a large proportion were examined between the 4th and 7th post-operative day. A small proportion of normal cells showed some signs of excitability and approximately 2% produced overshooting action potentials. The reason for this latter observation is not clear although it is possible that the cells could have received some injury to their axons several days prior to the experiment.

Input resistances of approximately 4.8 M ohms were obtained for both normal and axotomised cell bodies. Electrotonic potentials in the cells decayed exponentially with a time constant of approximately 14 m secs. This was also unchanged after axotomy indicating that the membrane capacitance was unaltered. Unfortunately no comparison can be drawn between these values of membrane resistance and time constant and those of other preparations since the surface area of each cell was not determined.

It is interesting that although the cell bodies of axotomised neurones were often excitable the membrane resistance measured at subthreshold levels of depolarisation was within the normal range (see Figure 21b). The same applied for the membrane time constant. It would appear that the change in membrane conductance produced by suprathreshold depolarisation of the cell bodies of axotomised neurones is a strictly all or none event; subthreshold depolarisations produce

only minimal changes in membrane conductance.

Axotomised spinal motoneurones of the cat show an increase in the excitability of their dendrites; the normally passive dendritic membrane produces spike-like partial depolarisations in response to afferent stimulation (Kuno and Llinas, 1970). The input resistances of normal and chromatolysed motoneurones are not significantly different. However the situation is complicated in such vertebrate preparations by the remoteness of the dendrites from the cell body. Since the microelectrode is implanted in the cell body and since the input resistance is presumably higher in the dendrites than in the cell body, the input resistance recorded would principally be that of the perikaryal membrane. Measurement of the membrane time constant of mammalian motoneurones is normally made by examining the decay time of synaptically evoked excitatory post-synaptic potentials (e.p.s.p.'s). However this technique is complicated in axotomised motoneurones by the loss of fibres making synapses on the soma and proximal dendrites - 'somatic stripping' (Blinzinger and Kreutzberg, 1968; Hamberger, Hansson and Sjostrand, 1970; Sumner and Sutherland, 1973). This results in a prolongation of the rise times and durations of the e.p.s.p.'s (Kuno and Llinas, 1970; Mendell, Munson and Scott, 1976). The available evidence indicates that axotomised motoneurones of the cat show no increase in time constant (Alley and Llinas, 1976). In accord with

this Mendell et al (1976) observed that axotomised motoneurones, whilst losing e.p.s.p.'s with short rise times, the rise times of the remaining e.p.s.p.'s were no longer than the longest rise times in normal motoneurones. Horridge and Burrows (1974) working on an identified motoneurone in the locust were able to indicate changes in the membrane time constant after axotomy by examining the decay of antidromic spikes recorded in the cell body. Initially the axotomised cell showed a shorter time constant than its intact contralateral homologue. Three days or more after axotomy the time constant became larger than normal in some cases increasing by as much as 6 ms (normal range, 4-12 ms). This increase in the apparent time constant may have been a direct result of an increase in the membrane resistance although this was not measured.

2) Sites of Drug Action.

Early experiments performed by Twarog and Roeder (1956; 1957) indicated that the fibrous sheath surrounding the central ganglia was partly responsible for the apparent insensitivity of cockroach neurones to topically applied ACh. Working on the cercal nerve-giant fibre synapses in the terminal ganglion of Periplaneta americana, these authors observed that transmission was unaffected by ACh at concentrations of up to 10^{-2} M when the sheath was intact. After removal of the sheath 10^{-3} M ACh was often effective in blocking

transmission. O'Brien and co-workers produced evidence that the nerve sheath provides a barrier to ionised compounds (O'Brien and Fisher, 1955; O'Brien, 1957). Working on the assumption that in mammals there is no such barrier, these authors by comparing the toxicity of various nitrogenous drugs in mice and cockroaches, were able to demonstrate that the effectiveness of the compounds was inversely proportional to the fraction ionised at physiological pH. It was proposed that this barrier could account for the relative insensitivity of the cockroach CNS to ACh. However Treherne and Smith (1965a) suggested that the increased sensitivity of the desheathed ganglion to ACh results from secondary changes produced by the desheathing process (Treherne, 1962a; 1962b). Rapid block of conduction through the terminal ganglion does not occur when ACh is injected beneath the nerve sheath. In addition Treherne and Smith (1965a) demonstrated that ^{14}C-labelled ACh penetrates rapidly into the tissues of the intact terminal ganglion. Smith and Treherne (1965) have postulated a biochemical barrier to the penetration of ACh through the central regions of the ganglion. Using the electron microscope, these authors demonstrated sites of strong eserine sensitive ChE activity "in association with axon membranes within the neuropile". This is in accordance with the conclusion made earlier by Wigglesworth (1958), that large quantities of a specific AChE are present within the neuropile. The significance of this high level of

ChE activity within the cockroach CNS will be discussed later.

A thin layer of glial cells lying immediately beneath the outer sheath of the ganglion forms another barrier for drug access to the soma membrane of cell 28. It is believed that for the most part this barrier was effectively removed by the 'clearing' process (methods page 42). The glial cell layer could be seen through the binocular microscope; after clearing the glial layer above cell 28 the outer soma membrane appeared to be free of any attached structures. However it is unlikely that the neurone cell body was completely cleared of glial tissue since the inner layer of the glial sheath comprises, in some areas, membranous structures in tight apposition to the perikaryon which frequently form finger-like invaginations within the nerve cell body (Smith and Treherne, 1965). In spite of this, access of ACh perfused over the ganglion to the soma of cell 28 would presumably be facilitated by removal of both the nerve sheath and much of the somatic glial covering.

3) The Action of Acetylcholine.

Acetylcholine appliedeither by perfusion of the ganglion or by iontophoresis produced a depolarisation of the cell membrane. The lowest concentration of bath applied ACh to produce a reliable response was in the order of 10^{-5} M. Although the exact concentration of

ACh reaching the cell is not known, this value compares favourably with that obtained by Kerkut, Pitman and Walker (1969) for DUM cells of the sixth abdominal ganglion. These authors reported a threshold concentration of approximately 5×10^{-5} M. Although their application technique was more direct (drugs were directed towards the cell body through a glass tube) the cell responded some 4-5 seconds after the initial application of ACh; in this study the delay between the time of application and the first signs of depolarisation was even longer (8-12 seconds). This indicates that in both cases the ACh applied to the bath had probably undergone considerable dilution before reaching the cell body. Callec and Boistel (1967) were able to reduce the application-response latency by ejecting ACh through a syringe controlled glass pipette placed close to the recording electrode. DUM cells of the sixth abdominal ganglion responded within 1 second after ACh ejection and were accordingly responsive to lower concentrations; the cells were sensitive to 5.5×10^{-6} M (initial) concentrations.

Repeated doses of ACh produced significant desensitisation of the cell even when applied at threshold concentrations (10^{-5} M). This posed a great problem in evaluating dose response data for individual cells. The same complication has been encountered in determining the dose response relationship of the giant fibre post-synaptic membrane in the terminal ganglion of Periplaneta

(Callec, Dowson and Sattelle, 1976; Sattelle, McClay, Dowson and Callec, 1976). Since most cells Produced a depolarisation in response to 10^{-4} M ACh, attenuation of the response by desensitisation could be avoided by applying a single dose to the preparation. Sattelle et al (1976) have shown that this method can provide a fairly consistent indication of the sensitivity of the postsynaptic membrane of the cercal nerve-giant fibre preparation. However the approximately two fold increase in the mean response of axotomised cells to 10^{-4} M ACh does not necessarily indicate a sensitivity change of the same order; this would depend on the shape of the dose response curve for the normal and axotomised cells.

It was possible to determine the relationship between dose and response by applying ACh iontophoretically to the surface of the soma membrane. The magnitude of the ejecting current is widely used as a measure of dose in iontophoresis experiments (Kuffler, Dennis and Harris, 1971; Brenner and Martin, 1976; Roper, 1976). This procedure rests on the assumption that a linear relationship exists between current intensity and rate of drug relaease and between rate of drug release and concentration at a fixed point in the tissue (Curtis, 1964). ACh applied to the soma membrane at 2 minute intervals produced no significant desensitisation using ejecting pulses of up to 10^{-6} coulombs. This was probably due to the relatively short exposure of the cell to iontophoretically applied ACh compared to that for bath application.

Since the slopes of the dose response curves for normal and axotomised cells were virtually the same, the shift of the curve to the left following axotomy is indicative of an increase in the sensitivity to ACh. Following axotomy the increase in the mean ACh sensitivity calculated from the steepest part of the dose response curve was approximately 3 fold.

4) The Action of GABA.

GABA applied to the cell by perfusion of the bath produced a hyperpolarisation of the membrane of motoneurone cell 28. The lowest initial concentration of GABA applied to the bath which produced a response was approximately 10^{-5} M which was similar to the value reported by Kerkut, Pitman and Walker (1969) for DUM cells of the sixth abdominal ganglion; when applied topically in the manner already described (page 88) 5×10^{-6} M GABA produced a temporary cessation of 'spontaneous' action potentials whilst 2.5×10^{-5} M GABA produced a hyperpolarisation of the cell membrane. Kerkut and co-workers have applied GABA iontophoretically to the soma membrane of DUM cells (Kerkut, Pitman and Walker, 1969; 1969a; Pitman and Kerkut, 1970). These neurones had similar sensitivities to iontophoretically applied GABA and ACh.

A biochemical or physical barrier to the penetration of GABA through the ganglion tissue has not yet been demonstrated. Considering the evidence outlined

above one would expect that such a barrier might exist since transmission across the cercal nerve-giant fibre synapse in the terminal ganglion is only blocked by concentrations of topically applied GABA above 10^{-2} M (Gahery and Boistel, 1965). Sittler and DeRemer (1967) reported a complete cessation of 'spontaneous' activity recorded in the connectives of cockroach ganglia after the application of 10^{-3} M GABA. Concentrations of GABA in the order of 10^{-1} M are required to inhibit synaptic transmission through the prothoracic ganglion of the grasshopper, Gampsocleis buergeri (Suya and Katsuki, 1961).

No significant difference was observed between the response of normal and axotomised cells to 10^{-4} M GABA applied by the single dose method. However it is not possible to state with certainty whether or not there is a change in the sensitivity of axotomised cells to GABA since the mean response was greater after axotomy but the difference was not significant; a significant difference may have been obtained had more preparations been examined.

5) The Action of Acetylcholine in the Presence of Anticholinesterases.

The anticholinesterases physostigmine and neostigmine caused potentiation of the ACh response when applied to the bath in 10^{-6} M concentrations. The ACh response was also increased in the presence of 10^{-7} M

neostigmine. Potentiations of up to 10 fold were observed indicating a high level of ChE in the region of the iontophoretic electrode. This is in accord with the observations of Winton, Metcalf and Fukuto (1958) who reported an intense staining for ChE beneath the nerve sheath and at the surfaces of the neurones. Smith and Treherne (1965) obtained similar results using the electron microscope; significant quantities of ChE were localised on the surface of the cell bodies in association with the surrounding glial tissue. This ChE appeared to be entirely absent from tissue pretreated with physostigmine.

The response of sixth abdominal DUM neurones to iontophoretically applied ACh is significantly increased in the presence of 1.5×10^{-5} M physostigmine (Pitman and Kerkut, 1970). Likewise the sensitivity of DUM cells of the terminal ganglion to ACh applied by perfusion is increased over 100 times after pretreatment with 10^{-6} M physostigmine (Pitman, 1968). Sattelle, McClay, Dowson and Callec (1975) employed the mannitol-gap technique of Callec and Sattelle (1973) to record potentials from the partly desheathed terminal ganglion of Periplaneta. The sensitivity to topically applied ACh was increased approximately 100 fold in the presence of 10^{-6} M physostigmine.

The effect of anticholinesterases on synaptic transmission in the cockroach was first described for the sixth abdominal ganglion by Roeder, Kennedy and

Samson (1947). Yamasaki and Narahashi (1960) described the first synptoms as an increase in the frequency and intensity of 'spontaneous' activity and the appearance of an 'after discharge' following cercal stimulation. Quantitative results have been obtained from extracellular recordings of 'spontaneous' activity in the metathoracic ganglion (Kerkut, Pitman and Walker, 1969); the lowest concentration of physostigmine to produce an effect was in the order of 1.5×10^{-5} M.

Synaptic transmission through vertebrate sympathetic ganglia is generally considered to be cholinergic (Eccles, 1952; Eccles and Libet, 1961). ACh perfused through these ganglia in approximately 4×10^{-4} M concentrations will normally produce action potentials in the postganglionic nerve trunk (Bronk, 1939). Following pretreatment with physostigmine the threshold ACh concentration falls to approximately 5×10^{-7} M (Emmelin and MacIntosh, 1952). This constitutes a similar increase in ACh sensitivity to that observed in the cockroach CNS following pretreatment with physostigmine. Bell (1967) noted that preganglionic stimulation of the guinea-pig hypogastric ganglion was more effective in producing a postsynaptic response after perfusion with physostigmine in concentrations ranging from 7×10^{-7} to 1.5×10^{-6} M. This is approximately 10 times less than the concentration necessary to enhance transmission in the cockroach. However it is not

yet possible to state whether or not this difference is of any significance.

Treherne and Smith (1965b) observed the metabolism of ^{3}H-labelled ACh by the intact central nerve cord of Periplaneta; ChE was only completely inhibited in the presence of 10^{-4} M physostigmine. It is therefore unlikely that 10^{-6} M physostigmine or neostigmine completely inhibited the ganglionic ChE activity in this study. Unfortunately higher concentrations could not be used; both physostigmine and neostigmine blocked the response of cell 28 to iontophoretically applied ACh. This block appeared to be competitive (Paton and Perry, 1953) in that it was produced without any initial stimulation of the cell by the anticholinesterases. In addition, some preparations became excitable such that action potentials could be induced in the cell body. A similar increase in excitability has been observed in DUM cells of the sixth abdominal ganglion when physostigmine was applied in concentrations of more than 1.5×10^{-6} M (Pitman, 1968).

Desensitisation by high doses of physostigmine has been observed in the vertebrate nervous system. This was first shown by Eccles (1934) who, by applying "large doses" of physostigmine, produced a reduction or abolition of action potentials recorded from the postganglionic nerve trunk of the cat superior cervical ganglion. Feldberg and Vartiainen (1935) obtained quantitative information using the same preparation. Shortly

after injecting 10 mls of 2 x 10^{-3} M physostigmine into the arterial blood supply the response to preganglionic stimulation rapidly declined and dissapeared within a few minutes. Lower doses of physostigmine produced a potentiation of the response. Neostigmine at approximately 10^{-5} M concentrations produces a competitive block of the ACh response of leech motoneurones (Sargent, 1977). When applied at 1.6-3.3 x 10^{-6} M concentrations, neostigmine produced a 50% reduction in the ACh response of identified sensory neurones. Similar concentrations (about 6.6 x 10^{-6} M) were required to produce a 50% reduction in the synaptic potential.

The idea that physostigmine and neostigmine in high concentrations compete with ACh for the receptor site is supported by the binding studies made on isolated ACh receptors from Torpedo (Eldefrawi, Eldefrawi, Seifert and O'Brien, 1972). Physostigmine or neostigmine applied at 10^{-3} M concentrations blocked the binding of ACh to soluble ACh receptors by 30 and 82% respectively.

When cell 28 was exposed to relatively low concentrations (10^{-6} M) of either physostigmine or neostigmine for more than about 5 minutes the resting potential showed a gradual decline. Similar concentrations of physostigmine have been shown to cause a gradual depolarisation of cockroach DUM cells (Pitman, 1968) and giant interneurones (Sattelle, McClay, Dowson and Callec, 1976). Prolonged application of relatively low doses of physostigmine (2 x 10^{-5} M) have been shown to

depress the response of cat superior cervical ganglia to preganglionic stimulation (Feldberg and Vartiainen, 1935). Whether this is due to the synaptic blocking action of physostigmine or depolarisation of the post-synaptic membrane is not clear.

A significant potentiation of the response to iontophoretically applied ACh was observed in both normal and axotomised cells after 2-4 minutes exposure to 10^{-6} M physostigmine or 10^{-7} to 10^{-6} M neostigmine. In such experiments the ACh response of normal cells was potentiated to a greater degree than that of axotomised cells. Since the ACh response is potentiated by the anticholinesterases one would assume that the response is normally limited to a significant extent by tissue ChE. The differences in the degree of potentiation observed are therefore taken to indicate that axotomised preparations contain less ChE in the area of the tip of the ACh micropipette and the site of drug action.

The same principle has been successfully used to demonstrate changes in ChE levels of the vertebrate CNS. Srebro and co-workers have shown, using biochemical techniques, that denervation of the rat hippocampus by lesions of the medial septum causes a large decrease in hippocampal AChE (Srebro, Oderfeld-Novak, Klodas, Dabrowski and Narkiewicz, 1973). A parallel fall in the potentiation by physostigmine of the ACh response of hippocampal pyramidal cells was observed by Bird and Aghajanian (1975) who applied the anticholinesterase

iontophoretically.

