


NEURONAL TRANSMISSION MECHANISMS

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SUMMARY

Several techniques were used to study the morphology of the salivary apparatus of the cockroach, *Nauphoeta cinerea*. A general survey of the ultrastructure was made. The acinar cells were of two distinct types : peripheral and central cells. The ducts that these cells give rise to could be classified into three morphologically distinct areas. The fine structure of the reservoir ducts was also studied.

Intracellular injections of Procion yellow dye and the use of lanthanum, as an electron dense marker, showed that there were many intercalated gap-junctions between the septate desmosomes of the acinar cells.

The innervation of the apparatus was studied in detail and it was observed, using techniques of fluorescence histochemistry and electron microscopy, that the salivary nerves, which arise from the suboesophageal ganglion, branch over the surface of the acini. The axons associated with the acini were found to be of two morphologically distinct types, designated type A and type B. Several histochemical tests indicated that type A axons contained a catecholamine.

It was attempted to stimulate the salivary nerves. This resulted in structural changes within peripheral cells and type A axons. When the salivary nerves were cut several degenerating axon profiles could be identified in association with the gland cells. This was not observed when the stomatogastric nerve was cut.

(iii)

Finally, enzyme-inhibiting drugs were used to interrupt the synthetic pathways of catecholamines. These experiments led to a number of unexpected results including the observation that some of the actions of these drugs appeared to be post-synaptic.

PART 1 : GENERAL INTRODUCTION

Section 1 - The salivary apparatus of the cockroach *NAUPHOETA CINEREA* (Olivier)

All the observations reported in this thesis were made on the salivary apparatus of adult cockroaches (*Nauphoeta cinerea* Olivier). The apparatus is a bilaterally symmetrical structure which consists of acini and ducts, as is the case with several other species of cockroach (eg, Lebedev, 1899; Whitehead, 1971). Caudal to the acini is a pair of reservoirs each of which gives rise to a duct. These ducts form a final common duct with a pair of secretory ducts which run from the acini. In the cockroach the salivary apparatus is positioned on each side of the gut; with the acini occupying some of the dorsal regions of the gut and joined across the midline with connective tissue. Nerves from the suboesophageal ganglion and the stomatogastric nerve supply the salivary apparatus. A diagram of the salivary apparatus and its relationship with the other organs of the cockroach is given in figure 1.

Section 2 - Previous work on the salivary apparatus of the cockroach

In this account only work performed on the cockroach, *Nauphoeta cinerea* shall be reported. Throughout the thesis comparison will be made with different species (eg, *Periplaneta americana*, *Blatta orientalis*).

The literature on the salivary apparatus of this cockroach begins in 1971 with the publication of a paper by Bland and House on the structure and function of the salivary glands of the

cockroach. In this paper some preliminary electron microscopy was reported and the authors suggested that the glands of the cockroach consist of four types of cell : peripheral cells; central cells; secretory duct cells and non-secretory duct cells. These observations are borne out by the present study. Bland and House also attempted some histochemistry upon these various cells and concluded that central cells contained amylase whereas secretory duct cells contained mucus. They were unable to attribute a precise function to the remaining two types of cell.

Bland et al (1973) examined the innervation of the gland using the Falck-Hillarp fluorescence histochemical technique, and observed that the cockroach gland was invested by axons which fluoresced and could be categorised as containing a catecholamine by microspectrofluorimetry. This is in keeping with the work of Fry et al (1974) who showed, by their radiochemical assay, that the glands contained quantities of dopamine. Bland et al (1973) also reported that hyperpolarising electrical potentials could be recorded from the acini of the gland in response to perfused dopamine (discussed below).

The innervation of the gland was further studied by Bowser-Riley (1978a) who observed methylene blue stained axons running from the suboesophageal ganglion, down the reservoir ducts to branch profusely over the acini and form a dense network of axons. He also noted that a limited number of axons from the stomatogastric nerve came within the vicinity of the acini and the reservoirs. Scanning electron microscopy of the acini has shown that many of

the axons have swellings in them which Bowser-Riley suggests are varicosities. Maxwell (1978) has performed transmission electron microscopy upon these axons and categorised them into two types : Type A and Type B. No further discussion of this paper shall be made as all of this work is presented in this thesis. Bowser-Riley (1978b) has back-filled the salivary nerves with the enzyme horseradish peroxidase and has identified the cell bodies of these nerves within the suboesophageal ganglion. It seems there may be two cell bodies on each side of the ganglion.

A considerable volume of electrophysiological data is now available on this preparation. In 1973, House reported that a microelectrode could be inserted into the cells of the acini and that a membrane potential of -32 mV could be recorded. If the salivary nerve was stimulated by field stimulation a slow transient hyperpolarisation could be observed. This has been called the 'secretory' potential and is characterised by a latency of about 1 S, a hyperpolarisation of up to 30 mV and a time course of about 10 S. Often a depolarisation of a few millivolts is observed after the secretory potential. House (1975) demonstrated by marking cells with Procion yellow that secretory potentials could be recorded from both central and peripheral cells. The nature of the hyperpolarising potential was investigated by Ginsborg, House and Silinsky (1974) who showed that it was accompanied by a rise in membrane potassium conductance. In this paper they also reported electrical coupling of the cells of the acinus ; a current injecting electrode could be placed in one cell while voltages were recorded from another. Ginsborg and House (1976) were now using

suction electrodes to stimulate the salivary nerves. Either both or just one salivary nerve could be placed within the electrode and stimulated. By this means they were able to demonstrate that there was a limited amount of contralateral innervation of the gland mainly at the midline region. In this paper they also claimed that the after-depolarisation was an independent response as it could be separated from the hyperpolarisation. The response of cells to trains of stimuli and to increasing voltages was also tested and it was found that the size of the response varied with the number of stimuli to some extent although maximal responses (-80 mV) could be recorded with between 2-50 pulses each of 50 V. Increasing the voltage showed that there was a threshold response at about 3.7 V which increased in jumps until a maximum response was recorded. The authors concluded that axons of different thresholds were being recruited.

Stimulation of the salivary nerves also elicits secretion (Smith and House, 1977). Pulses of 0.5 mS in duration of 50 V at 10 Hz would elicit a maximal rate of secretion of 80 nl/min for over 10 minutes until fatigue occurred. After a period of rest electrical stimulation again produced maximal secretion. The fatigue is thought to occur within the nerve as applied dopamine is able to produce a maximal secretory rate for over half an hour. The rate of secretion is also dependent upon the number of stimuli delivered (House and Smith, 1978).

A number of agonists have been tested on the salivary gland and of these, dopamine was the most potent and could elicit electrical

responses in concentrations as low as 10^{-10} M (Bowser-Riley and House, 1976). The log dose response curves for dopamine and noradrenaline were parallel but those for 5-hydroxytryptamine and adrenaline were not. This would suggest that 5-hydroxytryptamine and adrenaline probably combine with different receptors than the two other biogenic amines. In this study there was also some evidence that acetylcholine could modify the transmitter output of the salivary nerves. Ginsborg, House and Silinsky (1976) tested several alpha and beta agonists upon the preparation and found that the alpha agonists amidephrine and methoxamine had no effect whereas isoprenaline, a beta agonist, could only elicit electrical responses in concentrations greater than 10^{-4} M. Tyramine, an indirectly acting sympathomimetic, was able to increase the rate of the spontaneous hyperpolarisations that are observed from time to time. This evidence, and further evidence from antagonists (see below), led to the conclusion that the cockroach gland does not have 'classical' alpha and beta receptors.

Very similar results were found by Smith (1977) when he tested a number of agonists on the preparation for potency in the production of secretion. He found that dopamine was the most potent of a group of dopamine, noradrenaline, adrenaline and 5-hydroxytryptamine. Again the observation was made that dopamine and noradrenaline had parallel dose/response curves, whereas those for adrenaline and 5-hydroxytryptamine were different. As has been mentioned above, it is possible to stimulate the gland for 30 minutes with dopamine at 10^{-6} M and elicit a constant flow of saliva. Brief responses to dopamine may be elicited over a considerable period of time, each

response being similar in magnitude if the same dose is used (House and Smith, 1978). House and Smith (1978) also offered evidence to suggest that dopamine receptors were distinct from those for noradrenaline and adrenaline. Simultaneous doses of dopamine and noradrenaline or adrenaline were delivered to the preparation and the reasoning was that a matched dose (ie, the same molar concentration with $\frac{1}{2}$ a test dose of dopamine + $\frac{1}{2}$ the same dose of adrenaline or noradrenaline) of mixed agonists should produce a secretory response that is smaller than a test dose of dopamine of the same concentration because of the degree of joint occupancy of receptors by the less potent agonist. This prediction was not observed experimentally, indeed a modicum of addition was seen thus suggesting that there may be receptors with dopamine specific binding sites.

A number of antagonists have also been tried on the gland, with the eventual hope of distinguishing between noradrenaline and dopamine receptors. The beta antagonist, propranolol, had no effect upon the nerve response, whereas the alpha antagonist, phentolamine, did and also reduced electrical responses to applied dopamine (Ginsborg, House and Silinsky, 1976). Phentolamine was also found to reduce the amount of secretion elicited by nerve stimulation (Bowser-Riley, House and Smith, 1978). Applied dopamine, noradrenaline and adrenaline responses were reduced in a competitive manner, whereas those of 5-hydroxytryptamine were more complex, again providing further evidence of different receptors for 5-hydroxytryptamine.

The neuroleptic α -flupenthixol also inhibits the nerve response (House and Ginsborg, 1976) and was found to antagonise the secretory responses to dopamine and noradrenaline in a competitive manner, but not the secretory response to adrenaline, which seemed to be non-competitive (Breward, 1977). This would suggest that adrenaline operates by different receptors than dopamine and noradrenaline.

The response to ionophoretically applied dopamine to the isolated salivary gland is similar to the electrical response recorded after stimulation to the salivary nerves (Blackman, Ginsborg and House, 1979a), except that they tended to decay more slowly and have a more rapid onset. The effect of temperature changes to ionophoretically applied dopamine was similar to that of nerve evoked responses. The very slow latency of the electrical response to nerve stimulation was also investigated by Blackman et al (1979b). They used a micropipette which could deliver an ionophoretically applied pulse of dopamine to the surface of the acinus. It was not possible to reduce the latency greatly and the authors concluded on the basis of mathematical models that the long latency could not be explained in terms of slow diffusion or receptor kinetics. They decided that this effect must be due to some process that occurs after receptor activation. An interesting observation has been made by House, Ginsborg and Mitchell (1978) who ionophoresed dopamine inside cells and were able to record potentials similar to those when dopamine was ionophoresed on to the surface of the acinus. It is therefore possible that receptors are located inside the cells as well as on the surface. One objection to this interpretation of these results is that it may be that dopamine diffuses out of the cells to activate surface receptors.

Finally, in this resume of previous work, I wish to consider the composition of cockroach saliva. Smith and House (1979) have made an extensive study of the ionic composition of cockroach saliva and have shown that secretion is dependent upon the presence of external sodium ions, but not potassium or chloride. They have proposed a model of saliva elaboration which shows active transport of sodium at the inner membrane of the acinus as externally applied ouabain (an inhibitor of sodium pumps) has no effect. They propose that sodium is accompanied by a passive flux of chloride. Potassium also enters the lumina of cells: it is known that the final potassium concentrations in saliva are higher than those of the external bathing fluid. In this report the authors also showed that the ionic content of the reservoirs was low.

Section 3 - Dopamine as a neurotransmitter

There are four 'classical' conditions that have to be satisfied if a substance is to be considered to be a neurotransmitter :

- (1) the substance must be present in the presynaptic nerve ending;
- (2) it must be released from the ending;
- (3) it must react with postsynaptic receptors, initiating a chain of events;
- (4) it is inactivated after this process is completed.

There are three methods available for the identification of dopamine in nerve cells : (1) fluorescence histochemistry; (2) biochemical assays; and (3) labelling techniques. The method of fluorescence histochemistry was developed by Falck et al (1962) and depends upon the observation that formaldehyde gas converts catecholamines into 6,7 dihydroxy-3,4 dihydroisoquinolones

which are strongly fluorescent. This fluorescence differs significantly from background fluorescence as Corrodi et al (1964) were able to abolish it with sodium borohydride which reduces dihydro- compounds. The fluorescent method of Falck and Hillarp could be used to distinguish between the emission spectra of catecholamines and 5-hydroxytryptamine, but not noradrenaline and dopamine. Björklund^{et al} (1968) has developed a technique which is able to differentiate between the two catecholamines. This involves reaction of the tissue with hydrogen chloride gas after paraformaldehyde treatment, resulting in the production of fluorophores which have different excitation spectra. Lindvall et al (1975) have recently claimed that reaction of the tissue with glyoxylic acid and paraformaldehyde also results in the production of different fluorophores which have different excitation spectra.

Biochemical assays have the immediate drawback that homogenised tissue is used and it is therefore not possible to assign any dopamine found to a neuronal location. Cuello et al (1973) have developed a radiochemical assay using the enzyme catechol-o-methyl transferase which converts dopamine into 3-methoxytyramine.

Radioactive dopamine precursors have been employed in studies that have used a combination of autoradiography and electron microscopy. This has given some insight into the sorts of vesicles that dopamine may be stored in (eg, see Pentreath and Berry, 1975). Such studies have been the obvious objection that there is no reason why dopamine precursors should not be synthesised into another compound and enter vesicles.

PREPARATION	ORGAN	METHOD	REFERENCE
cockroach (<i>Periplaneta</i>)	sub and supra-oesophageal ganglion	biochemical* assay	Frontali (in Pitman, 1971)
<i>Manduca sexta</i> LEPIDOPTERA	neuroglanglular synapse	biochemical* assay and F	Robertson 1975
cockroach (<i>Periplaneta</i>)	brain	F	Frontali and Norberg (1966)
cockroach (<i>Nauphoeta</i>)	neuroglanglular synapse	F and biochemical* assay	Bland et al (1973) Fry et al (1974)
<i>Planorbis cornelius</i> MOLLUSCA	large cell	F*	Kerkut (1973)

F = fluorescence histochemistry

TABLE 1

Table 1 below is a list of invertebrates that have catecholamines present within neuronal elements. In some cases dopamine has been positively identified (*).

The best known example of a dopaminergic system in the mammalian C.N.S. is the nigro-neostriatal system. In adult animals a direct nigro-neostriatal system has not yet been demonstrated with the Falck-Hillarp method and the evidence has come mainly from degeneration studies (eg, Goldstein et al, 1969). But in the foetal mouse, Golden (1972) has produced evidence of a direct pathway with the fluorescence histochemical technique. Björklund and Lindvall (1975) have identified dopamine in the dendrites of substantia nigra cells using the glyoxylic method. It was previously thought that structures containing dopamine were equivalent to axons and cell bodies.

Dopamine is synthesised from the amino acid tyrosine and there is now evidence to suggest that a rate limiting step exists at the tyrosine hydroxylase conversion of tyrosine to dopa. This may be controlled by end-product inhibition in noradrenaline systems (Spector et al, 1967) but there is no evidence of such a system in dopamine synthesis. Immunofluorescent techniques have shown that the enzyme dopamine- β -hydroxylase (which converts dopamine into noradrenaline) is not found in the cell bodies of neurones that are known to be dopaminergic. On the other hand L-Dopa decarboxylase (the enzyme that converts Dopa to dopamine) is found universally in catecholamine containing cells. Centrifugation methods have shown that tyrosine hydroxylase and L-Dopa decarboxylase are found

in the supernatant whereas Dopamine- β -hydroxylase is found within the sediment (ie, in some structures, possibly the storage granules). This evidence would suggest that dopamine is synthesised in the cytoplasm and is taken up into the storage granules. (These ideas have been reviewed by Blaschko, 1973). In keeping with the idea that dopamine is transported down the axons of dopamine containing cells is the observation made by Fibriger et al (1973) that ^{14}C -Dopa labelling of cells results in peaks of activity along the axons of cells.

Electro-convulsive shock delivered to rats increases the impulse activity of the brain and according to Musacchio et al (1969) also increases tyrosine hydroxylase activity and hence the level of catecholamine found within the brain. In keeping with this Fuxe and Gunne (1964) found that catecholamine levels in cat brains did not increase in response to stimulation when they had been given H 22/54 which is an inhibitor of catecholamine synthesis.

There have been few reports of direct release of dopamine in invertebrates, Ascher et al (1968) noted a release of ^3H -dopamine after stimulation of ganglion cells in *Helix* and *Aplysia*.

In mammals McLenan(1965) collected dopamine that had been released from the stimulated putamen with the aid of a push-pull cannula. Portig et al (1968) stimulated the caudate nucleus of the cat with tubocurarine and were able to collect dopamine and homovanillic acid (a break-down product of catecholamines) which were assayed with a fluorometric method.

One indirect way of inferring dopamine release is to see if dopamine mimics the postsynaptic events of the natural transmitter. In mammals the predominant action of dopamine on the post synaptic membranes is a depressant one (eg, Aimes and Pollen, 1969, in the retina; Krnjevic and Phillis, 1963, in the cat cerebral cortex; Biscoe and Straughan, 1966, on the rat hippocampus; Hirst and Silinsky, 1975, in the Guinea Pig submucous plexus). There have been a few reports of an excitatory action of dopamine; eg, York (1970) has shown that dopamine has an excitatory action in the cat putamen. There is some evidence that this effect can be blocked by alpha antagonists. In the small intensely fluorescent cells of rabbit sympathetic ganglia dopamine seems to act as a modulator; the muscarinic responses (slow inhibitory postsynaptic potentials, followed by slow excitatory post synaptic potentials) are greatly enhanced, and again alpha blocking agents seem to abolish these facilitatory responses (Libet and Tosaka, 1970).

In invertebrates dopamine may cause excitatory, inhibitory or biphasic responses (eg, Berry and Cottrell, 1975) in *Planorbis corneus*. It has been found that ergometrine and 6-hydroxydopamine specifically abolish the inhibitory responses to applied dopamine. This would indicate that in this system two types of receptor are in operation. Evidence to support this idea comes from the study that Ascher (1972) performed upon *Aplysia* neurones. He found that strychnine and tubocurarine blocked the excitatory response, whereas the inhibitory responses could be selectively blocked with ergot derivatives. In *Helix aspersa*, Kerkut⁽¹⁹⁷³⁾ and his collaborators have shown that dopamine could inhibit the spontaneous firing of neurones

in the isolated brain. This was associated with a membrane hyperpolarisation. Woodruff (1972) then investigated these effects and noted that only compounds which contained -OH groups on the 3 and 4 position of a benzene ring inhibited the firing. The cells did not respond to powerful alpha and beta stimulants and chlorpromazine had no effect. However, phentolamine and lysergic acid did block the dopamine responses.

There is some evidence to suggest that ^{action of} dopaminergic receptors may be mediated via a specific adenylate cyclase. Kebabian and Greengard (1971) noted that stimulation of the rabbit sympathetic ganglia produced increases in cyclic-AMP formation. It was found that dopamine was a much more potent stimulator of this than noradrenaline. Brown and Makman (1972) found that dopamine was ten times more potent in the production of increased c-AMP levels than other catecholamines. This increase in c-AMP could be blocked with phentolamine. McAfee and Greengard (1972) have shown that theophylline, an inhibitor of phosphodiesterase, increases the post-synaptic response to dopamine in the rabbit superior cervical ganglion. McAfee and Greengard also claimed that c-AMP, when applied to the post-synaptic membrane, results in a hyperpolarisation. However, it has not been possible to substantiate this claim (see Libet, 1979).

In mammals the ionic basis for the inhibitory effects of dopamine are not known; Krnjevic (1975) has offered several possibilities. In invertebrates it seems highly probable that dopamine causes a rise in potassium conductance (Ginsborg et al, 1974). Walker et al (1971) have calculated that 90% of the current of the dopamine induced inhibitory post synaptic potential in the visceral ganglia

of *Helix aspersa* is carried by potassium ions. Ascher (1972) has noted that in *Aplysia* the inhibitory postsynaptic potential in response to dopamine may be reversed at E_K so that a selective permeability for K has occurred.

In the rabbit caudate nucleus there is evidence that monoamine oxidase and catechol-O-methyl transferase are both involved in the catabolism of dopamine (Jonason and Rutledge, 1968). In *Aplysia* McCaman and Dewhurst (1971) could find no evidence for monoamine oxidase activity, although there was demonstrable catechol-O-methyl transferase activity.

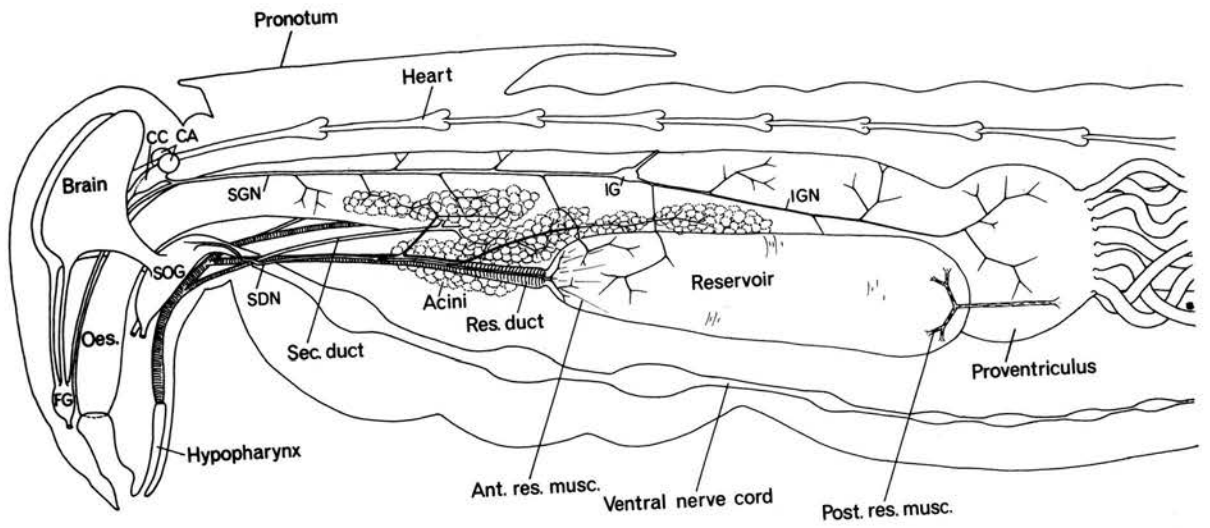
Another important method of inactivation of catecholamines is the reuptake of the transmitter by the presynaptic membrane. The mechanism for dopamine reuptake seems to be different from that of noradrenaline as desimipramine and chlorpromazine do not affect dopamine reuptake in the same way as they affect noradrenaline uptake (Hamberger, 1967). Iversen (1973) has shown that dopamine reuptake is inhibited by both + and - amphetamine whereas noradrenaline uptake is only affected by + amphetamine. This may be explained on the basis of dopamine not expressing optical isomerism and there being a higher affinity of uptake for - noradrenaline. Finally, Ascher et al (1968) have observed that in molluscs uptake of 3H -dopamine by non-neuronal structures is more prominent than uptake by the neurones themselves. Dopamine may be degraded in such structures.

In conclusion, although the four conditions that were presented at the beginning of this review have not been satisfied for any one specific system, it seems that there is a considerable volume of evidence from a variety of systems to suggest that dopamine is, in fact, a neurotransmitter. Likewise, at the cockroach neuroglandular synapse there is also evidence to suggest that dopamine acts as a neurotransmitter (see House, 1977 and House and Ginsborg, 1979). The remainder of this thesis is devoted to a study of this system.

PART I FIGURE I

This figure illustrates the salient features of the cockroach salivary apparatus (Sagittal section). (Reproduced with kind permission of Dr K P Bland).

Ant. res. musc.	anterior reservoir muscle
CA	corpus allatum
CC	corpus cardiacum
FG	frontal ganglion
IG	ingluvial ganglion
IGN	ingluvial nerve
Oes	oesophagus
Post. res. musc.	posterior reservoir muscle
Res. duct	reservoir duct
Sec. duct	secretory duct
SDN	salivary duct nerve (salivary nerves)
SGN	stomatogastric nerve
SOG	suboesophageal ganglion



	mM
Na Cl	160
K Cl	10
Ca Cl	5
TRIS	5
GLUCOSE	20

to pH 7.6 with H Cl

TABLE 1 COCKROACH RINGER (Smith and House, 1977)

PART 2 : GENERAL METHODS

Section 1 - Dissection of the cockroach salivary gland

The gross anatomy of the cockroach salivary gland is described in Part 1. Cockroaches were pinned out in a shallow vessel which contained a layer of Sylgard 184 (Dow-Corning). This Sylgard was stained with Sudan Black to enhance the contrast. The dorsal regions of the cockroach abdomen and pronotum, along with the wings, legs and tergites were removed. At this stage the preparation was immersed in cockroach ringer (see Table 1). The salivary apparatus was then carefully dissected away in its entirety along with the suboesophageal ganglion. Usually the small salivary nerves that have their origin in the ganglion were severed so that the salivary apparatus was free.

Section 2 - Electron microscopy

Unless otherwise stated, cockroach salivary glands were prepared for electron microscopy as follows. Whole glands, including reservoirs and ducts (part 2, section 1) were dissected out and pinned out in glass petri dishes which contained a layer of Sylgard 184 (Dow-Corning). Glutaraldehyde fixative was adjusted to a theoretical osmolarity of 370 mosm according to the method of Weakley (1972). The value of 370 mosm was chosen because cockroach ringer was found to have an osmolarity of 349 mosm when measured by depression of freezing point on an osmometer. It was therefore decided to adjust the fixative to 370 mosm as it is desirable that

the fixative should be slightly hypertonic to the tissue. The fixative consisted of 4% glutaraldehyde in 0.05 M sodium cacodylate buffer with 2.8% sucrose at pH 7.2. Glands were fixed in this for one hour at 4°C. The glutaraldehyde was then washed off the tissue by means of a washing buffer. This buffer was made from 0.05 M sodium cacodylate buffer and was adjusted to an osmolarity of 349 mosm with 8.3% sucrose. This had a pH of 7.2 and was used at 4°C. Osmication was achieved by immersing the glands in a 1% solution of osmium tetroxide in 0.1 M sodium cacodylate buffer pH 7.2 at 4°C for one hour. The glands were then washed again in buffer and placed in small bottles with plastic tops. Dehydration was achieved by taking the glands through 10, 30, 70 and 90% solutions of ethanol, for five minutes each, and then were placed in pure ethanol (stored with silica gel in it) for two periods of fifteen minutes. The glands were then given two changes of propylene oxide which lasted for fifteen minutes. The propylene oxide and glands were mixed throughout this stage on a TAAB araldite mixer.

The initial stages of embedding commenced with a mixture of 50% araldite and 50% propylene oxide in which the tissue was mixed for one hour. The araldite consisted of 19 mls of a 1:1 ratio of CY21D and HY964 resin with 0.6 mls of DBT plasticiser and 0.4 mls of DY062 accelerator. Glands were placed in this overnight and finally embedded the next day with fresh araldite in TAAB plastic capsules which were put in a 60°C oven for 48 hours.

When the block had cooled and hardened a pyramid was cut round the area of the tissue with a razor blade which was kept free of

grease by storing it under chloroform. One micron thick reference sections were cut on a Porter-Blum MT1 ultramicrotome using glass knives that were prepared by a LKB 7801B knife maker. The sections were spread using chloroform and transferred to a glass slide with a wire loop. The slide was then placed upon a Phototax dish warmer at 60°C and during the process of drying the sections became attached to the slide. The sections were stained with toluidine blue and viewed with a Nikon binocular microscope.

Thin sections (grey to pale gold) were cut using an OMU-3 microtome (Reichert) with the aid of a diamond knife (Walter Rawlins). The ribbons of sections were spread with chloroform and mounted on copper grids.

The grids were stained in uranyl acetate (a saturated solution in 50% alcohol which was centrifuged before use) for 20 minutes, washed in 10% alcohol and distilled water. Finally the grids were stained with lead citrate (Reynolds, 1963) for four minutes.

Sections were viewed with an AEI EM6B electron microscope. Photographic plates and prints were processed conventionally.

Section 3 - Fluorescence microscopy

Throughout this section the method of Laszlo (1974) was rigidly adhered to. Glands were dissected in the normal way, placed in small trays and quenched in isopentane which had been cooled in liquid nitrogen. The glands were then transferred to a dewar flask which contained liquid nitrogen (figure 1a).

The tissue was then freeze dried using a 'Speedivac' Pearce tissue drier, model 1. The samples were placed in the chamber with trays of phosphorus pentoxide and were freeze dried for two days. At the end of this period the temperature of the freeze drier was raised to 25⁰C (figure 1b). The glands were transferred to a box which contained silica gel.

The tissues were then exposed to paraformaldehyde vapour by placing them in a desiccator which contained paraformaldehyde at a relative humidity of 58%. This was heated in an 80⁰C oven for one hour (figure 1c).

The tissue was then vacuum embedded. This was achieved by placing it in a flask that contained solid paraffin wax which was lowered into a water bath at 60⁰C and evacuated (figure 1d). After 10 minutes air was allowed to enter the flask and the tissue was removed with a large pasteur pipette. The tissue was placed in a paraffin block and allowed to harden overnight. The following day the block was cut into the shape of a pyramid and mounted on a cube of wood. Ribbons were cut (8 microns thick) on a microtome and were placed in slide containers which were stored in plastic boxes in the dark. These boxes contained silica gel.

Sections were mounted on slides which had been warmed on a hot plate. On average, 3 sections were mounted on each slide which was covered by coverslip using Entellan as a mountant.

The sections were viewed in ultraviolet light with a Zeiss Universal microscope with an SP 50 (Zeiss) and a BG3 (Schott)

filter combination. These were photographed with Ilford HP4 film at 5-1½ minute exposures.

Section 4 - Secretion

The method employed for secretion experiments is a modification of that of Smith and House (1977). Glands were dissected out according to the method of Part 2, Section 1 and placed in a chamber (figure 2). This chamber differs from that of Smith and House (1977) in that the perfusion bath is disposable so glands may be fixed 'in situ' during an experiment. The perfusion bath was constructed from a tissue culture dish in which two celluloid barriers were placed. The base of the dish contained a layer of Sylgard 184 (Dow-Corning). The entire dish slotted into a perspex base, which prevented movement of it during experiments (figure 3). This base was then bolted down onto a metal plate as is shown in figure 4. Glands were pinned down in the perfusion chamber and their secretory ducts were drawn through a hole in one of the celluloid barriers into a pool of paraffin. The ducts were then held in place with a piece of silver wire. Reservoir ducts were sucked up into suction electrodes.

Salivary glands were perfused with a cockroach ringer (Table 1, Part 2, Section 1), at a rate of 2.5 mls/min from a Watson-Marlowe flow inducer. Drugs were administered to the gland via this route (see, for example, Part 7); test doses of dopamine (10^{-7} M) (Sigma) were frequently given. The salivary nerves (along with the reservoir ducts) were stimulated by 0.5 mS square pulses delivered from a Grass stimulator (SD5). The frequency and voltage of these varied,

depending upon the type of experiment that was being performed.

The droplets of secretion that were produced by the secretory ducts were collected in the paraffin pool at 5 minute intervals throughout experiments. A glass sucker was used for this purpose. This was coated with a non-stick surface to prevent the droplets adhering to the surface of it. The droplets were blown out of the sucker into another paraffin pool where the diameter of the secreted droplet was measured with an eyepiece graticule under a Zeiss binocular microscope. The volume of secretion was then calculated using the equation $V = \frac{\pi d^3}{6}$ (d = diameter). Hence, the rate of secretion per minute was calculated by dividing this value by 5. At the conclusion of many of the experiments the tissue was fixed by introducing fixative into the bath (Section 2, Part 2). Tissue could then be used for electron or light microscopy. The experimental set-up is illustrated in figure 4.

Section 5 - Intracellular staining

Electrophysiological experiments were performed with a view to the intracellular staining of cells of the cockroach acini. Isolated salivary glands were prepared and were bathed in a modified cockroach ringer (pH 7.6, 160 mM NaCl, 1 mM KCl, 5 mM CaCl, 5 mM TRIS, 4 mM HCl). These were mounted on a clear perspex chamber which contained Sylgard 184 (Dow-Corning) (figure 5a). The glands were pinned over a knob in the middle of the chamber and were illuminated from below to enable them to be viewed through a Zeiss binocular microscope. The chamber was perfused at a rate of 2.5 ml/min using a Watson-Marloweflow inducer.

Impalements were made using glass microelectrodes filled with a 4% solution of Procion yellow dye. These had an average resistance of 50 Megohms. The electrodes were connected to the bridge circuit of a BAK wide band electrometer (figure 5b). A silver/silver chloride reference electrode was used in conjunction with this. The potentials that were recorded from the acini were displayed on a Tektronix 502A oscilloscope and recorded with a Devices M2 recorder. The electrode was brought to the surface of the acini using a Naharishi micro-manipulator. Resting potentials were recorded as the electrode entered cells after a slight tap was given to the table that the preparation rested upon. It was considered that an impalement was made when a resting potential of about -30 mV was recorded. The salivary nerves and reservoir ducts were sucked into a suction electrode and were stimulated with a Devices isolated stimulator which delivered 40 V pulses of 0.5 mS in duration. If a successful impalement had been made a hyperpolarising secretory potential could be observed (see Section 3, Part 2). At this point in the procedure a Tektronix pulse generator, type 161, was switched on. This produced square waves of 100 mS duration at a frequency of 0.2 Hz. When the bridge circuit was balanced this resulted in a hyperpolarising current of 5×10^{-8} A being passed through the electrode which is sufficient to cause an ionophoretic flow of Procion dye into the impaled cell (Stretton and Kravitz, 1968). Cells were injected for 30 minutes and frequently stimulated to ensure that the electrode was still in place.

Glands were fixed immediately at the end of an injection with 10% formal-saline. They were dehydrated in a series of alcohols and cleared in xylene. After embedding in paraffin wax, 20 micron thick paraffin sections were cut and were mounted in serial order on glass slides. Coverslips were placed upon the sections with Gurr's UV-inert mountant. The tissue was viewed with a Zeiss 'Universal' fluorescence microscope using the SP41 and BG3 filters. Sections were also viewed under a Nikon microscope with phase contrast in order to identify the cells that had been filled with Procion dye.

Section 6 - Collection of data

After several experiments had been performed, it soon became clear that the collection of data from micrographs was tedious and time consuming. It was therefore decided to develop some form of semi-automatic method of data collection that could be used with the departmental PDP-12 computer. The solution was found in the form of a linear transducer (figure 6) which was able to measure and scale with respect to magnitude, data from micrographs. The data were then stored in files.

The linear transducer ($\pm 0.3\%$, DC, Model D2/500A; RDP Electronics Ltd) has a working distance of 2.5 cms and was made into an instrument which resembles a pair of vernier calipers. It uses the principle of a differential transducer and requires a voltage supply of +6.00 V at 50 mA. The output produced by this device is 1.22 V/cm with a zero output at the centre of movement.

A sectional drawing of the instrument is shown in figure 8. The distal point of the instrument is fixed, while the proximal point is connected through a slot to the movable core of the instrument which is spring loaded. The instrument can be held in one hand, while the operator can manipulate a micrograph with the other. The jaws of the caliper can be placed on the margin of the object to be measured and a reading entered by the depression of a foot switch which is connected to the external sense lines of the computer. This signal instructs the computer to make an analog-digital conversion of the output voltage. Other sense-lines are used to reject the previous reading or to terminate the process.

The electronic interface between the instrument and the computer consists of a regulated 6 V supply and a buffering amplifier with preset gain and off-set controls. The signal was scaled with these so that when the jaws were closed the output voltage was -1.00 V and when they were at 2 cms, the voltage was +1.00 V. This conforms to the requirements of the analog to digital converter of the computer. A full circuit diagram is given in figure 7.

The interfacing program consists of several sections (figure 9). It begins with a request for the magnification factor of the micrograph. It then asks for a calibration of the instrument first taking a reading at 0 cms and then at 2 cms. The device is then ready to start taking measurements. This process is repeated for as many times as is deemed necessary. If a mistake is made a correction can be performed with the sense lines. When the data has been collected another sense line is activated which stores the

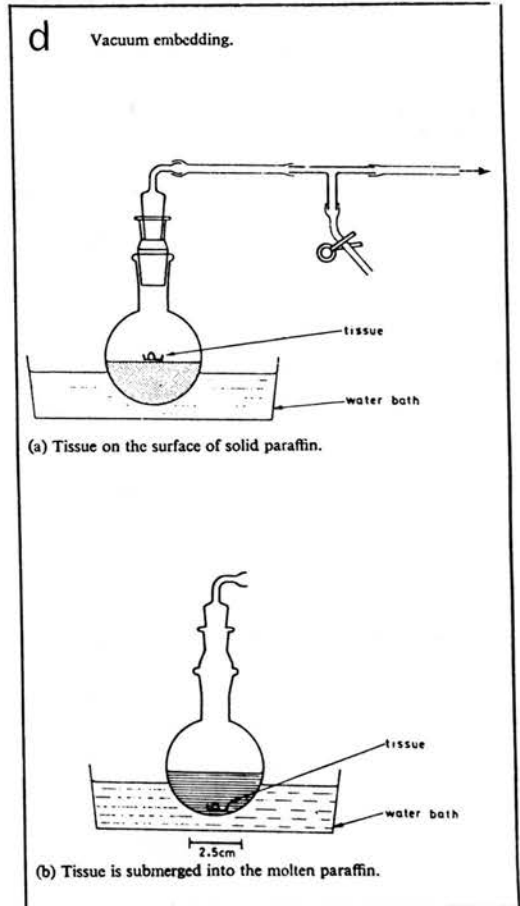
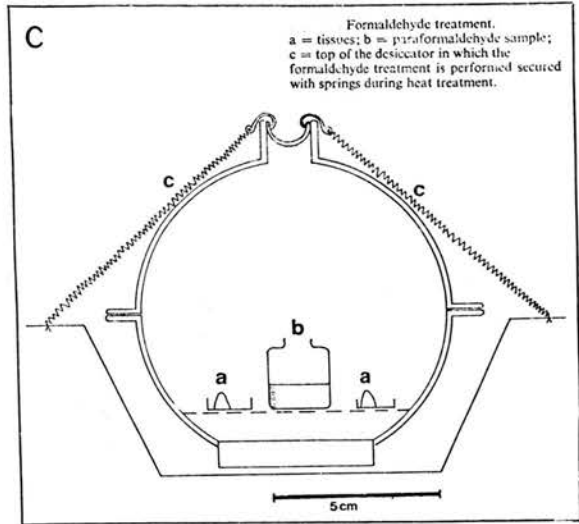
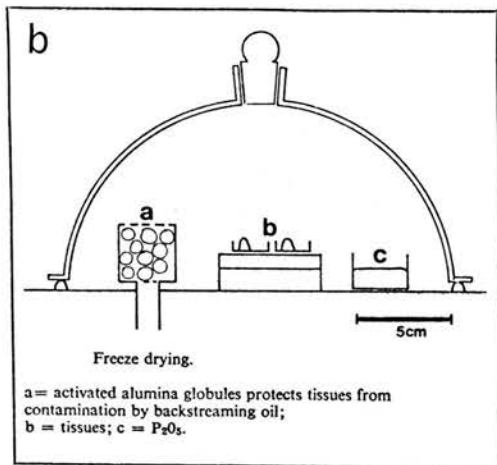
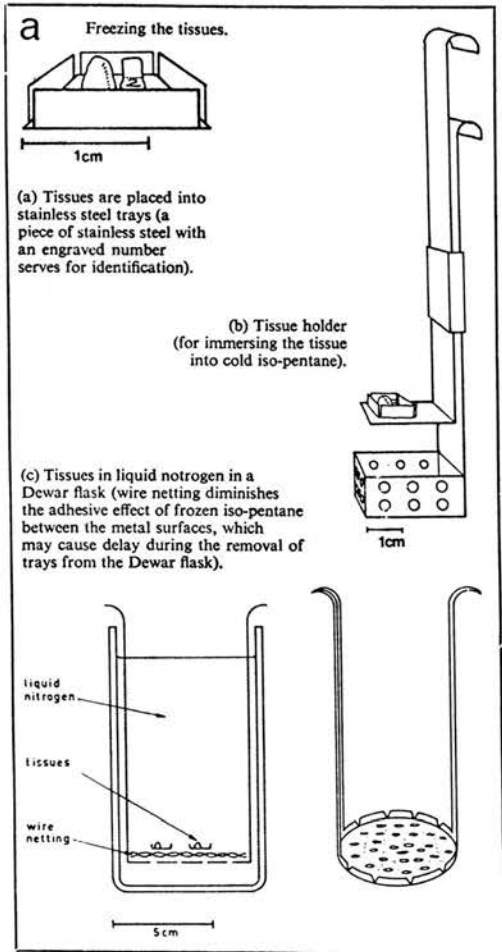
collected data in a file and then stops. Three further programs have been written to examine the data in the files; one calculates the mean and standard deviation of the data and another calculates ratios and logarithms of it (figure 10). The third program compares two files and performs a t-test upon them (Figure 11). In addition to these programs, a histogram plotting program by Dr Ensor was also employed.