The fact that the ACh response of the soma of axotomised cell 28s is potentiated by anticholinesterases indicates that the ChE activity falls but does not reach zero (c.f. Miledi, 1962). Unfortunately however, quantitative information on the amount of ChE present in normal and axotomised preparations cannot be obtained using this technique. The observation that ChE is markedly reduced but still present in significant quantities is consistent with the histochemical and biochemical studies made on axotomised vertebrate neurones. In the rat, whole autonomic ganglia show an approximately 50% reduction in total ChE after post-ganglionic nerve section (Brown, McLennan and Pascoe, 1952b; McLennan, 1954; Dhar, 1958) and a much greater although incomplete reduction in the ChE activity of the ganglion cell bodies and dendrites (Brown, 1958; Taxi, 1961; Harkonnen, 1964). Similar changes have been observed in cat autonomic ganglia (Fredricsson and Sjoqvist, 1962; Gromadzki and Koelle, 1965). Central neurone cell bodies have also been shown to preserve some ChE activity after axotomy. These include spinal motoneurones of the dog (Hard and Peterson, 1949; 1950), the rat (Schwarzacher, 1958) and bullfrog and common toad (Chacko and Cerf, 1960) and hypoglossal neurones of the rat (Lewis and Shute, 1965).

6) The Action of Carbamylcholine.

Carbamylcholine (CCh) produced a depolarisation of the soma membrane when applied either by perfusion of the bath or by iontophoresis. The cell was more responsive to bath applied CCh than ACh; the depolarisation produced by perfusing 10^{-4} M CCh through the bath was approximately 1.6 times that for the same concentration of ACh. When applied iontophoretically the cell appeared to be less sensitive to CCh than to ACh. The threshold doses of ACh tended to be higher than of CCh but the slope of the ACh dose response curve was appreciably higher than the curve for CCh (see Figures 30 and 31b). The reduction in the slope of the CCh dose response curve is most probably a result of desensitisation. Sattelle et al have shown that ACh is intrinsically more potent than CCh (Sattelle, McClay, Dowson and Callec, 1976). Recording from the postsynaptic membrane of the cercal nerve-giant fibre preparation in Periplaneta, the dose response curve for ACh in the presence of physostigmine was placed to the left of the curve for CCh. However the potency of CCh was greater than ACh alone. The dose response relationships were calculated from single doses applied (by perfusion) only once to a number of preparations, each preparation receiving a different dose. In this way problems of desensitisation were avoided. Similarly in this study, by challenging each preparation with only one test concentration of CCh or ACh, the soma membrane of cell 28

appears to be more responsive to CCh than ACh. It is therefore assumed that the relatively low sensitivity of the cell to iontophoretically applied CCh is due to desensitisation of the membrane.

The response of axotomised cells to CCh applied either by bath perfusion or by iontophoresis was not significantly different from that of normal cells. If it is assumed that CCh mimicks ACh, this observation supports the conclusion drawn from the anticholinesterase experiments; that axotomy results in a decreased inactivation of ACh by ChE. In the vertebrates this principle has been used successfully in predicting reductions of ChE activity in denervated rat diaphragm muscle (McConnell, 1974; Kiauta, Brzin and Dettbarn, 1977) and rat hippocampal pyramidal cells (Bird and Aghajanian, 1975; Srebro, Oderfeld-Novak, Klodas, Dabrowski and Narkiewicz, 1973).

7) Receptor Distribution and Density.

The increase in ACh sensitivity of axotomised neurone cell bodies could also result from an increase in the number of ACh receptors on the cell membrane within the region of the applied drug or of a redistribution of receptors. The rise time of the depolarisation produced by iontophoretically applied ACh was observed to increase linearly with the iontophoretic charge. Since the concentration of ACh along the membrane surface is proportional to the ejecting charge (DelCastillo and

Katz, 1955), increasing gradations of dose appear to progressively reach more distant receptors. One would therefore expect some indication of the receptor density from the rise time of the response to a given dose of ACh. Similarly, by examining the rise times of the depolarisations produced by applying ACh to different areas on the cell membrane, some information may be obtained about the distribution of receptors. However it should be born in mind that the rise time of the ACh response would be dependent on any diffusion barrier between the ACh micropipette and the ACh receptors, as well as the density of the receptors themselves.

The ACh response rise times were slow in comparison to those reported for ACh applied to extra-syanptic areas on vertebrate parasympathetic ganglion cells (Harris, Kuffler and Dennis, 1971; Roper, 1976). This is taken to indicate either a relatively low density of receptors or the presence of a diffusion barrier to ACh. There appeared to be no change in these parameters after axotomy since the rise times were similar. It is possible however that the failure to detect any difference may have been because this difference was too small to be resolved. Never the less, if either a proliferation of receptors or the removal of a diffusion barrier are responsible for the approximately 3 fold increase in ACh sensitivity one would expect a significant reduction in the ACh response rise time. Harris and co-workers observed that in parasympathetic neurones of the frog

atrium the sensitivity of the synaptic membrane to applied ACh was approximately 1.7 times that of the extrasynaptic membrane (Harris, Kuffler and Dennis, 1971). This was attributed to a difference in the receptor density. The times to peak of these potentials were however strikingly different; those produced by application of ACh to synaptic regions were about twice as fast as those produced at extrasynaptic regions.

In support of the conclusion that the density of ACh recepotrs is unchanged after axotomy is the observation that following pretreatment with anticholinesterases the sensitivity of normal and axotomised cells were the same. Certainly from the observations made on axotomised neurones (Mathews and Nelson, 1975; Brenner and Martin, 1976) one would expect if anything, a reduction in the density of ACh receptors after axotomy.

The surface membrane of cell 28 appears to be uniformally sensitive to ACh. Within the limits of the resolution by iontophoretic application the sensitivity of the membrane to ACh and the rise times of the response were remarkably consistent wherever the iontophoretic electorde was placed on the cell body. A similar uniformity of ACh receptor distribution has been demonstrated in extrasynaptic areas of other invertebrate preparations. ACh receptors appear to be present on the entire soma membrane of monopolar molluscan neurones (Tauc and Bruner, 1963) and specific sensory and motoneurones of the leech (Sargent, Yau and Nicholls, 1977).

Analysis of the distribution of extrasynaptic receptors in the vertebrates is complicated by the presence of synaptic areas on the soma membrane. In some vertebrate preparations synaptic spots can be visually identified, for example on the surface membrane of parasympathetic neurones in the interatrial septum of the heart of the frog (Harris, Kuffler and Dennis, 1971) and mudpuppy (Roper, 1976a). These authors were able to demonstrate the presence of extrasynaptic receptors using iontophoretically applied ACh. The spatial distribution of extrasynaptic receptors appeared to be more scattered on the soma membrane of these preparations than on the soma membrane of cell 28. However it is not possible to determine to what extent the junctional receptors contributed to the ACh response. In addition in making such comparisons the relatively limited spatial resolution of the technique used in this study should be acknowledged. Non-uniformity of the distribution of extrasynaptic receptors is even more pronounced on the surface of vertebrate muscle fibre, for example the rat diaphragm and the frog sartorius muscles (Miledi, 1962). In both cases there is a peak of ACh sensitivity in the region of the endplate which falls sharply on either side; falling to about one tenth within 200 μ. A similar distribution of chemosensitivity (to glutamate) has been shown in the locust retractor unguis muscle (Usherwood, 1969).

The apparent uniformity of chemosensitive

areas on the soma membrane of cell 28 was not changed appreciably by axotomy. As with the normal preparation the sensitivity to ACh and the rise times were about the same wherever the iontophoretic electrode was placed. A change in receptor distribution and density following axotomy would not have been altogether too surprising since axotomised cells appear to undergo radical changes in protein synthesis (see Introduction, page, 60).

8) The Acetylcholine Reversal Potential.

Since the ACh response shows a 10-fold reduction for a change in $(Na)_o$ from 214 mM to 20 mM, ACh must increase the permeability of the cell 28 soma membrane to sodium ions. This is consistent with observations made by Kerkut, Pitman and Walker (1969) recording from DUM cells of the cockroach metathoracic ganglion. It is interesting that the resting potential is increased by perfusion of saline containing low concentrations of sodium; in 20 mM $(Na)_o$ the resting potential is hyperpolarised by approximately 15 mV. This is probably due to a permeability of the resting membrane to sodium ions. When the $(Na)_o$ is reduced, E_{Na} (the sodium reversal potential) is shifted to a more negative value, the rate of entry of sodium ions into the cell would be reduced and so the resting potential would increase.

If for the moment we assume that ACh acts by increasing the permeability to sodium ions only, then the ACh reversal

potential should be equal to the sodium equilibrium potential. Treherne (1961) estimated the internal sodium concentration of cockroach giant axons to be 75.6 mM. This correlates well with the values obtained by direct measurement of the intracellular concentrations by determinations on extruded axoplasm, such as has been carried out with squid axons (e.g. 50 mM - Hodgkin, 1958). From the Nernst equation the sodium reversal potential for cockroach nervous tissue would then be:

$$E_{Na} = \frac{RT}{ZF} \log_e \frac{214}{75.6}$$

or $$58 \log_{10} 2.83 = 26.2 \text{ mV at } 18^{\circ}C$$

A reduction in the internal sodium concentration would result in a more positive value for E_{Na}. Thus if ACh opened the same number of sodium channels in each case the depolarisation produced would be greater with a lower (Na)i. The increase in ACh sensitivity following axotomy could possibly have been a result of a shift of the ACh reversal potential to a more positive value.

In the majority of preparations of nervous tissue studied the reversal potential could not be measured directly since a large positive shift in membrane potential produced action potentials and so masked the ACh response (e.g. Brenner and Martin, 1975; Hagiwara, Watanabe and Saito, 1959). In this preparation although the cell was not normally excitable, depolarisations in

excess of about 20 mV produced a dramatic fall in the input resistance and membrane oscillations. However provided that the overall depolarisation of the cell membrane did not exceed about 20 mV with respect to the resting potential the cells showed an approximately linear relationship between membrane potential and the magnitude of the depolarisation induced by iontophoretically applied ACh.

Therefore in order to estimate the reversal potential of the ACh response it seemed justified to extrapolate from points around the resting potential into the region of the reversal potential. Using this technique a value of approximately -35 mV was obtained for the ACh reversal potential. This value is rather more negative than those obtained from other preparations which usually lie in the region of 0 to -20 mV (see Ginsborg, 1967).

This deviation from the value predicted for the sodium equilibrium potential suggests that the ACh potential involves a significant influx of anions and/or an efflux of cations in addition to the influx of sodium ions suggested. Potassium ions present the most likely candidate for the carrier of an outward current. At the frog neuromuscular junction for example the value of the reversal potential of the end-plate current is dependent on both the extracellular sodium and potassium concentrations (Tackeuchi and Tackeuchi, 1960). Therefore the potassium conductance of the end-plate

membrane must also increase during the end-plate current. In cockroach giant axons the intracellular concentration is approximately 132 mM (Treherne, 1961). This is considerably higher than the extracellular concentration (about x 40) and so the potassium reversal potential would be negative with respect to zero. Therefore assuming that ACh acts by increasing the permeability of the membrane to cations so that sodium ions flow inward and potassium ions flow outward, the ACh reversal potential would be more negative than the sodium equilibrium potential. It is interesting that Feltz and Mallart (1970) obtained an even more negative value for the ACh reversal potential at extrasynaptic regions of frog muscle fibres. The reversal potential for extrasynaptic receptors was about -44 mV whereas the value for junctional receptors was about -15 mV.

Mallart, Dreyer and Peper (1976) point out the errors of determining the ACh reversal potential values of extrasynaptic receptors. The relationship between membrane potential and response was shown to be linear when the ACh was applied close to a synaptic area (on frog skeletal muscle). However if the iontophoretic electrode was moved away from the synaptic area (either vertically away from the entire preparation or horizontally and so into an extrasynaptic area) then the relationship between membrane potential and response became non-linear. This non-linearity is explained by the fact that the ACh concentration on the membrane outlasts the mean

life time of the ionic channels (about 10 ms) and that the mean life time of the channels is increaseased with hyperpolarisation. Therefore at more hyperpolarised levels the response is larger than would be expected for a linear relationship between membrane potential and response. A linear extrapolation of the membrane potential-ACh response relationship produced a reversal potential value more negative for extrasynaptic receptors than for synaptic receptors. This may explain the difference in the values obtained earlier by Feltz and Mallart (1970) between synaptic and extrasynaptic ACh reversal potentials.

It is interesting that in this preparation the membrane potential-ACh response relationship appeared to be linear. This may be because in the extrapolation a greater emphasis was placed on the ACh response at values of membrane potential positive with respect to the resting potential (Figure 37b); Feltz and Mallart (1970) extrapolated through ACh responses at -60 to -140 mV membrane potential. Alternatively the opening and closing times of the ionic channels may not be influenced by the membrane potential as is the case with frog muscle fibres. Obviously further experimentation is required to resolve this question.

In spite of these possible errors in determining the ACh reversal potential, using this technique a significant difference in the true values for normal and axotomised cells should be revealed. The mean estimated value for the ACh reversal potential of axotomised cells was -33 mV. This was

not significantly different from the value obtained for normal cells. These results indicate that the increase in the sensitivity of axotomised cells is not due to a shift in the ACh reversal potential to a more positive value.

9) The Number of Acetylcholine Molecules Required to Activate a Single Receptor.

Dreyer, Peper and Stertz (1978) were able to describe the relationship between dose and response at the frog neuromuscular junction (to ACh and CCh) using three constants: (i) the Hill coefficient (n, the average number of ACh molecules required to activate a single receptor), (ii) the dissociation constant (K_A) and (iii) the maximum conductance change which can be produced by the drug, Δg_{max}. This relationship can be expressed by the equation:

$$\Delta g = \frac{Q^n}{K_A + Q^n} \Delta g_{max}$$ (from Brenner and Martin, 1976)

where Q is the amount of the drug (ACh) applied to the preparation and is therefore proportional to the dose received and Δg is the response produced by Q.

For reasons already mentioned (Results p.102) a value for Δg_{max} could not be obtained. The dissociation constant K_A is defined as the concentration of ACh required to open 50% of the maximum fraction of channels that can be opened at high concentrations. A value for the constant K_A could not be determined since it has been assumed that Δg is proportional to the number of channels opened.

In the majority of preparations studied (see Rang, 1971; Table 1, p. 92) the slope of the Hill plot (log $E/E_{max} - E$ against log concentration, where E is the effect produced by a given dose of drug and E_{max} is the response to a maximal dose) is greater than one. In many cases the slope is greater than two. Rang (1971) suggested that these observations may indicate a value of n greater than unity. A reduction in the value of n could conceivably result in an increase in the sensitivity of a preparation to an applied drug.

A value of n was not obtained for the action of ACh on this preparation. However the slopes of the double logarithmic plots (log Δg/log dose) obtained for normal and axotomised preparations were not significantly different (see Results p.102). This is taken to indicate that the increase in ACh sensitivity produced by axotomy is not due to a reduction in the average number of ACh molecules required to activate a single receptor. It should be noted that a change in the constant K_A which would represent a change in the affinity of the receptors for ACh cannot be excluded.

These results are similar to those obtained by Brenner and Martin (1976) working on axotomised chick ciliary ganglion cells. These authors using the same analytical technique, observed no significant difference in the value of n between normal and axotomised cells. However in the chick preparation, axotomy resulted in a reduction rather than an increase in the sensitivity of the cells to iontophoretically applied ACh.

10) The Time Course.

The soma of cell 28 showed an increase in sensitivity to applied ACh within about 4 days after axotomy and this was apparently maintained for up to 60 days. Since it is suggested that this supersensitivity directly results from a reduction in ChE activity, changes in enzyme activity and ACh sensitivity should follow the same time course.

The reduction in ChE activity observed in axotomised vertebrate neurones seems to occur during the period of increased RNA and protein production (see p. 71 of Introduction). For example Pearse (1955) found parallel changes in cytoplasmic nucleic acid and nsChE activity in rat motoneurones following axotomy. In axotomised superior cervical ganglion cells of the rat the fall in AChE and nsChE activity followed the disappearance of Nissl substance containing cytoplasmic RNA. The RNA of normal cockroach motoneurones is in a dispersed state similar to that found in chromatolysed vertebrate neurones. Following axotomy the RNA forms an aggregate around the nucleus and begins to disappear after about 2-5 days (Cohen and Jacklet, 1965; Cohen, 1967; Byers, 1970). Experiments with tritiated uridine indicate that dispersal of the perinuclear aggregate is associated with an increase in RNA synthesis (Byers, 1970).

It therefore appears that the situation in axotomised cockroach central neurones resembles that of axotomised vertebrate neurones; RNA synthesis begins some 3 days after axotomy at approximately the same time as the reduction in ChE activity. This is possibly paralleled by

an increase in protein production. Since axons emerge from the cut nerve stump about 10 days after nerve section (Bodenstein, 1957; Guthrie, 1962), protein synthesis presumably starts before this.