PART 2 FIGURE I

Processing of tissue for fluorescence histochemistry.

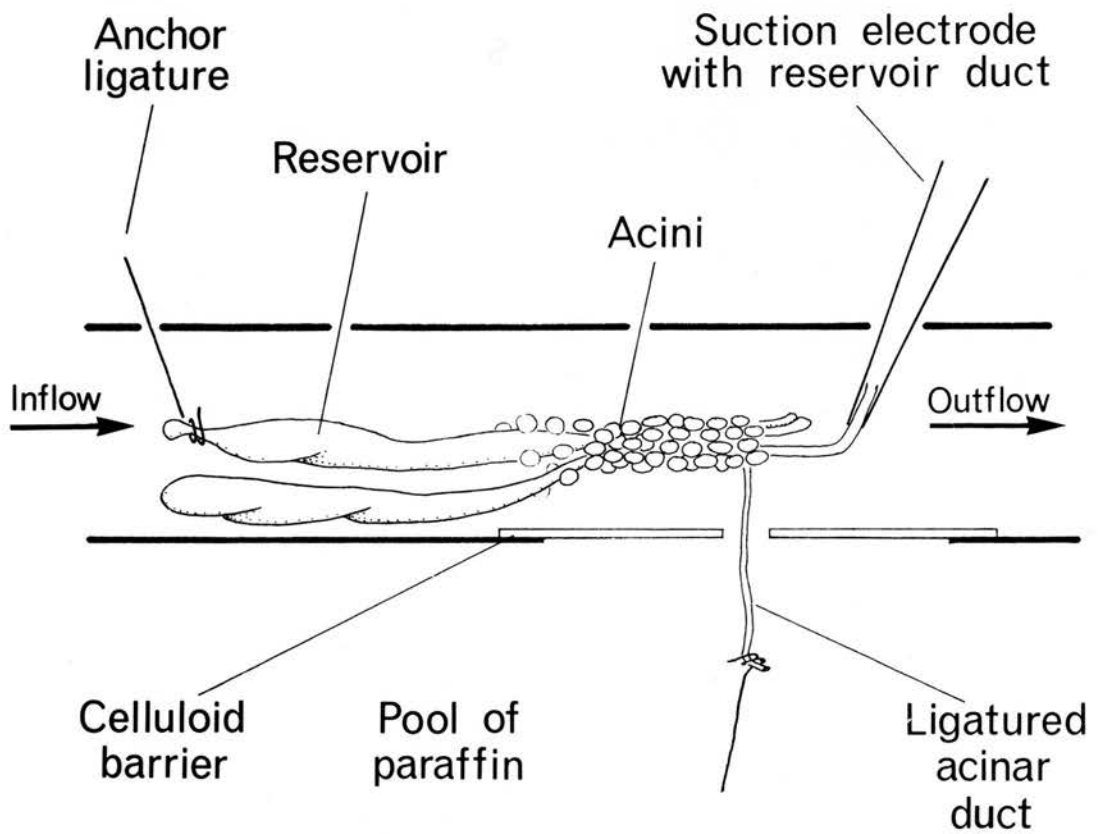
(Reproduced with kind permission of Dr I Laszlo.)

- a. The quenching and freezing of tissue with liquid nitrogen.
- b. Freeze drying.
- c. Exposing the tissue to paraformaldehyde.
- d. The process of vacuum embedding.



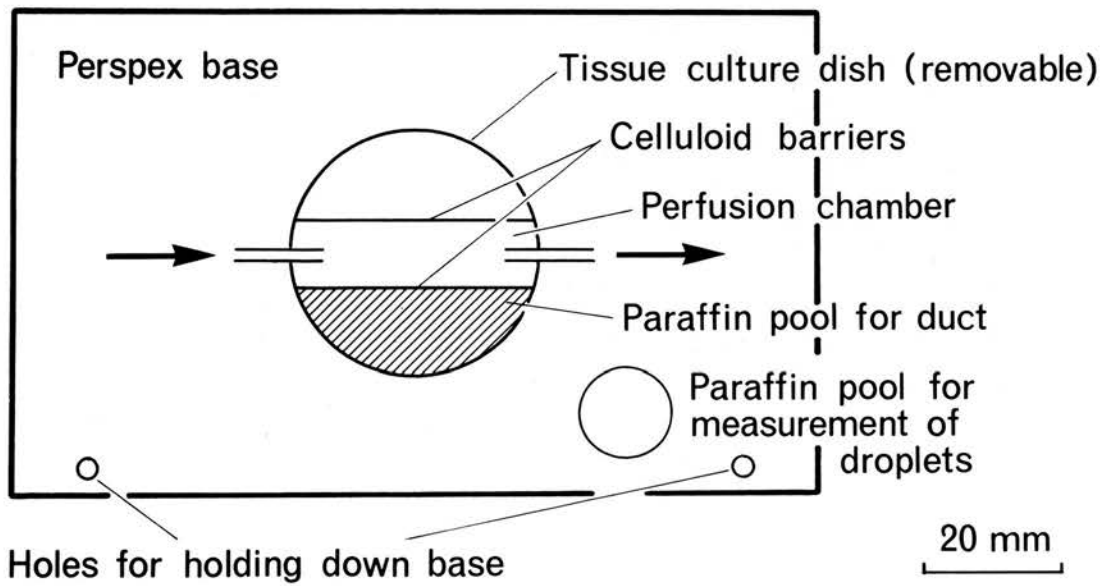
PART 2 FIGURE 2

The salivary apparatus of the cockroach in position in the perfusion chamber during a secretion experiment. The nerves can be stimulated via the suction electrode and secretion collected from the secretory duct in the paraffin pool. The preparation is perfused with cockroach Ringer.



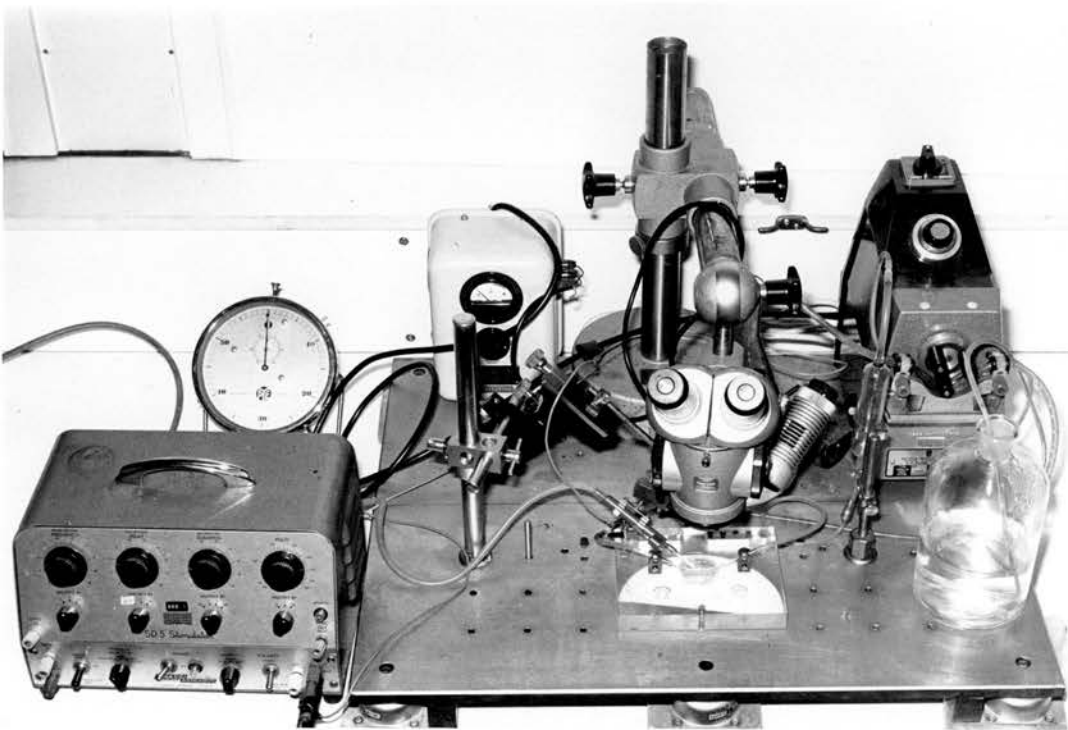
PART 2 FIGURE 3

Plan view of the perfusion chamber for secretion experiments.
Note that the centre of the apparatus is removable and disposable
so that fixative can be introduced into this chamber during the
course of an experiment without contaminating subsequent
experiments.



PART 2 FIGURE 4

The apparatus used for secretion experiments. A Grass stimulator is seen at the bottom left corner of the picture. Above this is a clock that was used for timing the experiments. The suction electrode was held in position with a micro-manipulator and the secretion could be collected under a Zeiss dissecting microscope. The preparation was perfused using a Watson-Marlowe flow inducer.

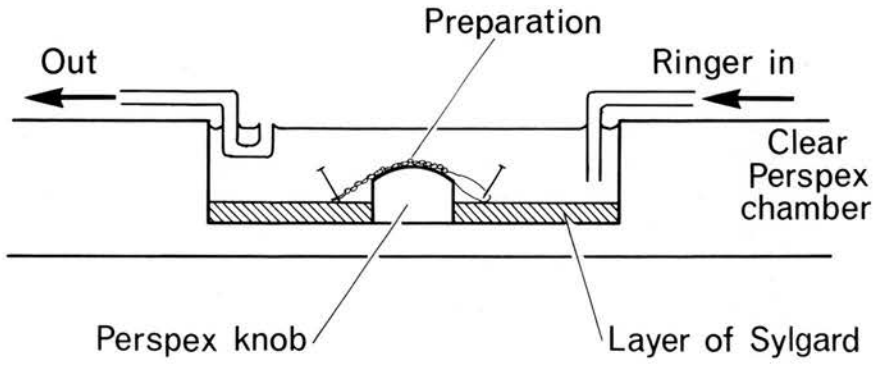


PART 2 FIGURE 5

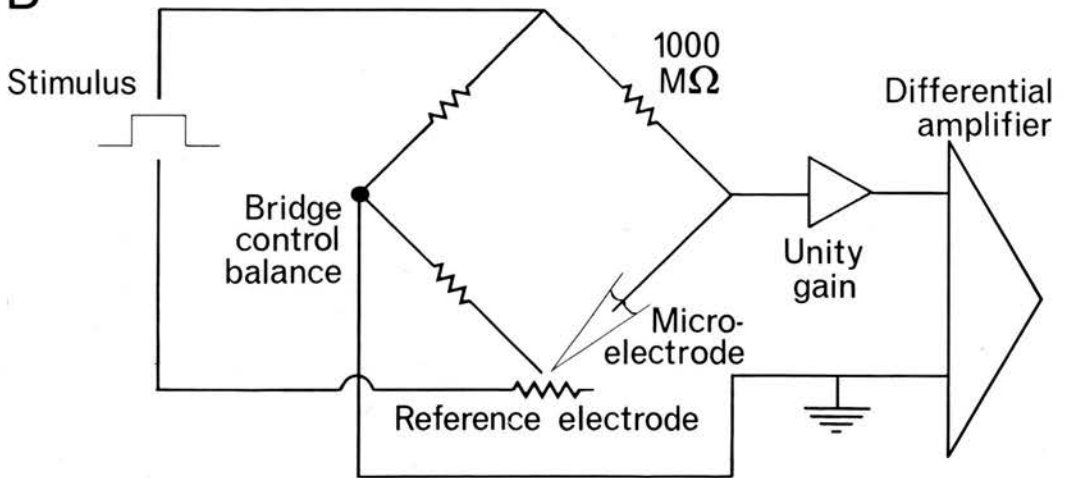
Figure 5a is the experimental chamber that was used in the electrophysiological experiments. The preparation was pinned over a perspex knob in the chamber and could be illuminated from below. It was continuously perfused with Ringer.

Figure 5b. The circuit that was used for injecting current and recording through a microelectrode. The amount of current passed could be calculated from Ohm's law when the bridge circuit was balanced.

A

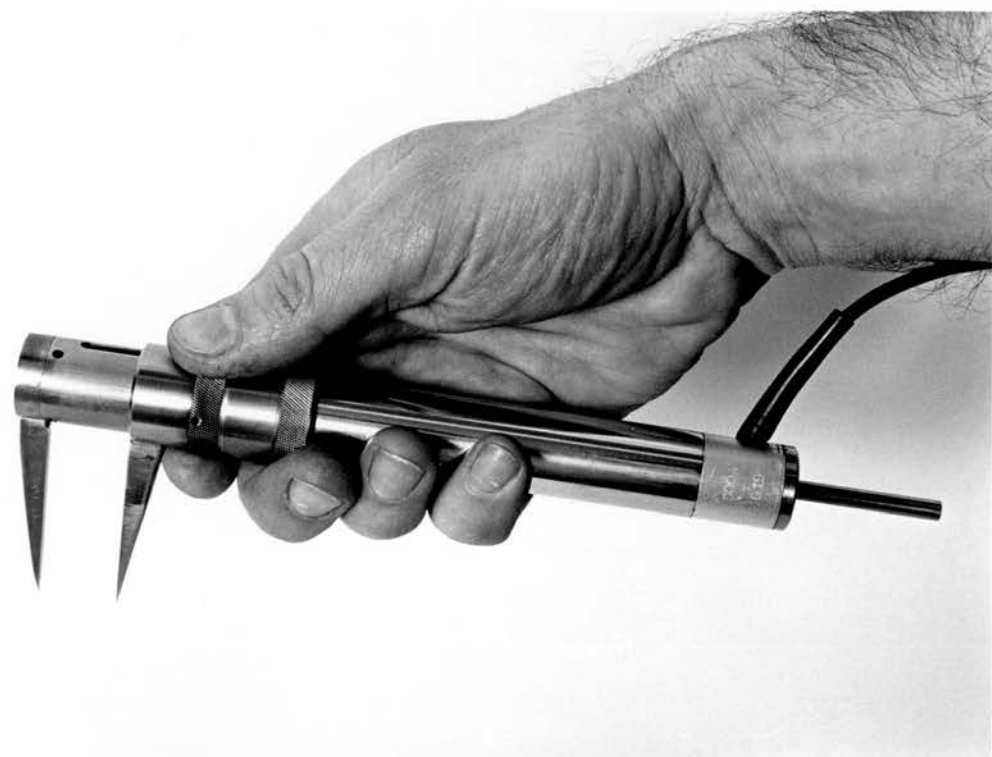


B



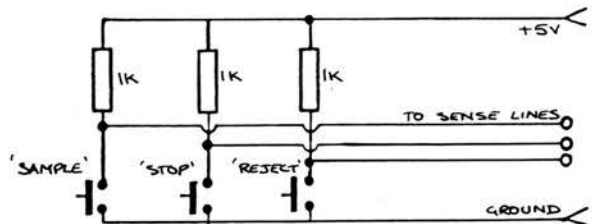
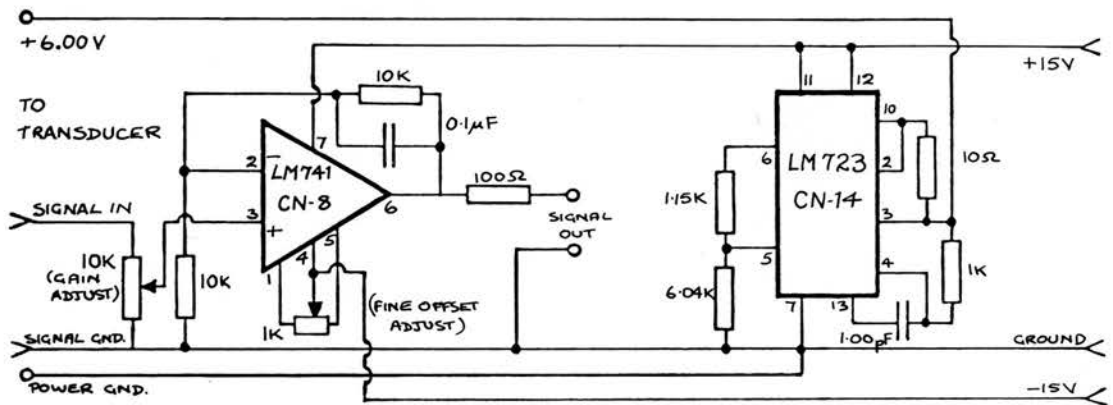
PART 2 FIGURE 6

The measuring device as it was held in an operators hand.
The other hand was free to manipulate micrographs, etc.



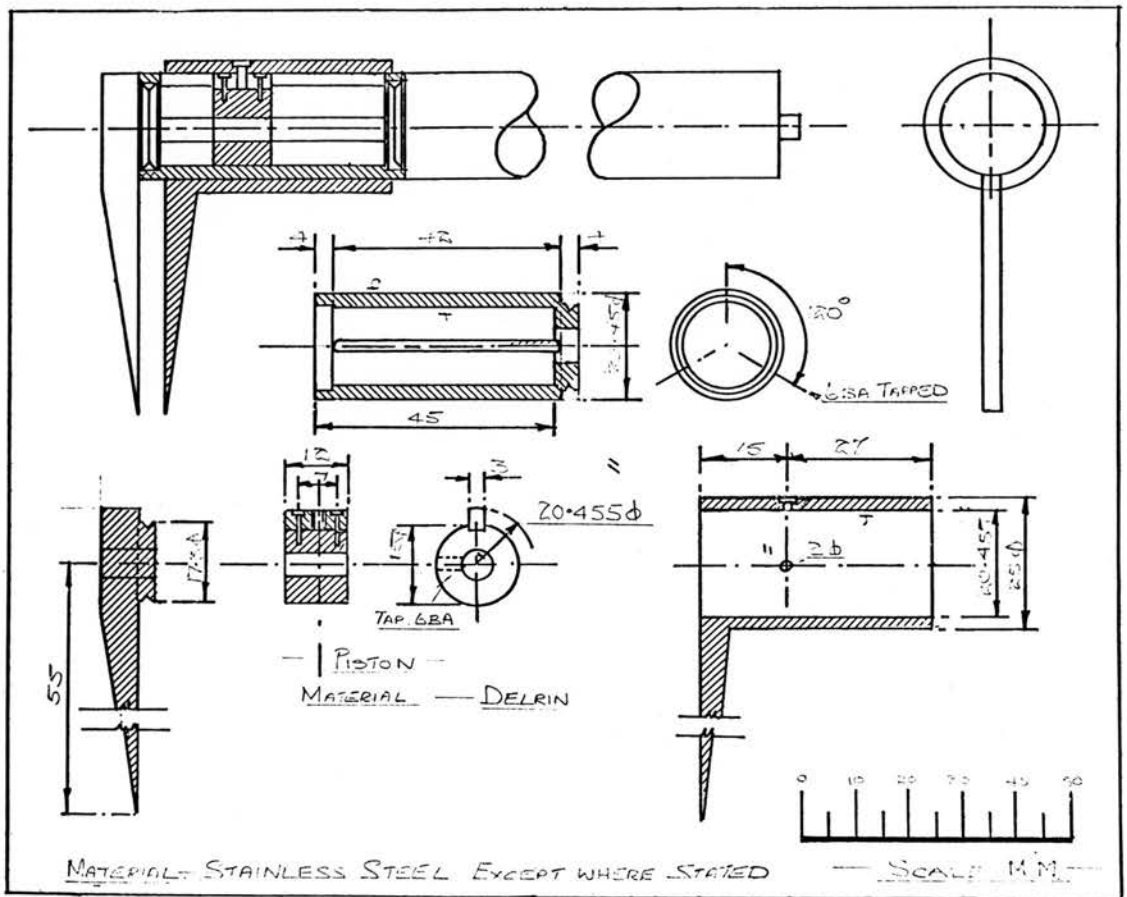
PART 2 FIGURE 7

The upper circuit is the interface circuit for the transducer and the analog to digital converter of the PDP-12 computer. The circuit reduces the ± 15 volt inputs from the computer power supply to +6 volts which supplies the transducer. The signal from the transducer is then converted into values between $I V$ and $-I V$ and fed into the computer. The lower diagram is the sense line activating circuit.



PART 2 FIGURE 8

A sectional drawing of how the transducer was made into the caliper instrument.



PART 2 FIGURE 9

The interface Fortran program for the machine. The various sections of it are illustrated on the right.

```

C TY MAX.FT
  GROTESQUE PROGRAM BY D. MAXWELL
  DIMENSION NAME(3)
  DIMENSION X(2),Z(500)
  EXTERNAL WAITXL,WXLOFF
  A=0
  L=448
  ICOUNT=0
  NC=10
  P=-99999
  WRITE(4,100)
  READ(4,200)M
  100 FORMAT('MAGNIFICATION ',S)
  200 FORMAT(I6)
  GOTO 1

  70 CALL MODE 8(WXLOFF,L)
  CALL MODE 8(WAITXL,L,NL)
  IF(NL.EQ.6)GOTO 2

  IF(NL.EQ.7)GOTO 3

  IF(NL.EQ.8)GOTO 4

  STOP
  1 WRITE(4,105)
  105 FORMAT(' SET POINTS TO ZERO THEN TO 2.000 CM')
  DO 99 I=1,2
  CALL MODE 8(WXLOFF,L)
  CALL MODE 8(WAITXL,L,NL)
  IF(NL.NE.6)GOTO 9

  9 X(I)=ADC(NC)
  IF(I.EQ.1)WRITE(4,300)X(I)
  IF(I.EQ.2)WRITE(4,400)X(I)
  300 FORMAT(' ZERO READING',F6.3)
  400 FORMAT(' 20 MM READING',F6.3)
  99 CONTINUE
  W=X(2)-X(1)
  Y=20*10**6/M
  GOTO 70

  2 A=ADC(NC)
  ICOUNT=ICOUNT+1
  I=ICOUNT
  Z(I)=Y*(A-X(1))/W
  WRITE(4,500)ICOUNT,Z(I)
  500 FORMAT(I3,F12.2,' NM')

  3 ICOUNT=ICOUNT-1
  WRITE(4,600)
  600 FORMAT(' CORRECTION MADE')
  GOTO 70

  4 WRITE(4,700) ICOUNT
  WRITE(4,800)
  READ(4,601)J
  700 FORMAT(' NO OF READINGS=',I10)
  800 FORMAT(' PHOTO NO=',S)
  601 FORMAT(I3)
  WRITE(4,802)
  802 FORMAT(' ENTER OUTPUT DEVICE:FILENAME')
  READ(4,297)NAME
  297 FORMAT(3A6)
  CALL USR(9,NAME,3,ERR)
  WRITE(4,601)ERR
  WRITE(9,301)ICOUNT
  WRITE(9,302)M
  DO 298 K=1,ICOUNT
  WRITE(9,299)Z(K)
  298 CONTINUE
  WRITE(9,303)P
  299 FORMAT(F8.2)
  301 FORMAT(I3)
  302 FORMAT(I10)
  303 FORMAT(I6)
  CALL USR(9,NAME,4,ERR)
  WRITE(4,601)ERR
  DO 777 I=1,25
  K=2/20
  777 CONTINUE
  STOP
  END

```

Magnification
request

Sense line
sampling
section

Calibration
section

Measurement
section

Correction
section

Data storage
and
terminating
section

PART 2 FIGURE 10

These two Fortran programs were used to analyse data that were collected by the machine. The first program accesses files and gives means and standard deviations of the data found within the files. The second program also accesses files and constructs ratios with the data.

```

.TY SMEAN.FT
C MEAN AND STANDARD DEVIATION (FILES) D.MAXWELL
  DIMENSION NAME(3),A(500)
  SUM=0
  ZMEAN=0
  SOS=0
  SD=0
  WRITE(4,100)
  READ(4,200)NAME
  CALL USR(9,NAME,2,ERR)
  READ(9,300)ICOUNT
  READ(9,400)M
  DO 1 I=1,ICOUNT
  READ(9,500)A(I)
  SUM=SUM+A(I)
1 CONTINUE
  ZMEAN=SUM/ICOUNT
  WRITE(4,600)ZMEAN
  DO 2 I=1,ICOUNT
  SOS=SOS+(A(I))**2
2 CONTINUE
  SD=SQRT((SOS-SUM**2/ICOUNT)/(ICOUNT-1))
  WRITE(4,700)SD
100 FORMAT(' FILENAME')
200 FORMAT(3A6)
300 FORMAT(I3)
400 FORMAT(I10)
500 FORMAT(F8.2)
600 FORMAT(' MEAN='F8.2)
700 FORMAT(' SD='F8.2)
  STOP
  END

```

```

.TY LRAT.FT
C LOG RATIO PROGRAM BY D.MAXWELL
  DIMENSION X(2,500),RL(500),ONAME(3),NAME(3)
  A=0
  B=0
  IJ=0
  DO 3 I=1,3
  NAME(I)=0
3 ONAME(I)=0
  WRITE(4,400)
  READ(4,300)ONAME
  CALL USR(9,ONAME,2,ERR)
  READ(9,500)ICOUNT
  READ(9,800)M
  A=ICOUNT/2
  IJ=IFIX(A)
  READ(9,100) ((X(J,I),J=1,2),I=1,IJ)
  DO 15 I=1,IJ
  IF (X(1,I).GE.X(2,I)) GOTO 15
  Z=(X(1,I))
  X(1,I)=X(2,I)
  X(2,I)=Z
15 WRITE(4,600)X(1,I),X(2,I)
  WRITE(4,900)
  READ(4,500)P
  IF (P.EQ.1) GOTO23
  DO 20 I=1,IJ
  RL(I)=(X(1,I)/X(2,I))
20 WRITE(4,700)RL(I)
  GOTO 66
23 DO 21 I=1,IJ
  RL(I)=ALOG10(X(1,I)/X(2,I))
21 WRITE(4,700)RL(I)
66 WRITE(4,200)
  READ(4,300)NAME
  CALL USR(9,NAME,3,ERR)
  WRITE(4,500)ERR
  WRITE(9,500)IJ
  WRITE(9,800)M
  DO 25 I=1,IJ
25 WRITE(9,700)RL(I)
  PO=-99999
  WRITE(9,950)PO
  CALL USR(9,NAME,4,ERR)
  WRITE(4,500)ERR
100 FORMAT(F8.2)
200 FORMAT(' ENTER FILENAME')
300 FORMAT(3A6)
400 FORMAT(' FILENAME REQUIRED ?')
500 FORMAT(I3)
600 FORMAT(F8.2) 'F8.2)
700 FORMAT(F7.4)
800 FORMAT(I10)
900 FORMAT(' FOR LOG RATIO TYPE 1')
950 FORMAT(I6)
  STOP
  END

```

PART 2 FIGURE 11

This Fortran program compares two separate files and performs a t-test between the data.

```

.TY VTEST.FT
C T-TEST PROGRAM FOR INDEPENDENT SAMPLES, (FILES) BY D. MAXWELL
  DIMENSION A(500),B(500),NAMEA(3),NAMEB(3)
  SOSA=0
  SOSB=0
  SUMA=0
  SUMB=0
  Z=0
  Y=0
  W=0
  X=0
  IMEANA=0
  IMEANB=0
  C=0
  D=0
  WRITE(4,200)
  READ(4,100)NAMEA
  CALL USR(9,NAMEA,2,ERR)
  READ(9,300)NA
  READ(9,400)M
  DO 1 I=1,NA
  READ(9,500)A(I)
  SUMA=SUMA+A(I)
  SOSA=SOSA+A(I)**2
1 CONTINUE
  Z=SOSA-(SUMA**2)/NA
  WRITE(4,200)
  READ(4,100)NAMEB
  CALL USR(9,NAMEB,2,ERR)
  READ(9,300)NB
  READ(9,400)M
  DO 2 I=1,NB
  READ(9,500)B(I)
  SUMB=SUMB+B(I)
  SOSB=SOSB+(B(I))**2
2 CONTINUE
  Y=SOSB-(SUMB**2)/NB
  X=(NA-1)+(NB-1)
  C=(1.0)/NA
  D=(1.0)/NB
  W=C+D
  IMEANA=SUMA/NA
  IMEANB=SUMB/NB
  T=(IMEANA-IMEANB)/SQRT(((Z+Y)/X)*W)
  WRITE(4,600)T
  WRITE(4,700)X
100 FORMAT(3A6)
200 FORMAT(' FILENAME? ')
300 FORMAT(I3)
400 FORMAT(I10)
500 FORMAT(F8.2)
600 FORMAT(' T=',F8.4)
700 FORMAT(' DF=',I3)
  STOP
  END

```


PART 3 : MORPHOLOGY OF THE COCKROACH SALIVARY APPARATUS

Section 1 - The general structure of the salivary apparatus

Introduction.

The salivary apparatus of the cockroach consists of glands, ducts and reservoirs. The glands are a racemose type structure and do not possess the thick capsule of mammalian salivary glands. The acini of these glands consist of two types of cell (Bland and House, 1971) which Lebedev (1899) referred to as central and peripheral cells. A similar arrangement is to be found in *Periplaneta americana* (Whitehead, 1971; Kessel and Beams, 1963; Day, 1951). There is still some confusion over the function of these cells. This problem was discussed fully by Bland and House (1971).

The ducts of the gland may be divided into two classes; reservoir ducts and secretory ducts. The reservoir ducts arise from the reservoirs and form a common duct with the secretory ducts which are connected to the acini. The latter ducts may play a role in the elaboration of the secretory product (Smith and House, 1979).

Finally, Sutherland and Chillseyzn (1968) have attributed a storage function to the reservoirs but Smith and House (1979) have demonstrated that the ionic composition of fluid within the reservoirs is different than that of saliva. Raychaudhuri and Ghosh (1964) have published some light micrographs of the reservoir structure.

Methods.

See Part 2, Section 2 (electron microscopy).

Results.

Acinar cells -

The observations of previous writers were confirmed; acini were found to consist of two distinct types of cell. These cells are illustrated in figure 1. Peripheral cells may be identified by the microvilli that surround the ductule that each one possesses and by the high density of mitochondria. Central cells possess many secretory granules. Plate 1c and d demonstrate that peripheral cells are commonly seen in pairs and are fused together by a septate desmosome. The external surfaces of acini consist of a basement membrane (figure 1c).

Peripheral cells -

Peripheral cells characteristically possess internal ductules which are lined with a high density of microvilli (figure 2a and b). These cells contain many mitochondria which are often surrounded by infoldings of the plasma membrane (figure 2a and b). This observation was also made by Kessel and Beams (1963) during their investigation of the peripheral cells in *Periplaneta*. The apical regions of peripheral cells open out into collecting ducts (figure 2b). On average, peripheral cells are 20 microns in diameter.

Central cells -

Central cells do not always have the same appearance, but go through a cycle (Bland and House, 1971). The first stage of the cycle is the formation of endoplasmic reticulum (figure 3a). There is then an accumulation of dense endoplasmic reticulum (figure 1b) and finally the appearance of secretory granules. Central cells seem to communicate directly with the ductule, forming villi-like processes which project into the lumen (figure 1c). Occasional layers of chitin are seen between the villi and the lumen of the ductule; this does not seem to prevent secretory granules entering the ducts directly (figure 1d). Central cells tend to measure 50 microns in diameter.

Secretory ducts -

Bland and House (1971) have described the intercalated secretory ducts of the acini which may be morphologically divided into two types : the secretory duct and the non-secretory duct. In addition to these there is also a common secretory duct which is morphologically different from the other two ducts. The latter duct joins up with the reservoir ducts to form the final common duct. Figure 4 illustrates the secretory duct. Initially this duct is surrounded by the cells of the acinus but is easily identifiable by the thick layer of chitin and by the microvilli that project towards the lumen (figure 4a). The duct cells become engorged with secretory granules (figure 4b and c) which are

of a denser variety than those of central cells. The non-secretory duct characteristically has a chitin lined lumen and infoldings of the plasma membrane (figure 5). The cells are rich in mitochondria and are coupled together with septate desmosomes.

The common secretory duct (figure 6) has an innermost layer of cells that is also rich in mitochondria but, unlike the non-secretory duct cells, possesses many microvilli behind the luminal layer of chitin. This layer of cells is in turn surrounded by another group of cells which frequently have vacuole-like structures within them. These cells are surrounded by a thick basement membrane. The plasma membrane of the cells is highly invaginated just below the basement membrane.

The reservoirs and reservoir ducts -

Raychaudhuri and Ghosh (1964) have described the reservoir structure in *Periplaneta*. This seems to be an identical structure in *Nauphoeta* which has a wall of epithelial cells and connective tissue fibres (figure 8a).

The reservoir duct, like the common secretory duct, is a paired structure, but is larger (figure 8b). It is composed of a layer of cells which appears to secrete a cuticle lining to the lumen (figure 9). The cells and the cuticle resemble those of the crop of *Calliphora* (Smith, 1968). The cuticle consists of epi- and endo-cuticle (figure 9b and c). There are also periodic thickenings within the structure of the cuticle which resemble 'teeth'; these probably add strength to the structure of the duct as the thickening forms a spiral within the intact duct.

Discussion.

Peripheral cells occur in pairs usually joined by a septate desmosome. Berridge and Oschman (1972) held the erroneous point of view that peripheral cells possessed a double ductule. Evidence shall be presented in this thesis that the peripheral cells are responsible for the production of a copious flow of water which enables secretion to be initiated.

The high density of endoplasmic reticulum within central cells and the formation of large granules suggest that they are involved in the production of protein. This idea is supported by the evidence of Bland and House (1971), Raychaudhuri and Ghosh, (1964) and Day (1951) who were able to demonstrate the presence of amylase in central cells by histochemical means.

The secretory duct cells have been shown by Bland and House (1971) to contain glucosaminoglycans, ie, mucus. Their structure indicates that they have a secretory role; they possess dense granules and microvilli around the area of the lumen. The function of the non-secretory duct is unknown. The numerous mitochondria of these cells indicates that their energy requirement is high. The infoldings of the plasma membrane close to the lumen are also a mystery. It could be that these cells indulge in secretion or in absorption. A micropuncture type study may yield more information on these ducts.

The common secretory ducts also possess numerous mitochondria, again suggesting that their energy requirements are high. The

function of the microvilli of these ducts is unknown; the vacuole-like objects within some of the cells may indicate that the secretory ducts are transporting substances into or out of the saliva.

The reservoirs possess a solution that is low in ionic content compared with that of saliva. Edney (1977) suggests that the reservoir is a store for water. However, thirsty cockroaches do not directly fill their reservoirs with water (Smith and House, 1979). There is little indication from the structure of the reservoirs of what their function may be. The fibres may be involved in some form of secretory process. Sutherland and Chilseyzn (1968) have reported that emptying of the reservoir is controlled by a muscle. The innervation of this muscle shall be considered later. The reservoir duct appears to be a transporting tube; the cuticle interior probably has only a barrier function.

Section 2 - Cell junctions

Introduction.

The electrophysiological experiments of Ginsborg, House and Silinsky (1974) have demonstrated the presence of low resistance junctions between the cells of the acini. In these experiments two glass micropipettes were placed within a single acinus. One electrode delivered pulses of current, the other recorded voltages. It was found that these pulses could be recorded in other cells with relative ease. When both electrodes were used as recording electrodes, a secretory potential could be recorded in both cells.

The coupling resistance of these cells was estimated to be less than 0.2 M ohms.

It was decided to investigate these low resistance pathways to try and determine what sort of structure corresponds with them. Payton et al (1969) have claimed that the morphological correlate of such pathways is the gap junction. Many studies have confirmed this claim. However, the cockroach salivary gland is rich in septate desmosomes (Bland and House, 1971), as is also the case in many other insects (see Smith, 1968). These structures were first described by Wood (1959) in *Hydra*, and are characterised by their ladder-like form which repeats at a periodicity of about 15 nm. At one time several authors believed that septate junctions also represented a form of low resistance junction between cells (Loewenstein, 1966; Gilula et al, 1970). This idea has become less popular since the discovery that gap junctions may be frequently found, dispersed among septate desmosomes (Berridge and Oschmann, 1972; Satir and Gilula, 1973; Noiroth-Timothee et al, 1978). However, Rose (1971) has calculated that the number of gap junctions observed in the glands of *Chironomus* cannot account for the low resistance offered by cell junctions to injections of current. It therefore seems that the problem of low resistance junctions between cells is not completely resolved.

There are several ways to investigate such junctions. The electrophysiological approach of Ginsborg et al (1974) has already been discussed. Payton et al (1969) injected procion dye into their cells and allowed it to diffuse for a few hours. They observed

that the dye could pass freely from cell to cell which demonstrated the existence of low resistance couplings between these cells. The dye did not pass into the extracellular fluid of the cells. They also found that it was possible to pass current pulses between the cells and to measure voltages in the neighbouring cells, demonstrating that the cells were also electrically coupled. Finally, the cells were fixed and examined under the electron microscope and it was found that gap junctions were present in regions of cell contact. Gap junctions possess a central space between the membranes which is 2-3 nm wide. If a tangential section is made through this region, a lattice which consists of hexagonal structures which repeat at a periodicity of 9 nm, may be seen. Revel and Karnovski (1967) have shown that gap junctions can be penetrated by lanthanum; a heavy metal which forms a colloidal precipitate in narrow junctions between cells and which is electron dense.

Lanthanum has also been used to stain the hexagonal structure of gap junctions. Noiro-Timotheé et al (1978) have also employed this technique to investigate the properties of septate desmosomes and have shown that they also possess a sub-unit. There is disagreement between authors concerning the nature of the sub-unit (Satir and Gilula, 1973).

The work of Loewenstein (Loewenstein et al, 1967; Nakas et al, 1966; Rose and Loewenstein, 1971) and his collaborators has shown that decreasing the calcium level of the extracellular medium increases the coupling resistance of cells. If 10^{-4} M EGTA* is added the coupling is usually abolished. It is not known if this process

*EGTA = Ethyleneglycol-bis-(β -aminoethyl ether)N, N'- tetra-acetic acid

results in morphological uncoupling. The spread of fluorescein (a dye like Procion) from cell to cell is prevented by this procedure (Oliveira-Casto and Loewenstein, 1971) but there is no evidence that gap junctions can be opened by this method (Bullivant and Loewenstein, 1968) although bathing in hypertonic solutions does increase coupling resistance and causes gap junctions to open. Perachia (1977) has suggested that calcium-free solution, along with a chelating agent, results in a breakdown of the gap junction hexagonal sub-unit.