11) Recovery of ChE Activity.

Many axotomised vertebrate neurones show a recovery of ChE activity, returning to normal within the regenerative period. (e.g. Schwarzacher, 1958; Huikuri, 1966). Harkonen (1964) has shown that in the superior cervical ganglion of the rat, AChE activity not only recovers but becomes significantly higher than in normal cells. Although the evidence is slender, it is possible that the cells investigated in this study on the cockroach also show a recovery of ChE activity; 4 out of the 6 cells examined more than 30 days following axotomy showed no sign of an elevated ACh sensitivity. Of the two cells examined between 110 and 120 days after axotomy, both showed an ACh sensitivity well below normal. It is possible that this was due to degeneration; Jacklet and Cohen (1967) observed that cell bodies of axotomised cockroach neurones which had not made connection with the muscle within 150 days appeared to be reduced in size. On the other hand the legs which these cells would normally innervate showed signs of functional innervation. Obviously more work would have to be done on the long term effects of axotomy on the ACh sensitivity of these cell bodies before this problem can be resolved.

12) Axoplasmic Flow.

All neurones studied show axonal transport. Proteins are most probably transported to and away from the cell body of cell 28 by axoplasmic flow; Treherne and Smith (1963) have demonstrated the presence of neurotubules in the axons of central neurones of the cockroach - structures usually associated with axonal transport. The structural and biochemical changes that occur in axotomised cell bodies may be explained by the fact that axonal transection interrupts axoplasmic transport - both orthograde (Koenig, 1958; Taylor and Weiss, 1965; Bray and Austin, 1968) and retrograde movement (Lubinska, 1964; McEwen and Grafstein, 1968). The products of the cell body would consequently accumulate in the proximal nerve stump and similarly materials from the distal nerve stump would no longer be able to reach the perikaryon. Pitman, Tweedle and Cohen (1972) have shown that colchicine, which blocks axoplasmic flow, when applied locally to the nerve induces morphological and electrophysiological changes in the soma of cell 28 similar to those produced by nerve section. The time taken for these changes to develop are about the same for nerve section and treatment with colchicine. It is therefore possible that an alteration in axoplasmic flow may be the mechanism by which the cell learns of the axonal injury. This is supported by the observation first made by Marinesco (1897) that the closer the site of injury to the cell body the sooner and more severe the chromatolytic reaction.

There are two possible sites at which ChE located

in the axonal membrane and synaptic structures of cockroach neurones may be synthesised; the perikaryon and the axon. Although axonal RNA has not been demonstrated in the insect CNS, axonal protein synthesis cannot be ruled out. Evidence for such a mechanism has been obtained from vertebrate preparations, for example the hypoglossal nerve of the cat (Koenig, 1965) and the giant axon of the Mauthner neurone of goldfish (Edstrom, 1964). However since the rate of axonal synthesis in these cases is relatively slow, the most likely location for the synthesis of ChE would seem to be the perikaryon. In the vertebrates the greater proportion of neuronal ChE appears to be synthesised in the cell body and migrates down the axon by fast axonal flow - about 100 to 500 mm/day (Lubinska and Niemierko, 1971; Krygier-Brevart et al, 1974). ChE synthesised in the cell body of a cockroach central neurone would probably be carried by a similar transport mechanism. This is probably maintained after axotomy; Ochs and Ranish (1969;1970) have shown that the velocity of movement of tritiated leucine in cat sciatic nerves is unchanged after section of the nerve. The fact that in vertebrate preparations migration of a significant fraction of ChE along the axon is by the fast component of axoplasmic flow would suggest that turn-over of this enzyme is fairly rapid.

Thus it can be postulated that the fairly rapid reduction in the ChE activity of this cockroach neurone is due to a reduction in enzyme synthesis coupled with a depletion of already existing stores of the enzyme by orthograde

axoplasmic flow. The possibility that axotomy causes an increase in the rate of enzyme degradation cannot be ruled out however.

13) Deafferentation by Nerve Five Section.

The fifth nerve (N5) of the metathoracic ganglion, in addition to innervating some of the muscles, innervates most of the sense organs of the hind leg (see Guthrie and Tindall, 1968). Thus transection of N5 would undoubtedly reduce the afferent input to the ganglion and presumably to cell 28 itself. In the locust the axons of the fast extensor tibiae motoneurones of the metathoracic ganglion pass through N5. Horridge and Burrows (1974) have shown that these neurones receive a sensory input through the same nerve. However this sensory input appears to be indirect, involving one or more interneurones.

The possibility that the reduction in ChE activity observed in this preparation results from deafferentation rather than axotomy cannot be ruled out. Certainly in some vertebrate preparations deafferentation has been shown to produce a reduction in ChE activity. Giacobini (1971), using biochemical techniques, measured the ChE activity of single neurones of the seventh lumbar sympathetic ganglion of the cat; preganglionic denervation produced a reduction in the ChE activity of the postsynaptic cell. Denervation of hippocampal pyramidal cells in the rat results in an increase in the sensitivity of these cells to iontophoretically applied ACh. Bird and Aghajanian (1975) attributed this supersensitivi

to a reduction in ChE activity. However in this vertebrate preparation most of the ChE is located presynaptically (Shute and Lewis, 1966; Kuhar and Rommelspacher, 1974).

Synaptic input to cell 28 may have been disrupted by the response to axotomy itself. In the vertebrates it is known that when the axon of a motoneurone is cut the synapses are physically removed by proliferating microglial cells (Blinzinger and Kreutzberg, 1968; Sumner and Sutherland, 1973) and retraction of the dendritic branches (Sumner and Watson, 1971). Horridge and Burrows (1974) produced electrophysiological evidence of synaptic disruption in an axotomised motoneurone of the locust although the mechanism underlying this change was not investigated. However, Tweedle, Pitman and Cohen (1973), using cobalt impregnation, were unable to detect any modification in the gross dendritic branching pattern of cell 28 after axotomy. This could have been because small changes in synaptic morphology escaped detection due to the relatively low resolution of the cobalt technique. Similar experiments using the more recently developed cobalt intensification techniques (Strausfeld and Obermeyer, 1976; Bacon and Altman, 1977; Pitman, 1979) should help resolve this question.

SUMMARY - SECTION I.

1) The cell body of the fast coxal depressor motoneurone (D_f) in the metathoracic ganglion of the cockroach, Periplaneta americana, has been used to examine the electrical characteristics and the response to topically and iontophoretically applied drugs of normal cells and cells that have been axotomised from 4-118 days. The majority of axotomised cells were examined between 4-10 days after operation.

2) Resting potentials, membrane resistances and membrane time constants were the same in axotomised cells as in normal cells. Using microelectrodes filled with 1 M potassium acetate these averaged 65.8 ± 1.6 mV, 4.8 ± 0.5 M ohms and 14.3 ± 1.2 ms respectively.

3) Normal cells were electrically inexcitable. After nerve section many cells became electrically excitable; producing all or none overshooting action potentials in response to a small depolarising current. The threshold depolarisation for these cells was in the region of 10-15 mV.

4) Bath applied ɣ-aminobutyric acid (GABA) produced a hyperpolarisation of the cell membrane. There was no significant difference ($P > 0.6$) between normal and axotomised cells in the degree to which the cell membrane was hyperpolarised by 10^{-4} M bath applied GABA. This value averaged 4.6 ± 1.1 mV (n=11) for normal cells and 5.4 ± 1.7 mV (n=4) for axotomised cells.

5) When applied topically or by iontophoresis on to the soma membrane, acetylcholine (ACh) caused depolarisation of the cell membrane. Repeated application of large doses resulted in relatively rapid desensitisation and depression of the response. The response to 10^{-4} M ACh applied to the bath was higher in axotomised cells than in normal cells by a factor of about two ($P < 0.005$); average values were 6.0 ± 0.9 mV (n=22) for normal cells and 13.9 ± 2.2 mV (n=19) for axotomised cells. Sensitivity to iontophoretically applied ACh (calculated from the dose response curves) was 0.077 ± 0.020 mV/nC (n=33) for normal cells and 0.246 ± 0.039 (n=20) for axotomised cells. These values were significantly different ($P < 0.001$).

6) The rise times of the iontophoretically applied ACh potentials were the same in the two groups indicating that the increased sensitivity was not due to either a redistribution of cholinoceptors on the soma membrane or a diffusion barrier.

7) The slopes of the dose response curves plotted on a double logarithmic scale were not significantly different ($P > 0.5$) suggesting that there was no difference between normal and axotomised cells in the number of ACh molecules needed to combine with individual cholinoceptors to produce a response.

8) The estimated reversal potential (determined by extrapolation) for the action of ACh was unchanged after axotomy. The values for normal and axotomised cells respectively

averaged -35 ± 2.4 mV (n=5) and -33 ± 2.9 mV (n=5), ($P > 0.5$).

9) There was no significant difference in the sensitivity of control and axotomised neurones to carbamylcholine (CCh) applied either by bath perfusion or iontophoresis indicating that a fall in acetylcholinesterase activity may be responsible for the increase in sensitivity to ACh.

10) The anticholinesterases physostigmine and neostigmine potentiated the ACh response when applied to the bath in 10^{-6} M concentrations. However both produced a greater potentiation of the ACh response in normal than in axotomised neurones; supporting the hypothesis that the supersensitivity to ACh is due to a decreased inactivation of ACh by acetylcholinesterase.

11) The time course of the increase in sensitivity to ACh is correlated to the time course of increased RNA and protein production.

SECTION II

The effect of denervation on the cholinesterase activity of metathoracic ganglia

PLAN OF EXPERIMENTS - SECTION II.

In the previous section it was concluded that the increase in sensitivity of an identified motoneurone cell body to applied ACh was due to a reduction in ChE activity in the region of the applied drug. Since the axons of a relatively large number of motoneurones leave the metathoracic ganglion through the fifth nerve trunk (Cohen and Jacklet, 1967) one would expect a detectable fall in the total ChE content of the ganglion after transection of this nerve.

Detection of a fall in the ganglionic ChE activity would be limited by two parameters: (i) the variation in the total enzyme activity from one control ganglion to another and (ii) the sensitivity of the enzyme assay. Should the change in ChE activity following nerve section be relatively small then the difference between control and denervated ganglia would tend to be masked by the variation between the controls. In order to help overcome this problem both left and right fifth nerve trunks were severed so that any difference in enzyme activity would (presumably) be doubled.

From the options available for the assay of ChE activity, the photometric technique of Ellman, Courtney, Andres and Featherstone (1961) was chosen. This method is extremely sensitive and therefore applicable to small amounts of tissue. The substrate used is acetylthiocholine; an analogue of ACh which appears to substitute well for the natural transmitter (Tabachnik,

1956; Gal and Roth, 1957; Ellman, Courtney, Andres and Featherstone, 1961). The method is based on the measurement of the rate of production of thiocholine which results from the hydrolysis of acetylthiocholine by ChE. The thiocholine produced by this reaction in turn reacts with 5:5-dithiobis-2-nitrobenzoate (DTNB) to produce the yellow anion of 5-thio-2-nitro-benzoic acid:-

$$\text{acetylthiocholine} \xrightarrow{\text{ChE}} \text{thiocholine} + \text{acetate}$$

$$\text{thiocholine} + \text{DTNB} \longrightarrow \text{yellow colour}$$

Thus the rate of the reaction can be continuously monitored by reading the absorbance of the solution at 412 mμ in a photometer.

METHODS.

1) Preparation of the Ganglion.

The third thoracic ganglion was exposed as described on page 35 and removed by cutting all nerve trunks as close as possible to the ganglion. The ganglion was weighed, and in the case of the experiments involving more than one ganglion, was stored in a glass vial at 0°C. The ganglia were then homogenised in a 'Potter' homogeniser in cold phosphate buffer 0.1 M, pH 8.0, using 1 ml of buffer per ganglion, to contain 0.51-0.83 mg of tissue/ml. At these protein concentrations the ChE activity in the homogenate remained constant for about two hours if kept below 0°C.

2) Solutions.

Phosphate buffer was made up from 0.1 M solutions of di-sodium orthophosphate and sodium dihydrogen phosphate and adjusted to the desired pH. The substrate acetylthiocholine iodide was dissolved in 0.1 M phosphate buffer, pH 8. This was diluted with the same buffer to yield a 10^{-2} M stock solution. Dithiobisnitrobenzoate (DTNB) was dissolved in 0.1 M phosphate buffer, pH 7.0, to give a 10^{-3} M solution which was kept in the dark. DTNB was made up in buffer of pH 7.0 since it is more stable than in that of pH 8.0 (Ellman, Courtney, Andres and Featherstone, 1961). The specific esterase inhibitor neostigmine bromide was dissolved in phosphate buffer, pH 8.0, to give a solution of 8.25×10^{-4} M.

3) The Reaction Vessel and Application of Solutions.

The assay was carried out in semi-micro glass cuvettes of 1 cm path length, the relatively thick walls of which minimised changes in temperature caused by the application of cold solutions. The cuvettes were held in a metal block through which water was circulated at 37°C. Solutions were added with micropipettes and mixed using 1 ml disposable syringes.

4) The Reaction Mixture.

The assay was performed as follows: 0.17 mls of the homogenate, 0.63 mls of phosphate buffer, pH 8.0 and 0.1 mls of DTNB were added to the cuvette. The solutions were mixed and left one minute for the temperature to stabilise. The blank cuvette (B) received 0.8 mls of phosphate buffer, pH 8.0, and 0.1 mls of DTNB. The reaction was initiated by the addition of 0.1 mls of acetylthiocholine to both cuvettes and the solutions were mixed and left for one minute before reading. This period of time was necessary before the rate of the reaction became linear. The disturbance was probably due to several factors such as equilibration of temperature, the settling of particles from the homogenate and air bubbles in the solution.

After obtaining a linear rate of hydrolysis for two minutes the reaction was terminated by the addition of 0.6 mls of neostigmine bromide and the rate of any non-cholinesterase substrate hydrolysis determined using the same technique.

The substrate concentration was altered by adding different amounts of either 10^{-2} or 10^{-3} M stock acetylthiocholine solutions. The volume of the reaction mixture was maintained at 1 ml by the addition of the appropriate amount of buffer.

A reagent blank was not included in the assay as no hydrolysis of any endogenous thiocholine esters could be detected.

5) Monitoring the Rate of Hydrolysis.

The rate of colour production was measured by continuously reading the absorbance of the reaction mixture against the blank (B) at 412 nm on a 'Beckman' model 24 spectrophotometer equipped with a 'Beckman' chart recorder.

6) Monitoring the Rate of Hydrolysis at Different pH Values.

Each assay was performed as described in (4) except that: (i) in place of the 0.63 mls of 0.1 M phosphate buffer, pH 8.0, various proportions (combined volume 0.63 mls) of 0.1 M solutions of di-sodium ortho-phosphate and sodium dihydrogen phosphate were added, and (ii) the reaction was not terminated by the addition of neostigmine. After the assay the pH of the contents of the cuvette containing the homogenate was measured at 37°C using a 'Radiometer' standard pH meter model PHM 62. This probably gave a true indication of the pH of the reaction mixture whilst in the photometer as the pH did not vary more than 0.01 units over a period of two minutes.

7) Protein Estimation.

The protein concentration of the homogenate was determined using the method of Lowry et al (1951) as modified by Maddy and Spooner (1970) to include the membrane bound protein. The following stock solutions were made up:

(i)	Sodium Carbonate	0.377 M
(ii)	Sodium Hydroxide	0.1 M
(iii)	Sodium Potassium Tartrate	0.095 M
(iv)	Cupric Sulphate	0.04 M
(v)	Sodium Deoxycholate	0.048 M

(made up in 0.25 M Sodium Hydroxide)

The procedure was as follows: a mixture of 2 mls (i), 2 mls (ii), 0.04 mls (iii) and 0.04 mls (iv) was made (in that order). 2.5 mls of this solution was added to a mixture of 0.2 mls of homogenate and 0.4 mls (v) to which was added 0.25 mls of 3 x diluted Folins' reagent (one part Folins': two parts water). The final mixture was left for one hour at room temperature before measuring its absorbance at 695 nm against a blank of 0.1 M phosphate buffer. Each estimation was done in triplicate.

A standard curve was prepared using the same method with bovine serum albumin substituted for the homogenate.

8) Statistics and Computing.

The data from normal and experimental animals were tested statistically using a Students' two tailed t-test. Straight line graphs were drawn using the method of 'Least Squares' to determine the line of best fit.

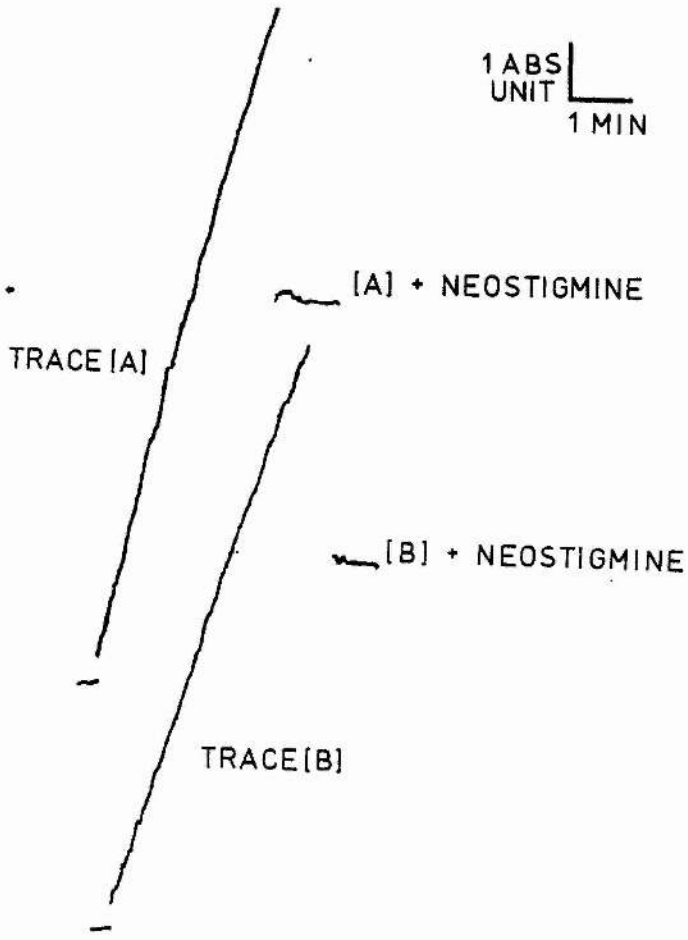


Figure 40. The rate of AThCh hydrolysis by a tissue homogenate made from 10 normal ganglia. Change in absorbance with time at pH 8.0 (trace A) and pH 7.5 (trace B). Hydrolysis is absent in the presence of 3.3×10^{-4} M neostigmine at pH 8.0, (A) + neostigmine and pH 7.5, (B) + neostigmine . Trace moves from left to right.

RESULTS.

1) The Preparation.

In the following experiments the homogenate from 10 metathoracic ganglia was used for each assay. The term preparation therefore refers to the combined homogenate made from 10 animals. 'Denervated' ganglia had both fifth nerve trunks cut 5-8 days prior to the experiment.

The result of a typical experiment is shown in Figure 40. The linear portion of the curve describing the hydrolysis is observed for over 15 minutes although normally the reaction was terminated after about 2 minutes. The rate of the reaction was determined almost exclusively by cholinesterases since the change in the absorbance was negliglible in the presence of 3.3×10^{-4} M neostigmine (Figure 40; also see Koenig, 1965). Neostigmine is a strong inhibitor of ChE. The contribution of non specific cholinesterases e.g. butyrylcholinesterase was not determined. However this is probably small since cholinesterase in cockroach nervous tissue is generally of the specific type (Iyatomi and Kaneshisa, 1958; Kerkut, Oliver, Rick and Walker, 1970). Woodson, Schlapfer and Barondes (1972) determined the ChE activity of cockroach thoracic ganglia by following the rate of hydrolysis of [1 - ^{14}C]ACh. Using a specific inhibitor of true ChE (BW 284C51) they demonstrated that the rate of [1 - ^{14}C]ACh hydrolysis by non-specific ChE

was negligible compared to the rate of hydrolysis by the normal tissue homogenate.

It was important to keep the activity of the esterase in the homogenate stable for periods of over an hour in order to examine some of the properties of the enzyme. Using the technique described, the sample was relatively stable (Figure 41) provided it was stored at temperatures below 0°C. At lower protein concentrations the enzyme activity fell dramatically after 15 minutes even if the homogenate was kept at -20°C.

The wet weights of the ganglia used in this study varied from 0.55 to 0.85 mg. There was no significant difference between the wet weights of normal and denervated ganglia. Similarly there was no difference between the protein concentrations. These varied from 11-19 μ gms per ganglion. The protein estimation included membrane bound protein since in the cockroach esterase is usually found in association with the cell membrane (Iyatomi, 1958; Smith and Treherne, 1965).

2) The Rate of the Reaction.

Since the extinction coefficient of the yellow anion 5-thio-2-nitro benzoic acid is known (Ellman, 1959) the rate can be converted to absolute units (Ellman, Courtney, Andres and Featherstone, 1961; Bergmeyer, 1963). The rate R in n moles/min/μ gm protein is given by the equation:

$$R = \frac{1000 \times A}{13.6 \times v \times p}$$

Figure 41. Stability of the homogenate: ordinate, rate of hydrolysis of AThCh (n moles/min/μ gm protein); abscissa, time after homogenisation of tissue. Protein concentration (μ gms/ml of test solution); • = 12, o = 5. The enzyme activity shows an appreciable fall with time at a protein concentration of 5 μ gms/ml test solution.

Figure 42. The effect of pH on the activity of ChE from the homogenate of 10 normal (o) and 10 experimental/ denervated (•) ganglia. Ordinate, velocity of reaction (n moles AThCh hydrolysed/min/μ gm protein); abscissa, pH of test solution.

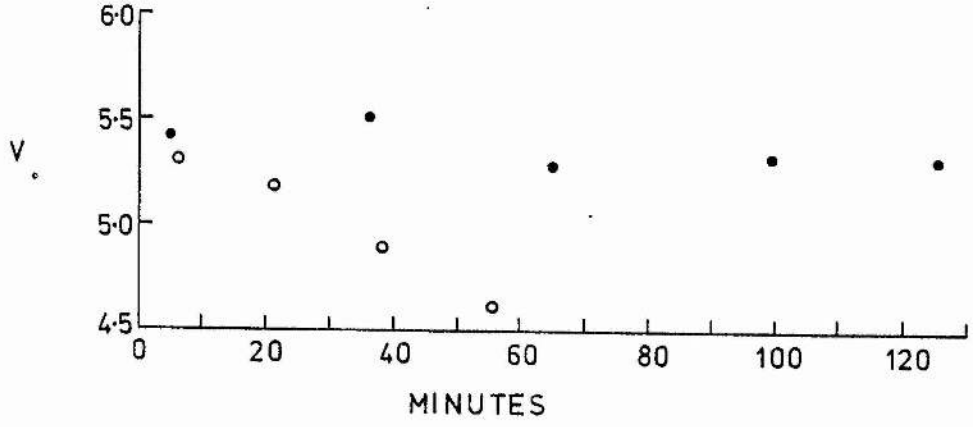


Figure 41.

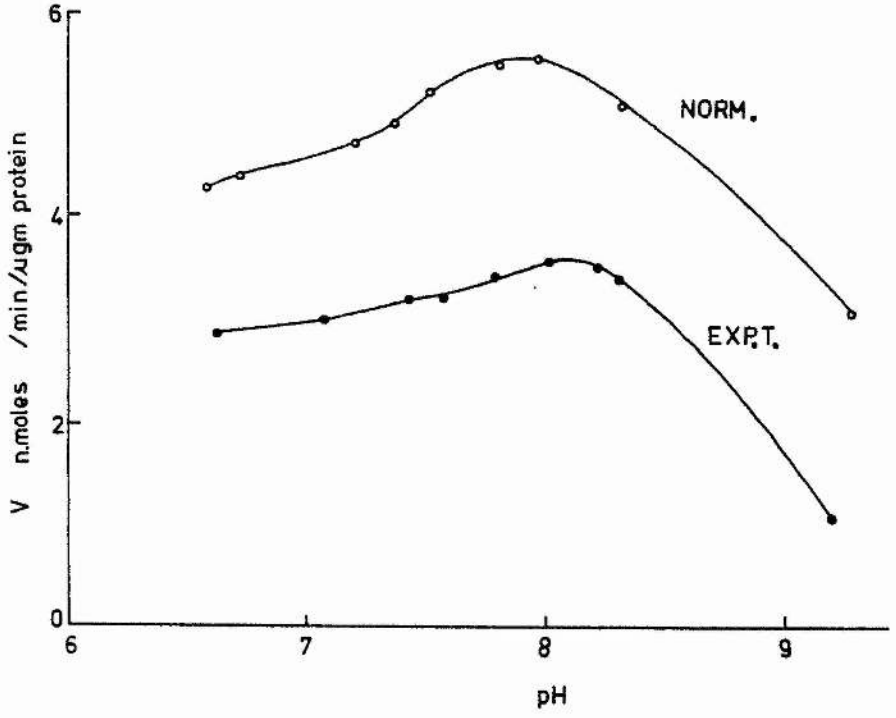


Figure 42.

where A is the change in absorbance per minute, v is the volume of the homogenate (o.17 mls) and p is the protein concentration in μ gms/ml.

3) Characteristics of the enzyme.

Since this study was in order to determine a quantitative (possibly small) difference in the activity of an ACh hydrolysing enzyme(s) it was desirable to determine first of all whether the basic properties of the enzyme(s) were affected by nerve section. It would then be possible for an assessment of the enzyme activity in ganglia before and after nerve section to be made under optimum conditions. Some indication of the affinity of the enzyme for the substrate was obtained by measuring the pH dependence of the reaction and the Michealis constant (Km) of the enzyme for this substrate.

a) Optimum pH.

Since enzymes are proteins containing many ionisable groups, their activity will be at least partially dependent on the pH of the bulk solution. The affinity of the enzyme for the substrate depends to some extent on the state of ionisation of the active groups and will be reflected in the rate of hydrolysis of the substrate at any one substrate concentration. A quantitative comparison of the enzyme activity before and after denervation cannot be made unless the enzyme from the two types of preparation is working at its optimum pH.

The optimum pH was determined by measuring the velocity of the reaction over a pH range of 6.5-9.5. Figure 42 gives the rate of hydrolysis at different pHs for a normal and a denervated preparation. The peaks of the curves occured at pH 7.9, 8.0 and 8.0 in three normal preparations and pH 8.0, 8.0 and 8.1 in three experimental preparations. The assays were accordingly performed at pH 8.0.

b) Determination of Km and Vmax.

Figure 43 shows the relationship between the rate of hydrolysis and the substrate (AThCh) concentration in a normal and a denervated preparation. As the AThCh concentration was increased up to 0.4 mM there was a linear correlation between velocity and the amount of substrate present. The reaction did not show substrate inhibition at substrate concentrations of up to 1 mM. However velocities at higher substrate concentrations were not investigated.

The reaction showed Michealis-Menton kinetics; when the substrate concentration is considerably below that required to produce half maximal velocity (the Km value) the velocity (v) depends upon the susbstrate concentration (s). This rendered it possible to determine an apparent affinity constant (Km) and maximal velocity (Vmax) for the enzymic process. Km and Vmax are related by the equation:

$$v = \frac{Vmax}{1 + \frac{Km}{s}}$$

from Dixon and Webb, 1958

Determination of Km and Vmax was made for normal and denervated ganglia by measuring the velocity of the reaction at different substrate concentrations and plotting $\frac{s}{v}$ against s (Eadie-Hofstee plot) which gives a straight line of slope $\frac{1}{Vmax}$ and an 'x' intercept of -Km. This is illustrated in Figure 44. Only points from the steepest part of the curve shown in Figure 43 were included. The Km was the same in normal and denervated ganglia; values for normal and denervated preparations were respectively 5.4 ± 1.1 x 10^{-5} M (n = 4) and 5.7 ± 0.7 x 10^{-5} M (n = 4). There was no significant difference between the two values ($P > 0.2$). These values are consistent with those reported by Beesley, Emson and Kerkut (1971) using the photometric technique of Gage and Litchfield (1966).

4) Enzyme Activity.

Denervated ganglia had a lower enzyme activity than the controls. Vmax values from these preliminary experiments were 5.68 ± 0.67 n moles/min/μ gm protein for the 4 control preparations and 4.26 ± 0.21 for the 4 denervated preparations. The two values were significantly different ($P < 0.05$). Vmax occured at substrate concentrations of 4.5-5.0 x 10^{-4} M. Further experiments were accordingly made on single ganglia using a substrate concentration of 5 x 10^{-4} M. Each assay was done in duplicate. The rates of hydrolysis were 5.24 ± 0.22 n moles/min/μ gm protein (n = 10) for normal ganglia and 3.53 ± 0.20 (n = 9) for denervated ganglia. The two values were significantly different ($P < 0.001$).

Figure 43. The effect of increasing substrate concentration (S) on the rate of hydrolysis of AThCh (V) by ChE from the homogenate of 10 normal (o) and 10 experimental / denervated (•) ganglia.

Figure 44. Eadie-Hofstee plot. S, substrate (AThCh) concentration; V, velocity of reaction (n moles AThCh hydrolysed/min/u gm protein). Linear regression line extrapolates to intersect abscissa at -Km. (•), Homogenate from 10 normal ganglia; (o) homogenate from 10 experimental /denervated ganglia.

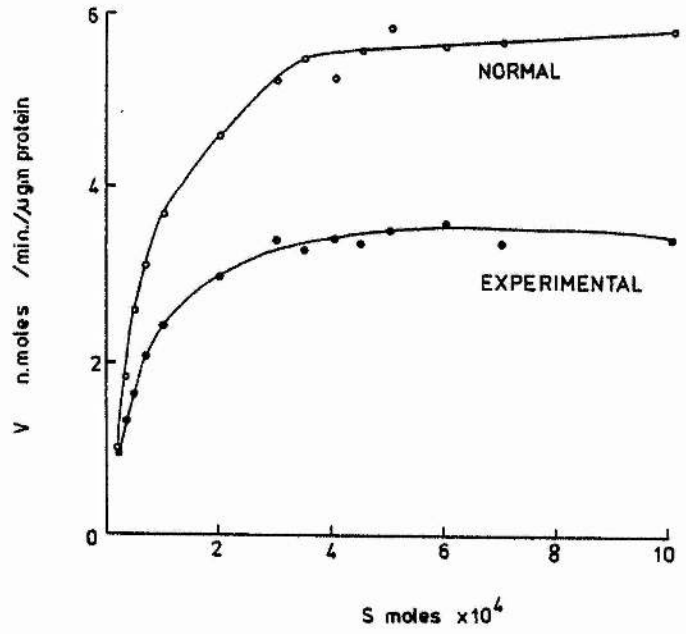


Figure 43.

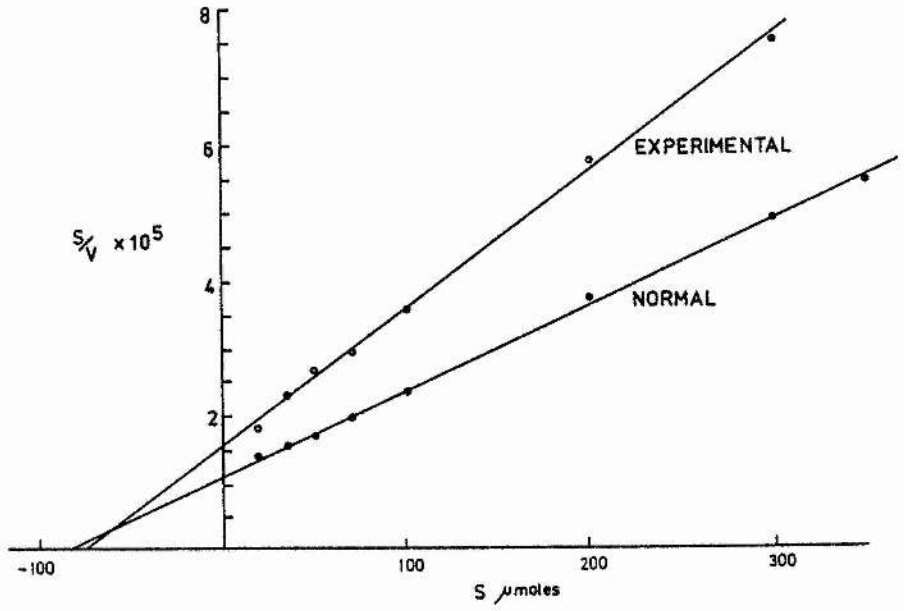


Figure 44.

DISCUSSION.

The metathoracic ganglion of the cockroach contains relatively large amounts of ChE. Using the technique described in this study the average ChE activity of normal ganglia was 5.24 nmoles AThCh hydrolysed/min/ µgm protein. Comparison with other insect preparations is severely limited by the large variations in the ChE activity recorded under different experimental conditions (see Silver, 1974). For this reason the majority of studies have been more qualitative than quantitative. This limitation is furthered by the fact that most values of ChE activity have been expressed in terms of wet weight of tissue. Using a manometric technique, Colhoun (1959) determined the ChE activity in various regions of the cockroach CNS. The value he obtained for the metathoracic ganglion was approximately 0.33 gms ACh hydrolysed/hour/gm tissue wet weight compared with an average value of 1.22 gms AThCh hydrolysed/hour/gm tissue wet weight obtained in this study. Unfortunately because Colhoun's experiments were generally for comparitive purposes the experimental conditions of his assay were not stated. However this discrepancy in values for the activity of ChE is probably because in this study the assay was performed at 37°C and at optimum pH and substrate concentration for the reaction.