The final method that has been employed in the investigation of cell junctions is freeze fracture. Fresh tissue is selected and frozen rapidly under liquid nitrogen after a brief incubation period in a cryoprotectant solution. A cold knife is drawn across the surface of the tissue, which fractures at its weakest point which is the space between cell membranes. A platinum replica is made of this under a high vacuum. When the replica has been prepared it may be mounted on a grid and viewed under the electron microscope. In this way the faces of cell membranes may be seen. The hexagonal sub-unit of gap junctions and the sub-unit of septate desmosomes have been observed via this method. Satir and Gilula (1973) and Noiro-Timothee et al (1978) have observed gap junctions between septate desmosomes with this method. These observations are important in that they demonstrate that two methods of fixation which depend upon completely different physical principles (ie, Glutaraldehyde fixation and freezing), can produce the same results which cannot simply be dismissed as being artefacts.

Some of these methods have been employed in the study that follows of junctions in the cockroach salivary glands.

Materials and Methods.

For the method of intracellular staining see Part 2, Section 5. Calcium-free solutions were modified cockroach ringer with no calcium chloride or were calcium chloride-free ringer plus 0.5 mM EGTA. Test doses of dopamine (10^{-7} M) were used under these circumstances as the nerve response could not be recorded as a result of the blocking action of calcium-free ringer upon chemical transmission. Several glands were also prepared for electron microscopy after being incubated in calcium-free ringer or in EGTA ringer for 2 hours.

Electron microscopy was performed upon the glands according to the method of Section 2, Part 2. The lanthanum technique was that of Revel and Karnovsky (1967). Tissues were fixed in the usual manner except for the osmication stage. A solution of 1% OsO₄ in 0.1 M s-collidine buffer containing 2% lanthanum nitrate at pH 7.4 was used. The s-collidine was prepared according to the method of Bennet and Luft (1959). Sections of tissue prepared in this manner were often viewed unstained.

Results.

Secretory potentials were recorded from impaled cells during Procion yellow injections (figures 1d and 2d). It was found that during the half hour period of an injection diffusion took place rapidly between cells (figures 1, 2, 3a and b). If the dye was

allowed to diffuse for more than 15 minutes after the injection was terminated it became difficult to identify the injection site as the dye concentration became low during diffusion through many cells. The rate of diffusion of the dye was therefore difficult to calculate and was not attempted. No evidence was found of the dye entering ducts and being disposed of in this manner. Procion was seen to have a high affinity with the nuclei of cells (figure 3b). No preference was noted for diffusion from cell to cell; both central and peripheral cells were found to contain Procion (see also House, 1975).

Calcium-free ringer and EGTA ringer were also employed in an attempt to uncouple the cells. The experiments were hampered by the effects that these solutions had upon the cell membranes. It was often found to be impossible to make an impalement as the cell membranes had become 'tough'. Only one experiment was successful and the results of this are shown in figure 3c. It appears that Procion dye was confined to a central cell only. During the procedure the cells began to look abnormal and opaque. An electron microscope examination was performed upon these cells (see below).

The ultrastructure of the salivary glands showed septate desmosomes to be a common feature. Figure 4a and b are examples of septate desmosomes between two peripheral cells. Such junctions are also to be found between central and peripheral cells (see Part 3, Section 1, figure 1d) and within the ducts of the acinus (see Part 3, Section 1, figure 4a, etc). The ladder-like septa repeat with a periodicity of 25 nm (figure 4b). The membranes

that enclose the septa appear to have a double structure. It is possible to impregnate sections with lanthanum and view the structure of the septate desmosomes. In tangential sections of these it is possible to see a sinusoidal-like structure (ie, the septa) which repeats at a periodicity of 22.66 ± 2.58 nm. As Noirot-Timotheé et al (1978) noted the septa are not strictly parallel but deviate somewhat.

In several sections it was noted that septate junctions had intercalated gap junctions (figure 5a). Such junctions were found to have a cytoplasm-to-cytoplasm distance of 12.5 nm and a central gap of 3 nm. They were found between both central and peripheral cells and also some of the duct cells. They were readily penetrated by lanthanum which showed the gap region distinctly in unstained sections (figures 5b, c and d). Figure 5b shows that these structures can be fairly large on occasions stretching up to a quarter of a micron in length.

Tissue that was incubated in calcium-free ringer was found to be abnormal in appearance (figure 6a). The chromatin of nuclei appeared to be deposited in 'lumps' and many of the cell membranes were non-existent. On the other hand tissue that had been incubated in EGTA ringer appeared to be remarkably well-preserved (figure 6b and c). Several gap junctions could be identified between the septate desmosomes of this tissue and there was no evidence of any form of morphological uncoupling.

Discussion.

Cell couplings in the cockroach salivary apparatus have been examined so that the low resistance pathways found by the electrophysiological experiments of Ginsborg et al (1974) could be categorised. Septate desmosomes have been found to be numerous, forming contacts between most of the acinar cells. Tangential sections through septate desmosomes have revealed a 'pleated sheet'-like contact between the cells (see Gilula et al, 1970). In this type of arrangement the septa form zig-zag patterns across the surface of cells which contact with the other cell. The rungs of the 'ladder' are sections through several of these septa. Noirot-Timothee et al (1978) have conjectured that septate junctions may act as diffusion barriers; they may also serve a function of cell cohesion. Payton et al (1969) have shown that Procion dye passes readily through gap junctions. There is no evidence to show that Procion dye can pass through septate junctions. This study has shown that septate junctions may possess intercalated gap junctions. Rose (1971) expressed some concern that few gap junctions were to be seen between the septate desmosomes in *Chironomus* salivary glands and calculated that the gap junctions that could be found would need to have a resistance of several orders of magnitude less than other known gap junctions in order to explain the low resistance of cell coupling. It is possible that septate desmosomes may contribute to the low resistance pathways between these cells but Noirot-Timothee et al (1978) claim that they could find no evidence to support the idea of inter-cellular channels in their freeze fracture experiments

on septate desmosomes. There seems to be little evidence to support the idea that septate desmosomes can act as low resistance junctions.

The results obtained from calcium-free ringer incubation are somewhat confusing. Other workers have experienced difficulties in making impalements within calcium-free ringer (Silinsky, 1978; Hurlburt et al, 1971). It is known that the gland will still continue to secrete in response to a dose of dopamine in calcium-free ringer (Smith and House, 1979). It appears that the gland is still physiologically functional in calcium-free ringer and that some interference with the fixative may explain the curious results that were obtained. Glands will also continue to secrete in EGTA ringer. EGTA does not abolish gap junctions even though Bulivant and Loewenstein (1968) have shown that calcium-free and EGTA ringer both cause electrical uncoupling of the cells. Peracchia (1977) has claimed that the hexagonal sub-units of gap junctions aggregate and that the intercellular channels within them cease to function.

Section 3 - Axons associated with the salivary apparatus

Introduction.

Bland et al (1973) have shown that the salivary glands of the cockroach have many axons which fluoresce after treatment by the Falck et al (1966) method. These axons have ultraviolet spectra which are consistent with the presence of catecholamines. Fry et al (1974) have also shown biochemically that there is dopamine present

within homogenates of the salivary glands. Bowser-Riley (1978a) has observed that axons stained with methylene blue arise from the suboesophageal ganglion, run down the reservoir ducts and branch profusely forming a dense network over the surface of the acini. It is possible to electrically stimulate these nerves giving rise to a secretory potential, which can be recorded with a micropipette in the acini (House, 1973) and to the production of saliva (Smith and House, 1977). Bowser-Riley (1978a) has claimed that scanning electron micrographs of the axons on the surface of acini show that they have varicosities which may represent the releasing sites for transmitter. There is now a considerable body of evidence to show that this transmitter is probably dopamine (see Part 1, Section 2).

Whitehead (1971) has performed an ultrastructural study of the innervation of the salivary apparatus of *Periplaneta* but apart from this pioneering study no others seem to exist. It was therefore decided to perform a systematic study of the innervation of the salivary apparatus of *Nauphoeta*.

Materials and Methods.

The innervation of the salivary apparatus was examined with electron microscopy (Part 2, Section 2) and fluorescence microscopy (Part 2, Section 3). In some cases stretch preparations were employed for fluorescence microscopy. Individual salivary glands were placed upon a microscope slide and left overnight in a

desiccator which contained silica gel. These were then reacted with paraformaldehyde and processed in the same manner as sections except that they were not embedded or sectioned.

Methods of stereological analysis were used to categorise the types of vesicle that could be identified within the axons (see Elias et al, 1970 and Weibel and Bolender, 1975). The machine and computer programs described in Part 2, Section 6 were used for this purpose. It became apparent after some time that many of the vesicles were not circular in their profiles. It was decided to test the types of profile that were observed against the theoretical curves of Elias et al (1970) which involved measuring the maximum and minimum dimensions of each profile and plotting the ratio of these against cumulative frequency (see figure 5c). It also became clear that it would be difficult to compare groups of these vesicles by statistical means so distributions of the log maximum/minimum dimensions were plotted as histograms. If the profiles of the vesicles were circular then the distribution would tend to cluster round about zero. If they were more of a tubular shape the distribution would be elongated (figure 5d).

Results.

Reaction of the salivary glands of the cockroach with paraformaldehyde results in the appearance of a network of axons which glow with an apple-green fluorescence when viewed under

ultraviolet light. This may be observed in whole-mount preparations (figure 1) or in sections (figure 2), but not in tissue that has not been reacted with paraformaldehyde. Figures 1a, b and c demonstrate how axons often seem to follow the contours of the acini and have the appearance of a net. Axons may often be seen passing from one acinus to the next, following the paths of connective tissue (figure 1d). These structures often have a beaded appearance, as do many of the axons which are to be found on the surface of the acini (figure 1e). These could correspond to the varicosities of Bowser-Riley (1978a).

Eight-micron thick sections give a similar pattern to the stretch preparations. Figure 2a is a section that has been reacted with paraformaldehyde which exhibits quite clearly specific fluorescence, especially around the edges of the acini. The section illustrated in figure 2b has not been reacted with paraformaldehyde and only exhibits non-specific fluorescence. In addition to fluorescent axons on the surface of acini sections also show such axons running between the cells of the acinus (figure 2c and d).

A thorough survey of the types of axon profile that were seen with the electron microscope was made and it was concluded that two types could be classified on the basis of the vesicles that they contained. These types were designated type A and type B.

A schematic diagram of these axons is presented in figure 3. In addition to these axons, some containing no vesicles were also found. The three types of axon are shown in figure 4. Type A axons were found to be 5 times more common than type B and contain small elliptical vesicles of mean dimension 40.8 nm and larger osmiophilic granules of mean diameter 91 nm. Type B axons contain only large osmiophilic granules of mean diameter 134 nm. A t-test confirms that the difference between the granule diameters of type A and type B axons is highly significant ($P < 0.0005$). T-tests were performed routinely between all vesicles that were measured to determine that either they were or they were not from the same category. Histograms of the diameters of type A and B axon granules are illustrated in figure 5a and b. It is obvious from these graphs that diameters of type A and B axon granules are distributed in a different way.

The small agranular vesicles of type A axons were examined with a stereological method (Elias et al, 1970) and compared with a theoretical curve. The graph of maximum/minimum dimension ratio against cumulative frequency correspond most closely with that of a prolate rotary ellipsoid (figure 5c). A log-ratio distribution of the same data that was used in figure 5c was also plotted (figure 5d). This shows that the agranular vesicles of type A axons are cylindrical, rather than spherical. This graph shall be used from time to time to illustrate similarities and differences in type A axon vesicles after various treatments.

It has already been stated that in addition to type A and type B axons there was a third category of axons which contained no vesicular profiles (figure 4c). Evidence shall be presented later that these are simply the intermediate areas of type A and B axons that contain no vesicles. They possess microtubules that are 25 nm in diameter.

Types A and B axons were not found to associate with any particular type of cell or other structure. Figure 6 illustrates the relationships of axons with other cells of the acinus. Both types of axon are commonly found within the basement membrane region of the acinus (figure 7). Such axons always have a glial coat and may be found in the vicinity of central and peripheral cells (figure 7a and b). On many occasions both type of axon were seen together (figure 7d) or accompanied by clear axons (figure 7c). A and B axons along with many clear axons were often observed within the nerves which cross from acinus to acinus (figure 8). The majority of axons contained within these nerves were surrounded by a dense, convoluted glial sheath which was often darker in appearance than those of the basement membrane axons. It was sometimes noted that type A axons in these nerves were free of glial sheaths and often found at the periphery (figure 8b). The majority of axons within the sheaths were not identifiable as type A or B axons, as they did not tend to possess vesicles. Like the acini, these nerves are surrounded with a basement membrane.

In addition to these superficial axons, axons are also found between the cell membranes of the acinar cells in the core of the acinus. These axons are not normally associated with glial cells and often only have a 20 nm gap between them and adjacent cells. These axons were found to be only of type A (figure 9). They have been found between central cells, central and peripheral cells, and duct cells and central cells.

Some evidence was found with the electron microscope to support the idea that transmitter may be stored in varicosities. Figure 10a illustrates a type A axon which has a swelling crammed with vesicles. This is not complete evidence in itself as serial sections of this axon are not available. However, figures 10b, c, d are adjacent sections through a type A axon. There are two regions of swelling, one which contains vesicles and another which contains a mitochondrion. In addition to this, figure 10d shows an accumulation of small vesicles in an area with no axonal swelling. These serial and longitudinal sections also show that type A axons have areas that contain no vesicles; these probably correspond to the transverse profiles of the clear axons.

No postsynaptic specialisations were observed in the vicinity of axons but on some occasions entities which could be presynaptic specialisations were seen. Some of these are illustrated in figure 11. In figure 11a there is a type A axon in the basement membrane region of the acinus which is only partially surrounded by a glial cell. In one corner of the axon that is free of glia there is an accumulation of vesicles around a dark object, which could be a presynaptic rodlet (see Osborne, 1977). In figure 11b and c there

is also an aggregation of vesicles close to an apposition with a central cell. In this case no particular presynaptic structure can be seen. An unusual observation is shown in figure 11d; this appears to be an axo-axonic synapse. The presynaptic region again possesses an object that resembles a presynaptic rodlet.

During the studies with paraformaldehyde gas condensation, axons associated with the ducts and the reservoirs were also observed. The reservoir duct was seen to possess at least two large axons which run over its surface (figure 12a). There were some smaller axons on the secretory duct, which fluoresced with an apple green colour and had a beaded varicose-like appearance. Sections through the reservoir duct also revealed a large nerve which had specific fluorescence (figure 12b). Fluorescing structures were also seen on the surface of the reservoir (figure 12e); these have a similar appearance to the axons associated with the reservoir that Bowser-Riley (1978a) stained with methylene blue. The reservoir is controlled by a muscle (Sutherland and Chylszeyn, 1968). This muscle has a very rich innervation, the nerves that innervate it were also seen to fluoresce (figure 12 c and d).

The reservoir duct nerve is connected to the reservoir duct by means of some connective tissue. It consists of two large axons (figure 13 a and b) which are surrounded with a wrapping of glial sheaths. The entire nerve is contained within a basement membrane. These axons are on average 7 microns in diameter and contain numerous microtubules and a few granules. These granules could be the kind that many authors have claimed are transported down catecholamine-

containing neurones from the cell bodies (eg, see Osborne, 1977). In addition to these large axons, smaller ones, often accompanied by glial cells are frequently seen in the basement membrane region of the nerves. These are usually clear axons but occasionally a type B axon has been observed in this region. Figure 13c shows two type B axons in close apposition to each other.

Discussion.

The ultimate aim of this study was to determine the types of axon present in the salivary apparatus and to correlate it with what is already known about the innervation of the gland from electrophysiological and secretion studies. Condensation with paraformaldehyde gas has demonstrated that the gland is richly innervated with axons that contain a catecholamine (this study and Bland et al, 1973). Electrophysiological (Bowser-Riley, and House, 1976) and secretion studies (Smith, 1977; Smith and House, 1977) have suggested that dopamine is the neurotransmitter in these glands. So do type A or type B axons employ dopamine as a neurotransmitter?

Previous ultrastructural studies on nerves that are known to contain catecholamines show that they are not unlike type A (Bloom, 1970; Bennet, 1972). Further evidence shall be presented in this thesis to support the idea that type A axons do indeed correspond to catecholaminergic neurones (Part 4).

There is no evidence to indicate that type B axons are in any way associated with catecholamines; indeed they appear to resemble the neurosecretory type of cell which are commonly found in insects

(eg, see Mancini and Frontali, 1967; Miller, 1975). The role of clear axons is unknown; they could be a separate category in their own right or, more probably, as adjacent sections show clear areas in types A and B, may be sections through these areas which are free from vesicular profiles.

It is possible that the elliptical vesicles observed in type A axons are a fixation artefact (Gray, 1977). However, some authors have noted elliptical and circular vesicles in the same section, but in different nerves (Wickelgren, 1977). This observation reduces the likelihood of their being artefactual. The problems of vesicular shapes shall be discussed more fully elsewhere.

Intracellular recording from acinar cells in the cockroach salivary gland has shown that a hyperpolarising potential is evoked by stimulation of the salivary nerves (Ginsborg and House, 1976; Ginsborg et al, 1976). This response has a latency of 1 second. This latency is reduced to not less than 380 msec when dopamine is ionophoresed on to the surface of the acini, rather than the nerve being stimulated. If one considers that the shortest distance that transmitter may have to diffuse is 20 nm (figure 9) until it encounters the membranes of the acinar cells, then this excessively long latency cannot be explained in conventional terms of diffusion. Even the proposed releasing site of a type A axon found within the basement membrane (figure 11a) should yield a theoretical latency of less than 1 msec, assuming that the transmitter must travel one micron to receptor sites on the acinar cell membranes and that the basement membrane does not impede its journey. These observations and

calculations may be consistent with the apparent absence of any postsynaptic specialisations in the vicinity of axons that contain vesicles. It may be that in some systems that employ catecholamines as neurotransmitter, including the cockroach salivary gland, they are released from varicosities (Elfvin, 1963; Bennet, 1972; Bowser-Riley, 1978a), and have to travel a considerable distance before coming in contact with the receptor sites. Since axonal swellings are not the only place where quantities of vesicles may be found (figure 10d), it is by no means established that a varicosity represents a releasing site.

Two possible axo-axonic synapses have been observed in this study and it is difficult to say what the function of such structures may be; it is possible that they serve to synchronise the conduction of impulses into the nerve net that surrounds the salivary glands.

Fluorescence microscopy and electron microscopy both indicate that a large nerve travels down the reservoir duct. Electrophysiological (Ginsborg and House, 1977; House, 1973), secretion (Smith and House, 1977), scanning electron microscopical and methylene blue (Bowser-Riley, 1978a) studies all indicate that these are the nerves which arise from the suboesophageal ganglion of the cockroach and innervate the acini. This nerve possesses two large axons and many small ones and it is not known if they all are involved in the innervation of the gland. More evidence supporting the idea that the large axons are involved in the innervation of the gland will be presented in Part 5.

Finally, the reservoir is controlled by a muscle which is richly innervated with axons that may also contain catecholamines. No electron microscopical data are available on this material. This is also the case with the axons on the surface of the reservoirs which Bowser-Riley (1978a) also observed with his methylene blue studies and claimed were stretch receptors.

Section 4 - The suboesophageal ganglion

Introduction.

The suboesophageal ganglion of the cockroach was investigated by Cholodowski in 1881 who discovered that it gave rise to nerve fibres which innervate the acini. These nerve fibres were also studied by Hoffer (1887) who attempted to identify the structures that gave rise to them. Bowser-Riley (1978b) investigated these salivary nerves by filling them with cobalt or horseradish peroxidase and allowing the retrograde transport mechanisms to carry the markers into the cell bodies he concluded that each salivary nerve had at least two cell bodies associated with it: anterior and posterior. These findings may be consistent with the observation in Part 3, Section 3 that the salivary nerves contain two large axons which would conduct the markers back to the cell bodies more easily than the smaller axons.

Frontali and Norberg (1966) conducted a fluorescence microscopy study upon the suboesophageal ganglion of the cockroach and found a few fluorescing cell bodies. It is possible that many of these cell

bodies contained dopamine as Frontali and Haggendal (1969) have demonstrated that the suboesophageal ganglion contains six times as much dopamine as noradrenaline.

In addition to the salivary nerves of the suboesophageal ganglion, the cockroach salivary gland also receives fibre branches from the stomatogastric nerve (Willey, 1961; Whitehead, 1971; Bowser-Riley, 1978a). There is no evidence that this has any functional significance (Smith, unpublished observations).

Materials and Methods.

Ganglia were prepared for light microscopy by fixing them in 10% formal saline. They were then dehydrated in alcohol, cleared in xylene and embedded in paraffin. Serial sections of these were cut and mounted on glass slides. The sections were brought down to water again and stained with haematoxylin and eosin or Palmgren's (1942) silver stain. The sections were viewed under a Nikon microscope.

Fluorescence histochemistry was performed according to the method in Part 2, Section 3, and electron microscopy also according to the method already outlined (Part 2, Section 2).

Results.

Serial sections through the suboesophageal ganglion show that in the dorsal regions of the ganglion (the area that gives rise to the salivary nerves), there is a large central area of nerve fibres

and a peripheral region of cell bodies (figure 1a). The entire ganglion is surrounded by a capsule. In the more ventral regions of the ganglion the fibre tracts are to be seen surrounding a central region of cell bodies and are themselves surrounded by a peripheral region of cell bodies (figure 1b). The nerve fibres seem to be arranged in fairly distinct trunks. Silver staining emphasises such trunks and in a longitudinal section through the ganglion (figure 1c) fibres may be seen entering the ganglion from the ventral nerve cord. Some of these fibres go towards the cockroach brain, others seem to arise from groups of cell bodies within the ganglion which, as would be expected from the H and E serial sections, are in a ventral region of the ganglion.

Serial 20 micron sections of ganglion that had been reacted with paraformaldehyde were observed. The specific fluorescence within the ganglion was fairly sparse although on some occasions whole fibre tracts fluoresced with a distinct apple green colour. On some occasions also, small objects of 25-50 microns in diameter were seen to fluoresce specifically. It was often noted that there was a dark circle in the middle of these objects which according to Frontali and Norberg (1966) are the nuclei of cell bodies which do not fluoresce. The plates shown in figure 3 are 3 adjacent transverse sections about half way through the ganglion. On each side of the midline of the ganglion a cell body may be seen; these, of course, are not necessarily the soma that give rise to the salivary nerves, but are of similar dimensions and also in a similar position to the cell bodies which were identified by Bowser-Riley (1978b).

Electron microscopy reveals that the fibre tract regions of the ganglion were often packed with neuronal structures which made numerous contacts with each other (figure 3a and b). 4 types of axon could be classified on the basis of the vesicles that they contained : round clear vesicles only; round clear vesicles and small granular vesicles; round clear vesicles and large granular vesicles; large granular vesicles only (similar to type B). The dimensions of these structures were not quantified. Cell bodies were found to be 20 - 50 microns in diameter and had large nuclei (figure 3c and d). Each individual cell body was wrapped in a glial sheath.

Discussion.

A preliminary attempt has been made to study the structure of the suboesophageal ganglion. The ganglion is a complex structure which consists of cell bodies and nerve fibres. No axons were observed within the ganglion which directly resembled type A axons except those with small clear vesicles and large granular ones. The basic difference between these vesicles and those of type A is that the agranular vesicles appeared to be circular in profile. This may be further evidence to indicate that the elliptical vesicles of type A are not fixation artefacts, as the same fixative was used for the ganglion. There is evidence to show that some of these fibres contain a catecholamine or 5-hydroxytryptamine as fluorescing fibre tracts were observed within the ganglion. Those that are similar to type A are probably the most likely candidates. The large granular vesicles illustrated in figure 3b are unlike type B vesicles

in that they have dense cores, rather than being simply granular. These structures correspond to the classical 'dense core vesicles' (see Itakura et al, 1975). Type B axons may be seen infrequently in the stomatogastric nerve (C R House, unpublished). Both the electron micrographs of cell bodies and the fluorescing cell bodies present a picture of a structure with a large nucleus and have similar shapes.

It would be useful to identify the position of the cell bodies that give rise to the salivary nerves as this would enable many experiments to be performed upon the nerve and its relationship with the cells that it innervates. For example, an orthograde injection of H.R.P. would yield information about the extent of innervation that one cell body provides; also injections of radio-active tracers would give much information concerning the storage and release of neurotransmitter.

PART 3 SECTION 1 FIGURE 1

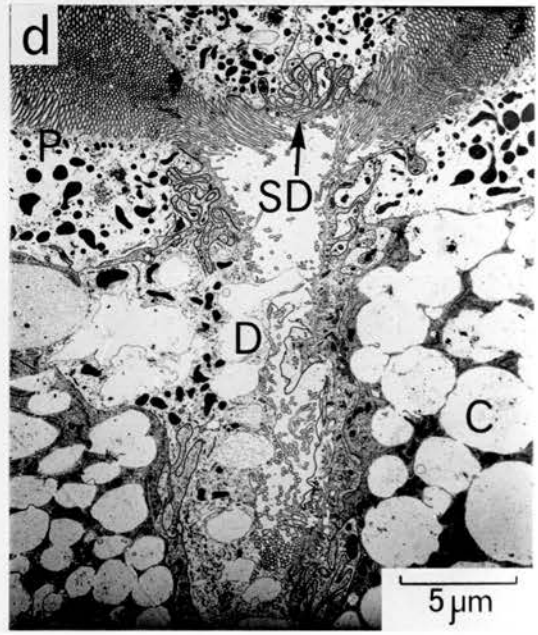
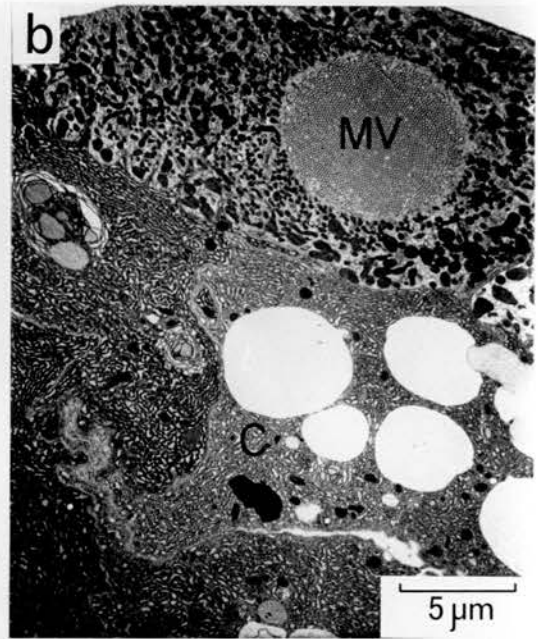
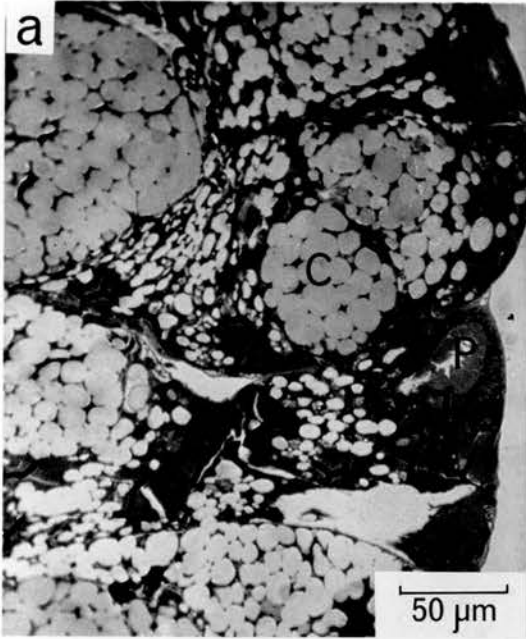
Central and peripheral cells

(a) A light micrograph of a one-micron-thick toluidine blue-stained section through an acinus. Peripheral cells (P) and central cells (C) can readily be seen. Note the many secretory granules within the central cells.

(b) Peripheral (P) cells contain many mitochondria and a brush border of microvilli (MV). The central cell (C) in this picture has a few secretory granules and contains much endoplasmic reticulum.

(c) Peripheral cells (P) are often found in pairs fused together by septate desmosomes (SD). The central cell (C) in this illustration has many microvilli which project into a duct, (asterisk). A granule is almost on the point of entering this duct.

(d) The arrangement of central (C) and peripheral (P) cells in an acinus in relation to a duct (D). Granules may be seen entering this duct which is partially surrounded with a layer of chitin (SD-septate desomosome).



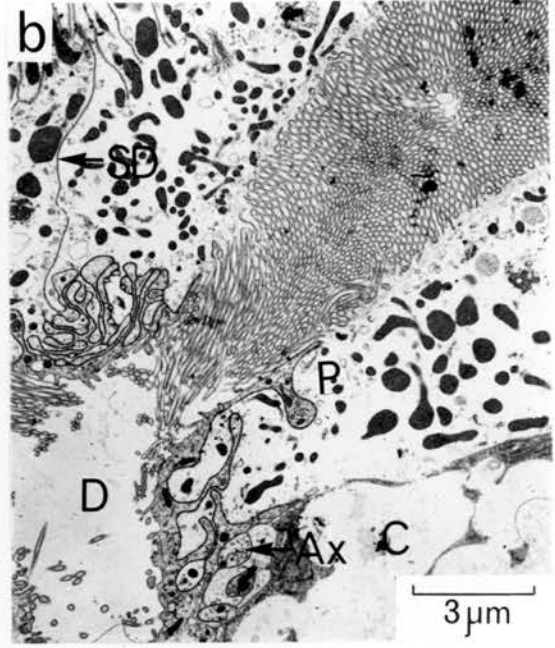
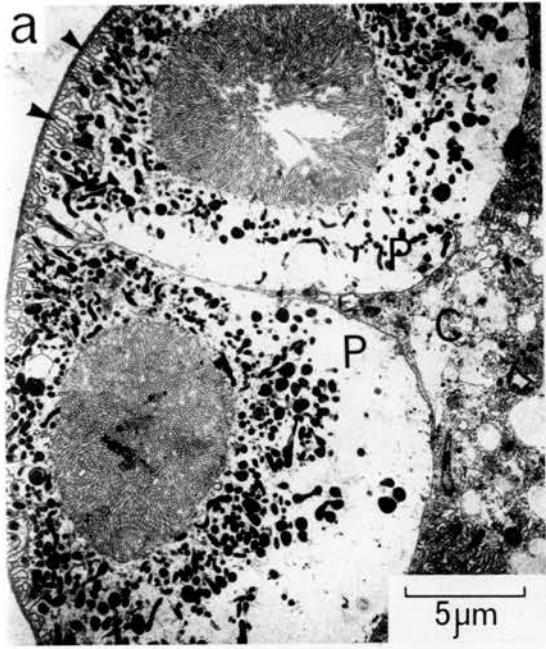
PART 3 SECTION 1 FIGURE 2

Aspects of peripheral cells

(a) The general structure of peripheral cells (P). They possess ductules with a brush border of microvilli, many mitochondria and have basal infoldings of the plasma membrane (at the arrows). (C-central cell).

(b) A section through a peripheral cell (P) at a different angle from figure (a). The ductule feeds into a larger duct (D) between central cells (C). A septate desmosome (SD) is found between this peripheral cell and its neighbour. A small axon is also visible (Ax).

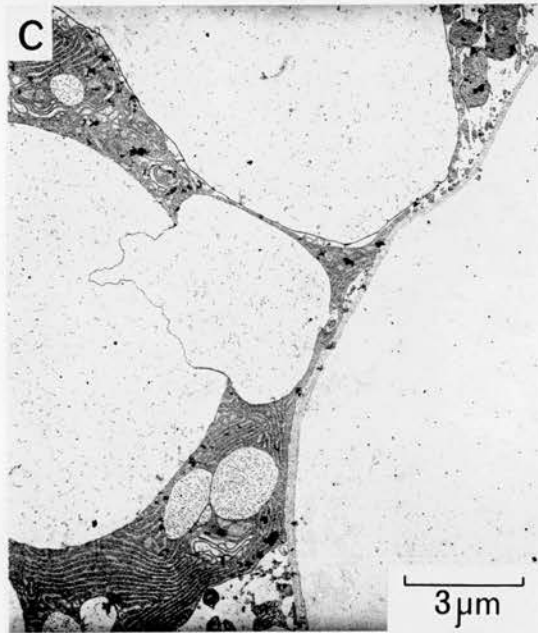
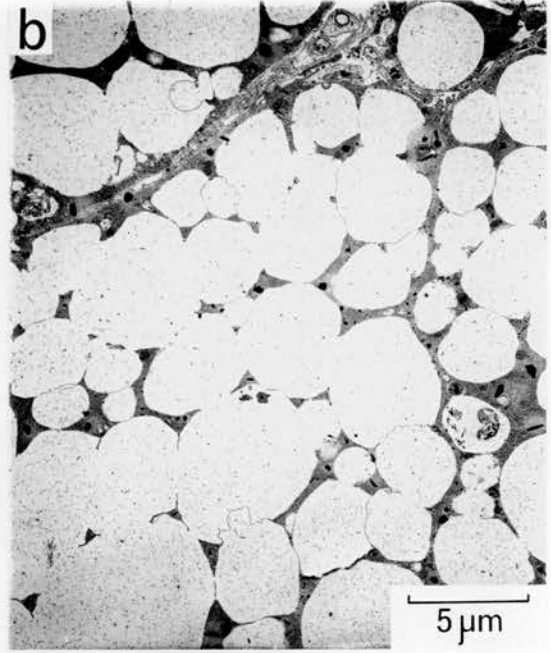
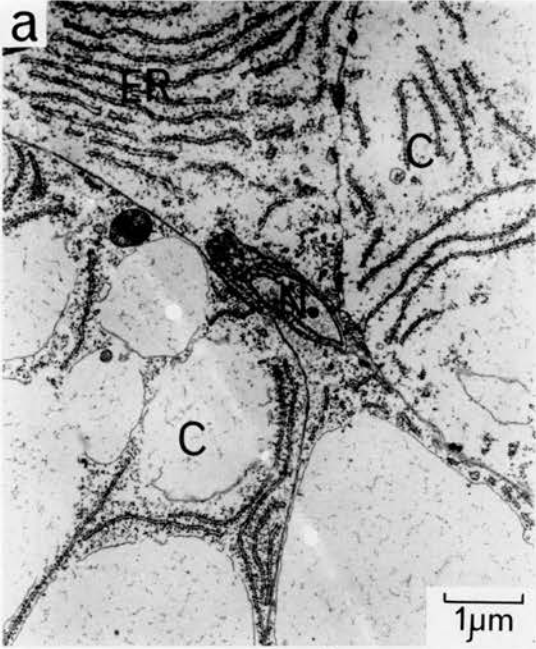
(c) The basal infoldings of the plasma membrane.



PART 3 SECTION 1 FIGURE 3

Aspects of central cells

(a) This picture illustrates the formation of endoplasmic reticulum (ER) in central cells (C). A small axon may also be seen (N). The endoplasmic reticulum eventually forms secretory granules, illustrated in figures (b) and (c).



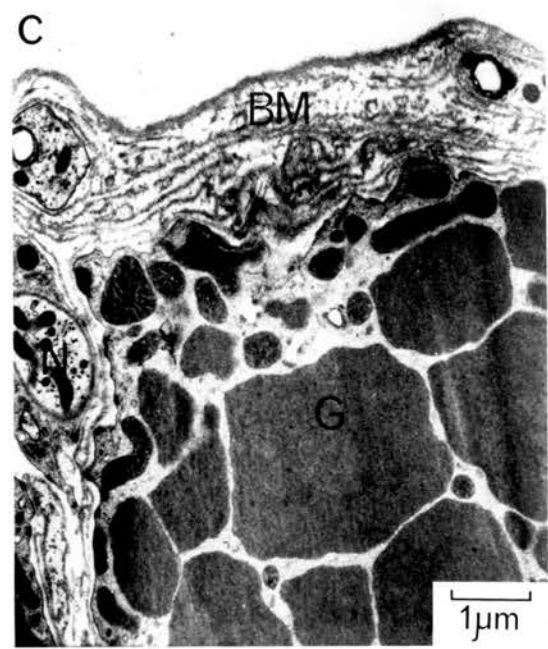
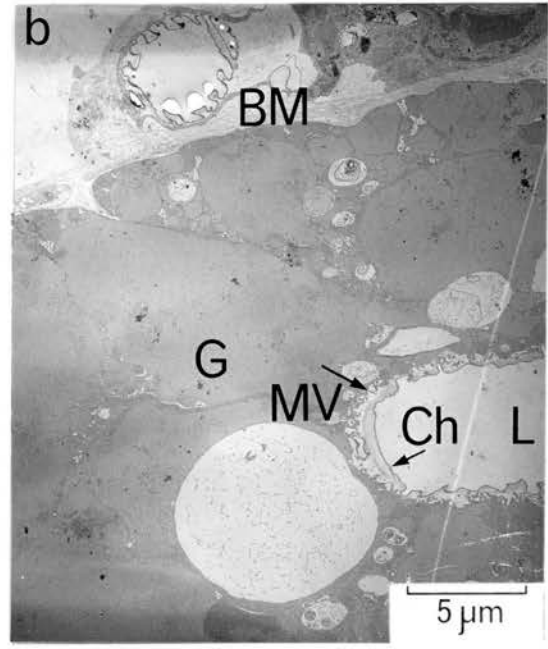
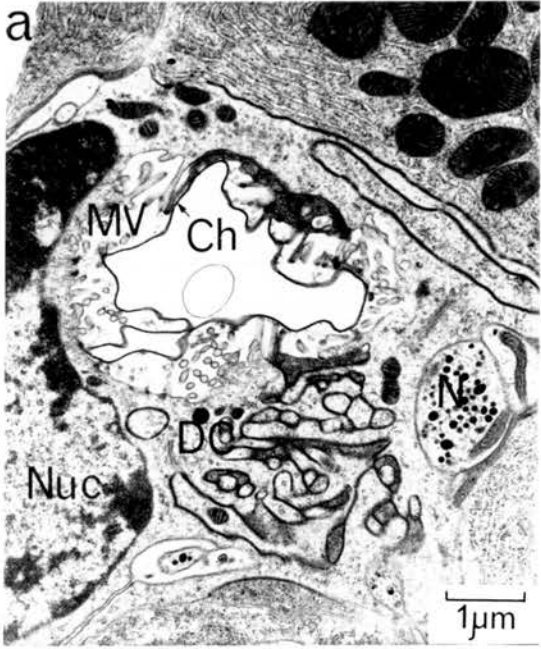
PART 3 SECTION 1 FIGURE 4

The secretory duct

(a) The beginning of the secretory duct within an acinus. It is identifiable by its lumen which has a coat of chitin (Ch) and the apical microvilli (MV). The nucleus (Nuc) of a duct cell (DC) and an axon are also visible (N).

(b) The secretory duct in transverse section. The lumen (L), Chitin (Ch), microvilli (MV) and dense secretory granules (G) are clearly visible. The duct is surrounded with a basement membrane (BM).

(c) The secretory granules (G) and the basement membrane (BM). There is also a nerve cell in this picture.



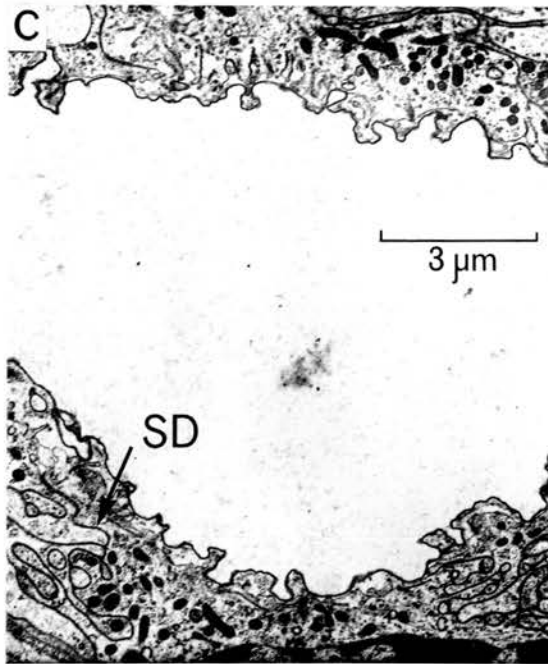
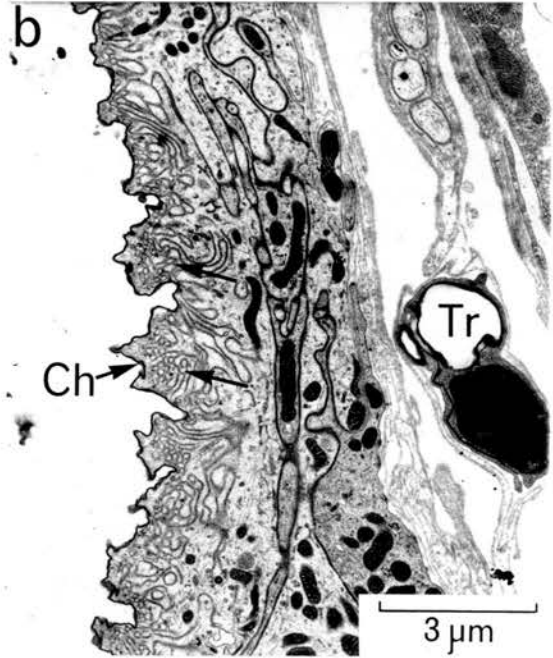
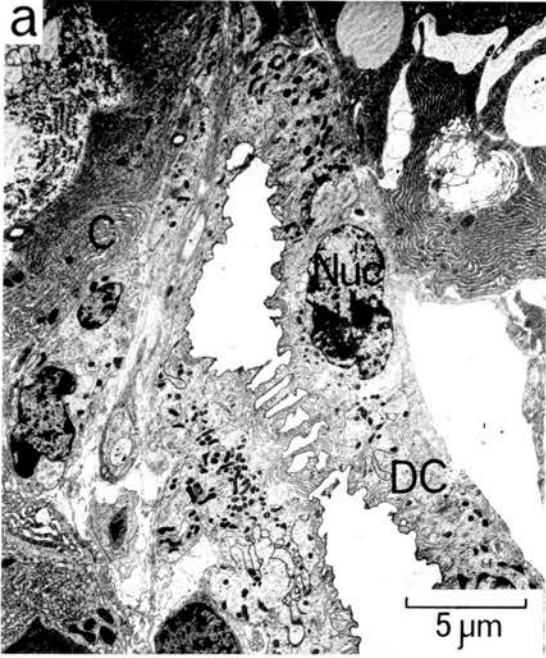
PART 3 SECTION 1 FIGURE 5

The non-secretory duct

(a) A longitudinal section through a non-secretory duct. A duct cell (DC) which contains many mitochondria and a nuclear (Nuc) may also be seen. A central cell (C) from a neighbouring acinus is also present.

(b) The lumen of the non-secretory duct is lined with chitin (Ch) and there are also apical foldings of the plasma membrane directly under this (arrows). (Tr-tracheole).

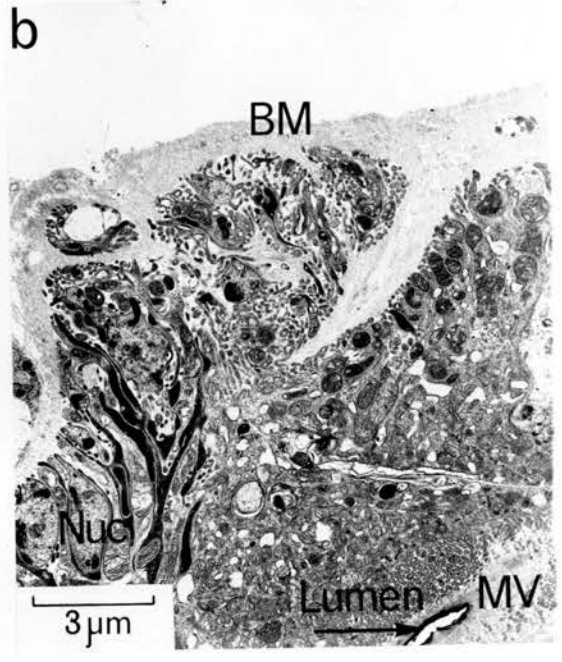
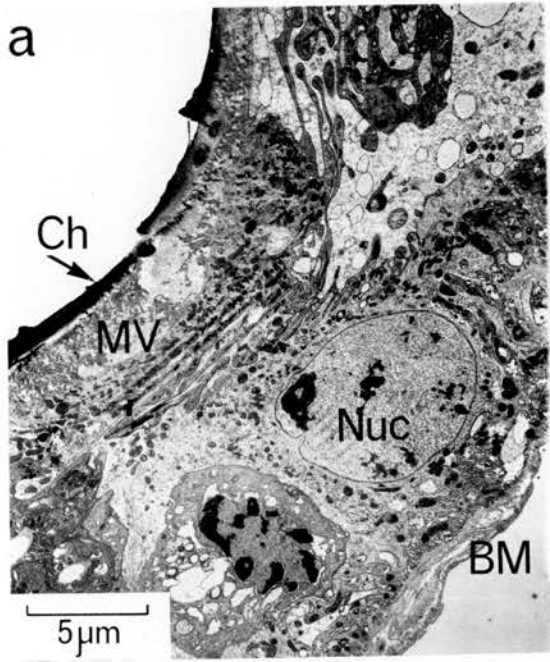
(c) A transverse section through a non-secretory duct which shows that many of the duct cells are held together with septate desmosomes (SD).



PART 3 SECTION 1 FIGURE 6

The common secretory ducts

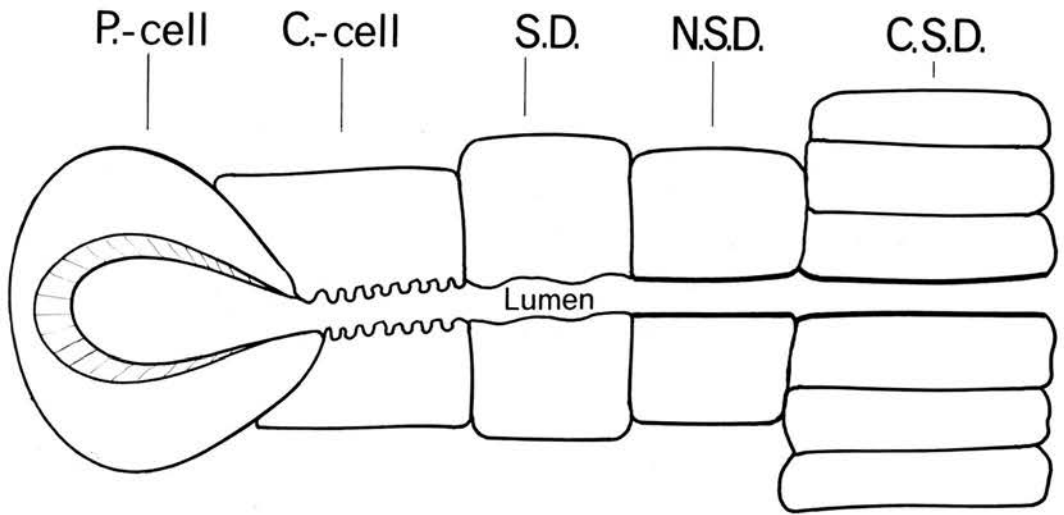
- (a) The general structure of the common secretory duct. The lumen is lined with chitin (Ch) under which is a layer of microvilli (MV). The duct is two or three cells thick and is surrounded by a basement membrane (BM). (Nuc-nucleus).
- (b) The cells of this duct contain many mitochondria, and, on some occasions, vacuole-like objects (BM-basement membrane, MV - microvilli, Nuc - nucleus).



PART 3 SECTION 1 FIGURE 7

A schematic diagram illustrating the duct systems of the cockroach salivary gland. The common secretory duct (CSD) is usually three cells thick.

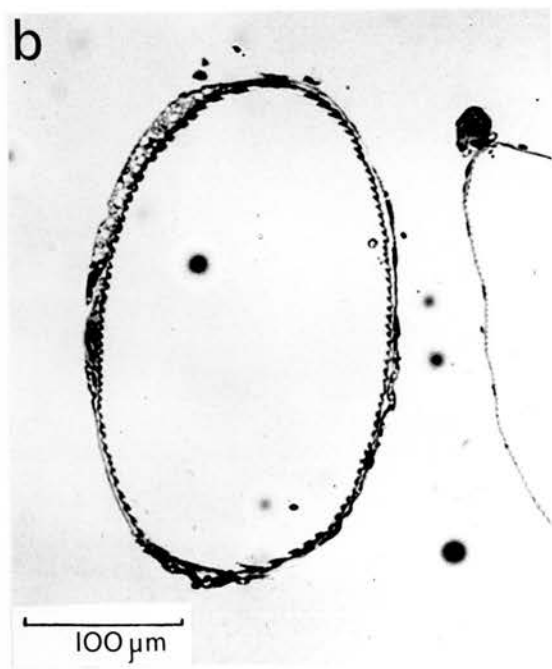
P-cell	peripheral cell
C-cell	central cell
S.D.	secretory duct
N.S.D.	non-secretory duct
C.S.D.	common secretory duct



PART 3 SECTION 1 FIGURE 8

(a) A section through the reservoir. The external surface of this structure consists of epithelial cells (EP), whereas the interior has numerous fibres (F) that project into it.

(b) A transverse section through the reservoir duct.

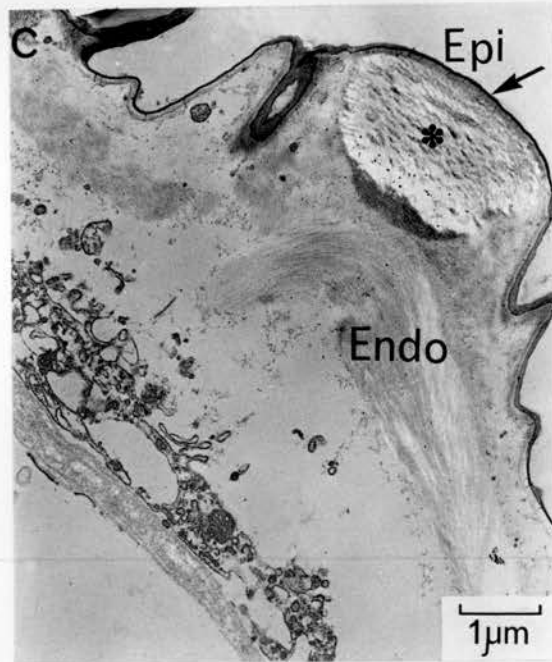
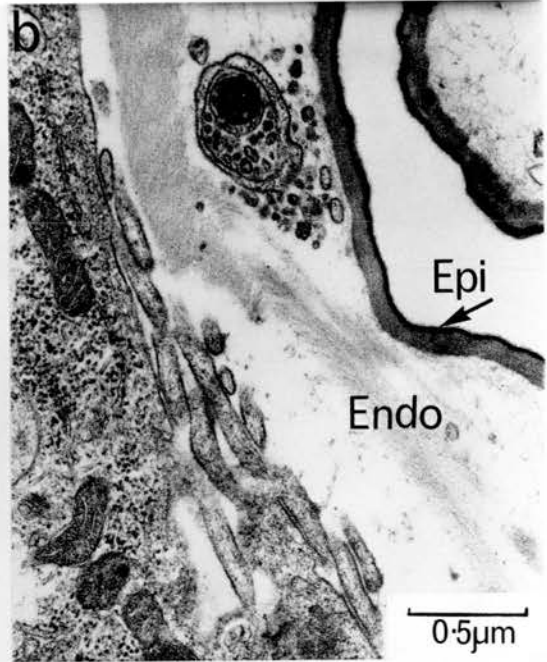
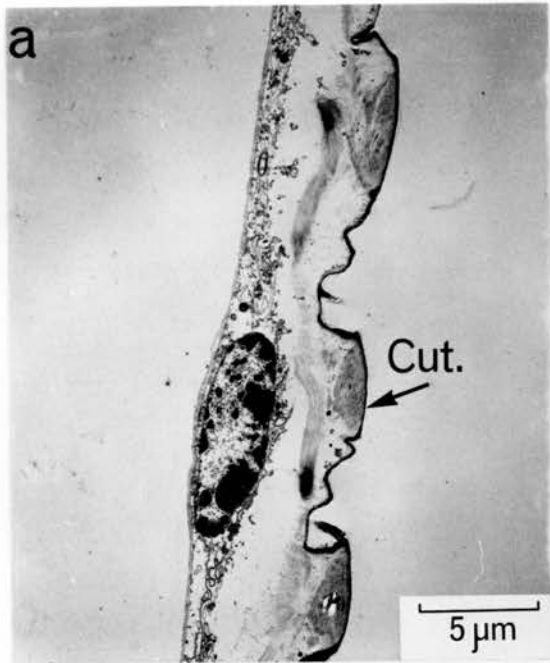


PART 3 SECTION 1 FIGURE 9

The reservoir duct

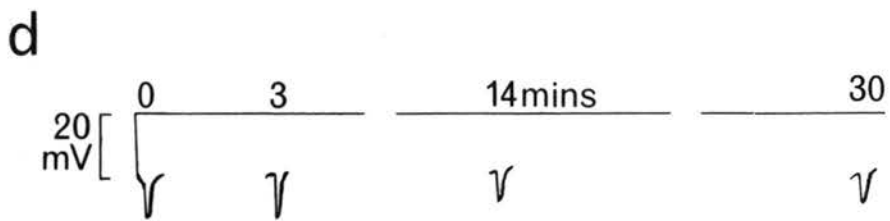
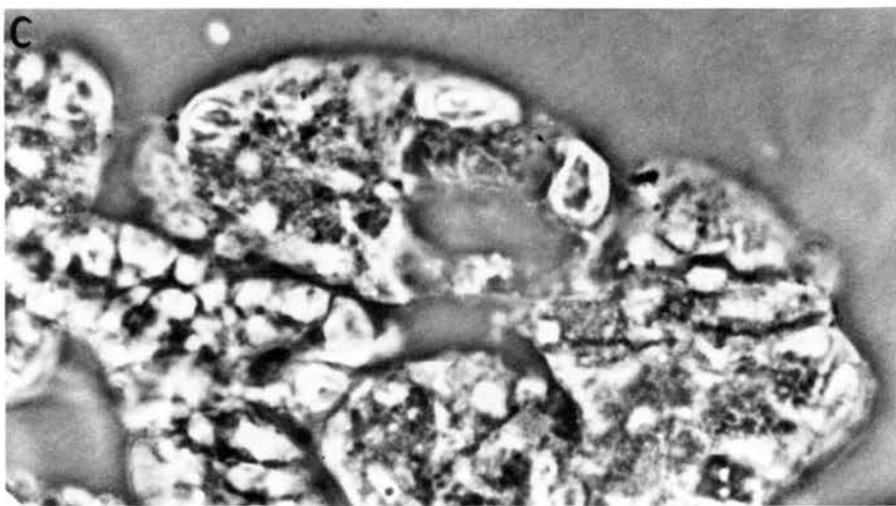
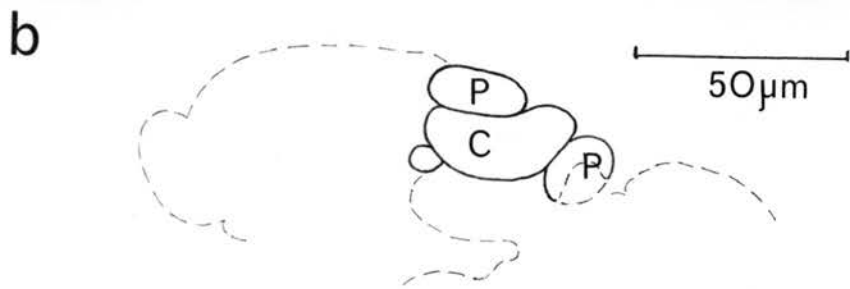
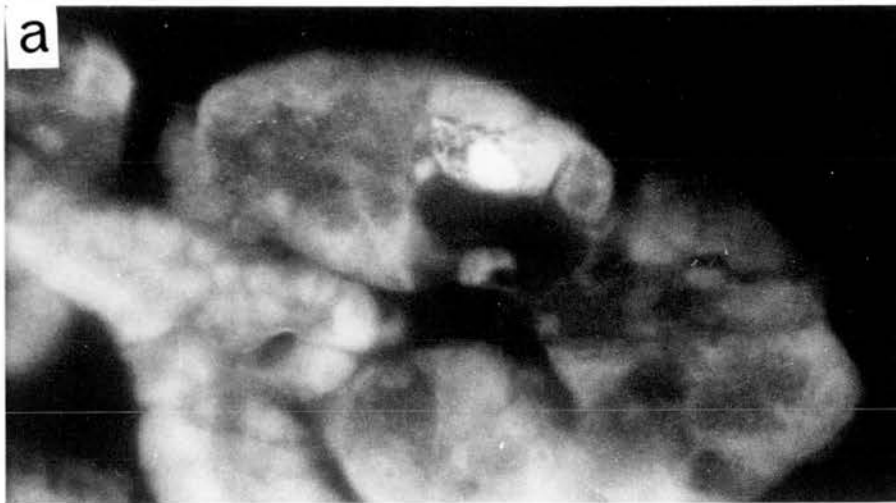
(a) One of the cells that secretes the cuticle (Cut) interior of the reservoir duct.

(b) This cuticle consists of epi and endocuticle (Epi and Endo). There is also another layer (the exocuticle) between these two layers. Figure (c) shows one of the 'teeth' (asterisk) of the duct which probably give strength to the structure (epi and endo- epi and endocuticle).



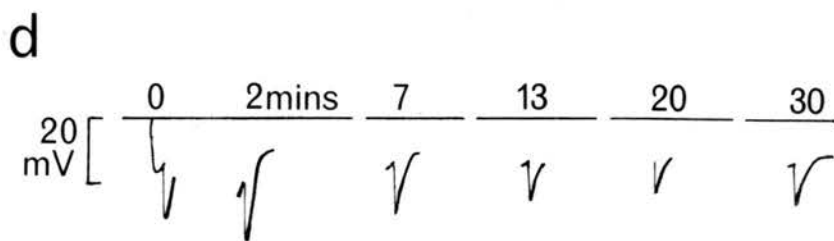
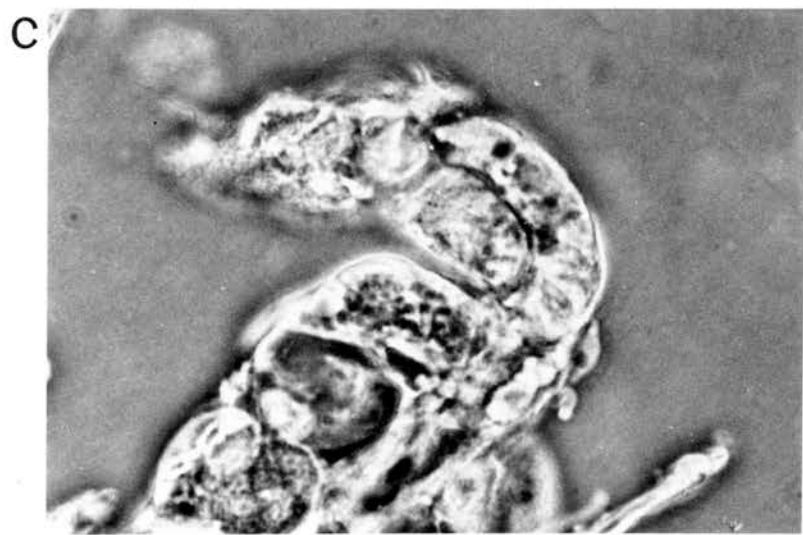
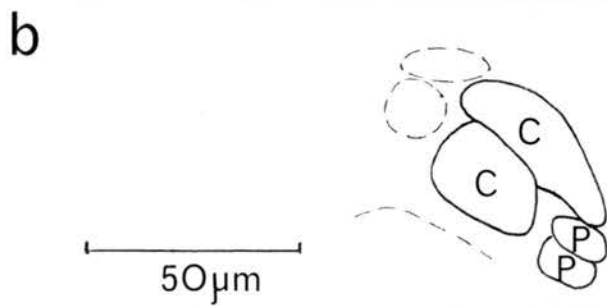
PART 3 SECTION 2 FIGURE 1

Figure (a) is a fluorescence micrograph of an acinus. Several of the cells in this micrograph glowed with the characteristic orange fluorescence after a 30-minute Procion infusion. Figure (b) is a tracing of the cells that the dye spread in to. (P - peripheral cell, C - central cell). The cells were identified from phase contrast pictures after Procion injections (figure c). Figure (d) is a tracing of the secretory potentials that were recorded during this experiment.



PART 3 SECTION 2 FIGURE 2

Figure (a) is a fluorescence micrograph of some Procion filled cells. (b) is a tracing of these cells and (c) is a phase contrast picture of the same cells. (d) is a tracing of the secretory potentials that were recorded during the impalement (C - central cell, P - peripheral cell).

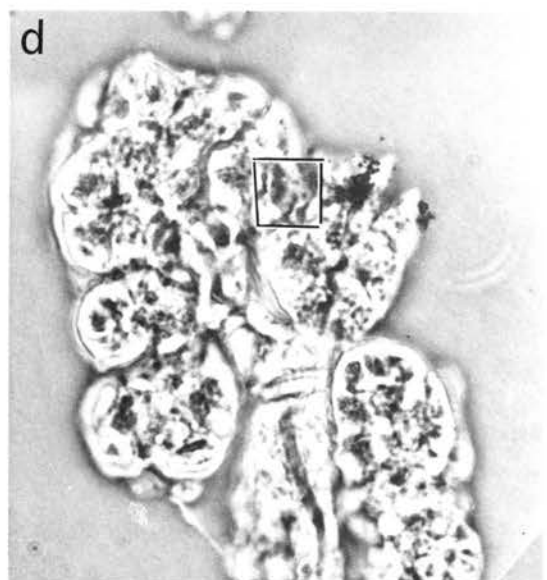
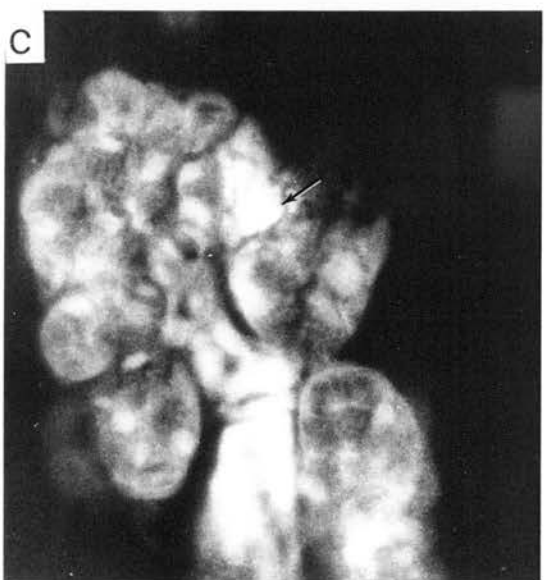
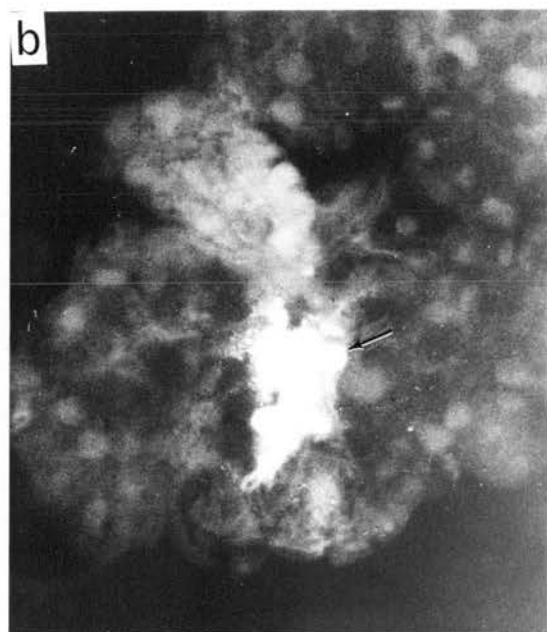
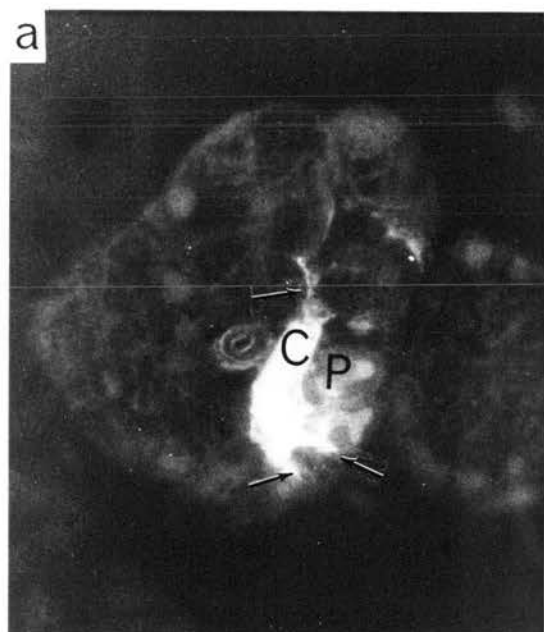


PART 3 SECTION 2 FIGURE 3

(a) A fluorescence micrograph of an acinus after an injection of Procion yellow, which spreads (at the arrows) during a half hour injection (C - central cell, P - peripheral cell).

(b) This is also a fluorescence micrograph of Procion filled cells. The arrow points to a nucleus which seems to have a high affinity for the dye.

(c) Is a Procion filled cell that has been incubated in calcium free ringer. In this case the dye has not spread from the cell to its neighbours. Figure (d) is a phase contrast picture of the same tissue; the box demarcates the Procion filled cell.



50 μ m

PART 3 SECTION 2 FIGURE 4

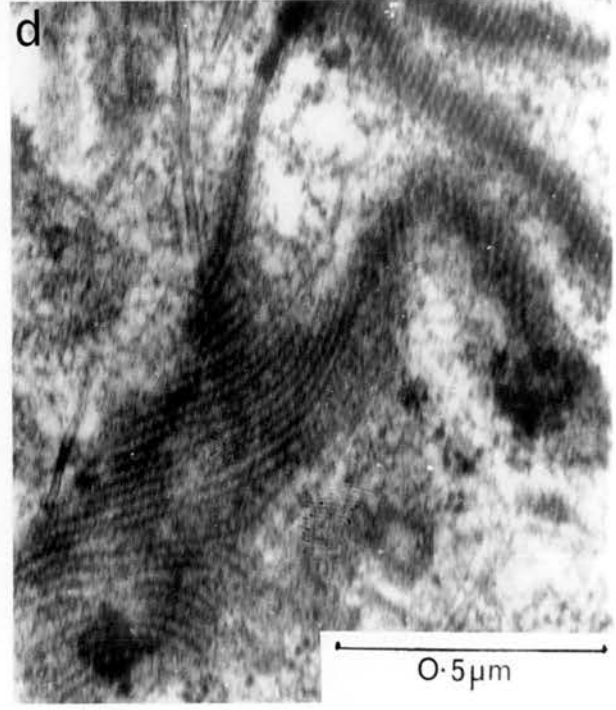
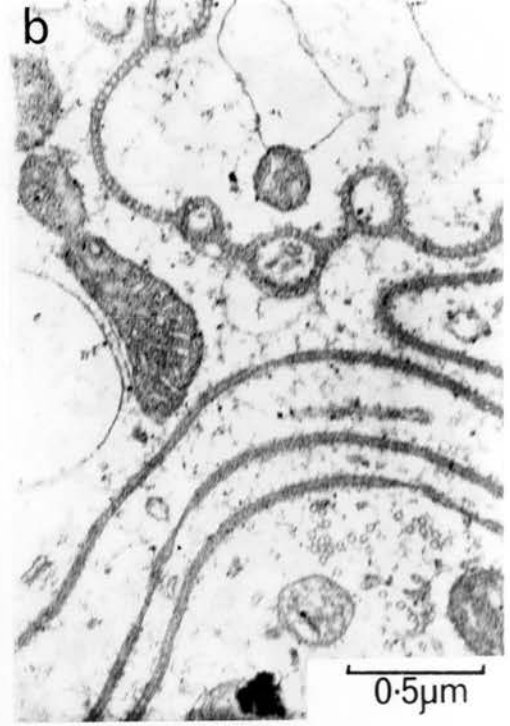
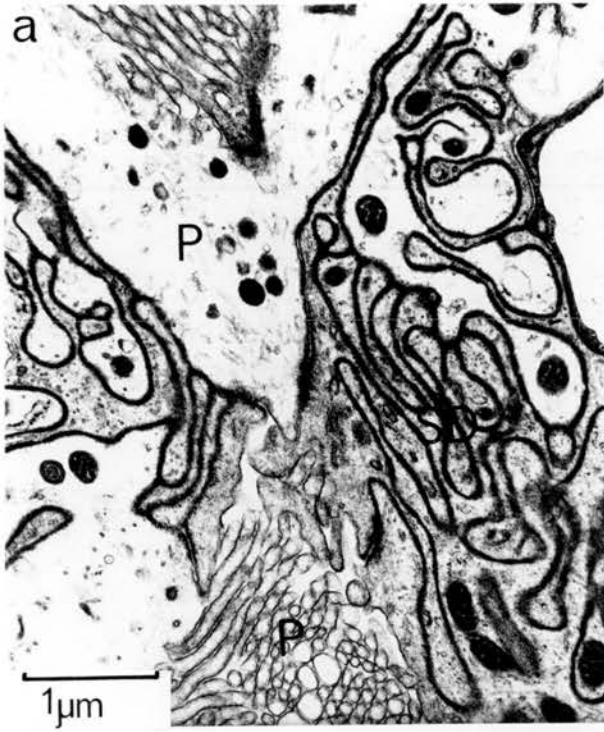
Septate desmosomes

(a) A micrograph of an area with septate desmosomes between two peripheral cells (P).

(b) This is from a similar area to figure (a), at a higher magnification.

(c) This micrograph illustrates the structure of a septate desmosome. The septa (ie, the rungs in the 'ladder') repeat at a periodicity of roughly 25 nm.

(d) This is a tangential section through a septate desmosome that has been impregnated with lanthanum. The electron dense areas are spaces between the septa that have been impregnated.



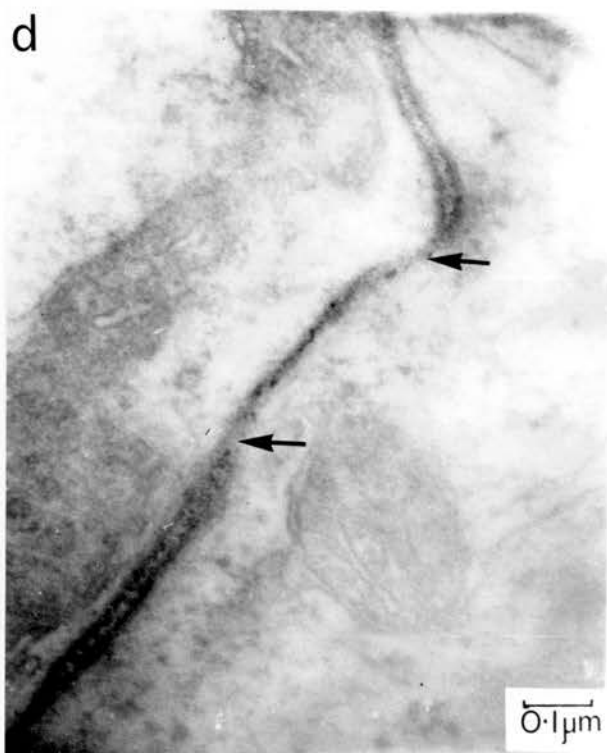
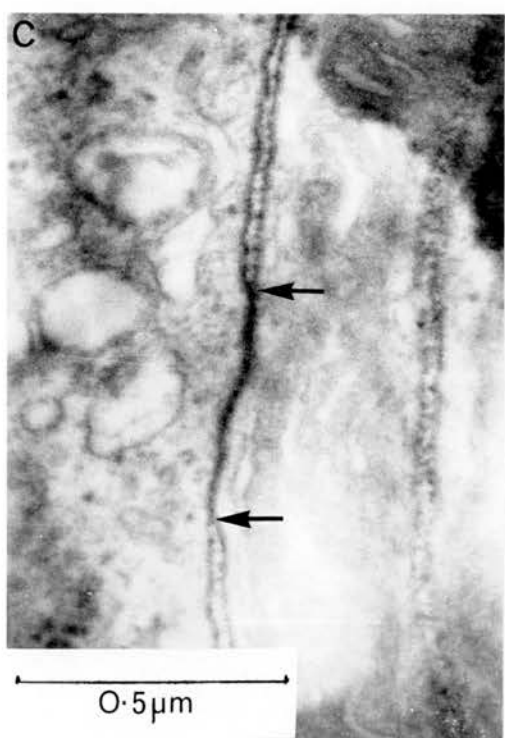
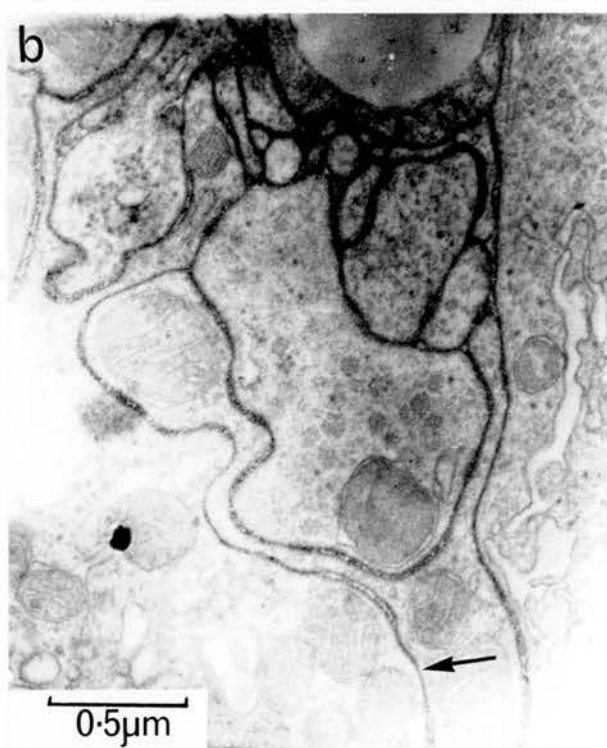
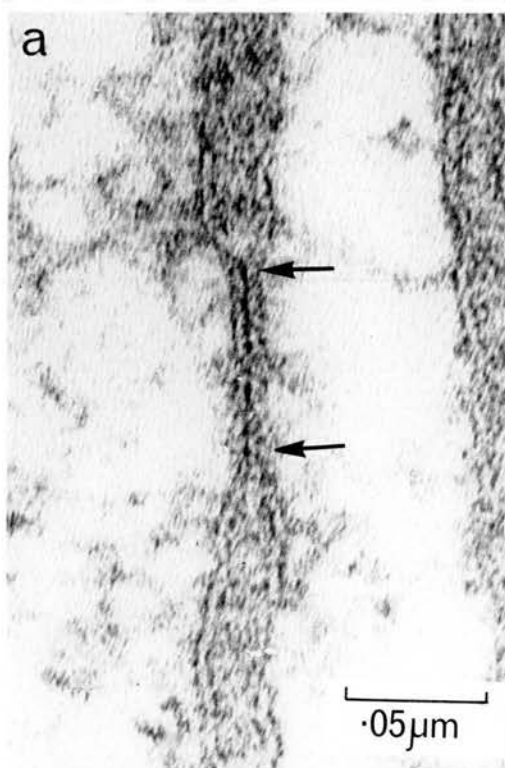
PART 3 SECTION 2 FIGURE 5

Gap junctions

(a) Shows a gap junction interposed in a septate desmosome (between the arrows).

(b) This is an unstained section (from a similar area to figure 4(a)) that has been impregnated with lanthanum. The arrow points to a large gap junction which is intercalated between septate desmosomes.

(c) and (d) These are unstained micrographs illustrating that lanthanum enters the spaces between the gap junctions (between the arrows).



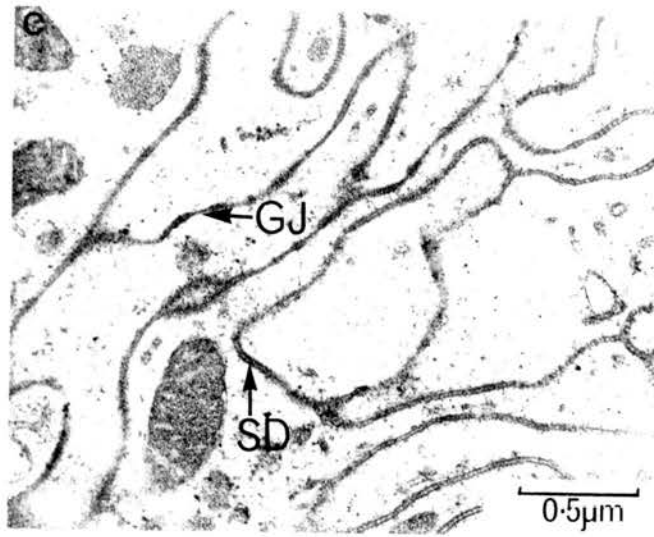
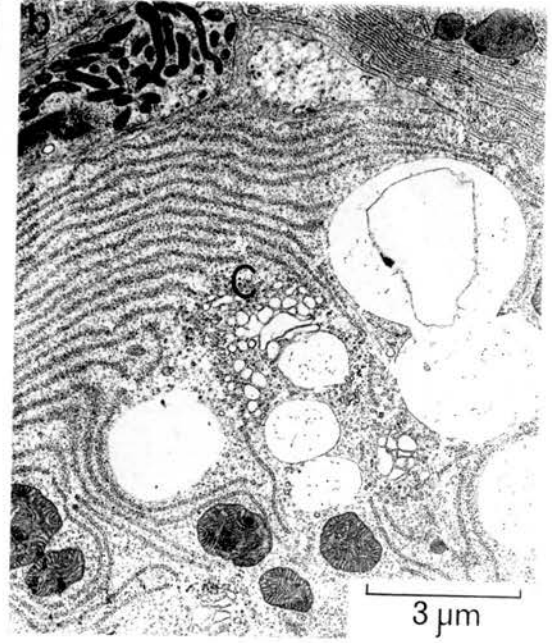
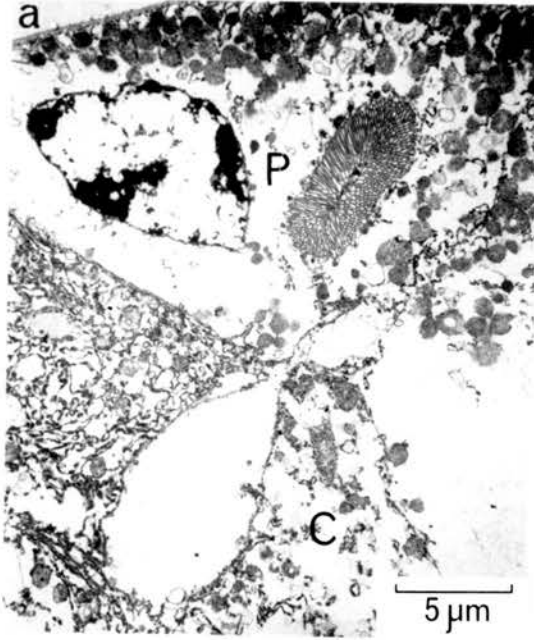
PART 3 SECTION 2 FIGURE 6

The effect of calcium free ringer upon acinar tissue

(a) Shows a micrograph of tissue that was incubated in calcium free ringer for two hours. Note the abnormal appearance of the tissue .