Klingman, Klingman and Poliszczuk (1968) determined the activity of ChE in rat superior cervical ganglia using the same technique as the one described for

this study. These authors obtained mean values of approximately 33 µmoles AThCh hydrolysed/min/gm tissue wet weight. This is about four times less than the values obtained for the cockroach metathoracic ganglion (112 µmoles AThCh hydrolysed/min/gm tissue wet weight).

The relatively high level of ChE activity in cockroach central ganglia is well documented; Treherne and Smith (1965) demonstrated the ability of the sixth abdominal ganglion to reduce the level of ACh in the extracellular fluid 100 fold after 30 minutes incubation in a 10^{-2} M solution of radioactively labelled ACh. Experiments performed on the whole ventral nerve cord (Treherne and Smith, 1965; 1965a) showed that this hydrolysis was considerably reduced after a 1 second exposure to physostigmine. This indicates that an appreciable amount of ChE is located at the surface of the ganglion and consequently close to the motoneurone cell bodies.

The optimum pH for the hydrolysis of AThCh by the tissue homogenate was in the region of 8.0. This is consistent with the histochemical observations of Iyatomi and Kanehisa (1958). In the vertebrates the optimum pH varies with the source of the enzyme but in most preparations with ACh as the substrate it is in the range of 8.0-8.5 (see Cohen and Oosterbaan, 1963). The bell shaped curve describing the relationship between ChE activity and pH has been taken as evidence that the esteratic site of the enzyme molecule contains

both a basic group and an acidic group (Dixon and Webb, 1958).

The reaction for the hydrolysis of AThCh by the tissue homogenate had a Km of approximately 5.4×10^{-5} M. This is similar to the value obtained by Beesley, Emson and Kerkut (1971) working on the same ganglion (5.9×10^{-5} M). Km values are usually in the region of 5 times higher when AThCh is hydrolysed by vertebrate tissue homogenates (approximately 2×10^{-4} M). This also seems to apply for the optimum substrate concentration (S); in this study S was about 5×10^{-4} M, in vertebrate preparations S varies from 5×10^{-4} to 1.2×10^{-2} M (see Silver; 1974, Table 2.4a). The values of Km and S quoted for vertebrate preparations are for the enzymic action of AChE only and so this discrepancy may be the result of the presence of other cholinesterases in the cockroach ganglion homogenate. Pseudocholinesterases are excluded as a likely possibility since the Km values of these enzymes are generally higher than those of AChE for choline esters (Silver, 1974). It is possible that the difference represents a true difference in the properties of cockroach and vertebrate ChE. Alternatively it may be a result of the experimental conditions under which the cockroach ChE was assayed. Obviously this point requires further investigation.

The Effect of Denervation on Ganglionic ChE Activity.

Denervated ganglia showed an approximately 33% reduction in their capacity to hydrolyse AThCh.

Since the pH optimum, Km and optimum substrate concentration for the enzymic reaction were unchanged in tissue homogenates from denervated ganglia, this probably represents a genuine reduction in the quantity of ChE.

Although the proportion of the cells affected by section of both fifth nerve trunks is unknown it is interesting to compare these results with those obtained for denervated vertebrate preparations. Harkonnen made a quantitative study of the ChE activity of the superior cervical ganglion of the rat (Harkonnen, 1964). 7-13 days after postganglionic denervation (axotomy) the total esterase activity was reduced by about 50% whereas the eserine sensitive esterase activity was reduced by about 61%. Preganglionic denervation produced an almost total loss of the ChE activity in the preganglionic axons and their terminals, although a quantitative value was not obtained. Similar results were obtained for the rat superior cervical ganglion by Klingman and Klingman (1969). These are compatible with the observation that approximately half of the total ganglionic ChE activity of rat superior cervical ganglia is associated with the preganglionic while the other half was associated with the postganglionic elements (McLennan, 1954; Dhar, 1958).

Section of both fifth nerve trunks of the metathoracic ganglion of the cockroach produced an approximately 33% loss of ChE activity. However since these nerves contain both sensory and motor axons, the fall in ChE activity in the ganglion could be a result

of both orthograde and retrograde changes. In both connectives (Hess, 1958) and peripheral nerves (Guthrie, 1962) of the cockroach, axons removed from their cell bodies by nerve section undergo pronounced degenerative changes, becoming surrounded and sometimes replaced by proliferating glial cells. This retrograde degeneration would undoubtedly account for some of the fall in ChE activity observed. However an enzyme histochemical study would be necessary in order to determine the relative contributions of orthograde and retrograde changes in the reduction in ganglionic ChE.

SUMMARY - SECTION II.

1) The cholinesterase (ChE) activity of normal and denervated metathoracic ganglia from the cockroach Periplaneta americana was measured by an adaptation of the colorimetric method of Ellman, Courtney, Andres and Featherstone (1951). Denervation was produced by transection of both fifth nerve trunks 5-8 days before the assay.

2) The optimum conditions for the hydrolysis of acetylthiocholine (AThCh) by homogenised ganglia were the same for normal and denervated preparations with respect to pH (8.0) and substrate concentration (5×10^{-4} M).

3) The rate of AThCh hydrolysis was negligible in the presence of 3.3×10^{-4} M neostigmine indicating that the reaction was determined almost exclusively by ChE.

4) Denervated ganglia showed a significant reduction in their capacity to hydrolyse AThCh. Average values were 5.24 ± 0.22 n moles AThCh hydrolysed/minute/ µgm of protein (n=10) for normal ganglia and 3.53 ± 0.20 (n=9) for denervated ganglia.

5) Km values for the enzymic reaction were the same for both normal and denervated ganglia (mean = $5.5 \pm 0.9 \times 10^{-5}$ M, n=8).

6) Since the pH optimum, Km and optimum substrate concentration for the enzymic reaction were unchanged in tissue homogenates from denervated ganglia, the reduction in the rate of AThCh hydrolysis probably represents a genuine reduction in the quantity of ChE.

CONCLUSION - CHAPTER II.

In the majority of preparations studied, axotomised cholinergic neurones have shown a reduction in their responsiveness to presynaptic stimulation. This has generally been attributed either to synaptic disruption (e.g. Horridge and Burrows, 1974; Mathews and Nelson, 1975) or to a reduction in cholinesterase activity (e.g. McLennan, 1954) and not to a reduction in the sensitivity of the postsynaptic membrane. The results obtained in this study are in accordance with this model.

The population of extrasynaptic receptors on the soma membrane of the cockroach fast coxal depressor motoneurone undergo no apparent change following axotomy. This stability of ACh receptors may also extend to the postsynaptic membrane.

Generally when a cholinergic neurone is axotomised, cholinesterase activity in the perikaryon is reduced and subsequently restored, the time course of the change approximating to the time course of chromatolytic changes in Nissl substance (see Schwarzacher, 1958; Chacko and Cerf, 1960; Taxi, 1961; Lewis and Shute, 1965). Axotomy of the fast coxal depressor motoneurone in the cockroach also reults in a reduction in cholinesterase activity in the region of the cell body which, as in the vertebrates, parallels the time course of the morphological changes in Nissl substance. This results in an increase in the response of the cell body to externally applied ACh. Experiments on the whole ganglion show that denervation results in a dramatic fall in the activity of cholinesterase. Thus section of a single nerve trunk may involve functional changes

BIBLIOGRAPHY.

Albuquerque, E.X., Schuh, F.T. and Kauffman, F.C. (1971).
Early membrane depolarisations of the fast mammalian muscle after denervation.
Pfluegers Arch. 328 36-50.

Aldridge, W.N. and Johnson, M.K. (1959).
Cholinesterase, succinic dehydrogenase, nucleic acids, esterase and gluthione reductase in sub-cellular fractions from rat brain.
Biochem. J. 73 270-276.

Alley, K. and Llinas, R. (1976).
Personal communication cited by Mendell, Munson and Scott (1976).

Atwood, H.L. (1967).
Variation in physiological properties of crustacean motor synapses.
Nature (Lond.). 215 57-58.

Augustinson, K.B. (1948).
Cholinesterases. A study in comparitive enzymology.
Acta Physiol. Scand. 15 Suppl. 52 1-182.

Axelsson, J. and Thesleff, S. (1959).
A study of supersensitivity in denervated skeletal muscle.
J. Physiol. (Lond.). 149 178-193.

Barlow, R.M. (1969).
Early morphological and histochemical consequences of peripheral nerve section in lambs of normal and low copper status.
J. Pathol. 99 153-160.

Barnard, E.A., Wieckowski, J. and Chiu, T.H. (1971).
Cholinergic receptor molecules and cholinesterase molecules at mouse skeletal muscle junctions.
Nature (Lond.). 234 207-209.

Barr, M.L. and Hamilton, J.D. (1948).
A quantitative study of certain morphological changes in spinal motoneurones during axon reaction.
J. Comp. Neurol. 89 (2) 93-121.

Beesley, P., Emson, P.L. and Kerkut, G.A. (1971).
Change in Km of insect ChE after behavioural training.
J. Physiol. (Lond.). 221 26-27P.

Bell, C. (1967).
An electrophysiological study of the effects of atropine and physostigmine on transmission to the guinea-pig vas deferens.
J. Physiol. (Lond.). 189 31-42.

Beranek, R. and Hnik, P. (1959).
Long term effects of tonotomy on spinal monosynaptic responses in the cat.
Science N.Y. 130 981-982.

Beranek, R. and Vyskogil, F. (1967).
The action of tubocurarine and atropine on the normal and denervated rat diaphragm.
J. Physiol. (Lond.). 188 53-66.

Berg, D.K., Kelly, R.B., Sargent, P.B., Williamson,P. and Hall, Z.W. (1972).
Binding of -bungarotoxin to acetylcholine receptors in mammalian muscle.
Proc. Nat. Acad. Sci. (Wash.). 69 147-151.

Bergmeyer, H.U. (1963).
Methods of enzymatic analysis. Vol. 2. N.Y. and Lond.

Bird, S.J. and Aghajanian, G.K. (1975).
Denervation supersensitivity in the cholinergic septohippocampal pathway: a microiontophoretic study.
Brain Res. 100 355-370.

Blinzinger, K. and Kreutberg, G. (1968).
Displacement of synaptic terminals from regenerating motoneurones by microglial cells.
Z. Zellforsch (Cell and Tissue Res.). 85 145-157.

Bodenstein, D. (1957).
Studies on nerve regeneration in Periplaneta americana.
J. Exp. Zool. 136 89-115.

Boeckh, J., Sandri, C. and Akert, K. (1970).
Sensorische Eingange und synaptische Verbindungen im Zentral nervensystem von Insekten.
Z. Zellforsch (Cell and Tissue Res.). 103 429-446.

Bradshaw, C.M. and Szabadi, E. (1974).
The measurement of dose in microelectrophoresis experiments.
Neuropharmacol. 13 407-415.

Brattgard, S.O., Edstrom, J.E. and Hyden, H. (1957).
Chemical changes in regenerating neurones.
J. Neurochem. 1 316-325.

Bray, J.J. and Austin, L. (1968).
Flow of protein and ribonucleic acid in peripheral nerve.
J. Neurochem. 15 731-740.

Brenner, H.R. and Johnson, E.W. (1976).
Physiological and morphological effects of postganglionic axotomy on presynaptic nerve terminals.
J. Physiol. (Lond.). 260 143-158.

Brenner, C. and Martin, A.R. (1976).
Reduction in acetylcholine sensitivity of axotomised ciliary ganglion cells.
J. Physiol. (Lond.). 260 159-175.

Brenner, C. and Merritt, H.H. (1942).
Effect of certain choline derivatives on electrical activity of the cortex.
Arch. Neurol. Psychiat. 48 382-395.

Bronk, D.W. (1939).

Synaptic mechanisms in sympathetic ganglia.

J. Neurophysiol. 2 380-401.

Brown, D.A. (1966a).

Depolarisation of normal and preganglionically denervated superior cervical ganglia by stimulant drugs.

Br. J. Pharmacol. 26 511-517.

Brown, D.A. (1966b).

Electrical responses of cat superior cervical ganglia in vivo to some stimulant drugs and their modification by hexamethonium and hyoscine.

Br. J. Pharmacol. 26 538-551.

Brown, D.A. (1969).

Responses of normal and denervated cat superior cervical ganglia to some stimulant compounds.

J. Physiol. (Lond.). 201 225-236.

Brown, G.L. (1937).

The actions of acetylcholine on denervated mammalian and frogs' muscle.

J. Physiol. (Lond.). 89 438-461.

Brown, G.L. (1965).
The release and fate of the transmitter liberated by adrenergic nerves.
Proc. R. Soc. Lond. (B) 162 1-19.

Brown, L.M. (1958).
Cholinesterase in the superior cervical ganglion of the rat after preganglionic denervation and axotomy.
J. Physiol. 142 7P and 8P.

Brown, G.L., Dale, H.H. and Feldberg, W. (1936).
Reactions of the normal mammalian muscle to acetylcholine and eserine.
J. Physiol. (Lond.) 87 394-424.

Brown, G.L., Dearnaley, D.P. and Geffen, L.B. (1967).
Noradrenaline storage and release in the decentralised spleen.
Proc. R. Soc. Lond. (B) 168 48-56.

Brown, G.L. and Harvey, A.M. (1938).
Reactions of avian muscle to acetylcholine and eserine.
J. Physiol. (Lond.) 94 101-117.

Brown, G.L., McLennan, H. and Pascoe, J.E. (1952a).
Failure of ganglionic transmission after postganglionic nerve section.
J Physiol. (Lond.) 117 28P.

Brown, G.L., McLennan, H. and Pascoe, J.E. (1952b).
Acetylcholine metabolism of axotomised sympathetic ganglia.
J. Physiol. (Lond.) 118 60P.

Brown, G.L. and Pascoe, J.E. (1952).
Conduction through the inferior mesenteric ganglion of the rabbit.
J. Physiol. (Lond.) 118 113-123.

Brown, G.L. and Pascoe, J.E. (1954).
The effect of degenerative section of ganglionic axons on transmission through the ganglion.
J. Physiol. (Lond.) 123 565-573.

Burt, P.E., Gregory, G. and Molloy, F. (1966).
A histochemical and electrophysiological study of the action of 'diazoxon' on cholinesterase activity and nerve conduction in ganglia of the cockroach Periplaneta americana.
Ann. appl. Biol. 58 341-354.

Butler, I.J., Drachman, L. and Goldberg, A.M. (1978).
The effect of disuse on cholinergic enzymes.
J. Physiol. 274 593-600.

Byers, M.R. (1970).
Chromatolysis in a pair of identifiable metathoracic neurones in the cockroach Diploptera punctata.
Tissue and Cell 2 255-279.

Cammermeyer, J. (1963).
Differential response of two neuron types to facial nerve transection in young and old rabbits.
J. Neuropathol. 22 594-616.

Campbell, B. (1944).
The effects of retrograde degeneration upon the reflex activity of ventral horn neurons.
Anat. Rec. 88 25-37.

Cangiano, A. (1973).
Acetylcholine supersensitivity: the role of neurotrophic factors.
Brain Res. 58 255-265.

Cannon, W.B. (1939).
A law of denervation.
Amer. J. Med. Sci. 198 737-750.

Cannon, W.B. and Haimovici, H. (1939).
The sensitisation of motoneurones by partial "denervation".
Amer. J. Physiol. 126 731-740.

Carbonell, C.S. (1948).
The thoracic muscles of the cockroach, Periplaneta americana.
Smithson. misc. Colls. 107 1-23.

Cavanagh, J.B., Thompson, R.H.S. and Webster, G.R. (1954).
The localisation of pseudocholinesterase activity in nervous tissue.
Quart. J. exp. Physiol. 39 185-197.

Cerf, J.A. and Chacko, L.W. (1958).
Retrograde reaction in motoneuron dendrites following ventral root section in the frog.
J. Comp. Neurol. 109 205-220.

Chacko, L.W. and Cerf, J.A. (1960).
Histochemical localisation of cholinesterase in the amphibian spinal cord and alterations following ventral root section.
J. Anat. (Lond.) 94 74-81.

Changeux, J.P., Meunier, J.C. and Huchet, M. (1971).
Studies on the cholinergic receptor protein of Electrphorus electicus: I. An assay in vitro for the cholinergic receptor site and solubilisation of the receptor protein from electric tissue.
Mol. Pharmacol. 7 538-553.

Cheah, T.B. and Geffen, L.B. (1973).
Effects of axonal injury on norepinephrine, tyrosine hydroxylase and monoamine oxidase levels in sympathetic ganglia.
J. Neurobiol. 4 443-452.

Chein, S. (1960).
Supersensitivity of denervated superior cervical ganglia to acetylcholine.
Amer. J. Physiol. 198 949-954.

Chu, N.-S., Rutledge, L. and Sellinger, O.Z. (1971a).
The effect of cortical undercutting and long-term electrical stimulation on synaptic acetylcholinesterase.
Brain Res. 29 323-330.

Chu, N.-S., Rutledge, L. and Sellinger, O.Z. (1971b).
Tubocurarine binding in undercut cerebral cortex and the effect of long-term electrical stimulation.
Brain Res. 29 331-337.