(b) This tissue was incubated in calcium free EGTA Ringer for two hours and has a normal appearance.

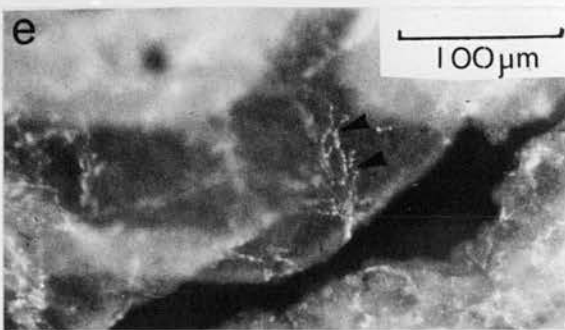
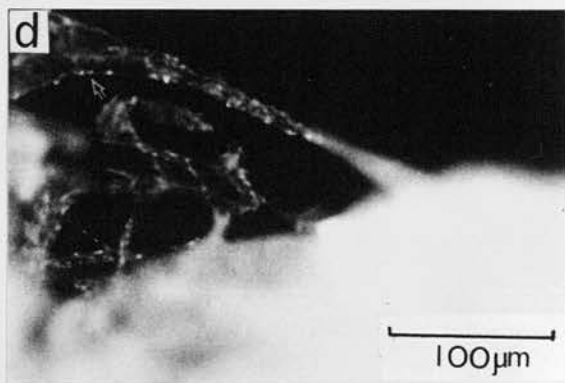
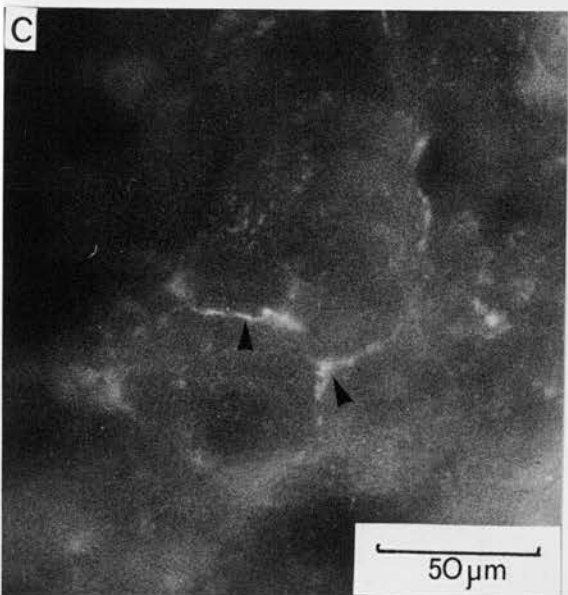
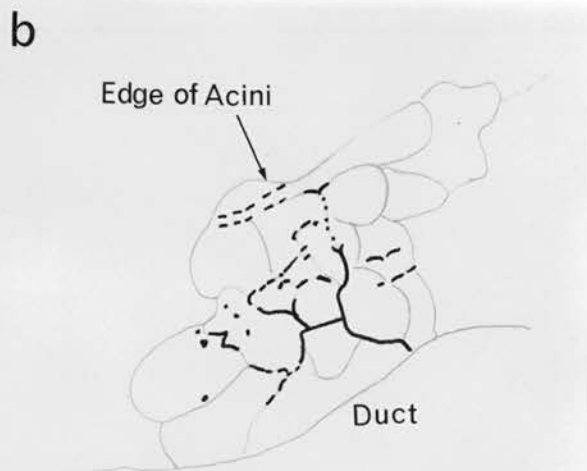
(c) This figure illustrates that incubation of tissue in calcium free EGTA Ringer for two hours does not destroy gap junctions (GJ). Septate desmosomes (SD) also have a healthy normal appearance .



PART 3 SECTION 3 FIGURE 1

Whole mount stretch preparations of cockroach salivary glands treated with paraformaldehyde.

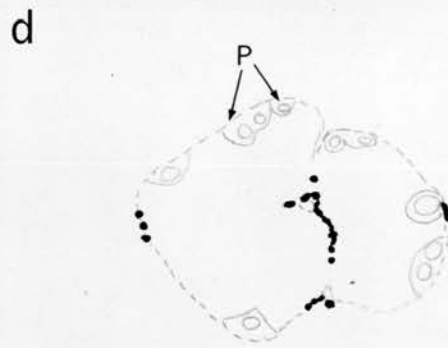
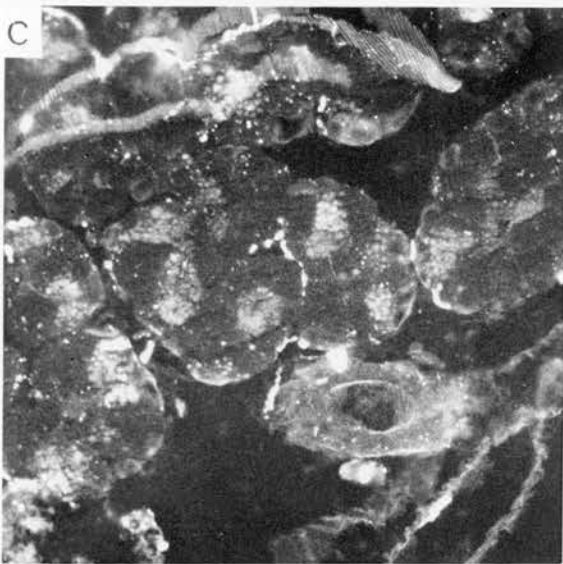
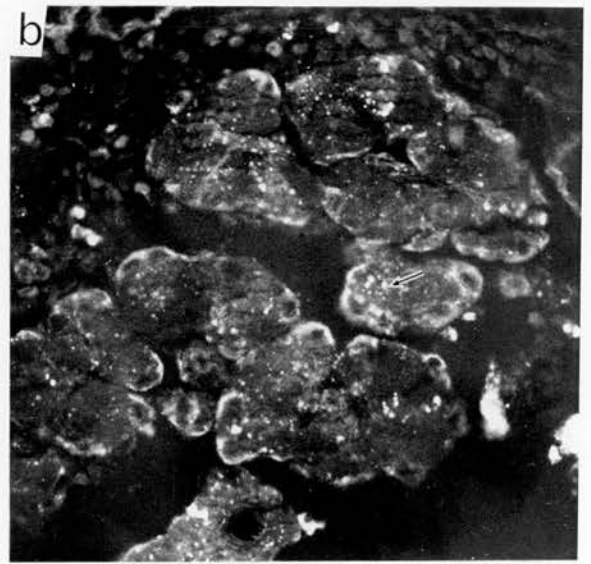
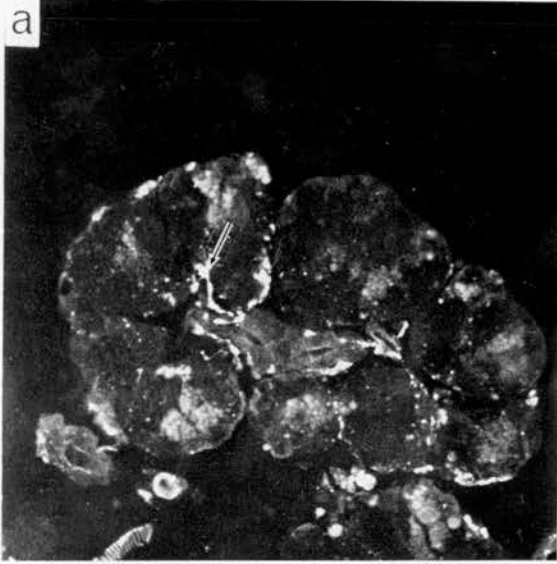
Figure (a) is a fluorescence micrograph of tissue that has been treated with paraformaldehyde (the Falck-Hillarp method). A network of several fluorescing axons may be seen across the surface of the acini. These have been traced in figure (b). Figure (c) is an enlargement of an area of figure (a) which shows the branching pattern of the axons (arrows). The axons may also be seen to outline the edges of the acini. Figure (d) illustrates one of the nerves that crosses from acinus to acinus. The arrow indicates a bright varicose area of specific fluorescence. Figure (e) shows several varicose areas of axons on the surface of some acinar cells (arrows).



PART 3 SECTION 3 FIGURE 2

Sections of cockroach salivary glands after treatment with paraformaldehyde.

Figure (a) illustrates the specific fluorescence obtained after paraformaldehyde treatment (arrow) whereas figure (b) is a piece of tissue that was untreated. The arrow indicates an area of unspecific fluorescence. Figure (c) illustrates several fluorescing axons which were traced in figure (d). The areas of peripheral cells (P) have been indicated. In this section axons run between the acinar cells.

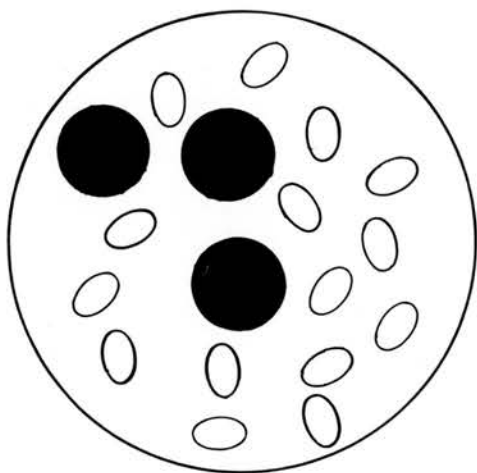



100 μ m

PART 3 SECTION 3 FIGURE 3

A diagram of the salient features of type A and B axons with their vesicular dimensions. The dark objects represent electron dense granules; elliptical objects represent the agranular vesicles of type A axons.

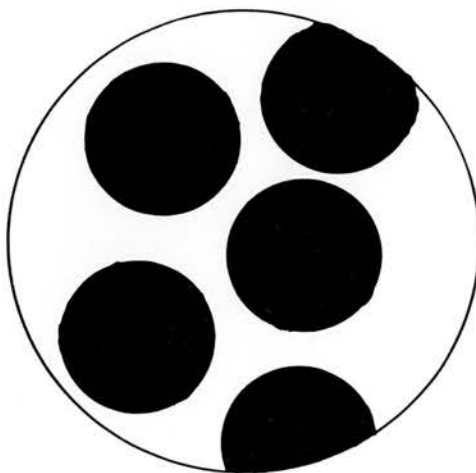
TYPE A



 $40 \pm 15.82 \text{ nm}$

 $91 \pm 27.47 \text{ nm}$

TYPE B



 $134 \pm 23.89 \text{ nm}$

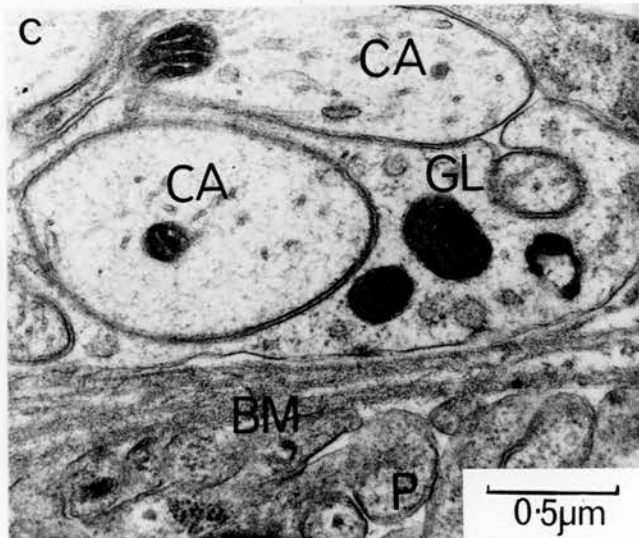
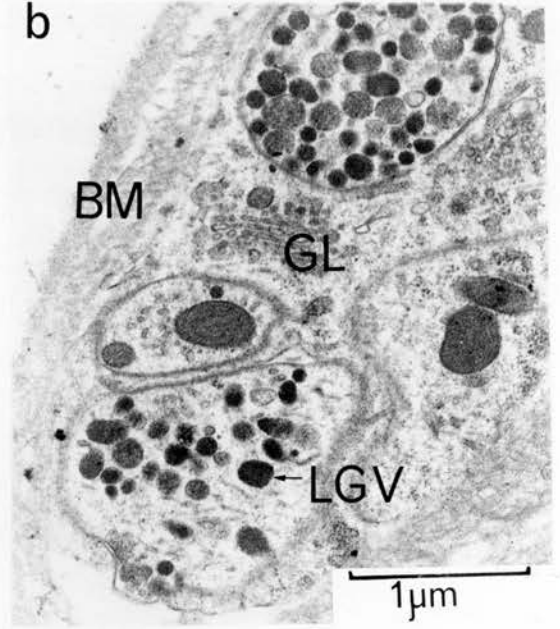
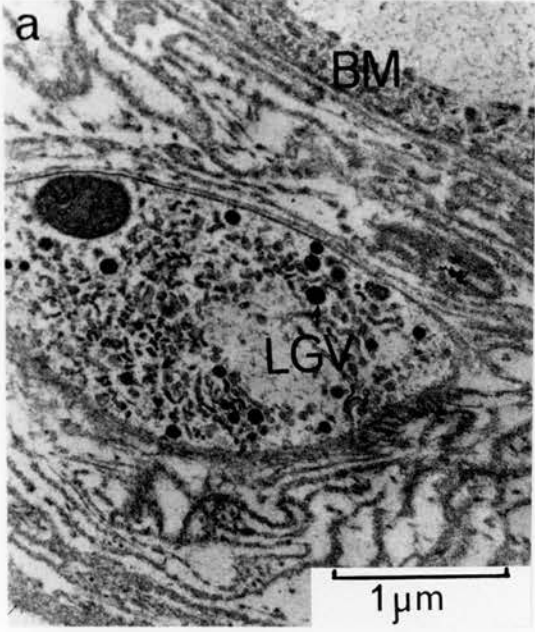
PART 3 SECTION 3 FIGURE 4

Type A, B and clear axons

(a) Shows a type A axon . Note the small elliptical agranular vesicles and the larger granular vesicles (LGV). This axon is situated in the basement membrane (BM) region of an acinus.

(b) Shows some type B axons with their characteristic large granular vesicles (LGV). A glial cell (GL) surrounds these axons which are situated in the basement membrane region of the acinus.

(c) This figure illustrates two clear axons (CA) in the vicinity of a peripheral cell. They also have a glial coat (GL) and are in the basement membrane region of the acinus. Note the microtubules within these axons.



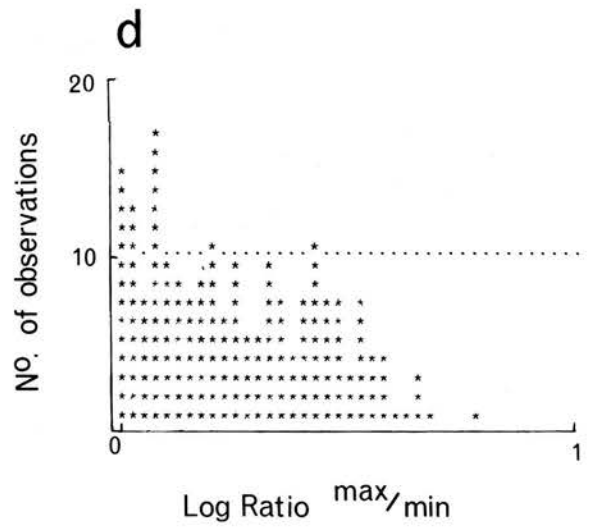
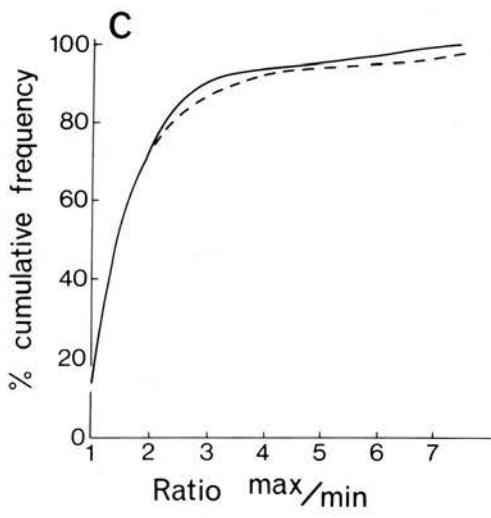
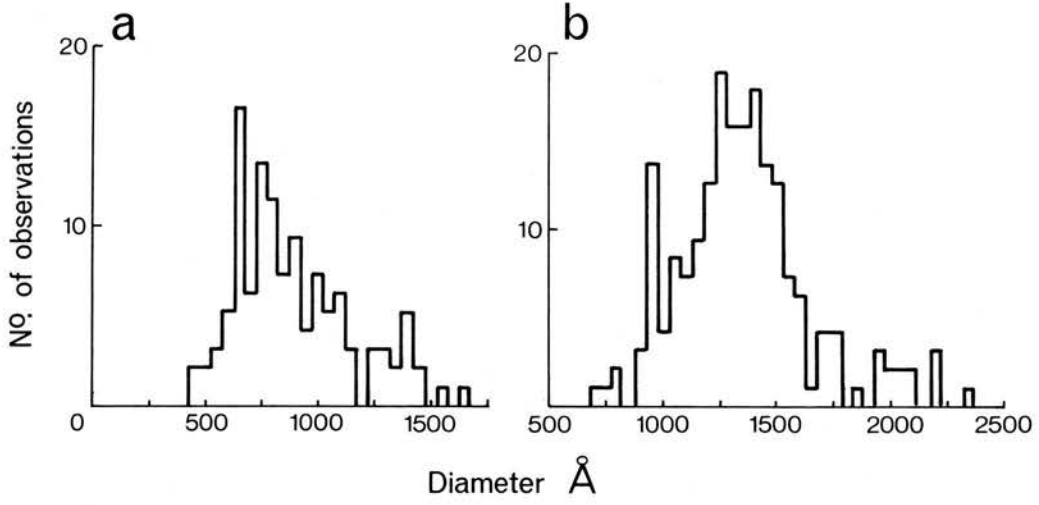
PART 3 SECTION 3 FIGURE 5

(a) A histogram of the diameters of 116 large granular vesicles as measured from several type A axons.

(b) A histogram of 187 large granular vesicles measured from several type B axons.

(c) A plot of cumulative frequency against the ratio of the maximum to the minimum dimension of the agranular vesicles of type A axons. The dotted line represents a curve produced by Elias et al (1970) for a prolate rotary ellipsoid.

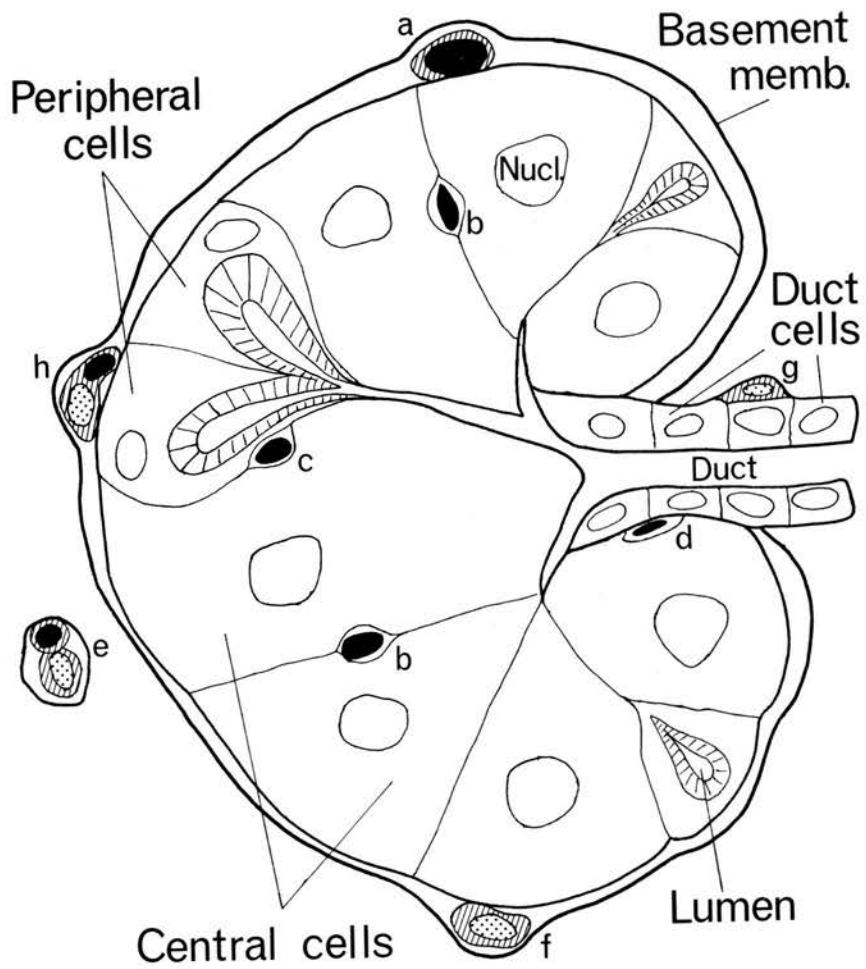
(d) This is a plot of the same data that were used in figure (c), except that the log of the ratio was plotted as a histogram.



PART 3 SECTION 3 FIGURE 6

A schematic diagram (not to scale) showing the main features of the cockroach acinus with special reference to the observed relationships of types A and B axons to the cells of the gland.

- (a) a type A axon in the basement membrane region of the acinus.
- (b) a type A axon between central cells.
- (c) a type A axon between central and peripheral cells.
- (d) a type A axon between central and duct cells.
- (e) a type A and B axon in a nerve in the vicinity of the acinus.
- (f) a type B axon in the basement membrane region of the acinus.
- (g) a type B axon on the surface of a duct.
- (h) type A and B axons running together through the basement membrane region of the acinus.

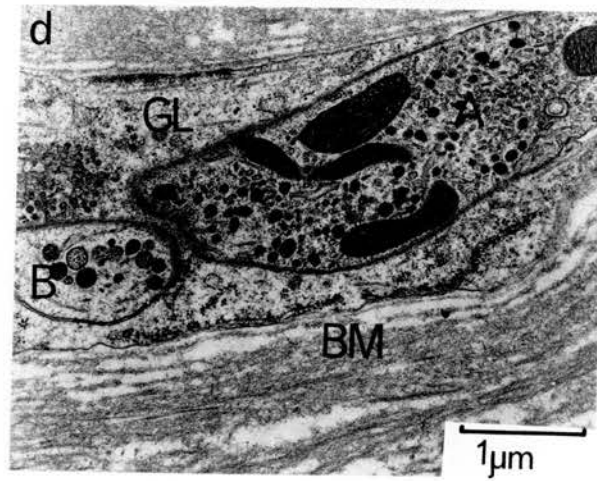
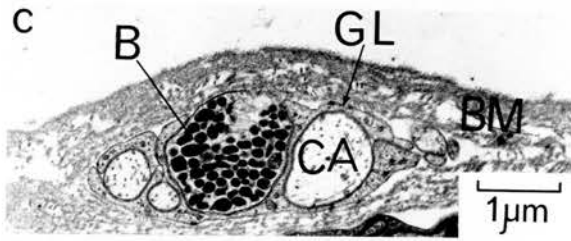
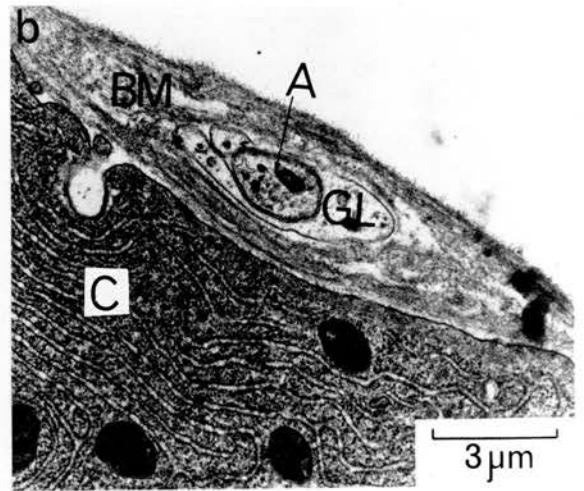
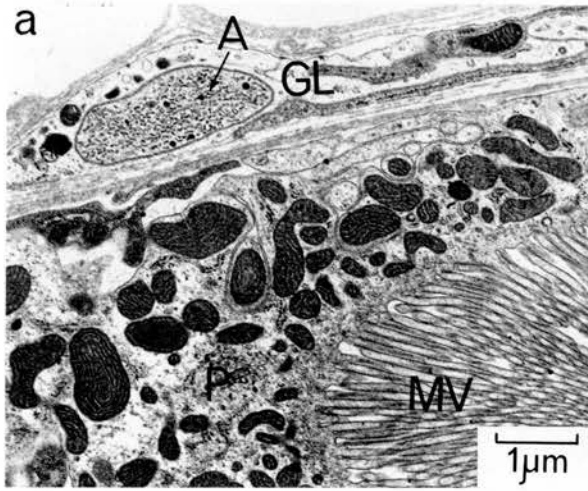


- Type A nerve
- Type B nerve
- Glial cell

PART 3 SECTION 3 FIGURE 7

Axons in the basement membrane of acini

- (a) A type A axon in the basement membrane in the vicinity of a peripheral cell (P). (GL - glial cell, MV - microvilli).
- (b) A type A axon in the basement membrane (BM) in the vicinity of a central cell (C) (GL - glial cell).
- (c) A type B and a clear axon (CA) in the basement membrane (BM) which have a glial surrounding (GL).
- (d) Shows a type A and B axon in the basement membrane (BM), also surrounded by glia (GL).



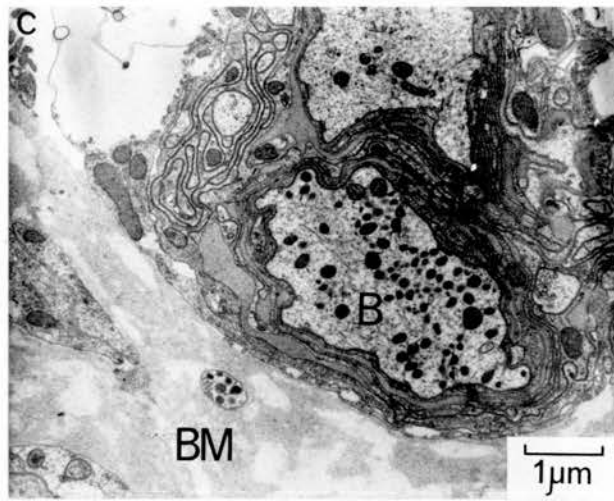
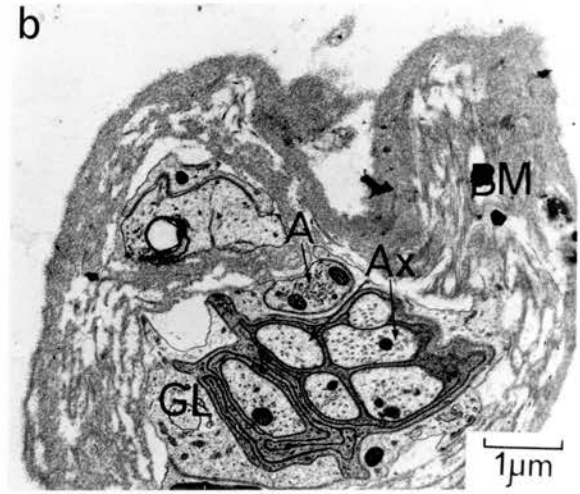
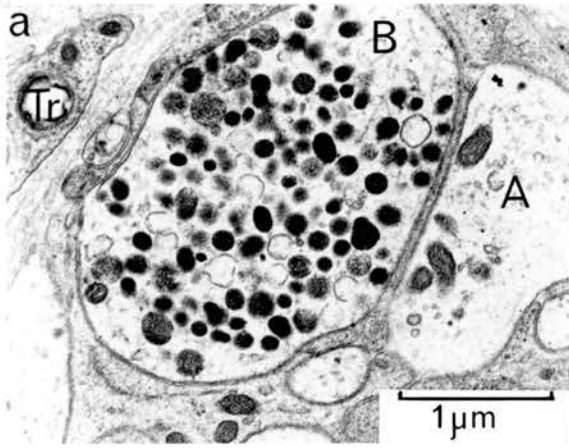
PART 3 SECTION 3 FIGURE 8

Axons in the nerves associated with acini

- (a) A type A and type B axon running together in the vicinity of an acinus (Tr - tracheole).

- (b) A nerve containing several axons (Ax). Note the thick glial sheath (GL) which surrounds them but does not surround a type A axon. (BM is the basement membrane of the nerve.)

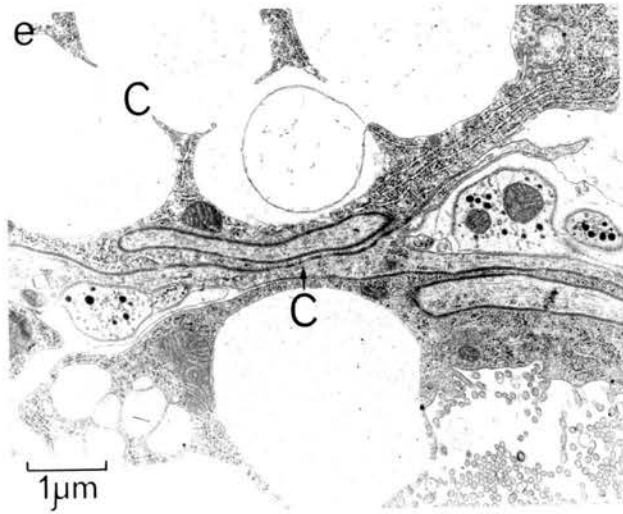
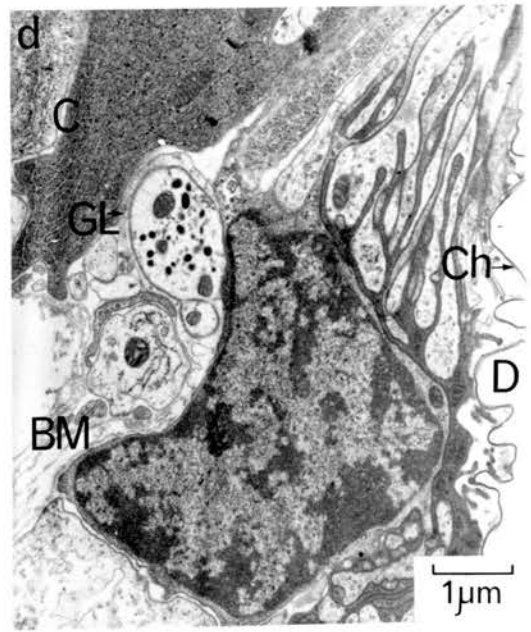
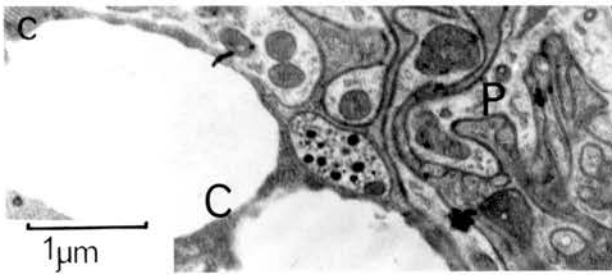
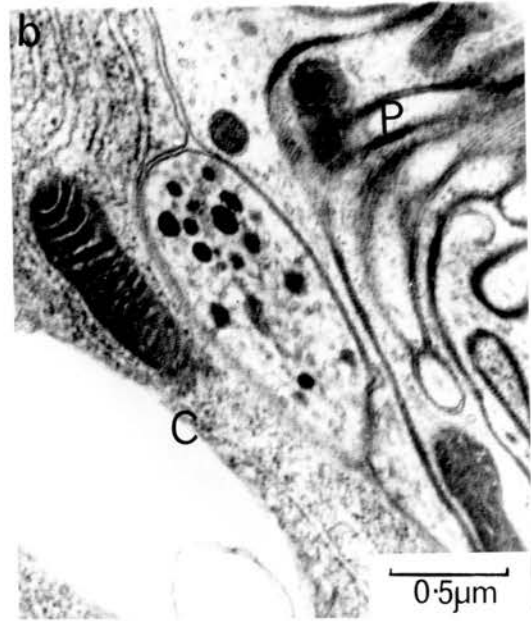
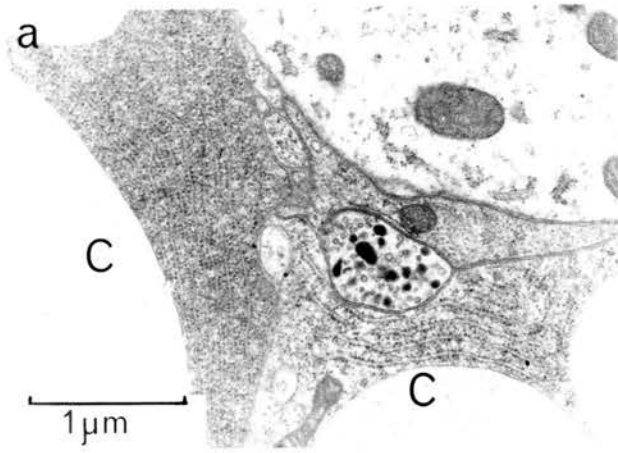
- (c) This is another nerve which contains a large type B axon. This axon seems to be wrapped up in a thick glial sheath (GL). (BM is the basement membrane of an acinus.)



PART 3 SECTION 3 FIGURE 9

Axons between the cells of the gland

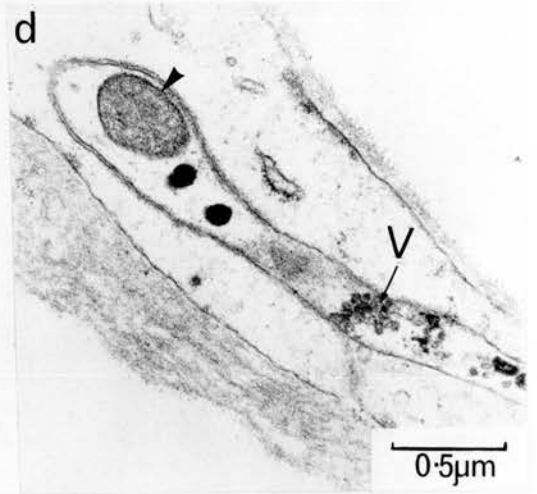
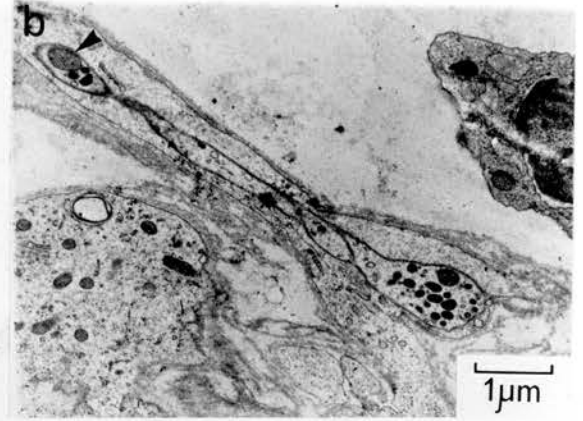
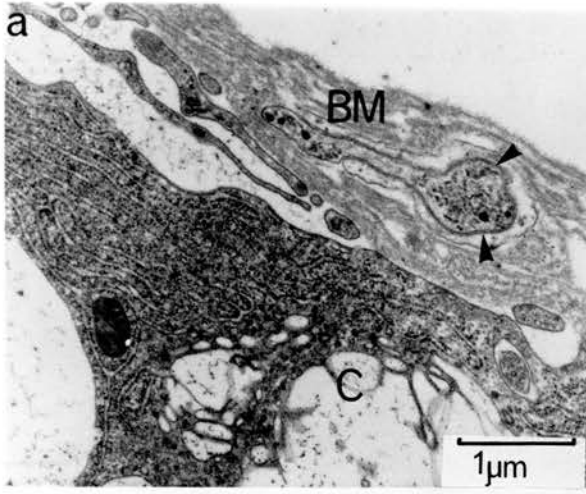
- (a) A type A axon surrounded by central cells (C) and lacking any glial coat. The distance between the axon membrane and the nearest cell membrane is roughly 20 nm.
- (b) A type A axon between a central cell (C) and a peripheral cell (P).
- (c) Another type A axon between a peripheral cell (P) and a central cell (C).
- (d) A type A axon between a central cell (C) and a secretory duct cell. This axon has a small glial coat (GL) between it and the central cell (D - duct, Ch - chitin).
- (e) Two groups of type A axons between central cells (C). Both of these groups seem to have glial cells on one side only and are in close proximity to the central cells on the other side.



PART 3 SECTION 3 FIGURE 10

Varicosities

(a) This is a type A axon in the basement membrane (BM) region of an acinus in the vicinity of a central cell. The arrows indicate an axonal swelling which is crammed with vesicles. Note that there is a glial sheath round this region. Figures (b), (c) and (d) are serial sections through a type A axon which has been cut in a longitudinal plane. There are two swellings: one contains vesicles while the other contains a mitochondrion (arrow). In figure (c) the small arrow indicates an area where the surrounding glial cell has obscured part of the axon from the observer's view. In figure (d) there is a group of vesicles which are found in a region of the axon with no swelling.