Cohen, M.J. (1967).
Correlations between structure, function and RNA metabolism in central neurons of insects.
In: Invertebrate Nervous Systems (Wiersma, C.A.G., ed.), 65-78. Univ. of Chicago Press, Chicago.

Cohen, M.J. and Jacklet, J.J. (1965).
Neurons of insects: RNA changes during injury and regeneration.
Science N.Y. 148 1237-1239.

Cohen, M.J. and Jacklet, J. (1967).
The functional organisation of motor neurons in an insect ganglion.
Phil. Trans. R. Soc. Lond. (B). 252 561-568.

Cohen, J.A. and Oosterbaan, R.A. (1963).
The active site of acetylcholinesterase and related esterases and its' reactivity towards substrates and inhibitors.
In: Cholinesterases and anticholinesterase agents (Koelle, G.B., ed.). Springer, Berlin. 299-372.

Colhoun, E.H. (1958).

Acetylcholine in Periplaneta americana.L. II. Acetylcholine and nervous activity.

J. Insect. Physiol. 2 108-116.

Colhoun, E.H. (1959).

Physiological events in organophosphorous poisoning.

Can. J. Biochem. Physiol. 37 1127-1134.

Cragg, B.G. (1970).

What is the signal for chromatolysis?

Brain Res. 23 1-21.

Cull-Candy, S.G. (1975).

Effect of denervation and local damage on extra-junctional L-glutamate receptors in locust muscle.

Nature Lond. 258 530-531.

Dale, H.H. and Gasser, H.S. (1926).

The pharmacology of denervated mammalian muscle. Part I. The nature of the substances producing contracture.

J. Pharmacol. and exp. Therap. 29 53-67.

Dhar, F.A. (1958).

Cholinesterase in decentralised and axotomised sympathetic ganglia.

J. Physiol. (Lond.) 144 27P-28P.

Diamond, J. and Miledi, R. (1962).
A study of foetal and new-born rat muscle fibres.
J. Physiol. (Lond.) 162 393-408.

Dixon, M. and Webb, E.C. (1958).
Enzymes. 2nd edition, Longmans, London.

Doane, B.K. (1961).
Changes in visual function with perceptual isolation.
Doctoral Thesis, McGill University. Reference cited by Heron, 1961.

Drake, C.G., Seguin, J.J. and Stavraky, G.W. (1956).
The effect of convulsant agents on partially isolated regions of the CNS.
Can. J. Biochem. and Physiol. 34 689-712.

Dresden, D. and Nijenhuis, E.D. (1953).
On the anatomy and mechanism of motion of the mesothoracic leg of Periplaneta americana.
Proc. K. Ned. Akad. Wet. C56 39-47.

Dresden, D. and Nijenhuis, E.D. (1958).
Fibre analysis of the nerves of the second thoracic leg in Periplaneta americana.
Proc. K. Ned. Akad. Wet. C61 213-223.

Dreyer, F., Peper, K. and Stertz, R. (1978).
Determination of dose response curves by quantitative ionophoresis at the frog neuromuscular junction.
J. Physiol. (Lond.) 281 395-419.

Droz, B. and Leblond, C.D. (1963).
Axonal migration of proteins in the central nervous system and peripheral nerves as shown by radioautography.
J. Comp. Neurol. 121 325-346.

Easson, L.H. and Stedman, E. (1937).
The specificity of cholinesterase.
Biochem. J. 31 1723-1729.

Eccles, J.C., Libet, R. and Young, R.R. (1958).
The behaviour of chromatolysed motoneurones studied by intracellular recording.
J. Physiol. (Lond.) 143 11-40.

Echlin, F.A. (1959).
The supersensitivity of chronically "isolated" cerebral cortex as a mechanism in focal epilepsy.
Electroencephalog. and clin. neurophysiol. 11 697-722.

Edwards, J.S. and Palka, J. (1971).
Neural regeneration: delayed formation of central contacts by insect sensory cells.
Science N.Y. 172 591-594.

Eldefrawi, M.E., Eldefrawi, A.T., Seifert, S. and O'Brien, R.D. (1972).
Properties of lubrol-solubilised acetylcholine receptor from Torpedo electroplax.
Archiv. Biochem. and Biophys. 152 210-218.

Ellman, G.L. (1959).
Tissue sulfhydryl groups.
Archiv. Biochem. and Biophys. 82 70-77.

Elliott, T.R. (1905).
The action of adrenaline.
J. Physiol. 32 401-467.

Ellman, G.L., Courtney, K.D., Andres, V.Jr. and Featherstone, R.M. (1961).
A new and rapid colorimetric determination of acetylcholinesterase activity.
Biochem. Pharmacol. 7 88-95.

Fambrough, D.M. (1970).
Acetylcholine sensitivity of muscle fibre membranes: mechanism of regulation by motoneurones.
Science N.Y.. 168 372-373.

Feltz, A. and Mallart, A. (1970).
Differences in the action of acetylcholine on the denervated muscle fibre and on the normal end-plate.
Brain Res. 22 264-267.

Feltz, A. and Mallart, A. (1971a).
Ionic permeability changes induced by some cholinergic agonists on normal and denervated frog muscles.
J. Physiol. (Lond.). 218 101-116.

Feltz, A. and Mallart, A. (1971b).
An analysis of acetylcholine responses of junctional and extrajunctional receptors of frog muscle fibres.
J. Physiol. (Lond.). 218 85-110.

Filogamo, G. and Candiollo, L. (1962).
Observations on the behaviour of acetylcholinesterase (AChE) in the nerve cells of the spinal reflex arc after section of the peripheral nerves (experimental investigations in Lepus cuniculus L).
Acta Anatom. 51 273-289.

Flack, W. and Yeoh, T.S. (1968).
The action of some cholinergic agonists and anti-cholinesterase agents in the dorsal muscle of the leech.
Br. J. Pharm. Chem. 33 145-153.

Flumerfelt, B.A. and Lewis, P.R. (1969).
Changes in the hypoglossal nucleus following axotomy.
Proc. Anatom. Soc. 104 587-588.

Frank, G.B. and Inoue, F. (1966).
Large miniature e.p.p.s in partial denervated skeletal muscle.
Nature (Lond.). 212 596-598.

Frank, E., Nothmann, M. and Hirsch-Kauffmann, H. (1922).
Uber die "tonische" kontraktion des quergestreifften saugetiermuskels nach Ausschaltung des motorischen Nerven.
Pflugers Arch. 197 270-287.

Fukura, T. and Koelle, G.B. (1959).
The cytological localisation of intracellular neuronal acetylcholinesterase.
J. Biophys. Biochem. Cytol. 5 433-440.

Gage, J.C. and Litchfield, M.H. (1966).
An automated determination of choline esterase activity in microsamples and low activity media.
Technicon International Symposium 92-102.

Gahery, Y. and Boistel, J. (1965).
Study of some pharmacological substances which modify the electrical activity of the sixth abdominal ganglion of the cockroach P. americana.
In: The Physiology of the Nervous System. (Treherne, J.E. and Beament, J.W.L., eds). 73-78. London. Academic Press.

Gepner, J.I., Hall, L.M. and Sattelle, D.B. (1978).
Insect acetylcholine receptors as a site of insecticide action.
Nature (Lond.). 276 188-189.

Gerebtzoff, M.A. (1959).
Cholinesterases. A histochemical contribution to the solution of some functional problems.
In: Modern Trends in Physiological Sciences Vol. 3.
Pergamon Press, London.

Gersh, I. and Bodian, D. (1943).
Some chemical mechanisms in chromatolysis.
J. Cell. Comp. Physiol. 21 253-280.

Giacobini, E. (1959).
The distribution and localisation of cholinesterases in nerve cells.
Acta Physiol. Scand. 45 Suppl. 156 1-45.

Ginsborg, B.L. (1967).
Ion movement in junctional transmission.
Pharm. Rev. 19 289-316.

Goldstein, A. (1951).
Properties and behaviour of purified human plasma cholinesterase. III. Competitive inhibition by prostigmine and other alkaloids with special reference to differences in kinetic behaviour.
Arch. Biochem. Biophys. 34 169-188.

Gomori, G. (1948).
Histochemical demonstration of sites of cholinesterase activty.
Proc. Soc. Exp. Biol. Med. 68 354-358.

Gomori, G. (1952).
Histochemistry of esterases.
Int. Rev. Cytol. 1 323-335.

Grampp, W., Harris, J.B. and Thesleff, S. (1971).
Inhibition of denervation changes in skeletal muscle by blockers of protein synthesis.
J. Physiol. (Lond.). 21 743-754.

Green, R.D. (1969).
The effect of denervation on the sensitivity of the superior cervical ganglion of the pithed cat.
J. Pharmacol. and Exp. Therapy. 167 143-150.

Guth, L., Albers, R.W. and Brown, W.C. (1964).
Quantitative changes in cholinesterase activity of denervated muscle fibres and sole plates.
Exp. Neurol. 10 236-250.

Guth, L. and Brown, W.C. (1965).
The sequence of changes in cholinesterase activity during reinnervation of muscle.
Exp. Neurol. 12 329-336.

Guthrie, D.M. (1962).
Regenerative growth in insect nerve axons.
J. Insect Physiol. 8 79-92.

Harkonen, M. (1964).
Carboxylic esterases, oxidative enzymes and catecholamines in the superior cervical ganglion of the rat and the effect of pre- and post-ganglionic denervation.
Acta Physiol. Scand. 63 Supplementum 237-243.

Harris, A.J., Kuffler, S.W. and Dennis, M.J. (1971).
Differential chemosensitivity of synaptic and extrasynaptic areas on the neuronal surface membrane in parasympathetic neurons of the frog, tested by microapplication of acetylcholine.
Proc. R. Soc. Lond. (B) 177 541-553.

Harris, J.B. and Marshall, D.W. (1973).
A study of action potential generation in murine dystrophy with reference to "functional denervation".
Exp. Neurol. 41 331-344.

Harris, J.B. and Ribchester, R.R. (1978).
Neuromuscular transmission is adequate in identified abnormal dystrophic muscle fibres.
Nature Lond. 271 362-364.

Hartmann, J.F. (1954).
Electron microscopy of motor nerve cells following section of axones.
Anat. Rec. 118 19-34.

Hartzell, H.C. and Fambrough, D.M. (1972).
Acetylcholine receptors: distribution and extra-junctional density in rat diaphragm after denervation correlated with acetylcholine sensitivity.
J. Gen. Physiol. 60 248-262.

Hebb, C.O. and Silver, A. (1963).
The effect of transection on the level of choline acetylase in the goats' sciatic nerve.
J. Physiol. Lond. 169 41-42P.

Hebb, C.O. and Waites, G.M.H. (1956).
Choline acetylase in antero- and retro-grade degeneration of a cholinergic nerve.
J. Physiol. 132 667-671.

Heron, W. (1961).
Cognitive and physiological effects of perpetual isolation.
In: Sensory Deprivation (Solomon, P., Kubzansky, P., Leiderman, P., Mendelson, J., Trumbullo, R. and Wexler, D., eds). Harvard Univ. Press, Cambridge, Massachusetts. 6-33.

Hess, .A. (1958).

The fine structure of nerve cells and fibres, neuroglia and sheaths of the ganglion chain in the cockroach Periplaneta americana.

J. Biophys. and Biochem. Cytol. 4 731-742.

Hess, A. (1958).

The fine structure and morphological organisation of the peripheral nerve fibres and trunks of the cockroach (Periplaneta americana).

Quart. J. Micr. Sci. 99 333-340.

Hess, A. (1960).

The fine structure of degenerating nerve fibres and their sheaths and their terminations in the central nerve cord of the cockroach (Periplaneta americana).

J. Biophys. and Biochem. Cytol. 7 (2) 339-344.

Hillman, D.E. (1970).

Cited by Kuno and Llinas (1970) as personal communication.

Holmstedt, B. and Taschi, G. (1959).

Enzymic properties of cholinesterases in subcellular fractions from rat brain.

Acta Physiol. Scand. 47 280-283.

Holtzman, E., Novikoff, A.B. and Villaverde, H. (1967).

Lysosomes and GERL in normal and chromatolytic neurons of the rat ganglion nodosum.

J. Cell Biol. 33 419-435.

Horridge, G.A. and Burrows, M. (1974).
Synapses upon motoneurones of locusts during retrograde degeneration.
Phil. Trans. R. Soc. Lond. (B). 269 95-108.

Howe, P.R.C., Telfer, J.A., Livett, B.G. and Austin, L. (1977).
Extrajunctional acetylcholine receptors in dystrophic mouse muscles.
Experimental Neurol. 56 42-51.

Howell, J.N., Fairhurst, A.S. and Jenden,D. (1966).
Alterations of the calcium accumulating ability of striated muscle following denervation.
Life Sciences 5 439-446.

Hudson, G., Lazarow, A. and Hartmann, J.F. (1961).
A quantitative electrom microscopic study of mitochondria in motor neurones following axonal section.
Exp. Cell Res. 24 440-456.

Huikuri, K.T. (1966).
Histochemistry of the ciliary ganglion of the rat and the effect of pre- and post-ganglionic nerve division.
Acta Physiol. Scand. 69 Suppl. 286.

Iles, J.F. (1972).
Structure and synaptic activation of the fast coxal depressor motoneurone of the cockroach.
J. Exp. Biol. 56 647-656.

Isaacson, A. and Sandow, A. (1967).
Caffeine effects on radiocalcium movement in normal and denervated rat muscles.
J. Pharmacol. 155 376-388.

Iyatomi, K. and Kanehisa, K. (1958).
Localisation of cholinesterases in the American cockroach.

Jacklet, J. and Cohen, M.J. (1967).
Nerve regeneration: correlation of electrical, histological and behavioural events.
Science N.Y. 156 1640-1643.

Jaffe, J. and Sharpless, S.K. (1965).
The rapid development of physical dependence on barbiturates.
J. Pharmacol. and Exp. Therap. 150 140-145.

Jansen, J.K.S., Muller, K.J. and Nicholls, J.G. (1974).
Persistent modification of synaptic interactions between sensory and motor nerve cells following discrete lesions in the central nervous system of the leech.
J. Physiol. (Lond.). 242 289-305.

Jenkinson, D.H. (1960).
The antagonism between tubocurarine and substances which depolarise the motor end-plate.
J. Physiol. 152 309-324.

Jenkinson, D.H. and Terrar.D. (1973).
Influence of chloride ions on changes in membrane potential during prolonged application of carbachol to frog skeletal muscle.
Br. J. Pharm. 47 363-376.

Kahn, R. and Le Yaouanc, -A. (1971).
An analysis of acetylcholine receptors of junctional and extra junctional receptors of frog muscle fibres. Appendix to Feltz and Mallart, 1971.
J. Physiol. (Lond.). 218 85-100.

Kalisker, A., Rutledge, C.O. and Perkins, J.P. (1973).
Effect of nerve degeneration by 6-hydroxydopamine on catecholamine-stimulated adenosine 3',5'-monophosphate formation in rat cerebral cortex.
Mol. Pharmacol. 9 619-629.

Karlin, A. (1967).
Permeability abd internal concentration of ions during depolarisation of the electroplax.
Proc. Nat. Acad. Sci. U.S.A. 58 1162-1167.

Katz, B. and Miledi, R. (1964).
Further observations on the distribution of acetylcholine reactive sites in skeletal muscle.
J. Physiol. (Lond.). 170 379-388.

Katz, B. and Miledi, R. (1964a).
The development of acetylcholine sensitivity in nerve free segments of skeletal muscle.
J. Physiol. (Lond.). 170 389-396.

Kawai, H. (1963).
Cytoplasmic granules in nerve cells of the spinal cord following transection of sciatic nerve of guinea-pig.
Exp. Neurol. 7 457-463.

Kerkut, G.A., Oliver, G., Rick, A. and Walker, R.J. (1970).
The effects of drugs on learning in a simple preparation.
Comp. and Gen. Pharmacol. 1 (4) 437-483.

Kerkut, G.A., Pitman, R.M. and Walker, R.J. (1969).
Iontophoretic application of acetylcholine and GABA onto insect central neurones.
Comp. Biochem. Physiol. 31 611-633.

Kerkut, G.A., Shapira, A. and Walker, R.J. (1965).
The effect of acetylcholine, glutaminc acid and GABA on the contractions of the perfused cockroach leg.
Comp. Biochem. Physiol. 16 37-48.

Kiauta, T., Brzin, M. and Dettbarn, W.D. (1977).
Synthesis of neuromuscular cholinesterases in innervated rat diaphragm.
Exp. Neurol. 56 281-288.

Klingman, G. and Klingamn, J. (1969).
Acetylcholinesterase of rat sympathetic ganglia after immunosympathectomy, decentralisation and axotomy.
J. Neurochem. 16 261-268.

Klingman, G., Klingman, J. and Poliszcu, K. (1968).
Acetylcholinesterase and pseudocholinesterase activities in sympathetic ganlgia of rats.
J. Neurochem. 15 1121-1130.