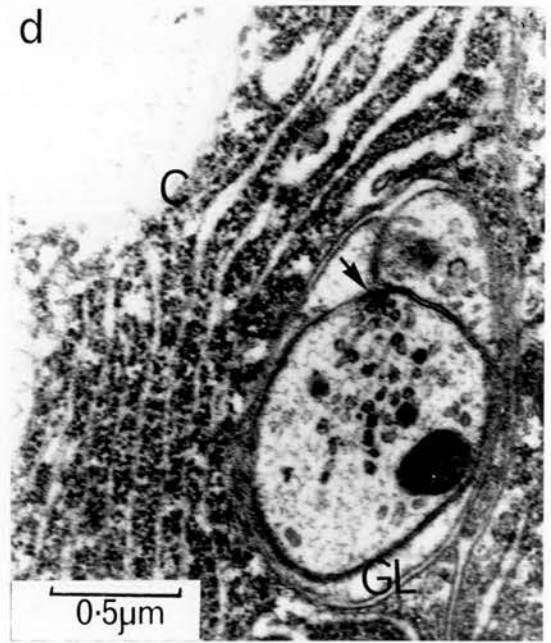
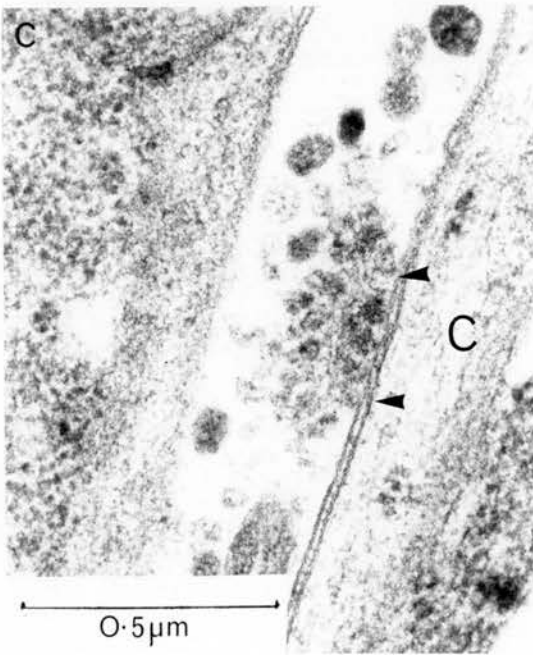
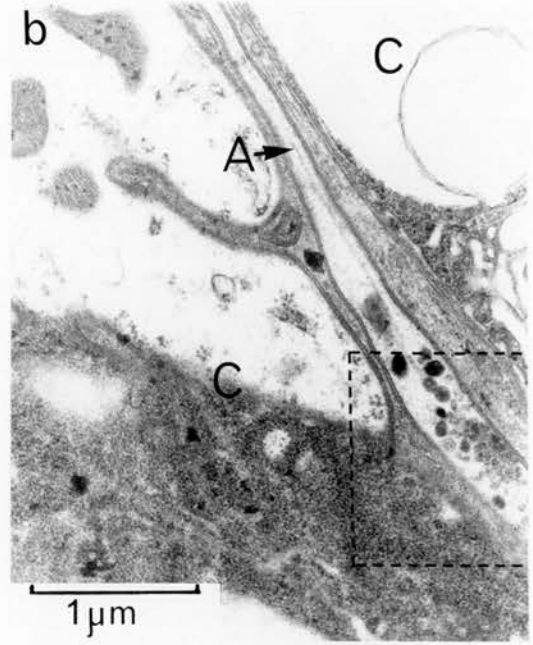
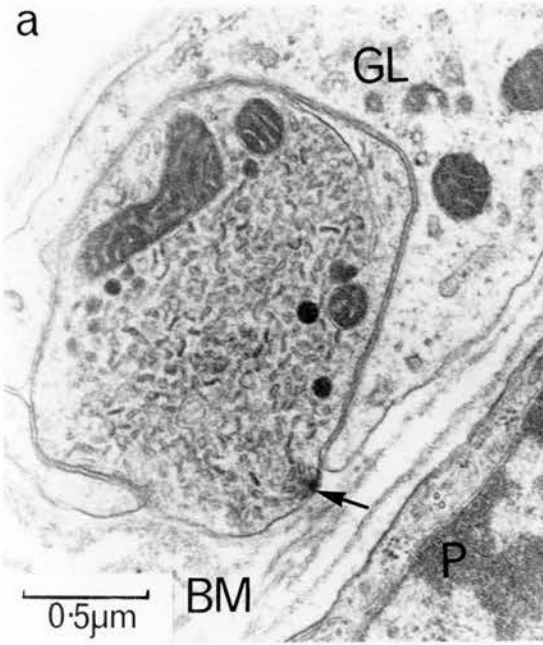
PART 3 SECTION 3 FIGURE 11

Presynaptic specialisations

(a) A type A axon in the basement membrane (BM) region of an acinus. This axon is only partially surrounded by glia (GL) and in one corner of the cell, that is free of glia, there is a dark object (ARROW) which is surrounded with vesicles. (P - peripheral cell).

(b) Is a type A axon that runs between some central cells (C). The area in the box has been enlarged in figure (c) to show a cluster of vesicles (between the arrows).

(d) A presumed axo-axonic synapse between two type A axons. There is a membrane specialisation at the arrow which is similar to the one in figure (a) (C - central cell, GL - glial cell).



PART 3 SECTION 3 FIGURE 12

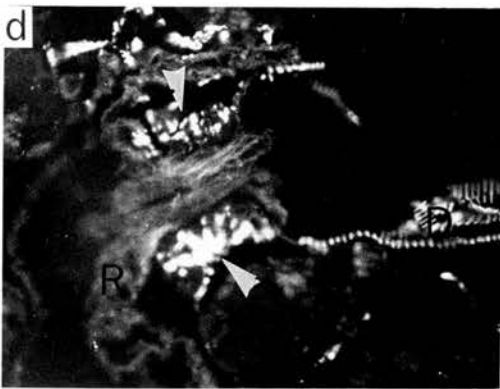
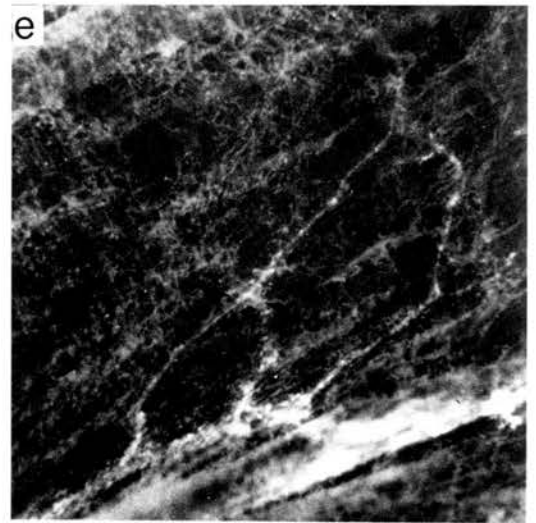
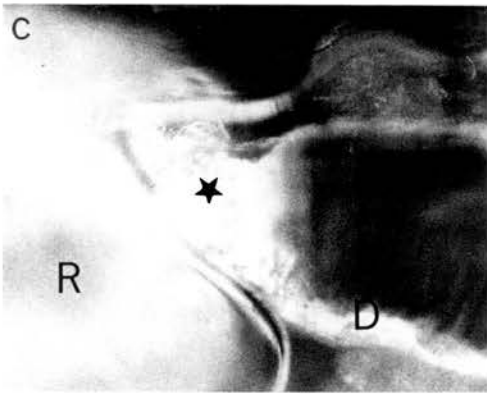
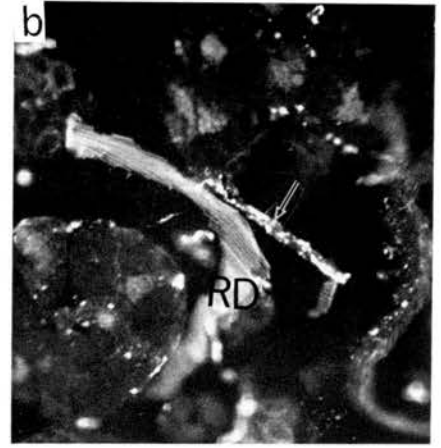
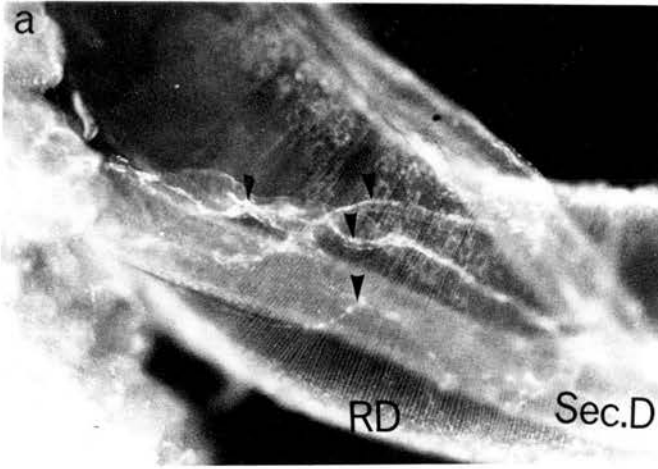
Fluorescence histochemistry of the reservoirs and reservoir ducts

Figure (a) shows a stretch preparation of the reservoir ducts (RD) and secretory ducts (Sec. D). The arrows point to several fluorescing axons on the surface of these two ducts. There are at least two axons on the reservoir duct.

(b) A section through a reservoir duct (RD). A large fluorescing nerve may be observed at the arrow.

(c) Shows a stretch preparation of the region that the reservoir duct (D) joins to the reservoir (R). A bright area of intense fluorescence may be observed around the muscle between the duct and the reservoir (star). A section through this (figure d) shows the many fluorescing nerve fibres that innervate this muscle (arrows).

(e) Some of the fluorescing structures that are seen on the surface of the reservoir.



100µm

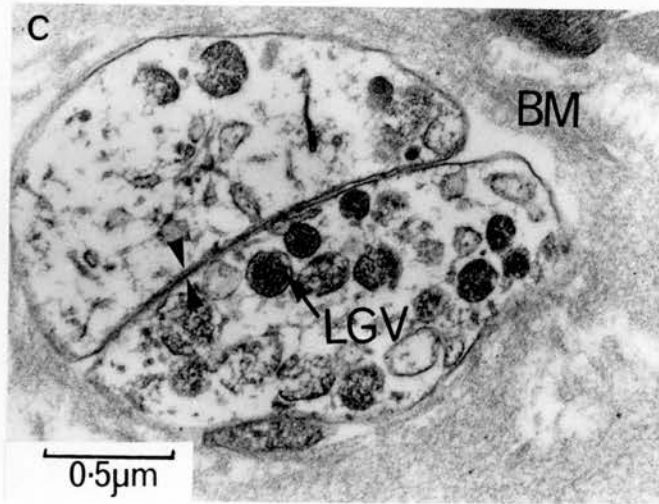
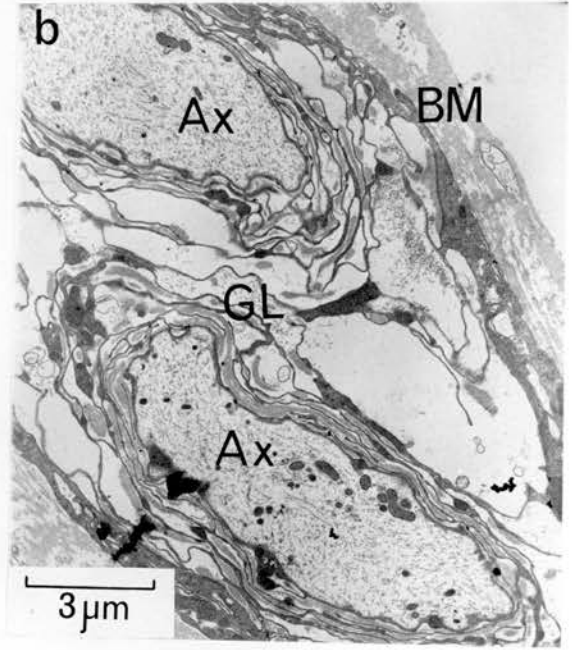
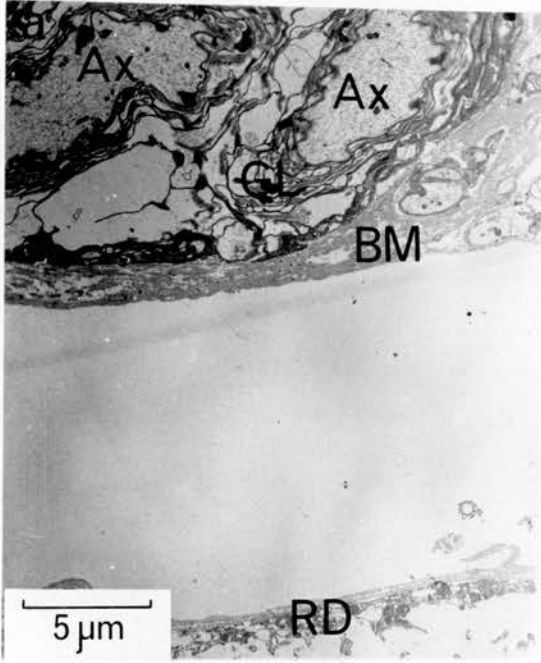
PART 3 SECTION 3 FIGURE 13

The salivary nerves

(a) The salivary nerve consists of two large axons (Ax) which are surrounded with a glial coat (GL). The nerve is surrounded by a basement membrane (BM) which contains several smaller axons (RD - reservoir duct).

(b) Another example of a salivary nerve similar to figure (a) (Ax - axon, GL - glial cells, BM - basement membrane of the nerve).

(c) Two type B axons in the basement membrane (BM) of the salivary nerves. The membranes of these two axons are in close apposition between the arrows. (LGV - large granular vesicle).



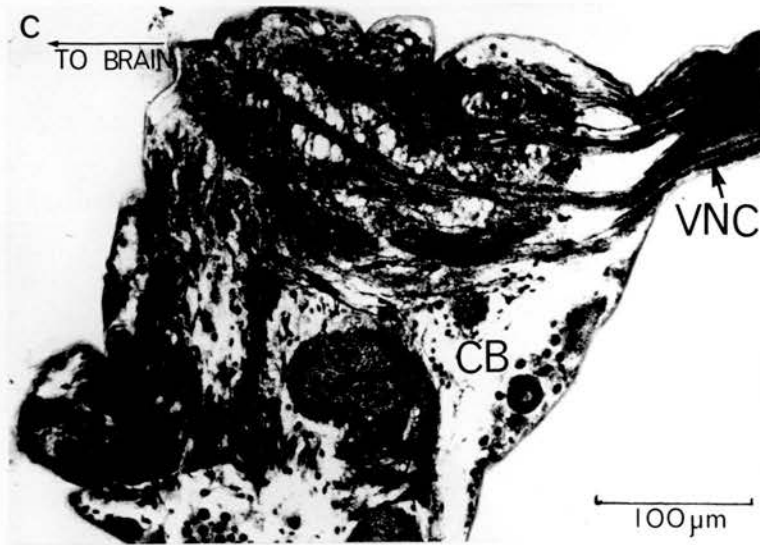
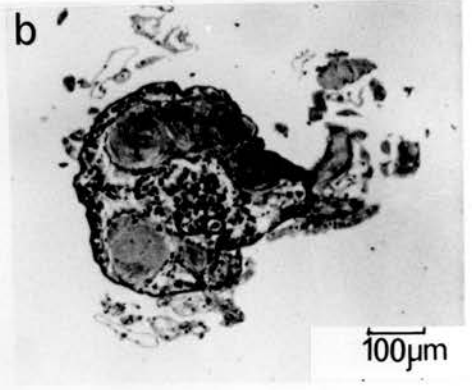
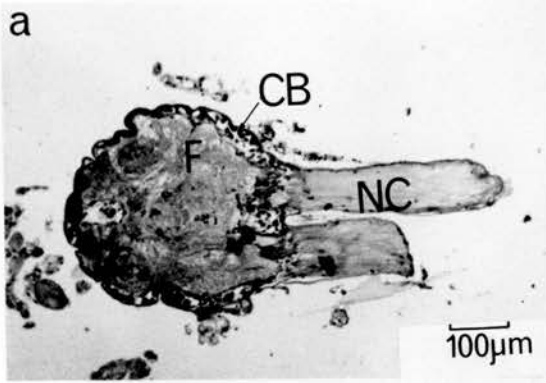
PART 3 SECTION 4 FIGURE 1

Light micrographs of the suboesophageal ganglion

(a) An Haematoxylin and Eosin stained horizontal section through the dorsal region of the ganglion. In the periphery a region of cell bodies (CB) may be seen. In the more central regions of this section there are many nerve fibres (F). The ventral nerve cord (NC) may be seen leaving the ganglion.

(b) This is a section through the ganglion in a ventral area. It can be seen that there is now a region of cell bodies (CB) in the middle of the ganglion in addition to that on the periphery. The fibres (F) are sandwiched between these two areas (H and E stain).

(c) Shows a silver stained longitudinal section (ie, at right angles to the sections in figure (a) and (b)) through the ganglion. Nerve fibres may be seen running from the ventral nerve cord (VNC) to the brain. In the more ventral regions many cell bodies may be seen (CB).



PART 3 SECTION 4 FIGURE 2

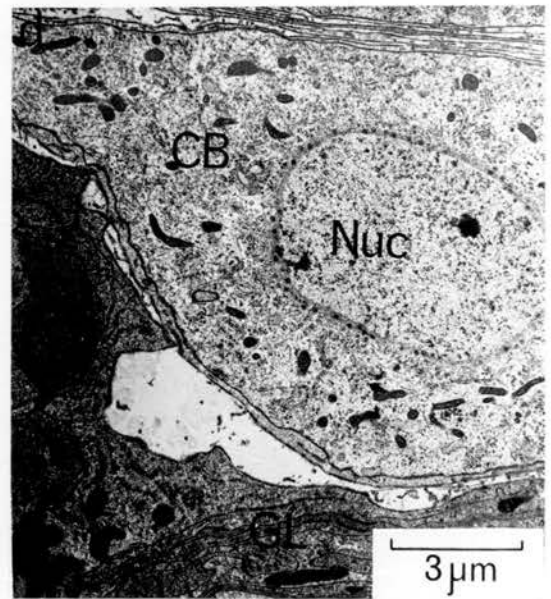
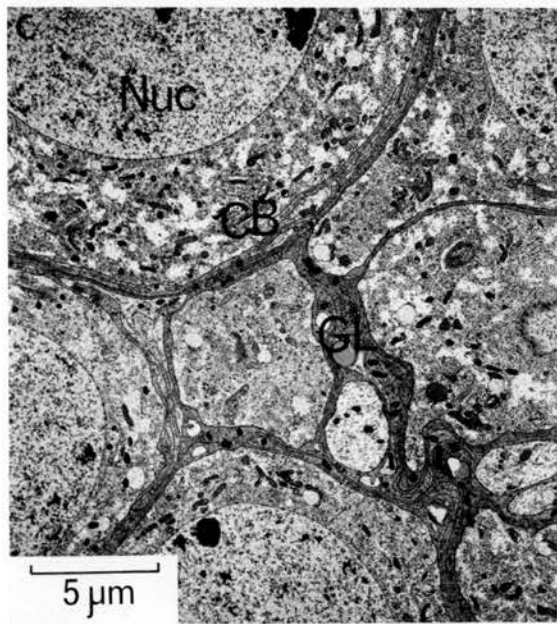
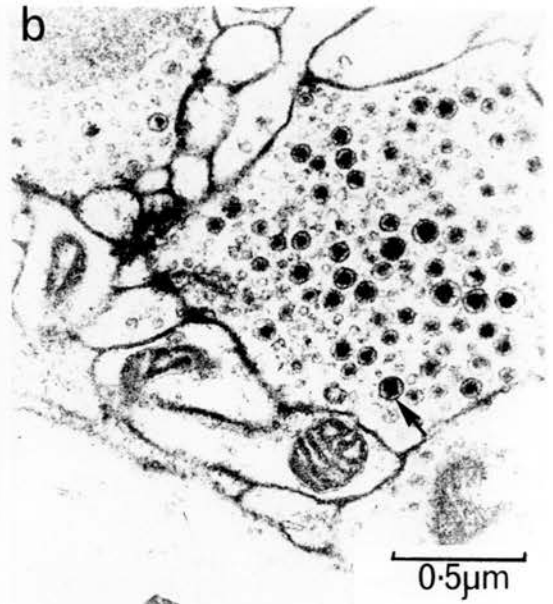
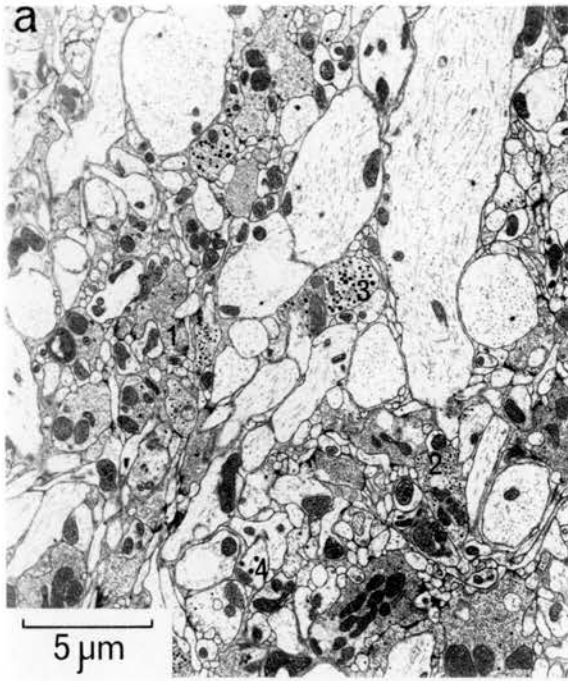
Electron micrographs of the suboesophageal ganglion

(a) The neuropil of the ganglion. In this figure at least four different types of axon may be classified on the basis of the vesicles that they contain :

- 1 - small agranular vesicles
- 2 - small agranular vesicles and small granular vesicles
- 3 - small agranular vesicles and large granular vesicles
- 4 - large granular vesicles

(b) Some axons from the ganglion. One of them contains large 'dense-cored' vesicles (arrow).

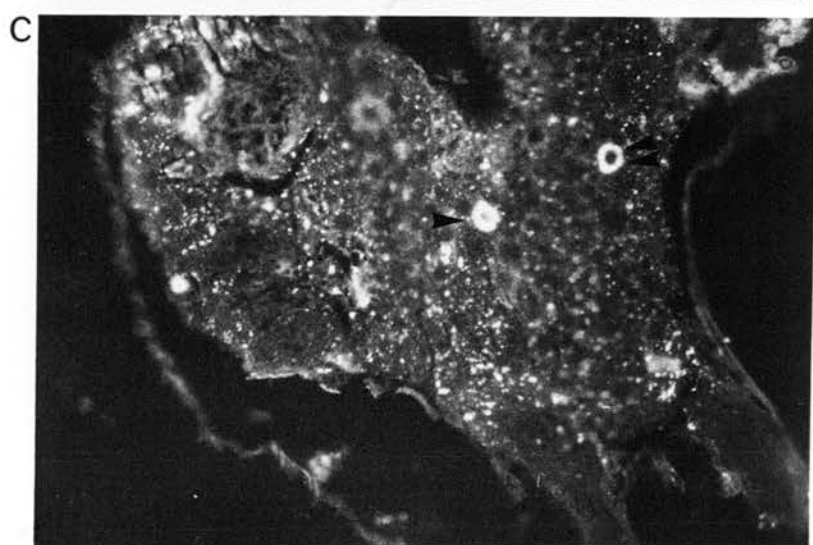
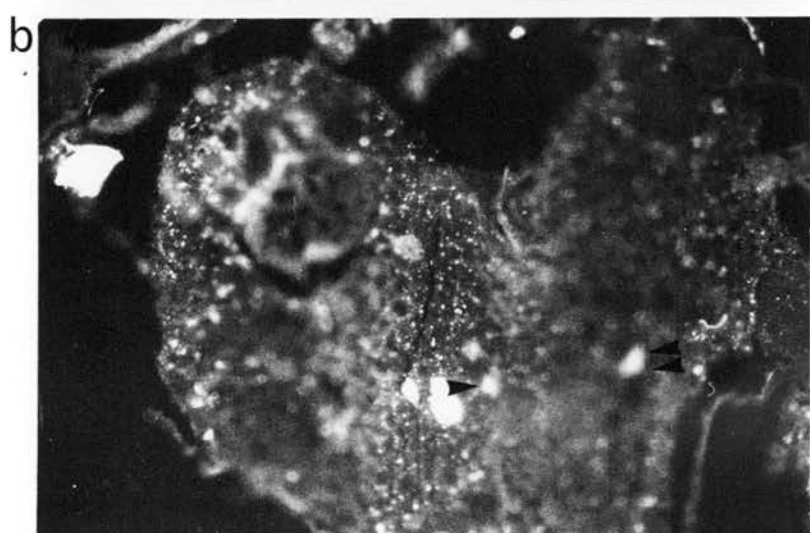
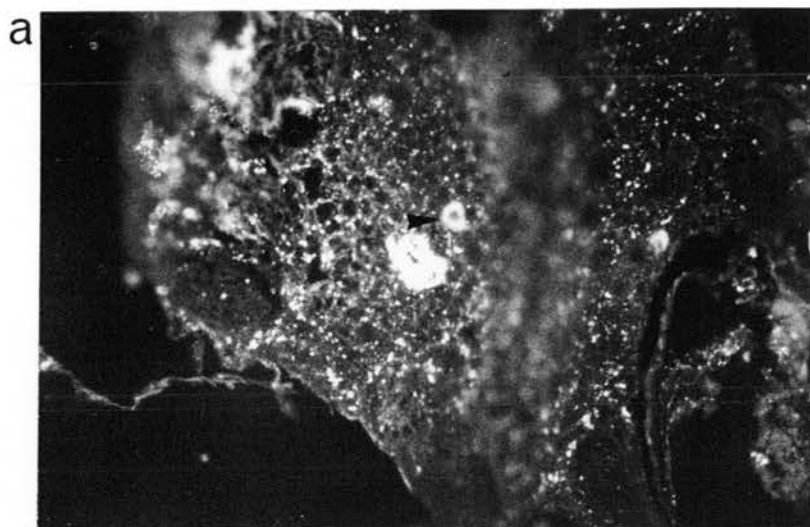
(c) and (d) The cell bodies (CB) of the ganglion. These are wrapped in glial sheaths (GL) and contain large nuclei.



PART 3 SECTION 4 FIGURE 3

Fluorescence histochemistry of the suboesophageal ganglion.

Figures (a), (b) and (c) are adjacent sections through the same ganglion. Two cell bodies are visible on each side of the ganglion midline (arrows). Many of these have a central dark region that does not fluoresce; this is the nucleus of the cell.



100 μ m

PART 4 : CYTOCHEMISTRY

Introduction.

Bland et al (1973) have categorised the transmitter substance of the cockroach salivary gland as a catecholamine, rather than 5-hydroxytryptamine, by microspectrofluorimetry. However, this does not differentiate between noradrenaline and dopamine. Several Swedish workers have developed a technique that employs HCl to modify the fluorophores so that different excitation spectra can be recorded for dopamine and noradrenaline (Eränkö and Eränkö, 1971; Lindvall et al, 1975; Björklund et al, 1968). This technique was applied to the cockroach salivary gland in the hope that it would be possible to demonstrate the presence of dopamine within the axons that innervate it.

Several techniques, used in conjunction with electron microscopy, are now available to demonstrate the presence of catecholamines within the vesicles and endings of axons. These have been reviewed by Hökfelt and Ljungdahl (1972). Potassium permanganate fixative has been used to demonstrate the presence of catecholamines in autonomic nerve endings (Richardson, 1966). Hökfelt and Jonsson (1968) have tested this compound 'in vitro' and claim that it reacts specifically with biogenic monoamines. Similar claims have been made for potassium dichromate fixative (Wood, 1966; Wood and Barnett, 1964). In both cases these compounds form an electron dense precipitate with monoamines in vesicles.

Another method of making the vesicles in monoamine systems electron dense is to incubate the tissue in a solution containing the 'false transmitter' 5-hydroxydopamine, (Tanzer and Theonen, 1967; Coyle and Molliver, 1977). This substance enters nerve endings via the catecholamine reuptake system and is not broken down by the enzymes that are present within the nerve endings. 5-hydroxydopamine reacts with osmium tetroxide to form an electron dense precipitate.

Materials and Methods.

Microspectrofluorimetry was performed with the equipment illustrated in figure 1. This equipment is a modified version of that described by Laszlo (1974). A xenon lamp was used as a source of excitatory wavelengths. The emissions from this were directed through a chopper, into a Zeiss monochromator and then through a diaphragm. The light was then reflected through a mirror into the microspectrofluorimeter (Zeiss). It then passed through a quartz dark field ultracondenser to the plane of a coverslip. A photomultiplier tube (EMI 625B) recorded the spectra of experimental material which could be observed on a galvanometer and recorded permanently with the aid of a Servoscribe pen recorder.

Fluorescence in tissues was located by using a mercury lamp (see Laszlo, 1974) and a X10 objective. A BG3 primary filter was employed along with an SP 50 secondary filter. Fluorescence was observed using the xenon light at 410 nm wavelength and the excitation spectra recorded using a 53 secondary filter.

Test droplets of noradrenaline and dopamine solutions in albumin were prepared to test the sensitivity of the equipment. 1 mg of noradrenaline bitartrate or dopamine hydrochloride were placed in 1 ml of 2% albumin solution. 5 microlitre drops of these were spotted onto slides and dried in a desiccator using silica gel. They were then exposed to paraformaldehyde gas at a relative humidity of 58% at 80°C for an hour. The slides were next placed in Coplin jars that contained HCl and were exposed to HCl vapour for 3 minutes. Tissue was prepared according to the method of Part 2, Section 3 and exposed to HCl vapour in the same way as the droplets. Before the spectra were recorded the tissues were deparaffinised according to the method of Erankö and Erankö (1971).

Tissue to be prepared for permanganate fixation was dissected out in the usual manner (Part 2, Section 1). It was fixed in permanganate according to Richardson's (1966) original method, ie, ice cold 3% KMnO_4 in 0.1 M phosphate buffer at pH 7.0 for 30-45 minutes. Several alternatives were tried, but this method produced the most satisfactory results. The tissue was washed in phosphate buffer and prepared for electron microscopy according to the method of Part 2, Section 2 with the exclusion of the initial glutaraldehyde and osmium fixation.

Dichromate fixative (Wood and Barnet, 1964; Wood, 1966) produces a better result than permanganate fixative as it combines the well tested techniques of glutaraldehyde and osmium fixation with a period of incubation of the tissue in potassium dichromate.

Tissues were dissected out and placed in the shallow fixation dishes (Part 2, Section 1). The fixative was modified from that of Wood and Barnett (1964) as it was felt that the osmolarity of this fixative would have been too great for the cockroach gland. Glands were initially fixed in glutaraldehyde fixative, washed and placed in a solution of 25% potassium dichromate with 1% sodium sulphate in 0.2 M acetate buffer at pH 4.1 at 4°C for 24 hours. The tissue was then washed and osmicated in the usual manner according to Part 2, Section 2.

Glands were also incubated in 100 µg/ml of 5-hydroxydopamine in cockroach ringer for one hour. They were then prepared for electron microscopy in the usual manner except some of the sections were not stained.

Results.

Figure 2 illustrates results obtained from droplets of noradrenaline and dopamine when recorded with microspectrofluorimetry. The percentage ratio of the trough to the peak was averaged over six experiments and for dopamine was found to be 88.75 ± 3.24 and for noradrenaline 48.3 ± 17.7 . There is a clear difference between the excitation spectra of the two amines.

Many attempts were made to record excitation spectra from the tissue itself but these were thwarted by the difficulty of recording spectra from axons of such a small size. This problem shall be discussed.

In figure 3a a typical product of permanganate fixation can be seen. Mitochondria are swollen and the general picture is not as good as those obtained with glutaraldehyde fixative. However, an axon can be seen that possesses small granular vesicles of a circular profile. These vesicles have a mean diameter of 46.48 ± 9.24 nm. Axons that contained small dense vesicles also possess larger more opaque vesicles which have a mean diameter of 98 nm. It seems highly probable that this type of axon is a type A axon. The change in shape of the small vesicles shall be discussed below. In figure 3b in addition to a type A axon there is another axon with large opaque vesicles which have a mean diameter of 139 ± 21.57 nm. A t-test has shown that there is no significant difference between these vesicles and the large granular vesicles of type B axons. Figure 3c illustrates another of these structures showing that they do not form a dense precipitate with permanganate fixative. It seems from these results that the small vesicles of type A axons react with permanganate to form a dense precipitate, whereas the larger vesicles of type B axons do not. It can be concluded that type A axons contain a biogenic monoamine.

Axons containing small dense vesicles were observed in similar positions to those type A axons that have been described in Part 3 (see figure 6, Part 3, Section 3). They have been observed in the basement membrane region in proximity to peripheral cells (figure 3a) and to central cells (figure 4a). Figure 3d is an axon that has small dense vesicles between two peripheral cells. This axon, like axons in a similar position viewed after glutaraldehyde fixation, has

no glial sheath and is no more than 20 nm away from the membrane of the peripheral cell. These axons have also been seen in the nerves which travel between the acini (figure 4b). Again the axon containing vesicles is on the periphery of the nerve.

The fixation obtained with dichromate was never very satisfactory but some positive results were obtained. Small granular objects were noted in type A axons (figure 4c) which at higher power were seen to be the small elliptical vesicles of such axons. Type B axons were not affected by this process (figure 4d).

Tissue that was incubated in 5-hydroxydopamine exhibited axons with small granular vesicles which were elliptical in shape. These axons also possessed larger granular vesicles and were consistent with type A axons.

Some of the sections were stained with lead citrate and uranyl acetate (figure 5a and b). Figure 5a shows a nerve that consists of several axons one of which contains many elliptical vesicles that have been filled with 5-hydroxydopamine. These vesicles were found to have an average dimension of 40 nm. Figure 5b shows two axons labelled with 5-hydroxydopamine in the basement membrane of an acinus, proximal to a peripheral cell. Some of the larger granular vesicles may be seen in these cells. Such axons are also found proximal to central cells (figure 5c and d). Axons of this type, free of any glial surrounding, were again noted between the cells of acini (figure 5e) and also within the nerves which cross from acinus to acinus (figure 5f). Type B axons do not appear to be affected by

incubation in 5-hydroxydopamine; figure 5g illustrates a type A axon and a type B axon running in close proximity within the basement membrane region of an acinus.

It was decided to compare the shapes of the small vesicles in type A axons after these various treatments with tissue that has been fixed in glutaraldehyde. Figure 7 shows three log ratio distributions : one for glutaraldehyde fixation (figure 7a); one for permanganate fixation (figure 7b); and one for 5-hydroxydopamine. These graphs illustrate that permanganate fixed vesicles are circular in their profile as the distribution tends towards zero, whereas the glutaraldehyde fixed and 5-hydroxydopamine filled vesicles (which have also been fixed in glutaraldehyde) are similar in their distribution and are more spread out. This may mean that permanganate fixative changes the shapes of type A agranular vesicles or that glutaraldehyde produces vesicles which are elliptical in profile.

Discussion.

From the results using dopamine and noradrenaline droplets it is clearly possible to differentiate between their excitation spectra. Unfortunately, this method was not technically possible when applied to measuring the spectra of small fluorescent axons in the cockroach salivary glands. In the literature (Björkland *et al* 1968, etc) areas for recording excitation spectra were $800 \mu^2$, the varicose areas of the cockroach salivary gland were roughly 2μ in diameter which would correspond roughly to a $7 \mu^2$ area.

Therefore, the area that the recording was attempted from was roughly 1/200 of that used by the Swedish workers. A specialised technology may have to be devised to record from such small areas. One possibility may be to try and repeat the experiment performed by Erankö and Erankö (1971). They attempted to measure the change in intensity of fluorescence before and after exposure to HCl vapour and found that dopamine and noradrenaline have different rates of fading. However, the same fluorescent axons would have to be positioned under the microspectrofluorimeter in exactly the same location twice in order to record the intensity of fluorescence before and after exposure to HCl. This again could prove to be technically difficult because of the size of the axons. It is unfortunate that it was not possible from the microspectrofluorimetry experiments to show that the axons contained dopamine. However, it is safe to conclude on the strength of the permanganate, 5-hydroxydopamine and dichromate experiments that type A axons contain catecholamines and there is no evidence of any kind that associates type B axons with catecholamines. The profiles produced by permanganate fixative are similar to those observed in the rat brain (Hökfelt, 1968), axons of the silk moth (Robertson, 1974) and in the brain of *Periplaneta* (Mancini and Frontali, 1970). All of these authors commented upon the poor quality of permanganate as a fixative.

The results obtained with dichromate were disappointing although the few type A axons observed had dense deposits within their vesicles. After this process much of the tissue appeared granular

and features were difficult to identify. This may well indicate that this method is not suitable for insect tissue in its present form; previous experiments with it have mainly been performed upon the mammalian CNS (Wood, 1966; Wood and Barnet, 1964).

Results with 5-hydroxydopamine were good and again confirmed the suspicion that type A axons contained catecholamines. The profiles observed were similar to those of Coyle and Molliver (1977) in the new-born rat cortex. In their experiments they were also able to deplete nerve endings of 5-hydroxydopamine by giving their animals injections of reserpine (see Part 7).

A curious result is the observation that permanganate fixation results in the production of round shaped vesicles in type A axons whereas glutaraldehyde and osmium fixation present elliptical profiles. Both 5-hydroxydopamine-filled vesicles and those that were reacted with dichromate were elliptical; these methods use glutaraldehyde fixation. There is no evidence to suggest that dopamine is stored in vesicles of an elliptical shape (eg, see Pentreath and Berry, 1975), but Van Orden et al (1966) claimed that dopamine-containing vesicles were asymmetrical. It was also seen in Section 4 of Part 3 that glutaraldehyde fixative did not affect circular vesicular profiles in the suboesophageal ganglion. Finally, as has been stated several times, it is known that permanganate is a poor fixative so that the differences may be explained by this observation. Evidence shall be presented in Parts 6 and 7 which indicate that the shapes of vesicles may be

important. Finally a note of caution : E G Gray (1977) has stated that fixatives may result in many artefacts of the vesicles in cholinergic nerve endings.

PART 4 FIGURE 1

Equipment for measuring excitation spectra

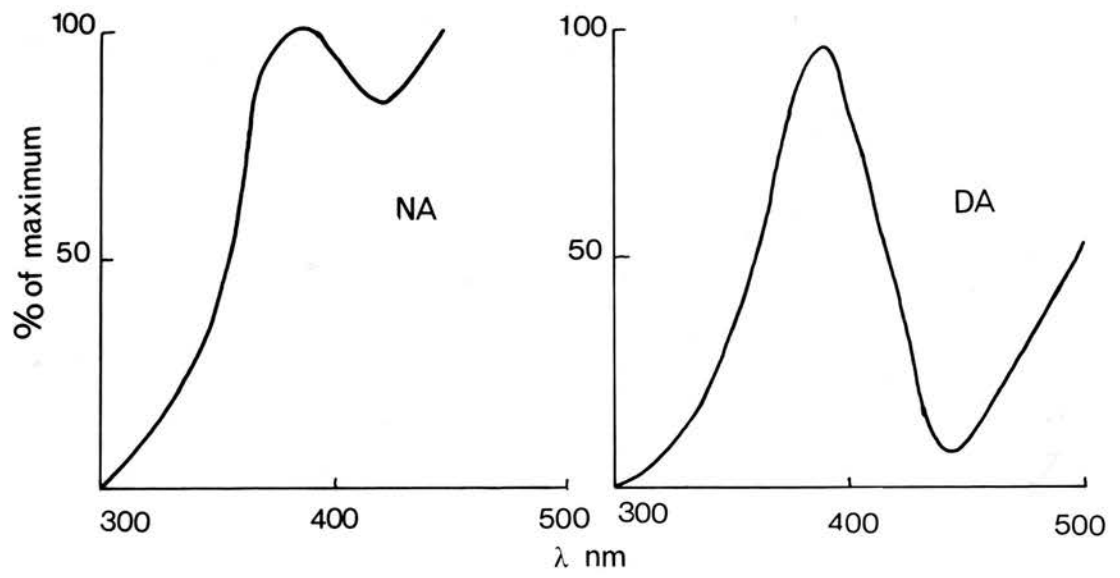
1. control box for xenon lamp
2. xenon lamp
3. monochromator
4. Zeiss microscope
5. photomultiplier tube
6. galvanometer
7. pen recorder



PART 4 FIGURE 2

Excitation spectra for droplets of noradrenaline (NA) and dopamine (DA). Note the differences between the troughs.

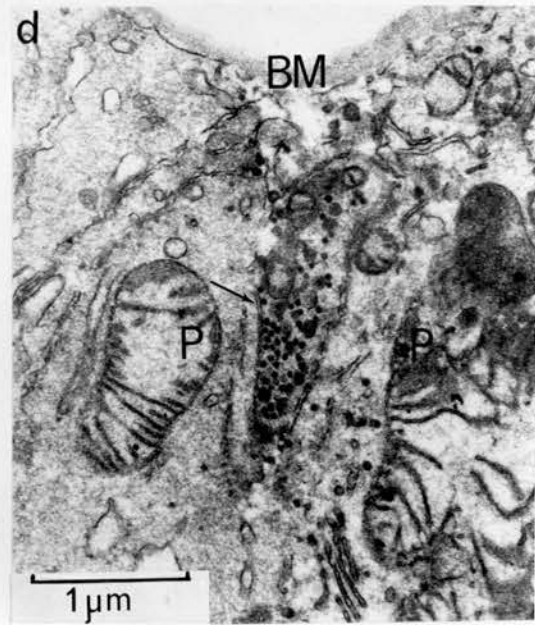
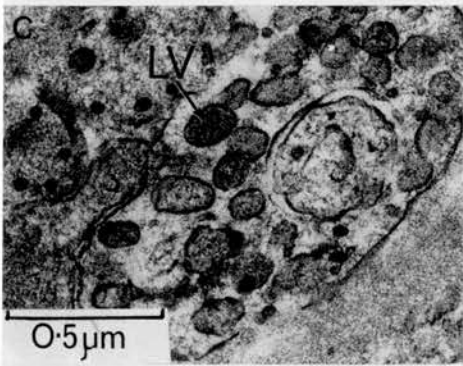
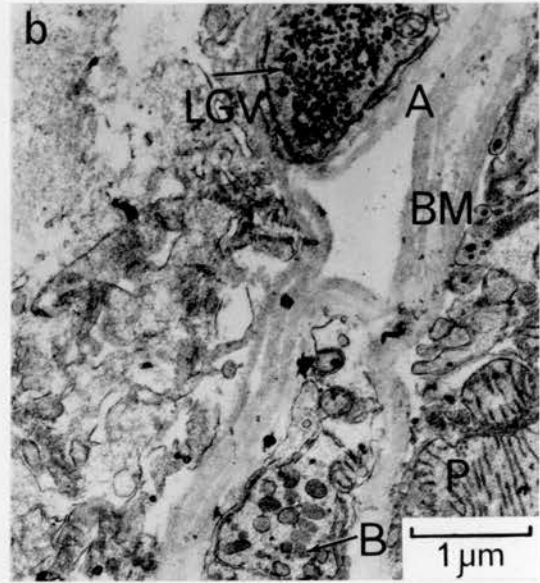
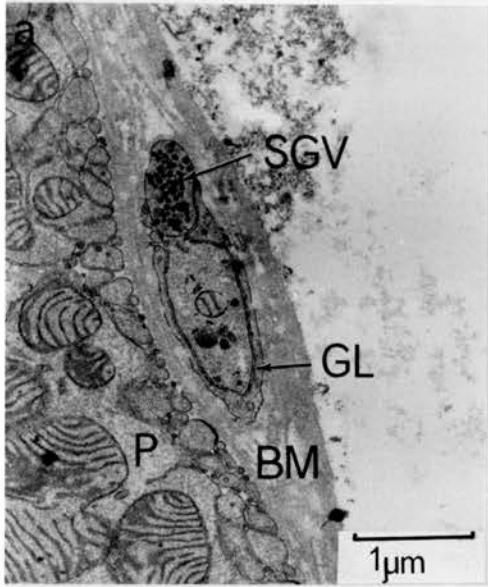
Excitation Spectra



PART 4 FIGURE 3

Permanganate fixation

- (a) An axon in the basement membrane (BM) region of an acinus which contains small granular vesicles (SGV). (GL - glial cell, P - peripheral cell).
- (b) A type A and B axon. Note that the type A axon also contains large granular vesicles (LGV). (BM - basement membrane).
- (c) This figure shows that type B axons do not react with permanganate to form a dense precipitate. (LV = Large vesicles)
- (d) A type A axon between two peripheral cells (P). The distance between the two cells at the arrow is 20 nm (BM - basement membrane).



PART 4 FIGURE 4

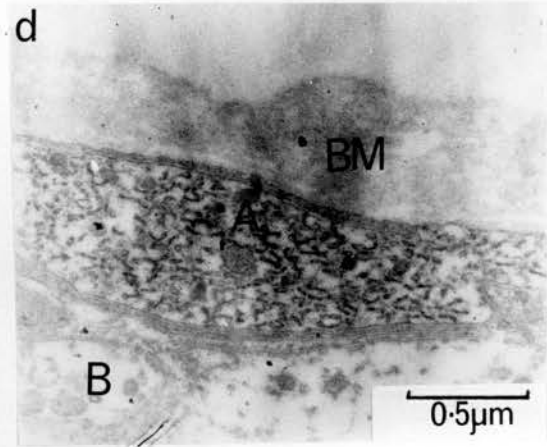
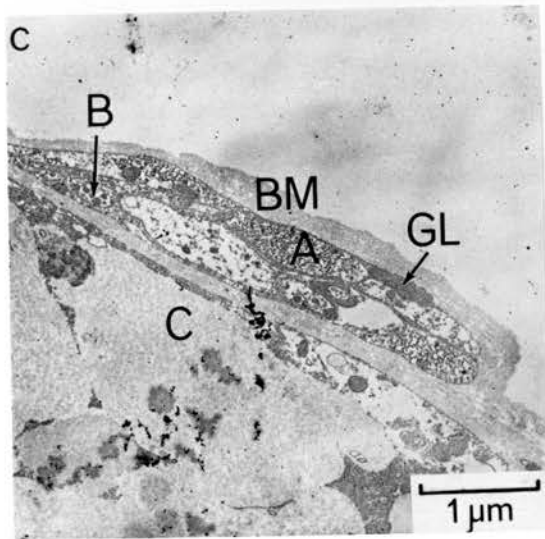
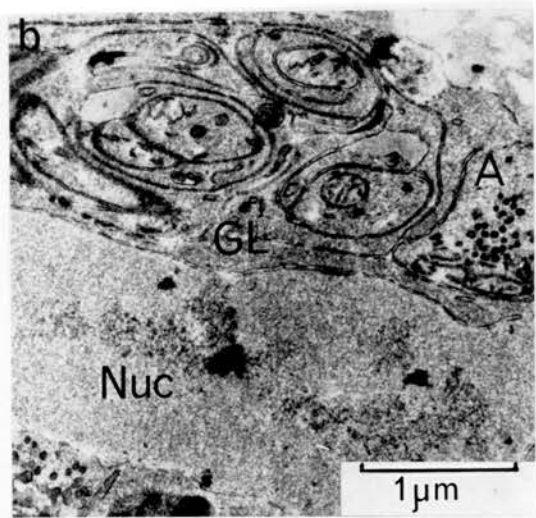
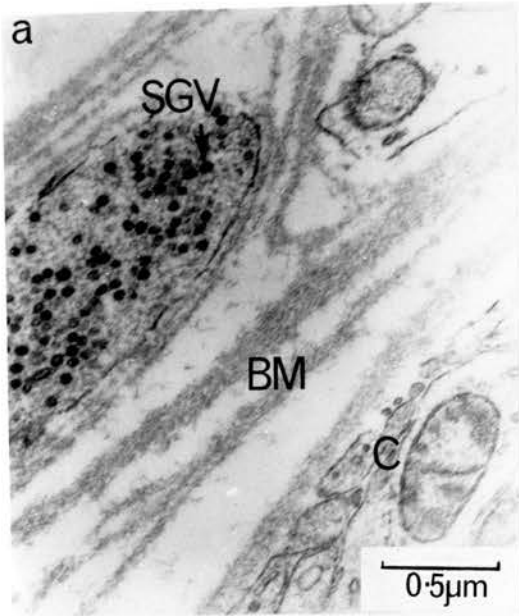
Permanganate and dichromate fixation

(a) Is a permanganate-fixed type A axon in the basement membrane (BM) region of an acinus, close to a central cell (C) (SGV - small granular vesicle).

(b) A section through a nerve in the vicinity of an acinus in which a type A axon is visible (GL - glial cell, Nuc - Nucleus). (Permanganate fixation.)

(c) Dichromate fixed tissue. Several type A axons are visible in this section, which have electron dense vesicles (B - type B axon, C - central cell, GL - glial cell, BM - basement membrane).

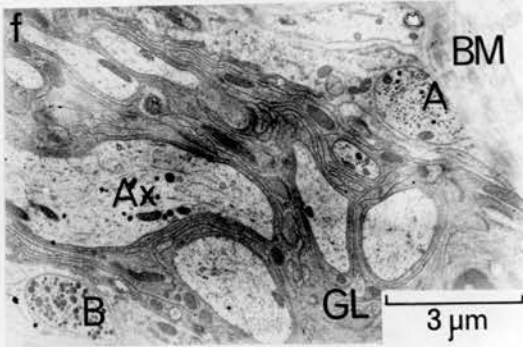
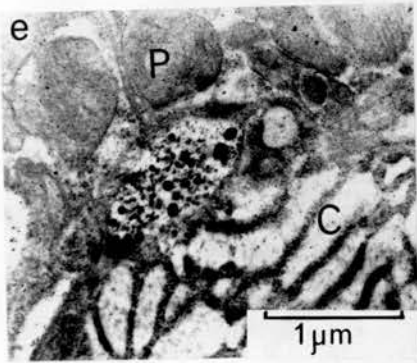
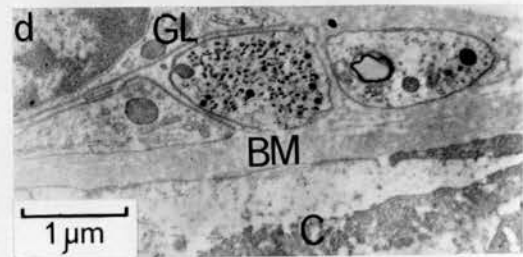
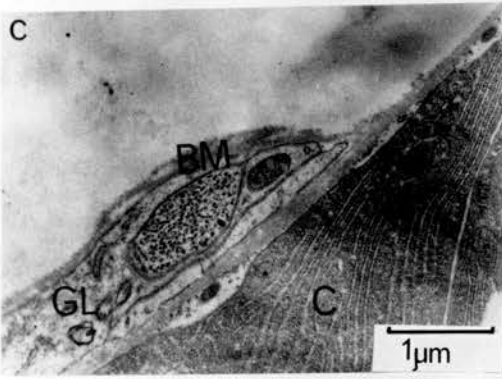
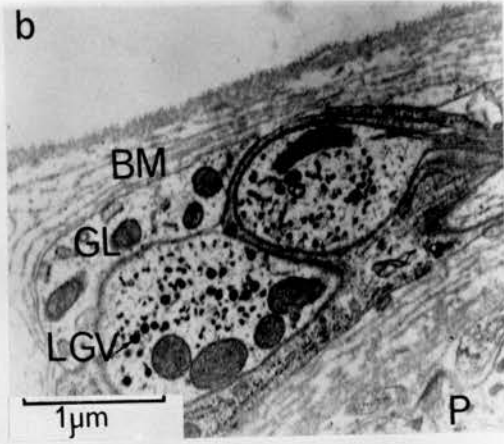
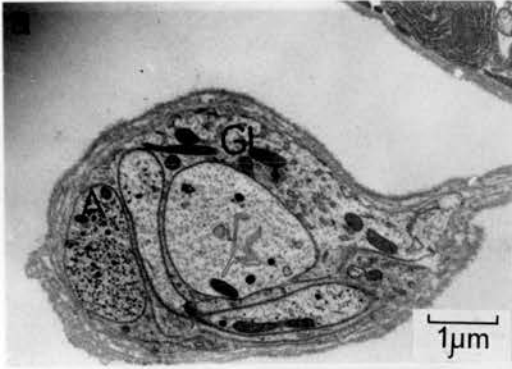
(d) This is an enlargement of part of figure (c) which shows that the small elliptical vesicles of type A axons contain electron dense material. A type B axon is visible in the corner of this photograph (BM - basement membrane).



PART 4 FIGURE 5

5-hydroxydopamine incubation

- (a) A section through a nerve which contains a type A axon with dense vesicles after incubation with 5-hydroxydopamine (GL - glial cells).
- (b) Two type A axons in the basement membrane (BM) of an acinus (P - peripheral cell, GL - glial cell, LGV - large granular vesicle).
- (c) An unstained section illustrating a type A axon (GL - glial cell, BM - basement membrane, C - central cell).
- (d) A section similar to (c) (unstained) (GL - glial cell, BM - basement membrane, C - central cell).
- (e) A type A axon between a central cell (C) and a peripheral cell (P) (unstained).
- (f) A nerve which contains a type A axon and a type B axon (BM - basement membrane of the nerve, GL - glial cell, Ax - axon) (unstained).
- (g) A type A and type B axon. The type B axons were not affected by incubation in 5-hydroxydopamine (unstained).



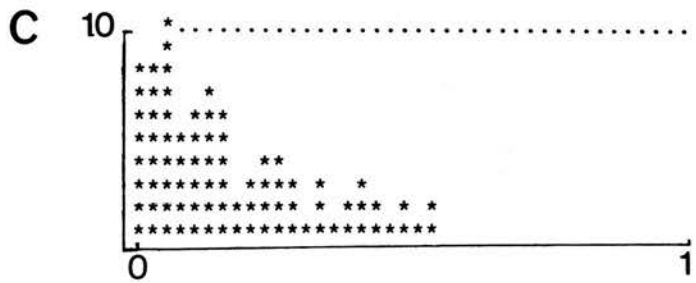
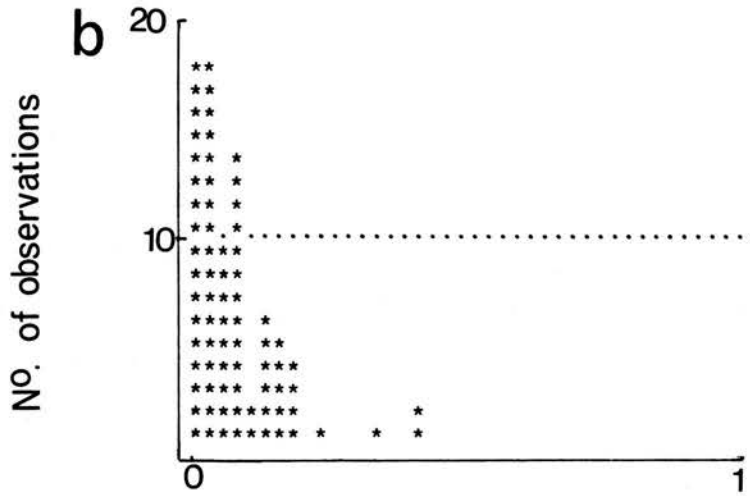
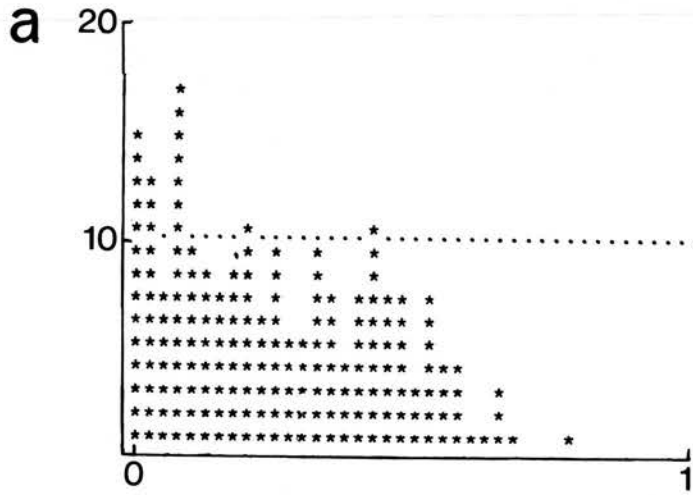
PART 4 FIGURE 6

Histograms of the logarithm of the maximum to minimum dimensions of the small vesicles of type A axons. Each histogram was constructed from data obtained from several different axons.

(a) A distribution of log ratios after glutaraldehyde and osmium fixation.

(b) A distribution of log ratios after permanganate fixation.

(c) A distribution of log ratios after incubation in 5-hydroxydopamine.



Log Ratio $\frac{\text{max}}{\text{min}}$

PART 5 : NERVE DEGENERATION

Introduction.

The cockroach salivary apparatus is a paired structure. The work of Bowser-Riley (1978a) and evidence presented in this thesis (Part 3, Section 3) shows that each of the reservoir ducts have nerves on their surfaces which go to the acini and branch profusely to innervate the acini. There is also considerable evidence to show that each of these nerves innervates mainly the ipsilateral side of the gland. In 1956 de Robertis used nerve degeneration as an anatomical tool for tracing neuronal pathways in the auditory system of the guinea-pig. It was decided to cut the nerve on one side of the gland only and to use the other side as a control. In this way it was hoped to discover the origins of type A and type B axons and also to see the extent that a single salivary nerve innervates the glands. It may also be the case that branches of the stomatogastric nerve innervate the acini (Willey, 1956; Whitehead, 1971; Bowser-Riley, 1978a). In an attempt to discover the extent to which the stomatogastric nerve is involved with acini it was decided also to allow this nerve to degenerate.

6-hydroxydopamine is known to destroy nerves that contain catecholamines (Berry et al, 1974; Wadsworth, 1973). It reduces the level of paraformaldehyde-induced fluorescence and increases the amount of electron dense material in electron micrographs (Bennet et al, 1970). It has also been claimed that 6-hydroxydopamine results in the production of dense cores in vesicles,

similar to those of 5-hydroxydopamine (Ajika and Hokfelt, 1973).

Materials and Methods.

10 experiments were performed involving surgical denervation of cockroach glands. Cockroaches were immobilised with hypothermia and placed under a Zeiss dissecting microscope with their ventral surface uppermost; a small longitudinal incision was made in the 'throat' of the animal with a piece of blue Gillette razor blade held in a pin tong. The reservoir duct was hooked out of this incision with a syringe needle with a slight bend in its tip. Once the reservoir duct had been identified it was severed, along with the salivary nerve. Only one duct and nerve were cut in each animal (cf Sutherland, 1962, who cut both ducts and nerves). Instruments were bathed in absolute ethanol between experiments to reduce the possibility of infection. Cockroaches were left for 7, 14 and 28 days after the time of operation. They were dissected in the usual way (Part 2, Section 1) except that great care was taken to identify the cut duct. The glands and ducts were then separated down the midline and placed in different dishes for fixation (Part 2, Section 2).

The stomatogastric nerve was also cut in a similar manner except on this occasion the gut was partially pulled through the incision with the syringe needle hook until the stomatogastric nerve could be identified and severed.

The survival rate of cockroaches after this procedure was low but some lived for two weeks and were dissected. Out of these only two could be positively identified as having severed stomatogastric nerves. Glands from these cockroaches were prepared for electron microscopy in the usual manner.

Cockroach salivary glands were also dissected and incubated in 4×10^{-3} M solution of 6-hydroxydopamine (Sigma) in cockroach ringer for 2 to 4 hours (see Berry et al, 1974), and were then prepared for electron microscopy (Part 2, Section 2) or for stretch preparations for the Falck-Hillarp fluorescence histochemistry method (Part 2, Section 3). Control glands were incubated in cockroach ringer solution for the same periods of time. It was also attempted to inject solutions of 6-hydroxydopamine into cockroaches but no results were obtained from these experiments.

Results.

In three out of the ten preparations where the salivary nerve was successfully cut on one side only it was noted on the experimental side of the gland that the acinar tissues had become opaque and contained red nodules, whereas the unoperated acini had a fairly normal appearance. Such tumours bear a remarkable resemblance to those reported by Sutherland (1962 and 1966) after denervation of the salivary nerves of *Periplaneta*. He also observed nodules which he claimed were inclusion cyst material. In figure 1a one of these pieces of tissue is illustrated; note the striking

differences between this piece of tissue and its opposite number from the unoperated side of the gland (figure 1b). In this tissue the salivary nerve was cut one month before. The only abnormality noted in the tissue from the unoperated side of the gland is an unusual number of blood cells surrounding the tissue. If tissue from glands that produced tumours was examined under the electron microscope it was usually not possible to identify peripheral or central cells (figure 1c). Cells resembling blood cells were present in great quantities and had vacuoles which probably contained the remains of acinar cells. Sutherland (1966) has also observed this form of haemocytic encapsulation. No relationship between tumour formation and time allowed for degeneration of the nerves could be determined.

In most cases when the nerve was cut tumours did not form but the ultrastructure of some cells appeared to be abnormal (figure 1d). Central cells became more electron dense and seemed to lose their endoplasmic reticulum. Also many vacuole-like objects were observed in their cytoplasm. On the other hand, peripheral cells had a comparatively normal appearance.

After the salivary nerve was severed and allowed to degenerate the two large axons were seen to form an amorphous mass of electron dense material (figure 2a). Many of the smaller axons found within the basement membrane region of the nerve did not seem to degenerate. The uncut nerve (figure 2b) was usually also fixed and embedded and was always normal in every respect when compared with its degenerate neighbour.

TYPE	DEGENERATE			
	A	B	CLEAR	NOT IDENTIFIABLE
NUMBER OF AXONS	5	7	20	12

TYPE	NORMAL			
	A	B	CLEAR	NOT IDENTIFIABLE
NUMBER OF AXONS	7	0	9	0

TABLE 1 Numbers of degenerate profiles compared to normal profiles identified in the operated side of glands from several experiments

When the axons that invest the salivary glands were examined after degeneration was allowed to occur, some surprising results were obtained. Firstly many completely normal axons were observed in the experimental side of the gland alongside axons which were obviously in a state of degeneration. The second observation was that no pattern of degeneration could be determined that was related to time. Both type A and B axons were seen to degenerate but one of the difficulties is that often degenerating axons no longer have any resemblance to their original form. Table 1 summarises the results obtained from the degeneration experiments. Only one degenerating axon was noted in the unoperated side of the glands. This result is slightly surprising as there is some electrophysiological evidence to suggest that there is a limited amount of contralateral innervation of the gland (Ginsborg and House, 1976).

The most obvious feature of a degenerating axon is an increase in electron density of the cytoplasm followed by a gradual break-up in vesicular material (figure 3a). Several axons were found to contain 'bull's eye' vesicles, which according to Glees and Hassan (1968) is a characteristic of synaptic degeneration (figure 3b). Another common feature of degenerating axons is that they are often surrounded by lamellated bodies (figure 3c and d). These were also observed by Garrett and Thulin (1975a), after denervation of the sympathetic nerve supply to the parotid glands of the rat.

Type B axons were seen to lose their electron dense cores during the process of degeneration (figure 3d, figure 4b), as well as accumulating electron dense material in their cytoplasm. Large quantities of amorphous electron dense material was often observed accumulating in the nerves which cross from acinus to acinus (figure 4a).

After the stomatogastric nerve was cut most glands and axons appeared to be normal in every respect. Only one degenerating axon could be found (figure 4c).

The results obtained after incubating glands in 6-hydroxydopamine for 2-4 hours were much more clear than those when the glands were surgically denervated. Virtually every type A axon observed showed some signs of degeneration, whereas type B axons were not affected. The signs of degeneration after 6-hydroxydopamine are similar to those of cut axons; much amorphous electron dense material accumulates within the axon cytoplasm (figure 5a) and the vesicles have a granular appearance (figure 5b and c). Many of the vesicles became dense cored as was also noted by Ajika and Hokfelt (1973). After four hours exposure to 6-hydroxydopamine, axons contained small vesicles which appeared to be coalescing (figure 5d and e). This is a typical phenomenon presented by degenerating axons (Smith et al, 1966). Large granular vesicles of type A axons do not seem to be affected in this way (figure 5f and g). Tissue incubated in 6-hydroxydopamine for four hours did not respond to paraformaldehyde-induced fluorescence whereas tissue incubated in

ringer solution for the same period of time did. These results indicate that type A axons contain catecholamines.

Discussion.

The formation of tumours after the salivary nerves were cut was an unexpected result and in some ways interfered with the original aim of this study; axons were difficult to find amongst the mass of encapsulated material. The mechanism of this tumour formation is unknown but it seems reasonable to assume that the nerves that innervate the gland exert some influence that maintains the cells of the gland. It is also not understood why this phenomenon was only observed in a few of the operated glands as there is plenty of evidence to show that axons also degenerated in glands that did not form tumours.

After the salivary nerves were cut the two large axons of the nerves degenerated. However, there was also evidence to demonstrate that some of the axons associated with the nerve were not affected by this procedure; it is possible that these axons do not arise from the same system as the larger axons. Not all of the axons associated with the acini degenerate after denervation of the glands. There may be several possible explanations for this : (i) that the salivary nerves are not the only source of innervation of the acini. For example, Bowser-Riley (1978a) has claimed that the stomatogastric nerve gives out branches to the acini. Kushner and Maynard (1977) have also shown that the stomatogastric nerve of the

lobster fluoresces after paraformaldehyde treatment and therefore may contain catecholamines. On the other hand this study has shown that cutting the stomatogastric nerve does not result in the production of degenerating axon profiles within the acini of the gland. Secretion studies have also indicated that the stomatogastric nerve, when stimulated, does not elicit secretion from the salivary glands (Smith, unpublished observation). (ii) it is possible that there is a considerable amount of crossover between the left and right salivary nerves. Ginsborg and House (1976) have indicated that electrophysiological experiments showed some evidence of contralateral innervation of the gland. However, tumours do not develop on the contralateral side of the gland and also only one degenerating axon was observed on the unoperated side. (iii) that the axons do not degenerate quickly. Further studies would be required to investigate this. (iv) that the axons regenerate quickly; again further investigation is required. It is not possible at this stage to decide upon any of the four options given above.

6-hydroxydopamine destroyed most of the type A axons observed after 2-4 hours. After two hours most axons contained dense amorphous material and had granular material within the small axons. After four hours the vesicles of many of the axons had coalesced. 6-hydroxydopamine also abolishes the paraformaldehyde-induced fluorescence of the axons. This evidence and the electron microscopical evidence cited above directly links type A axons with the fluorescent catecholamine containing axons of Bland et al (1973).

PART 5 FIGURE 1

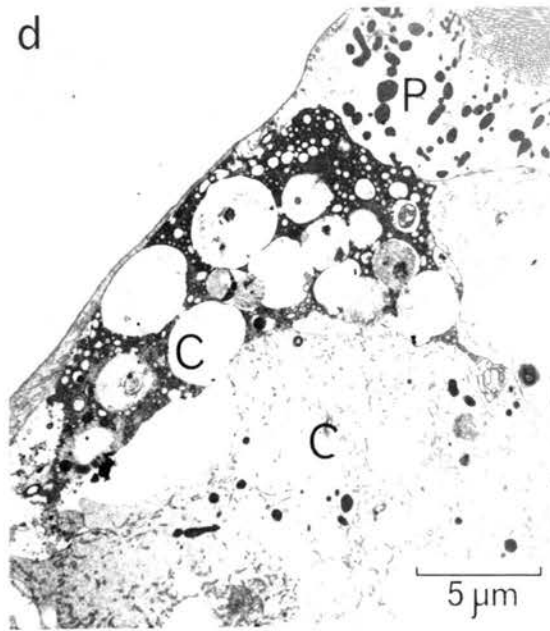
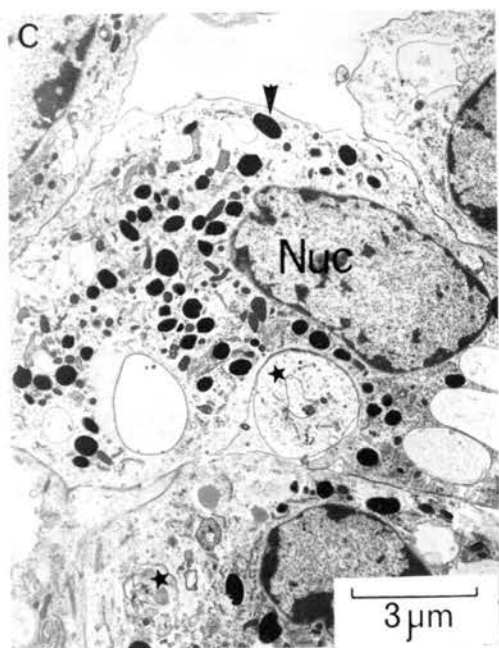
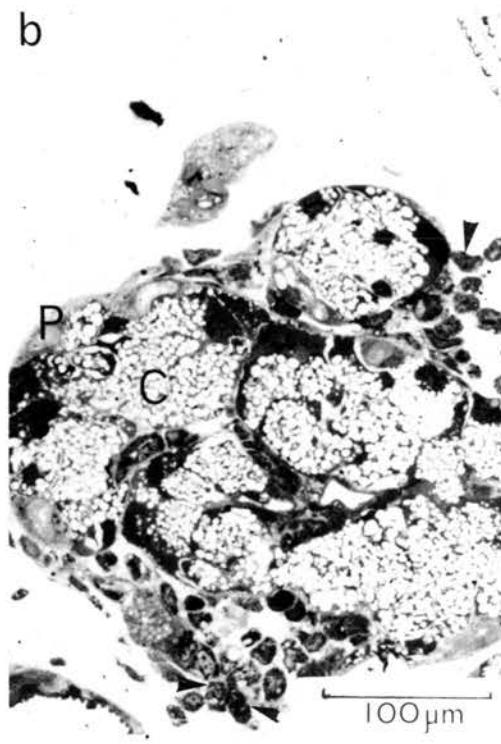
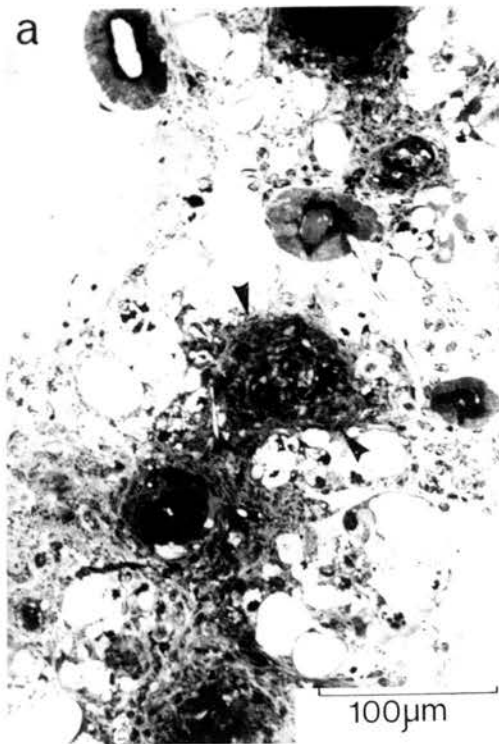
Tissue changes after denervation

(a) A section from a denervated gland one month after denervation. Note the unusual appearance of the tissue, and the absence of well defined cell boundaries. The arrows point to some inclusion cyst material.

(b) A section from the contralateral gland in the same animal as figure (a). The appearance of the cells is normal apart from an unusual number of haemocytes at the periphery of acini (arrows) (P - peripheral cell, C - central cell).

(c) This is an electron micrograph of some of the tissue from figure (a). Some haemocyte-like cells with large nuclei (Nuc) and granules (arrow) are visible. These cells seem to have encapsulated some material (stars) which is perhaps the remains of acinar cells.

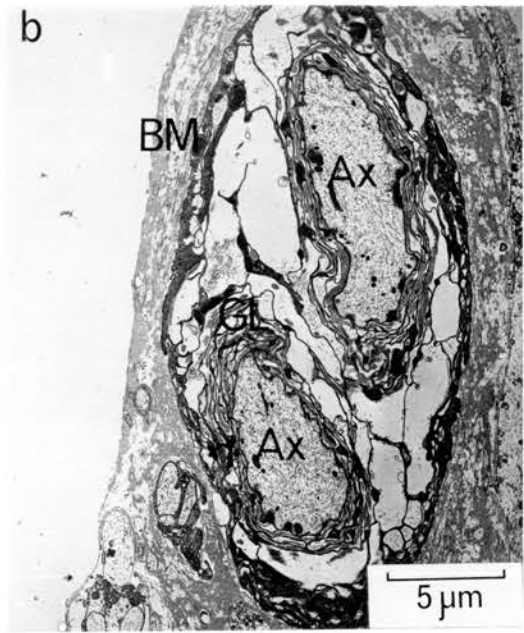
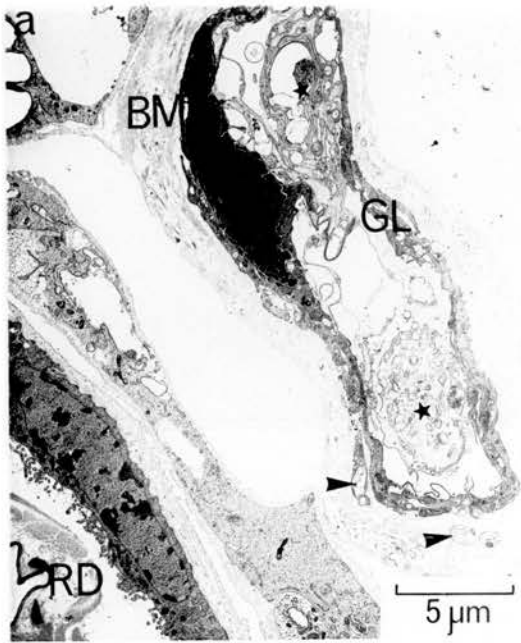
(d) Tissue two weeks after denervation. In this gland peripheral cells (P) had a fairly normal appearance, whereas central cells (C) were abnormal.



PART 5 FIGURE 2

Degeneration of the salivary nerve.

Figure (a) is a salivary nerve that was severed one month before dissection. The two large axons have completely degenerated (stars) whereas several small axons in the basement membrane region (BM) of the nerve (arrows) did not degenerate (RD - reservoir duct, GL - glial cells). Figure (b) is the uncut nerve on the contralateral reservoir duct in the same animal as figure (a). The two axons (Ax) are normal (BM - basement membrane of the nerve).



PART 5 FIGURE 3

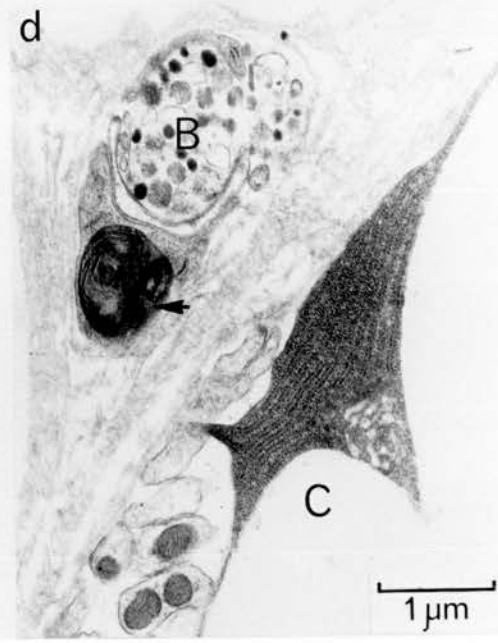
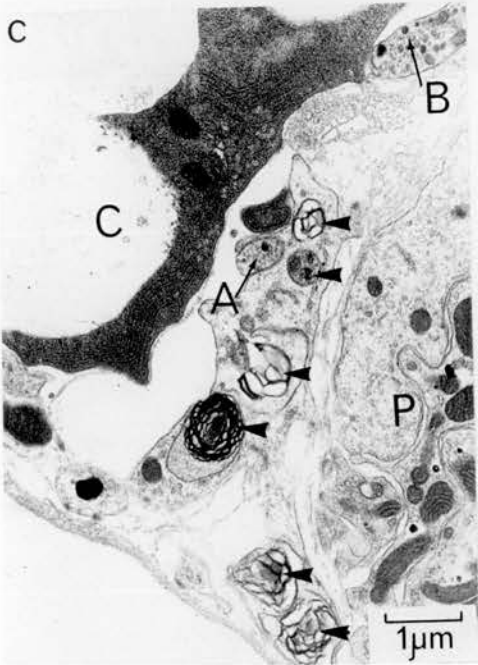
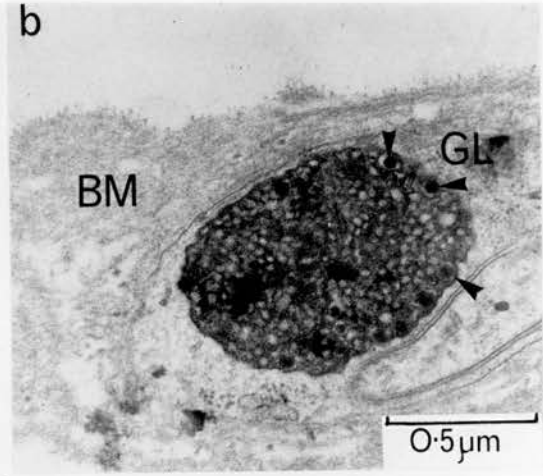
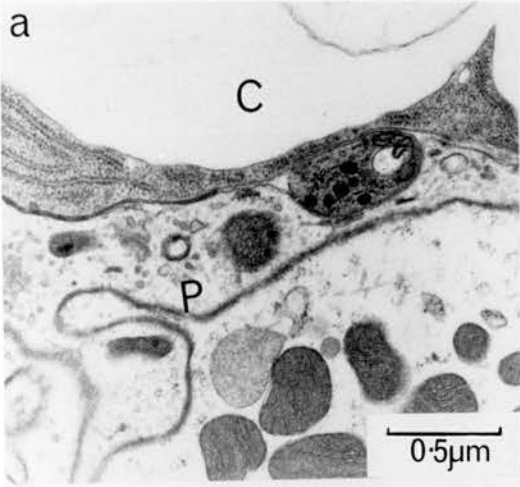
Degenerating axons

(a) This is a type A axon between a peripheral (P) and a central cell (C). The nerves supplying this gland were cut a week before the tissue was fixed. Note the unusual density of the cytoplasm.

(b) This axon was also allowed to degenerate for a week. Note the granular appearance of the cytoplasm and the large aggregation of 'bull's-eye' vesicles (arrows) (BM - basement membrane, GL - glial cell).

(c) Several degenerating profiles two weeks after denervation (arrows). A type A axon and a type B axon are identifiable (P - peripheral cell, C - central cell).

(d) A type B axon and a degenerating profile two weeks after denervation. The degenerating profile (arrow) has a lamellated structure; the type B axon seems to have lost much of the electron density of its granules (C - central cell).