Klodos, I. and Niemierko, S. (1968).
Influence of temperature on accumulation of acetylcholinesterase activity at the ends of transected nerves of the frog.
Acta. Biochem. Pol. 15 31-36.

Koelle, G.B. (1951).
The elimination of enzymatic diffusion artefacts in the histochemical localisation of cholinesterases and a survey of their cellular distributions.
J. Pharmacol. 103 153-171.

Koelle, G.B. (1955).
The histochemical identification of acetylcholinesterase in cholinergic, adrenergic and sensory neurons.
J. Pharmacol. Exp. Therap. 114 167-184.

Koelle, W.A. and Koelle, G.B. (1959).
The localisation of external or functional acetylcholinesterase at the synapses of autonomic ganglia.
J. Pharmacol. Exp. Therap. 126 1-8.

Koenig, E. (1965).
Synthetic mechanisms in the axon- I. Local axonal synthesis of acetylcholinesterase in the cat.
J. Neurochem. 12 343-355.

Koenig, E. (1965).
Synthetic mechanisms in the axon - II. RNA in myelin free axons of the cat.
J. Neurochem. 12 357-361.

Koplin, I.J. and Silberstein, S.D. (1972).
Axons of sympathetic neurons: transport of enzymes *in vivo* and properties of axonal sprouts *in vitro*.
Pharmac. Rev. 24 245-254.

Kostrzewa, R.M. and Jacobowitz, D.M. (1974).
Pharmacological actions of 6-hydroxydopamine.
Pharmac. Rev. 26 199-288.

Kozak, W. and Westerman, R. (1961).
Plastic changes of spinal monosynaptic responses from tenotomised muscles in cats.
Nature (Lond.). 189 753-755.

Krygier-Brevart, V., Weiss, D.G., Mehl, E., Schubert, P. and Kreutzberg, G.W. (1974).
Maintenance of synaptic membranes by the fast axonal flow.
Brain Res. 77 97-110.

Kuffler, S.W. (1943).
Specific excitability of the end plate region in normal and denervated muscle.
J. Neurophysiol. 6 99-110.

Kuffler, S.W., Dennis, M.J. and Harris, A.J. (1971).
The development of chemosensitivity in extrasynaptic areas of the neuronal surface after denervation of parasympathetic ganglion cells in the heart of the frog.
Proc. R. Soc. Lond. (B). 177 555-563.

Kuhar, M.J. and Rommelspacher, H. (1974).
Acetylcholinesterase staining synaptosomes from rat hippocampus: relative frequency and tentative estimation of internal concentration of free or 'labile bound' acetylcholine.
Brain Res. 77 85-96.

Kumamoto, T. and Bourne, G.H. (1963).
Experimental studies on the oxidative enzymes and hydrolytic enzymes in spinal neurons.
Acta Anat. 55 255-277.

Kuno, M. and Llinas, R. (1970a).
Alterations of synaptic action on chromatolysed motoneurones of the cat.
J. Physiol. (Lond.). 210 823-838.

Kuno, M. and Llinas, R. (1970b).
Enhancement of synaptic transmission by dendritic potentials in chromatolysed motoneurones of the cat.
J. Physiol. (Lond.). 210 807-821.

Langley, J.N. (1905-6).
On the reaction of cells and of nerve endings to certain poisons, chiefly as regards the reaction of striated muscle to nicotine and curari.
J. Physiol. (Lond.). 33 374-413.

Lasek, R.J. (1968).
Axoplasmic transport of labelled protein in rat ventral motoneurones.
Exp. Neurol. 21 41-51.

Lee, C.Y., Tseng, L.F. and Chiu, T.H. (1967).
Influence of denervation in localisation of neurotoxins from clapid venoms in rat diaphragm.
Nature (Lond.). 215 1177-1178.

Lewis, P.R. and Shute, C.D. (1965).
Changes in cholinesterase activity following axotomy.
J. Physiol. 178 25P.

Lewis, P.R. and Shute, C.C.D. (1966).
The distribution of cholinesterase in cholinergic neurons demonstrated with the electron microscope.
J. Cell Sci. 1 381-390.

Lieberman, A.R. (1971).
The axon reaction: a review of the principal features of perikaryal responses to axon injury.
Int. Rev. Neurobiol. 14 49-124.

Lieberman, A.R. (1971b).
In: Essays on structure and function in the nervous system. Academic Press.

Llinas, R., Precht, W. and Kitai, S.T. (1967).
Cerebellar Purkinje cell projection to the peripheral vestibular organ in the frog.
Science N.Y. 158 1328-1330.

Lomo, T. and Rosenthal, J. (1972).
Control of acetylcholine sensitivity by muscle activity of the rat.
J. Physiol. (Lond.). 221 493-513.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951).
Protein measurement with the folin phenol reagent.
J. Biol. Chem. 193 265-275.

Lubinska, L. (1964).
Axoplasmic streaming in regenerating and in normal nerve fibres.
Prog. Brain Res. 13 1-71.

Lubinska, L. and Niemierko, S. (1971).
Velocity and intensity of biderctional migration of acetylcholinesterase in transected nerves.
Brain Res. 27 329-342.

Lubinska , L., Niemierko, S. and Oderfield, B. (1961).
Gradient of cholinesterase activity and of choline acetylase activity in nerve fibres: gradient of cholinesterase activity.
Nature (Lond.). 189 122-123.

Lubinska, L., Niemierko, S., Oderfeld, B., Szwarc, L. and Zelena, J. (1963).
Biderctional movements of axoplasm in peripheral nerve fibres.
Acta Biol. exp. (Warszawa) 23 239-247.

Lunt, G.G., Stephani, E. and DeRobertis, E. (1971).
Increasec incorporation of (G - ^{3}H)-leucine into possible "receptor" proteolipid in denervated muscle *in vivo*.
J. Neurochem. **18** 1545-1549.

MacIntosh, F.C. (1959).
Formation, storage and release of acetylcholine at nerve endings.
Can. J. Biochem. Physiol. **37** 343-356.

MacIntosh, F.C. (1961).
Effect of HC3 on acetylcholine turnover.
Fed. Proc. **20** 562-565.

Maddy, A.H. and Spooner, R.O. (1970).
The erythrocyte agglutinability I. Variation in the membrane protein.
Vox. Sang. **18** 34-41.

Mallart, A., Dreyer, F. and Peper, K. (1976).
Current voltage relation and reversal potential at junctional and extrajunctional acetylcholine receptors of the frog neuromuscular junction.
Pflugers Archiv. **362** 43-47.

Marinesco, G. (1897).
Pathologie generale de la cellule nerveuse.
Lesions secondaires et primitives.
Presse Medicale. **5** 41-47.

Martin, A.R. and Pilar, G. (1964).
Quantal components of the synaptic potential in the avian ciliary ganglion.
J. Physiol. (Lond.). 175 1-16.

Mathews, M.R. and Nelson, V. (1975).
Detachment of structurally intact nerve endings from chromalytic neurones of the rat superior cervical ganglion during depression of synaptic transmission induced by postganglionic axotomy.
J. Physiol. (Lond.). 245 91-135.

McComas, A.J., Sica, R.E.P. and Campbell, M.J. (1971).
"Sick" motoneurones. A unifying concept of muscle disease.
Lancet 1 321-325.

McConnell, M.G. (1974).
Comparison of supersensitivity to acetylcholine and carbachol in chronically denervated rat diaphragm.
Soc. Neurosci. St. Louis, Mo. 331-337.

McEwen, B.S. and Grafstein, B. (1968).
Fast and slow components in axonal transport of protein.
J. Cell Biol. 38 494-508.

McIntyre, A.K., Bradley, K. and Brock, L.G. (1959).
Responses of motoneurones undergoing chromatolysis.
J. Gen. Physiol. 42 931-958.

McKinstry, D.N. and Koelle, G.B. (1967).
Acetylcholine release from the cat superior cervical ganglion by carbachol.
J. Pharmacol. Exp. Ther. 157 319-327.

McLennan, H. (1954).
Acetylcholine metabolism of normal and axotomised ganglia.
J. Physiol. (Lond.). 124 113-116.

Mendell,B., Mundell, D.B. and Rudney, H. (1943).
Studies on cholinesterase. 3. Specific test for true cholinesterase and pseudocholinesterase.
Biochem. J. 37 473-476.

Mendell, L., Munson, J.B. and Scott, J.G. (1976).
Alterations of synapses of axotomised motoneurones.
J. Physiol. (Lond.). 255 67-69.

Mendell, B. and Rudney, H. (1943).
Studies on cholinesterase.I. Cholinesterase and pseudocholinesterase.
Biochem. J. 37 59-63.

Miani, N. (1963).
Analysis of the somato-axonal movement of phospholipids in the vagus and hypoglossal nerves.
J. Neurochem. 10 859-874.

Miledi, R. (1960a).
The acetylcholine sensitivity of frog muscle fibres after complete or partial denervation.
J. Physiol. (Lond.). 151 1-23.

Miledi, R. (1960b).
Properties of regenerating neuromuscular synapses in the frog.
J. Physiol (Lond.). 154 190-205.

Miledi, R. (1962).
Induced innervation of end-plate free muscle segments.
Nature (London). 193 281-282.

Miledi, R. and Potter, L.T. (1971).
Acetylcholine receptors in muscle fibres.
Nature (London). 233 599-600.

Nandy, K. (1968).
Histochemical study on chromatolytic neurones.
Arch. Neurol. 18 425-428.

Nastuk, W.L. (1953).
Membrane potential changes at a single muscle end-plate produced by transitory application of acetylcholine with an electrically controlled microjet.
Fed. Proc. 12 102-104.

Nicholls, J.G. (1956).
The electrical properties of denervated skeletal muscle.
J. Physiol. (Lond.). 131 1-12.

Nijenhuis, E. and Dresden, D. (1952).
A micro-morphological study of the sensory supply of the mesothoracic leg of the American cockroach Periplaneta americana.
Proc. K. Med. Akad. Wet. C55 300-310.

Nissl, F. (1892).
Ueber die Veranderungen der Ganglienzellen am Facil-iskern des Kaninchens nach Ausreissung der Nerven.
Allg. Zeitschr. Psychiat. 48 197-198.

Nissl, F. (1894).
Ueber eine neue Untersuchngs methode des Central organs speciell zur Festellung der Localisation der Nernenzellen.
Zentrall. Nerv. Psychiat. 4 337-339.

O'Brien, R.D. (1957).
Esterases in the semi intact cockroach.
Ann. Ent. Soc. Amer. 50 223-229.

O'Brien, R.D., and Fisher, R.W. (1958).
The relation between ionisation and toxicity to insects for some neuropharmacological compounds.
J. Econ. Ent. 51 169-175.

Ochs, S., Johnson, J. and NG, M.-H. (1967).
Protein incorporation and axoplasmic flow in motoneurone fibres following intra-cord injection of labelled leucine.
J. Neurochem. 14 317-331.

Ochs, S. and Ranish, N. (1969).
Characteristics of the fast transport system in mammalian nerve fibres.
J. Neurobiol. 1 247-261.

Ochs, S. and Ranish, N. (1970).
Metabolic dependence of fast axoplasmic transport in nerve.
Science N.Y. 167 878-879.

Ord, M.G. and Thompson, R.H.S. (1952).
Pseudo-cholinesterase acitivty in the central nervous system.
Biochem. J. 51 245-251.

Palmer, G.C. (1972).
Increased cyclic AMP response to norepinephrine in rat brain following 6-hydroxydopamine.
Neuropharmacol. 11 145-149.

Paton, W.D.M. and Perry, W.L.M. (1953).
The relationship between depolarisation and block in the cats' superior cervical ganglion.
J. Physiol. (Lond.). 119 43-57.

Pearse, A.G.E. (1955).
Functional histochemistry of hypothalmic neurons.
In; Proc. 2nd Int. Congr. Neuropath. Lond. 601-605.

Pearson, K.G. and Bergman, S.J. (1969).
Common inhibitory motoneurones in insects.
J. Exp. Biol. 50 445-471.

Pearson, K.G. and Bradley, A.B. (1972).
Specific regeneration of excitatiry motoneurones to leg muscles in the cockroach.
Brain Res. 47 492-496.

Pearson, K.G. and Fourtner, C. (1973).
Identification of the somata of common inhibitory motoneurones in the metathoracic ganglion of the cockroach.
Can. J. Zool. 51 859-866.

Pearson, K. and Iles, J.F. (1970).
Discharge patterns of coxal levator and depressor motoneurones of the cockroach.
J. Exp. Biol. 52 139-165.

Pearson, K. and Iles, J.F. (1971).
Innervation of coxal depressor muscles in the cockroach.
J. Exp. Biol. 54 215-232.

Pearson, K.G., Stein, R.B. and Malhotra, S.K. (1970).
Properties of action potentials from insect motor nerve fibres.
J. Exp. Biol. 53 299-316.

Penfield, W.G. (1920).
Alterations of the Golgi apparatus in nerve cells.
Brain 43 290-305.

Pestronk, L., Drachman, D.B. and Griffin, J.W. (1976).
Effect of muscle disuse on acetylcholine receptors.
Nature (Lond.). 260 352-353.

Philipeaux, J.M. and Vulpian, A. (1863).
Note sur une modification physiologique qui se produit dans le nerf lingual par suite de l'abolition temporaire de la maturite dans le nerf hypoglosse du meme cote.
Compt. Rend. Acad. Sci. (Paris). 56 1009-1011.

Pitman, R.M. (1968).
Ph.D Thesis: Pharmacological studies on the cockroach central nervous system. Southampton University.

Pitman, R.M. (1971).
Transmitter substances in insects. A review.
Comp. Gen. Pharmacol. 2(7) 347-371.

Pitman, R.M. (1975).
The ionic dependence of action potentials induced by colchicine in an insect motoneurone cell body.
J. Physiol. (Lond.). 247 511-520.

Pitman, R.M. and Kerkut, G.A. (1970).
Comparison of the actions of iontophoretically applied acetylcholine and GABA with the epsp and ipsp in cockroach central neurones.
Comp. Gen. Pharmacol. 1 221-230.

Pitman, R.M., Tweedle, C.D. and Cohen, M.J. (1972).
Branching of central neurons: intracellular cobalt injection for light and electron microscopy.
Science 176 412-414.

Pitman, R.M., Tweedle, C.D. and Cohen, M.J. (1972a).
Electrical responses of insect central neurons: augmentation by nerve section or colchicine.
Science N.Y. 178 507-509.

Pitman, R.M., Tweedle, C.D. and Cohen, M.J. (1973).
The form of nerve cells: determination by cobalt impregnation.
In: Intracellular staining in neurobiology. (Kater, S.B. and Nicholson, C., eds). Springer-Verlag, New York. Chapter 6, 83-97.

Porter, K.R. and Bowers, M.B. (1963).
A study of chromatolysis in motoneurones of the frog, Rana pipiens.
J. Cell Biol. 19 56A.

Potter, L.T. (1970).
Methods in enzymology. (Fleischer, S. and Packer, L. eds). pp 309-323.

Pringle, J.W.S. (1940).
The reflex mechanism of the insect leg.
J. Exp. Biol. 17 8-17.

Purves, D.

Functional and structural changes in mammalian sympathetic neurones following interuption of their axons.

J. Physiol. (Lond.). 252 429-463.

Purves, D. and McMahan, U.J. (1972).

The distribution of synapses in a physiologically identified motoneuron in the central nervous system of the leech.

J. Cell Biol. 55 205-220.

Rall, W. (1967).

Distinguishing theoretical synaptic potentials computed by different soma-dendritic distributions of synaptic input.

J. Neurophysiol. 30 1138-1168.

Rang, H.P. (1971).

Drug receptors and their function.

Nature (Lond.). 231 91-96.

Rang, H.P. (1973).

In: Drug receptors, a symposium. Biological Council. Macmillan Press (London).

Ray, J.W. (1964).

The free amino acid pool of the cockroach (Periplaneta americana) central nervous system and the effect of insecticides.

J. Insect Physiol. 10 587-597.

Richter, D. and Croft, P.G. (1942).
Blood esterases.
Biochem. J. 36 746-757.

Roeder, K.D. (1967).
Nerve cells and insect behaviour.
Harvard University Press, Cambridge, Mass.

Roeder, K.D., Kennedy, N.K. and Samson, E.A. (1947).
Synaptic conduction to giant fibres of the cockroach and the action of anticholinesterases.
J. Neurophysiol. 10 1-11.

Rogowicz, N. (1885).
Ueber pseudomotorische Einwirkung der Ansa Vieussenii auf die Gesichtsmuskeln.
Pflugers Arch. ges. Physiol. 36 1-12.

Roper, S. (1976).
The acetylcholine sensitivity of the surface membrane of multiply-innervated parasympathetic ganglion cells in the mudpuppy before and after partial denervation.
J. Physiol. (Lond.). 254 455-473.

Roper, S. (1976a).
An electrophysiological study of chemical and electrical synapses on neurones in the parasympatheitic cardiac ganglion of the mudpuppy, Necturus maculosus: evidence for intrinsic ganglionic innervation.
J. Physiol. (Lond.). 254 427-454.

Rosenburg, P. and Echlin, F. (1968).
Time course of changes in cholinesterase activity of chronic partially isolated cortex.
J. Nervous and Mental Diseases. 147 56-63.

Ross, R.A., Joh, T.H. and Reis, D.J. (1975).
Reversible changes in the accumulation and activities of tyrosine hydroxylase and dopamine-B-hydroxylase in neurons of nucleus coerleus during the retrograde reaction.
Brain Res. 92 57-72.

Rowe, E.C. (1964).
Microelectrode records from an insect thoracic ganglion.
Diss. Abstr. 25 2766-2770.

Sakharov, D.A. (1970).
Cellular aspects of invertebrate neuropharmacology.
A. Rev. Pharmacol. 10 335-352.

Sargent, P. (1977).
Synthesis of acetylcholine by excitatory motoneurones in CNS of the leech.
J. Neurophysiol. 40 453-461.

Sargent, P., Yau, K-W. and Nicholls, J.G. (1977).
Extrasynaptic receptors on cell bodies of neurons in CNS of leech.
J. Neurophysiol. 40 447-452.

Sattelle, D.B., Gepner, J.I., and Hall, L.M. (1979).
Acetylcholine receptors in insect central nervous system.
Submitted for publication.

Sattelle, D.B., McClay, A.S., Dowson, R.J. and Callec, J.J. (1976).
The pharmacology of an insect ganglion: actions of carbamylcholine and acetylcholine.
J. Exp. Biol. 64 13-23.

Sawyer, C.H. (1946).
Cholinesterases in degenerating and regenerating peripheral nerves.
Amer. J. Physiol. 146 246-253.

Sawyer, C.H. and Hollinshead, W.H. (1945).
Cholinesterase in sympathetic fibres and ganglia.
J. Neurophysiol. 8 139-153.

Schultz, J. and Daly, J.W. (1973).
Accumulation of cyclic adenosine 3',5'-monophosphate in cerebral cortical slices from rat and mouse: stimulating effect of α- and B-adrenergic agents and adenosine.
J. Neurochem. 21 1319-1326.

Schwarzacher, H.G. (1958).
Der Cholineseraseghalt motorischer Nervenzellen wahrend der axonalen Reaction.
Acta Anat. 32 51-65.

Sharpless, S.K. (1975).

Disuse supersensitivity.

In: the developmental neurophysiology of sensory deprivation. Academic Press. pp125-152.

Sharpless, S.K. and Halpern, L. (1962).

The electrical excitability of chronically isolated cortex studied by means of permanently implanted electrodes.

Electroencephalography and Clinical Neurophysiology. 14 224-255.

Sherrington, C.S. (1893).

On the anatomical constitution of nerves of skeletal muscles; with remarks on recurrent fibres in the ventral spinal nerve root.

J. Physiol. 17 211-258.

Shute, C.C.D. and Lewis, P.R. (1961).

The use of cholinesterase technique combined with operative procedures to follow nervous pathways in the brain.

Bibl. Anat. 2 34-49.

Shute, C.C.D. and Lewis, P.R. (1966).

Electron microscopy of cholinergic terminals and acetylcholinesterase-containing neurones in the hippocampal formation of the rat.

Z. Zellforsch 69 334-343.

Silver, A. (1974).

The biology of cholinesterases.

Frontiers of Biology: Neuberger, A. and Tatum, E.L., (eds).

Sittler, O.D. and DeRemer, R.J. (1967).

Gamma aminobutyric acid induced changes in the spontaneous firing rates of insect neurones.

Experientia 23 284-285.

Smith, K.R. (1961).

The fine structure of neurons of the dorsal root ganglia after stimulating or cutting the sciatic nerve.

J. Comp. Neurol. 116 103-107.

Smith, D.S. and Treherne, J.E. (1963).

Functional aspects of the organisation of the insect nervous system.

Adv. Ins. Physiol. 1 401-484.

Smith, D.S. and Treherne, J.E. (1965).

The electron microscopic localisation of acetylcholinesterase activity in the CNS of Periplaneta americana.

J. Cell Biol. 26 445-459.

Soderholm, U. (1965).

Histochemical localisation of esterases, phosphatases and tetrazolium reductases in the motor neurones of the spinal cord of the rat anf the effect of nerve division.

Acta Physiol. Scand. 65 Suppl. 256.

Sporn, J.R., Harden, T.H., Wolfe, B.B. and Molinoff, P.B. (1976).
B-adrenergic receptor involvement in 6-hydroxydopamine induced supersensitivity in rat cerbral cortex.
Science N.Y. 194 624-626.

Srebro, B., Oderfeld-Novak, B., Klodas, I., Dabrowski, J. and Narkiewicz, O. (1973).
Changes in acetylcholinesterase activity in hippocampus produced by septal lesions in the rat.
Life Sci. 12 261-270.

Storm-Mathisen, J. (1972).
Glutamate decarboxylase in the rat hippocampal region after lesion of the afferent fibre systems. Evidence that the enzyme is localised in intrinsic neurones.
Brain Res. 40 215-235.

Stretton, A.O.W. and Kravitz, E.A. (1968).
Neuronal geometry: determination with a technique of intracellular dye injection.
Science N.Y. 162 132-134.

Suga, N. and Katsuki, Y. (1961).
Pharmacological studies on the auditory synapse in a grasshopper.
J. Exp. Biol. 38 759-770.

Sumner, B.E.H. and Sutherland, F.I. (1973).
Quantitative electron microscopy on the injured hypoglossal nucleus in the rat.
J. Neurocytol. 2 315-328.

Sumner, B.E.H. and Watson, W.E. (1971).
Retration and expansion of the dendritic tree of motor neurones of adult rats induced in vivo.
Nature (Lond.). 233 273-275.

Szentagothai, J. (1957).
Zum elementaren Bau der interneuronalen synapse.
Acta Anat. 30 827-841.

Tabachnik, I.I.A. (1956).
A rapid spectrophotometric assay of purified cholinesterase
Biochem. Biophys. Acta 21 580-581.

Takeuchi, A. and Takeuchi, N. (1960).
On the permeability of end-plate membrane during the action of transmitter.
J. Physiol. (Lond.). 154 52-67.

Tauc, L. and Bruner, J. (1963).
Desensitisation of cholinergic receptors by acetylcholine in molluscan central neurons.
Nature (Lond.). 198 33-34.

Taxi, J. (1961).
La distribution des cholinesterases dans divers ganglion du systeme nerveux autonome des Vertebres.
Bibl. Anat. 2 73-89.

Taylor, A.C. and Weiss, P. (1965).
Demonstration of axonal flow by the movement of tritium labelled protein in mature optic nerve fibres.
Proc. Nat. Acad. Sci. (Wash.). 54 1521-1527.

Thesleff, S. (1960).
Supersensitivity of skeletal muscle produced by botulinum toxin.
J. Physiol. (Lond.). 151 598-607.

Thesleff, S. (1963).
Spontaneous electrical activity in denervated rat skeletal muscle.
In; The effect of use and disuse on neuromuscular function. (Gutmann, E. and Hnik, P, eds.). Amsterdam. Elsevier. pp. 41-51.

Thorpe, W. and Seeman, P. (1961).
Effect of denervating skeletal muscle on calcium binding by isolated sarcolemma.
Exp. Neurol. 30 277-290.

Thron, C.D. and Waud, D.R. (1968).
The rate of action of atropine.
J. Pharmacol. 160 91-105.

Torack, R.M. and Barrnett, R.J. (1962).
Fine structural localisation of cholinesterase activity in the rat brain stem.
Exp. Neurol. 6 224-244.

Tower, D.B. (1960).
The neurochemistry of epilepsy. Thomas, Springfield. III.

Treherne, J.E. (1961).
Sodium and potassium fluxes in the abdominal nerve cord of the cockroach Periplaneta americana.
J. Exp. Biol. 38 315-322.

Treherne, J.E. (1962a).

The distribution and exchange of some ions and molecules in the central nervous system of Periplaneta americana.

J. Exp. Biol. 39 193-217.

Treherne, J.E. (1962b).

Some effects of the ionic composition of the extracellular fluid on the electrical properties of the cockroach abdominal nerve cord.

J. Exp. Biol. 39 631-641.

Treherne, J.E. and Smith, D.S. (1965a).

The penetration of acetylcholine into the central nervous tissue of an insect, Periplaneta americana.

J. Exp. Biol. 43 13-21.

Treherne, J.E. and Smith, D.S. (1965b).

The metabolism of acetylcholine in the intact central nervous system of an insect, Periplaneta americana.

J. Exp. Biol. 43 441-454.

Twarog, B.M. and Roeder, K.D. (1956).

Properties of the connective tissue sheath of the cockroach abdominal nerve cord.

Biol. Bull. 111 278-286.

Twarog, B.M. and Roeder, K.D. (1957).

Pharmacological observations on the desheathed last abdominal ganglion of the cockroach.

Ent. Soc. Amer. 50 231-236.

Tweedle, C.D., Pitman, R.M. and Cohen, M.J. (1973).
Dendritic stability of insect central neurons subjected to axotomy and de-afferentation.
Brain Res. 60 471-476.

Uretsky, N.J. and Iversen, L.L. (1970).
Effects of 6-hydroxydopamine on catecholamine containing neurones in the rat brain.
J. Neurochem. 17 269-278.

Usherwood, P.N.R. (1961).
The nature of "slow" and "fast" contactions in the coxal muscles of the cockroach.
J. Ins. Physiol. 8 31-52.

Usherwood, P.N.R. (1967).
Insect neuromuscular mechanisms.
Amer. Zoologist. 7 553-582.

Usherwood, P.N.R. (1969).
Glutamate sensitivity of denervated insect muscle fibres.
Nature (Lond.). 223 411-413.

Watson, W.E. (1965).
An autoradiographic study of the incorporation of nucleic acid precursors by neurones and glia during nerve regeneration.
J. Physiol (Lond.). 180 741-752.

Watson, W.E. (1966).

Some quantitative observations upon the oxidation of substrates of the tricarboxylic acid cycle in injured neurones.

J. Neurochem. 13 849-856.

Watson, W.E. (1968a).

Observations on the nucleolar and total cell body nucleic acid of injured nerve cells.

J. Physiol. (Lond.). 196 655-676.

Watson, W.E. (1968b).

Centripetal passage of labelled molecules along mammalian motor axons.

J. Physiol. (Lond.). 196 122P-123P.

Wigglesworth, V.B. (1960).

The nutrition of the CNS in the cockroach Periplaneta americana L. The role of the perineurium and glial cells in the mobilisation of reserves.

J. Exp. Biol. 37 500-512.

Wilson, D.M. (1965).

Proprioceptive leg reflexes in cockroaches.

J. Exp. Biol. 43 397-409.

Wine, J.J. (1972).

Slowly developing hypereflexia in the crayfish abdomen following nerve cord transection.

Soc. for Neurosciences, 2nd Annual Meeting, Abstract.

Wine, J.J. and Krasne, F.B. (1969).
Independence of inhibition and habituation in the crayfish lateral giant fibre escape reflex.
Proc. 77th Ann. Conv. A.P.A. 237-238.

Winton, M.Y., Metcalf, R.L. and Fukuto, T.R. (1958).
The use of acetyl thiochooine in the histochemical Study of the action of organophosphorous insecticides.
Ann. Ent. Soc. Amer. 51 436-441.

Woodson, P., Schlapfer, R.A., and Barondes, S. (1972).
Postural avoidance learning in the headless cockroach without detectable changes in ganglionic cholinesterase.
Brain Res. 37 348-352.

Yamasaki, T. and Narahashi, T. (1959a).
The effects of potassium ions and sodium ions on the resting and action potentials of the cockroach giant axon.
J. Ins. Physiol. 3 146-158.

Yamasaki, T. and Narahashi, T. (1959).
Electrical properties of the cockroach giant axon.
J. Ins. Physiol. 3 220-242.

Yamasaki, T. and Narahashi, T. (1960).
Synaptic transmission in the last abdominal ganglion of the cockroach.
J. Ins. Physiol. 4 1-13.

Yamamura, H.I. and Snyder, S.H. (1974).
Postsynaptic localisation of muscarinic cholinergic receptor binding in the rat.
Brain Res. 78 320-326.

Young, J.Z. (1932).
On the cytology of the neurons of cephalopods.
Q. J. Microsc. Sci. 75 1-47.

Zelena, J. and Lubinska, L. (1962).
Early changes of acetylcholinesterase activity near the lesion in crushed nerves.
Physiol. Biochem. 11 261-268.

ADDENDUM.

Bacon, J.P. and Altman, J.S. (1977).
A silver intensification method for cobalt-filled neurones in wholemount preparations.
Brain Res. 138 359-363.

Callec, J. and Boistel, J. (1967).
Les effets de l'acetylcholine aux niveaux synaptique et somatique dans le cas dernier ganglion abdominal de la Blatte Periplaneta americana.
Comptes Rendus Soc. Biol. 161 442-446.

Callec, J.J. and Sattelle, D.B. (1973).
A simple electrophysiological technique for monitoring the synaptic actions of pharmacological agents.
J. Exp. Biol. 59 725-738.

Crossman, A.R., Kerkut, G.A., Pitman, R.M. and Walker, R.J. (1971). Electrically excitable nerve cell bodies in the central ganglia of two insect species Periplaneta americana and Schistocerca gregaria. Investigation of cell geometry and morphology by intracellular dye injection. Comp. Biochem. Physiol., 40A, 579-594.

Grossman, Y. and Parnas, I. (1973).
Control mechanisms involved in the regulation of the phallic neuromuscular system of the cockroach Periplaneta americana.
J. Comp. Physiol. 82 1-21.

Curtis, D.R. (1964).
Microelectrophoresis. In: Physical Techniques in Biological Research, VA , 144-190. Nastuk, W.L. (Ed.). New York, Academic Press.

Del Castillo, J. and Katz, B. (1955).
On the localisation of acetylcholine receptors.
J. Physiol. (Lond.). 128 157-181.

Eccles, J.C. (1952).
The Neurophysiological Basis of Mind. Clarendon Press: Oxford.

Eccles, R.M. and Libet, B. (1961).
Origin and blockade of the synaptic responses of curarized sympathetic ganglia.
J. Physiol. (Lond.). 157 484-503.

Fatt, P. and Katz, B. (1953).
The electrical properties of crustacean muscle fibres.
J. Physiol. (Lond.). 120 171-204.

Feldberg, W. and Vartiainen, A. (1935).
Further observations on the physiology and pharmacology of a sympathetic ganglion.
J. Physiol. (Lond.). 83 103-128.

Fredricsson, B. and Sjoqvist, F. (1962).
A cytomorphological study of cholinesterase in sympathetic ganglia of the cat.
Acta Morphol. Neerl. Scand. 5 140-166.

Giacobini, E., Palmorg, B. and Sjoqvist, F. (1967).
Cholinesterase activity in innervated and denervated sympathetic ganglion cells of the cat.
Acta Physiol. Scand. 69 355-361.

Gromadzki, C.G. and Koelle, G.B. (1965).
The effect of axotomy on acetylcholinesterase of the superior cervical ganglion of the cat.
Biochem. Pharmacol. 14 1745.

Grossman & Parnas: see page 217.

Guthrie, D.M. and Tindall, A.R. (1968).
The Biology of the Cockroach. Edward Arnold (Publishers).

Hagiwara, S. and Watanabe, A. (1956).
Discharges in motoneurones of cicada.
J. Cell. Comp. Physiol. 47 415-428.

Hagiwara, S., Watanabe, A. and Saito, N. (1959).
Potential changes in syncitial neurons of lobster cardiac ganglion.
J. Neurophysiol. 22 554-572.

Hamberger, A., Hanson, H.A. and Sjostrand, J. (1970).
Surface structure of isolated neurones. Detachment of nerve terminals during axon regeneration.
J. Cell. Biol. 47 319-331.

Hard, W.L. and Peterson, A.C. (1949).
Studies on the histochemical localisation of cholinesterase in nerve tissue of the dog.
Anat. Rec. 105 494-495.

Hard, W.L. and Peterson, A.C. (1950).
The distribution of cholinesterase in nerve tissue of the dog.
Anat. Rec. 108 57-69.

Hodgkin, A.L. (1958).
Ionic movements and electrical activity in giant nerve fibres.
Proc. R. Soc. Lond. (B). 148 1-37.

Pitman, R.M. (1979).
Block intensification of neurones stained with cobalt sulphide: a method for destaining and enhanced contrast.
J. Exp. Biol. 78 295-297.

Strausfeld, N.J. and Obermeyer, M. (1976).
Resolution of intraneuronal and transynaptic migration of cobalt in the insect visual and central nervous system.
J. Comp. Physiol. 110 1-12.

Treherne, J.E. (1961).
Sodium and potassium fluxes in the abdominal nerve cord of the cockroach, Periplaneta americana.
J. Exp. Biol. 38 315-322.

Usherwood, P.N.R. and Machili, P. (1966).
Chemical transmission at the insect excitatory neuromuscular synapse. Nature 210 634-636.