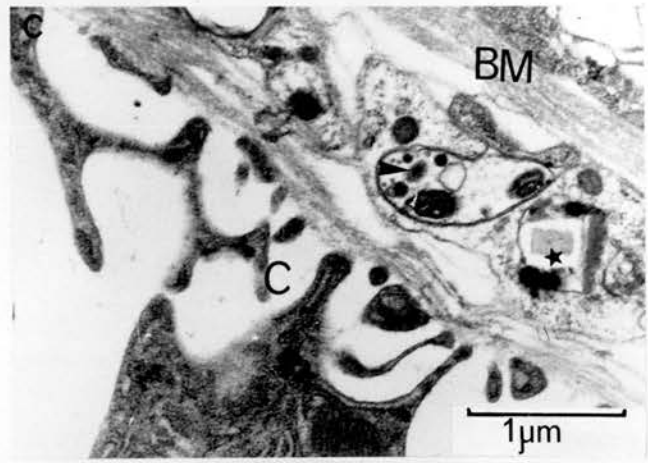
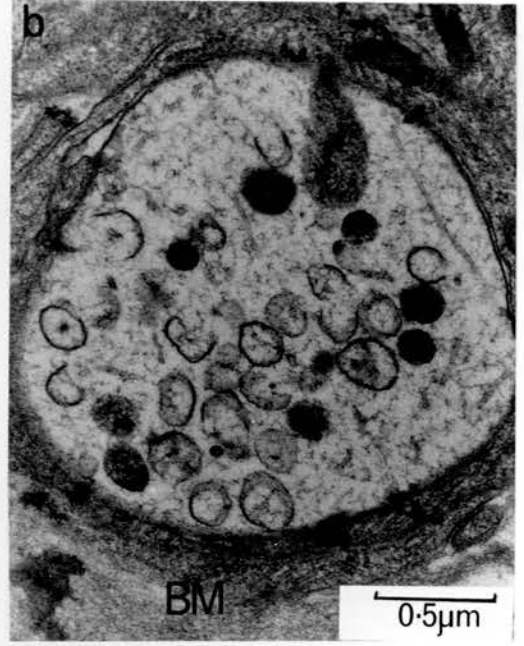
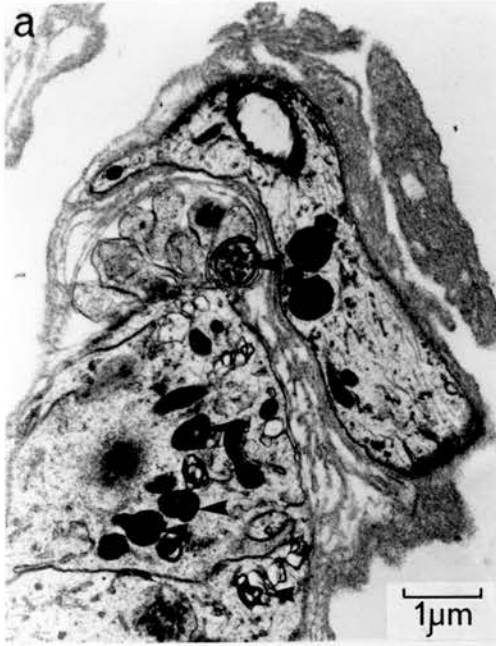
PART 5 FIGURE 4

Degenerating axons

(a) A section through a degenerating nerve two weeks after denervation. Note the many dense degenerating profiles (arrows).

(b) A type B axon two weeks after denervation. This axon again seems to have lost much of the electron density of its granules.

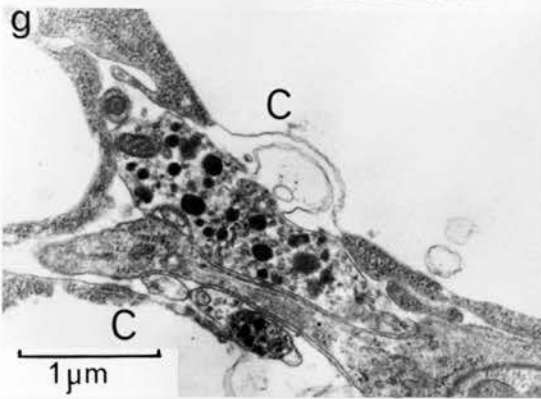
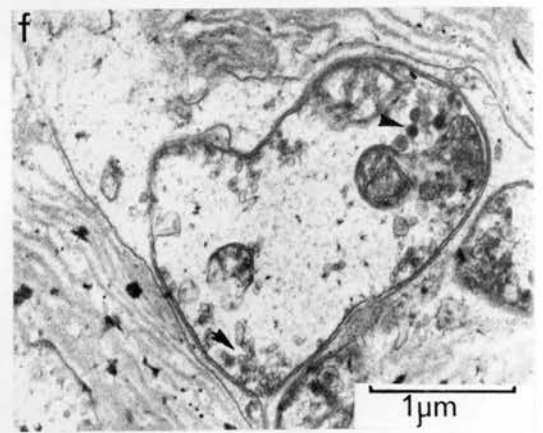
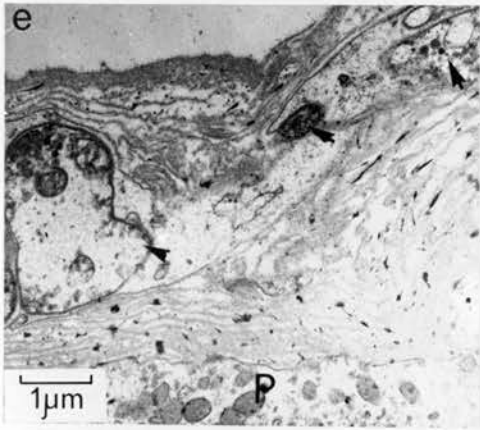
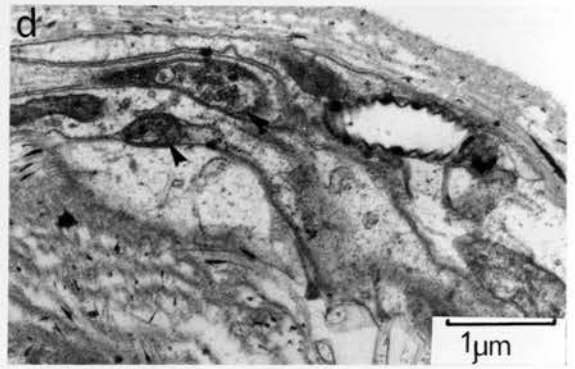
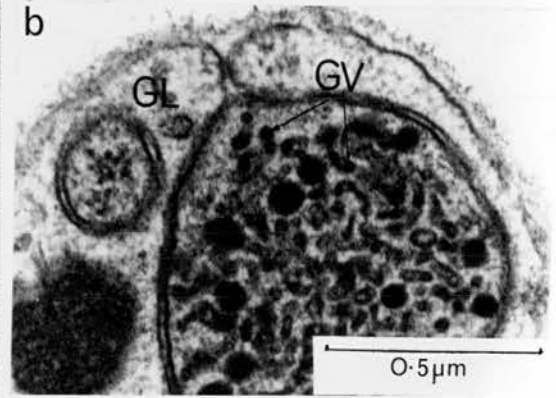
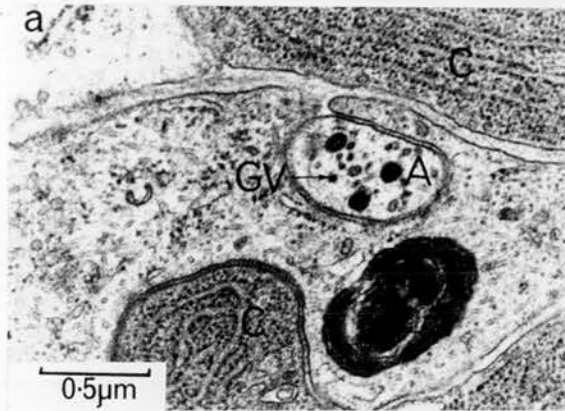
(c) Two weeks after the stomatogastric nerve was cut one possible example of degenerating axon profiles was noted (star). The arrow also indicates the possible accumulation of amorphous material within another axon.



PART 5 FIGURE 5

Degeneration after incubation in 6-hydroxydopamine

- (a) A type A axon that has been exposed to 6-hydroxydopamine for two hours. Some of the vesicles have become granular after this exposure (GV) and the cytoplasm of the axon contains dense amorphous material. There is also another profile in this picture which has become completely electron dense (C - central cell).
- (b) A type A axon in a nerve that has been exposed to 6-hydroxydopamine for 2 hours. Note the dense granular appearance of the cytoplasm and the granular cores of some of the vesicles (GV) (GL - glial cells).
- (c) An axon similar to (b).
- (d) Profiles after four hours of incubation (arrows).
- (e) As (d).
- (f) An enlargement of an area of (e) showing that the vesicles seem to be coalescing.
- (g) A type A axon between two central cells (C) after four hours exposure. Much of the cytoplasm of the axons seems to have degenerated.



PART 6 : STIMULATION OF THE SALIVARY NERVES

Introduction.

The effects of stimulating the salivary nerves of the cockroach have been reviewed in Part 1. Both electrical potentials (House, 1973) and secretion (Smith and House, 1977) may be elicited by stimulation of the nerve. Perfusion with dopamine also produces the same results except that the transient hyperpolarisation lasts for very much longer. Ginsborg et al (1974) have shown that the transient hyperpolarisation may arise as a result of an increase in membrane conductance to potassium ions. A recent study has also shown that secretion is dependent upon the presence of sodium in the external medium (Smith and House, 1979). It was decided to examine the morphology of the cockroach gland after stimulation and to determine if any morphological changes could be seen such as those observed by Diamond and Tormey (1966) and Tormey and Diamond (1967) in the transporting rabbit gall bladder. The cockroach gland is eminently suitable for studying such phenomena as it is a double structure and one side may be stimulated alone, while the other may be used as a control.

It was also decided to study the effects of stimulation upon the axons of the gland to see if any changes occurred within the synaptic vesicles and axons during transmitter release.

Materials and Methods.

Salivary glands were usually stimulated at 5 Hz, 40 V using pulses of 0.5 ms duration. The procedure for these experiments is described in Part 2, Section 4. Glands were fixed at various times during these experiments according to the method in Part 2, Section 2.

Results.

When the gland is stimulated it initially produces a copious flow of secretion which dissipates after some time. Figure 1 represents such an experiment where the gland was stimulated for some time with a half-maximal stimulus (see House and Smith, 1978). In this case the gland continued to secrete for roughly 40 minutes and then was no longer able to respond.

If the glands were fixed during the copious flow of secretion (about 10 minutes after stimulation had begun) it was noted that the unstimulated side of the glands had a perfectly normal appearance under the light microscope when 1 micron thick araldite sections were viewed (figure 2a). However, the stimulated side of the gland had peripheral cells with greatly swollen ductules (figure 2b). The lumina of these ductules were clearly visible, whereas those of the contralateral side of the gland were not. Out of the seven experiments of this type that were performed only on one occasion were distended peripheral cell ductules observed on the contralateral side of the gland, and this was confined to one group of acini. This is in keeping with the idea that there is a limited amount of

crossover of innervation to the contralateral side (Ginsborg and House, 1976). If the sizes of the ductules are measured from the edge of the brush-border and averaged, then the mean of this dimension in the stimulated side of the gland is 12.81 ± 3.4 microns and for the unstimulated side is 6.73 ± 2.09 microns. A t-test indicates that this is a highly significant difference ($P < 0.001$). ($N(\text{stimulated}) = 48$), ($N(\text{unstimulated}) = 25$)

Glands that were stimulated until secretion had ceased did not display this difference, although there was some evidence that the ductules had been swollen; there was often a clear area round the edge of the brush-border as though the mitochondria that were normally to be found in this region had been mechanically pushed towards the basal regions of the cells.

On some occasions it was also noted that dopamine could cause the lumina of the peripheral cells to increase in size (figure 3b) when glands were incubated in solutions of dopamine for various reasons. Finally there is no evidence to show that the extra-cellular spaces between the cells of the acini were involved in fluid transport; unlike the rabbit gall bladder (Tormey and Diamond, 1967) they did not increase in size during secretion (figure 3a).

No changes could be found between the central cells of the stimulated and unstimulated glands.

Stimulation of axons resulted in changes in the vesicular profiles which could not be related to the length of the period of stimulation; similar effects were observed in axons that were

stimulated for 10 minutes to those that were stimulated for 40. The most common feature was aggregation of the small agranular vesicles and the formation of large irregular cisternae (figure 4). Such effects have also been observed after stimulation of the locust neuromuscular junction by McKinlay and Usherwood (1973) and by Botham et al (1978). In some of the axon profiles the small agranular vesicles seem to have accumulated round a membrane structure. This structure is electron dense and resembles a presynaptic rodlet (Osborne, 1977) (figures 4c and d.). Such a structure is also to be seen in figure 5a where two axons, both apparently of type A, come into close apposition and form an axo-axonic contact. It is also the case that the vesicles in these axons assume a more circular profile after stimulation (figure 5b) when compared to the unstimulated side of the gland (figure 5c). A log ratio distribution confirms this claim (figure 6).

Discussion.

Evidence has been presented that after nerve stimulation and also after incubation in dopamine, the ductules of peripheral cells become swollen. In the case of nerve stimulation swollen ductules were observed only on the stimulated side of the gland. Only on one occasion swollen ductules were seen on the unstimulated side of the gland. This may be explained by the cross-over of axons at the midline region of the gland which seem to innervate a limited area of the contralateral gland.

Diamond and Tormey (1966) have proposed that since the spaces between the epithelial cells in the rabbit gall bladder become enlarged during fluid transport, water enters these spaces by passing through cells; the basal regions of these spaces are protected by tight junctions. Diamond proposes that sodium chloride accumulates inside these spaces and creates a hypertonic solution which would cause water movement into these spaces. This standing gradient theory of Diamond has been criticised by Hill (1977) who has calculated that the size of such channels are several orders of magnitude too wide to produce the required osmotic forces. Hill came to the conclusion that there was no adequate explanation of how water is transported through epithelia. This subject has been extensively reviewed by House (1974).

In the cockroach gland Smith and House (1979) have demonstrated that the presence of sodium ions in the bathing media is necessary for secretion to occur, and have proposed a model in which sodium passes into cells and is actively pumped into the ductules of peripheral cells. Ouabain does not affect secretion when applied to the basal surface of the acinus so it was concluded that any pump that operates by ATP must be situated on the inner surface of the cells. If sodium is accumulated inside the ductules of peripheral cells then it seems probable that chloride ions would accompany it passively, and that an accumulation of sodium chloride would build up in the ductules which would cause water to enter them and make them swell. Supporting evidence for these ideas can be inferred from the structure of the peripheral cells.

Firstly they possess many mitochondria which suggests that their energy requirements are high. Secondly, the lumen of the duct is lined with many microvilli which greatly increase its surface area; as these microvilli are not involved with absorption then it is highly probable that they are secreting substances into the ducts. Finally the basal invaginations of the peripheral cell plasma membrane may provide low-resistance routes of water transport.

Several further experiments could be performed to test these ideas. One obvious one would be to bathe the gland in a series of hypertonic solutions of cockroach ringer. It should eventually be possible to produce a reverse osmotic gradient under these conditions so that the gland should cease to secrete in response to electrical stimulation. The osmotic force produced by the peripheral cells could then be estimated. Also an X-ray microprobe analysis of the microvilli could be performed to determine the levels of sodium present within them.

No changes were noted in central cells after stimulation of the salivary nerves. Garrett and Thulin (1975b) also noted no marked depletion of secretory granules in the stimulated rat parotid gland. Central cells seem to contain amylase (Bland and House, 1971), but there is no data available on the amount of amylase secreted by these cells during saliva production.

Results obtained from the ultrastructural study of stimulated axons are similar to those which have already been reported (McKinlay and Usherwood, 1973; Botham et al, 1978). It is

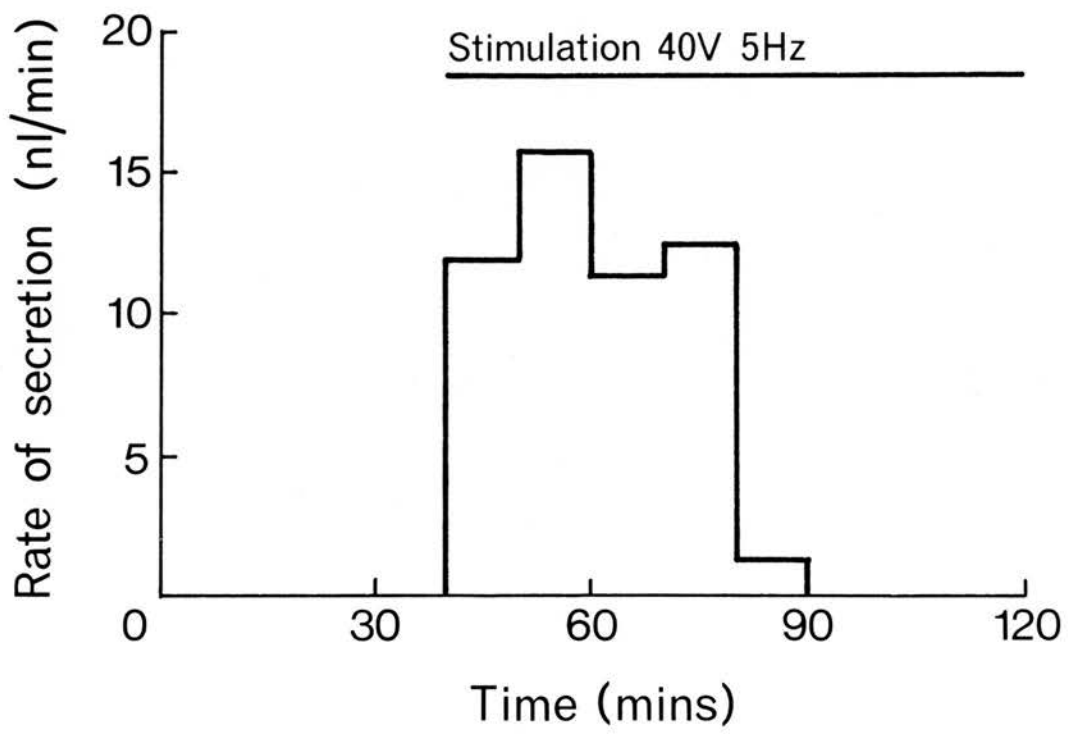
difficult to say if the number of vesicles was reduced by stimulation. Basbaum and Heuser (1979) have claimed that in adrenergic neurones innervating the vas deferens of the guinea pig, it is possible to deplete the nerve endings of the small agranular vesicles but not of the large dense vesicles. The large dense vesicles may vary considerably between axons and it is not possible to conclude for this reason if any change in their numbers had occurred.

The change in shape of the vesicles from elliptical to round may have some connection with transmitter release. Osborne (1977) has noted in photoreceptor cells that there is an interchange between flat and circular vesicles during stimulation. Van Orden et al (1966) have claimed that dopamine granules in vesicles are stored asymmetrically. This could lead to the formation of an elliptical shaped vesicle which may release its granule to adopt a circular profile.

Type B axons were not affected by stimulation. This observation may have little importance as many authors in the past have had difficulty in depleting axons of vesicular material. If type B axons are indeed neurosecretory cells it may be that they release their contents slowly to maintain a background tone.

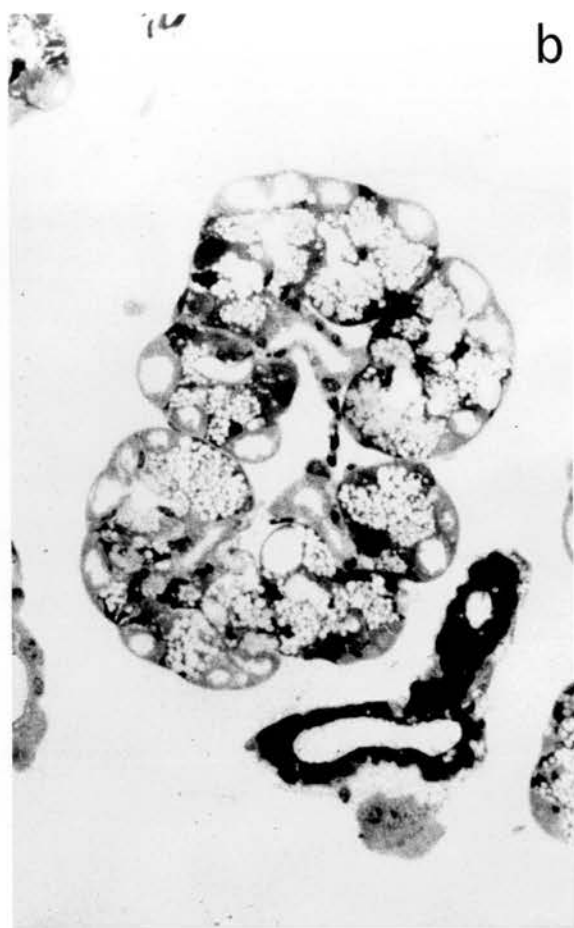
PART 6 FIGURE 1

This is a graph of secretion that was collected at 10 minute intervals when the salivary nerves were stimulated continuously for 80 minutes at 40V, 5 Hz. Note the initial copious flow of secretion which dies away after about 40 minutes of stimulation.



PART 6 FIGURE 2

These two sections were taken from a pair of glands that had been fixed during fluid secretion from one of them. Figure (a) is the unstimulated gland and figure (b) the stimulated contralateral gland. Note the swollen peripheral cell ductules in figure (b).

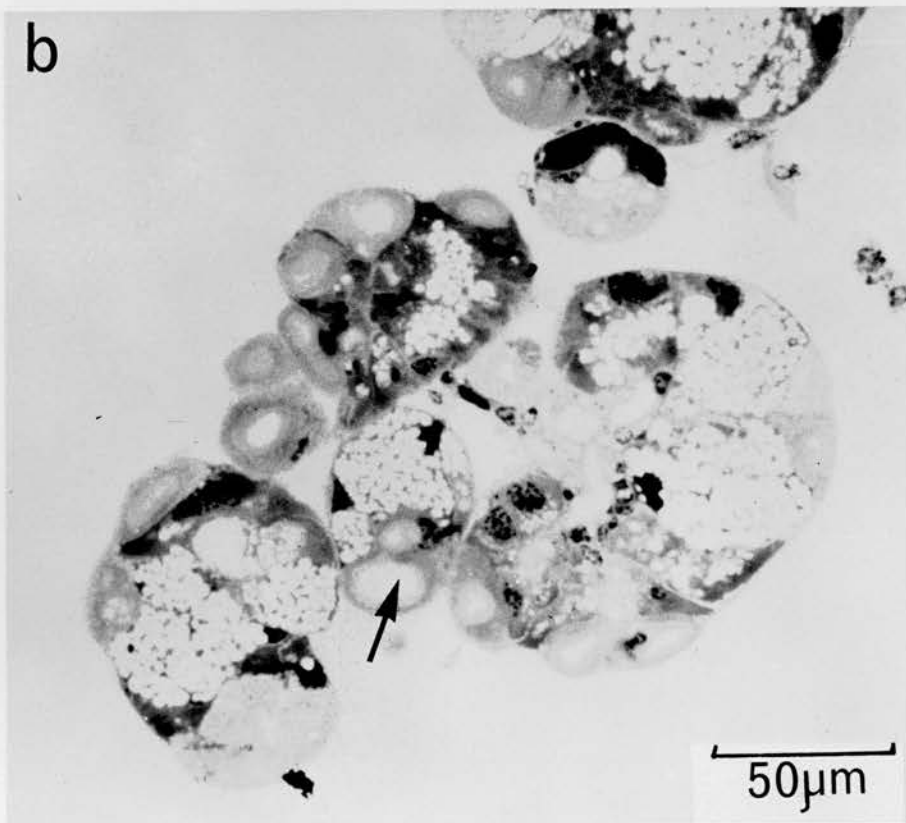
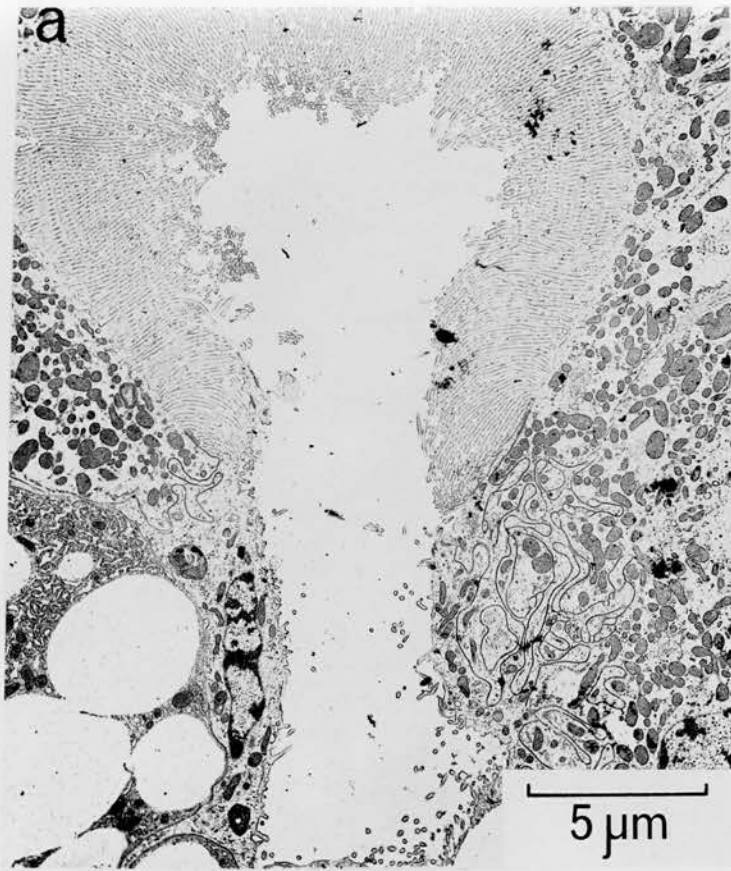


100μm

PART 6 FIGURE 3

(a) An electron micrograph of a stimulated peripheral cell from the tissue illustrated in figure 2(b). Note the obvious lumen of the peripheral cell and the lack of swelling in the septate desmosomes between the cells.

(b) This tissue was incubated in 10^{-7} M dopamine for half an hour before fixation. Note that many of the peripheral cell ductules are still distended (eg, arrow) at the time of fixation.

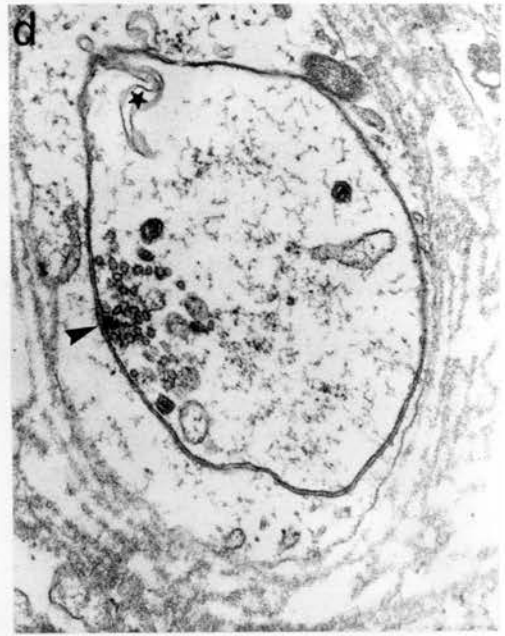
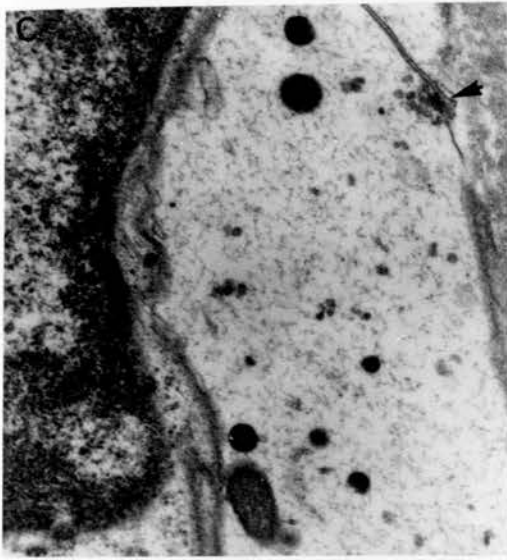
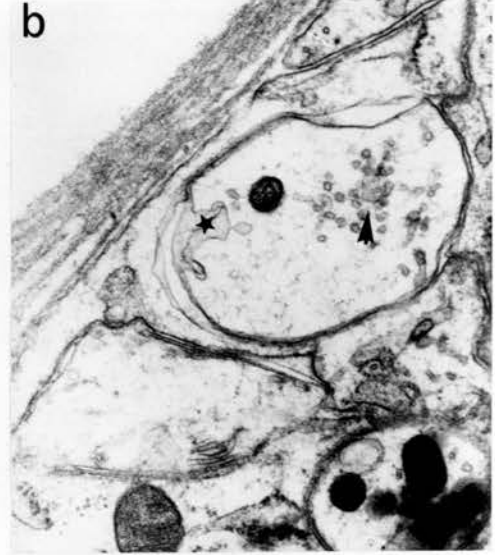
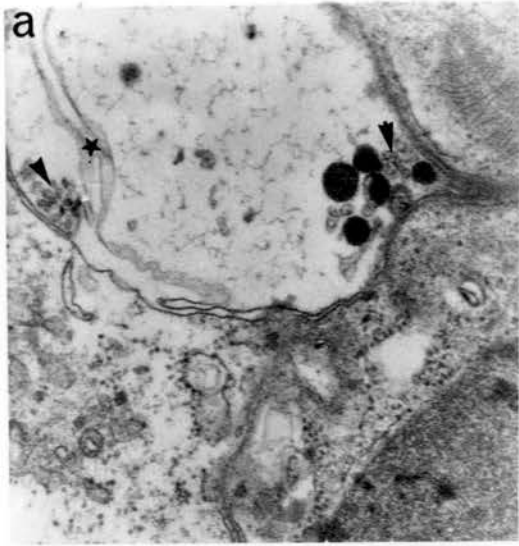


PART 6 FIGURE 4

Stimulated type A axons

(a) and (b) Two axons from preparations that were stimulated for several minutes; they possess enlarged cisternae (stars) and the vesicular material has aggregated (arrows).

(c) and (d) In these two axons vesicles have aggregated around dense objects on the cell membranes. The axon in figure (d) also has an enlarged cisterna.



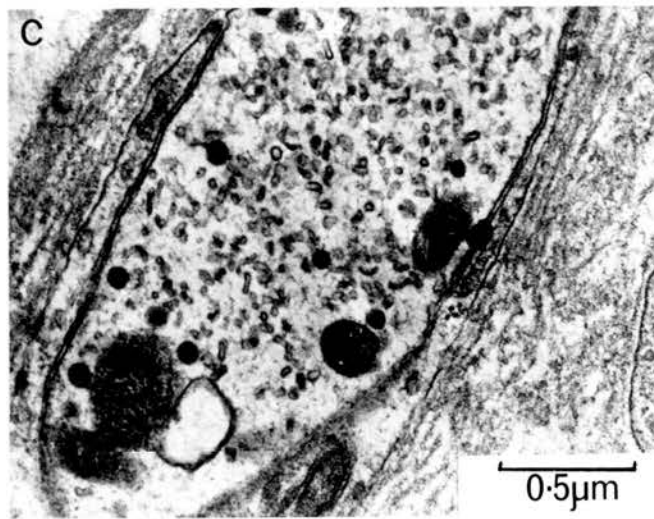
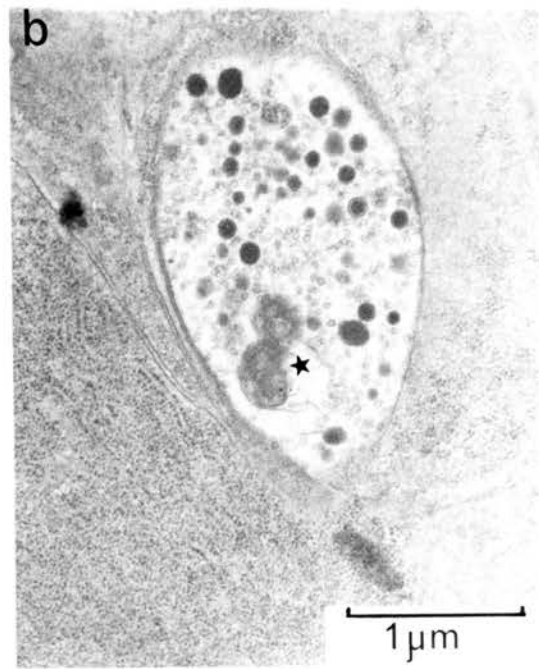
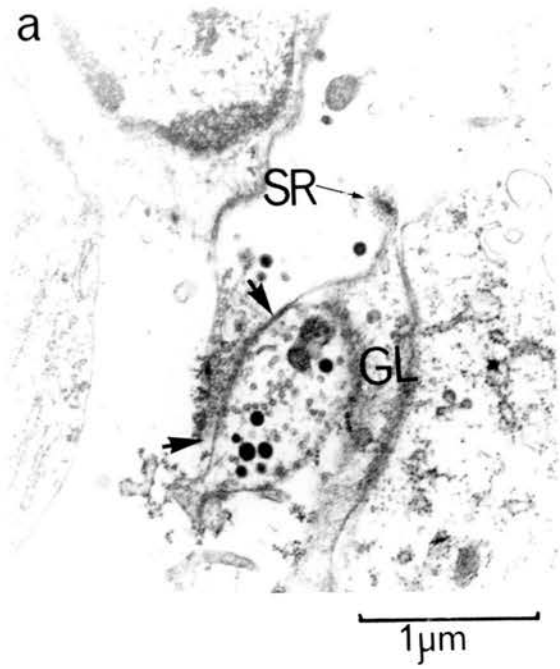
0.5 μ m

PART 6 FIGURE 5

(a) In this section two axons are in close apposition and seem to form an axo-axonic synapse. There is also another structure which has a cluster of vesicles around it and resembles a synaptic rodlet (SR) (GL - glial cell).

(b) This axon illustrates that after stimulation of the nerves many of the agranular vesicles contained within type A axons became circular in their profiles. An enlarged cisterna may also be seen (star).

(c) This is a type A axon from the contralateral (unstimulated) side of a gland that was stimulated. It still contains many elliptical vesicles.



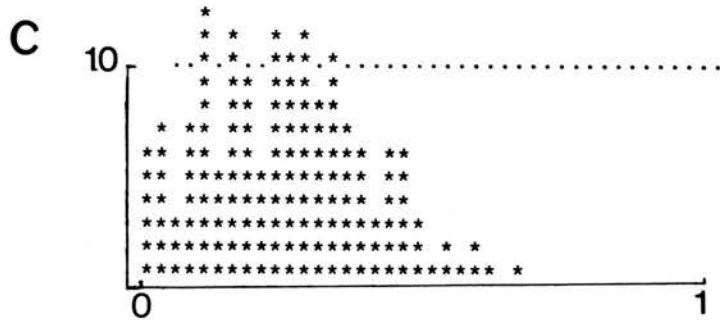
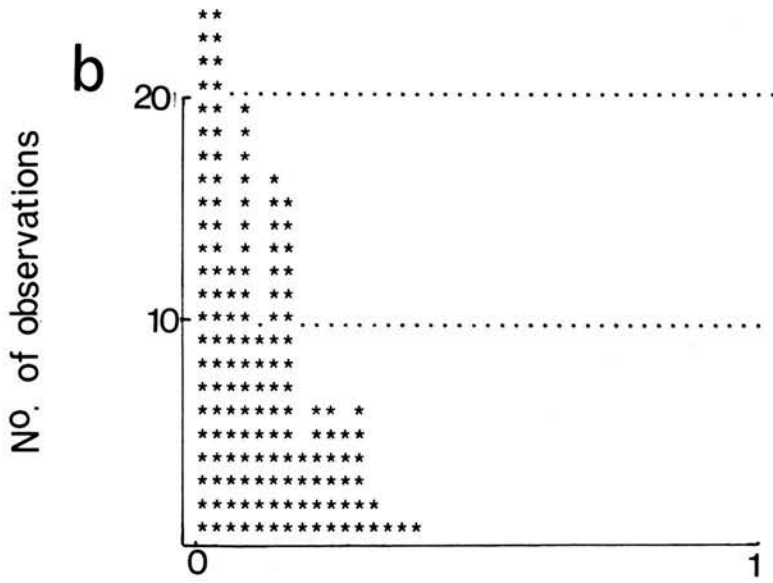
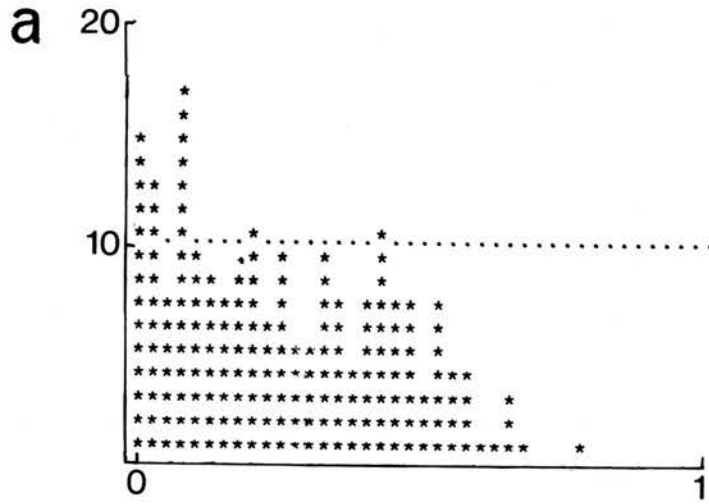
PART 6 FIGURE 6

Log ratio distributions of vesicles gathered from several different type A axons.

(a) Normal glutaraldehyde fixation.

(b) Stimulated axons.

(c) Unstimulated axons from the opposite sides of the same glands as in (b). From these distributions it may be seen that (c) resembles (a), but (b) has a narrower distribution indicating profiles that tend to be circular.



Log Ratio $\frac{\text{max}}{\text{min}}$

PART 7 : DRUGS WHICH ACT AT THE NEUROGLANDULAR SYNAPSE

Introduction.

In this section, three drugs were used which act on the presynaptic terminals of catecholamine-containing neurones : reserpine; alpha-methyl-p-tyrosine (AMPT); and fusaric acid.

The effects of reserpine on catecholamine containing neurones are well known. Hokfelt (1968) has demonstrated in several catecholamine containing structures in the rat that pre-treatment with reserpine results in a depletion of small granular vesicles after fixation with permanganate. Walker et al (1971) have observed that reserpine pre-treatment resulted in a reduction of the magnitude of the inhibitory postsynaptic potential in the brain of *Helix aspersa*. There is much evidence to suggest that dopamine is the neurotransmitter at this site. Finally, Fulton and Healy (1976) have shown that reserpine reduces the levels of catecholamines in whole-brain assays of rats. It was decided to test the effects of reserpine upon the secretory response of cockroach glands and then to examine the ultrastructure of the axons to determine if any alteration of synaptic vesicles had occurred. Bland et al (1973) used injections of reserpine on cockroach salivary glands to reduce the level of paraformaldehyde induced fluorescence.

Alpha-methyl-p-tyrosine is a blocker of tyrosine hydroxylase (Spector et al, 1965). Tyrosine hydroxylase converts tyrosine to dopa and hence is the first link in catecholamine synthesis.

In the presence of AMPT, ^{14}C tyrosine is not converted to catecholamines. AMPT also reduces the level of paraformaldehyde induced fluorescence and the number of granular vesicles observed after permanganate fixation (Itakura et al, 1975). It also reduces the whole-brain assay for catecholamines in the rat (Fulton and Healy, 1976) and the inhibitory postsynaptic potential in the brain of *Helix* (Walker et al, 1971).

There is some evidence to suggest that fusaric acid is an inhibitor of dopamine β -hydroxylase (Nagatsu et al, 1972; Ishii et al, 1976), the enzyme that converts dopamine to noradrenaline. Both fusaric acid and AMPT have been tested clinically to assess their effects on psychiatric patients (Goodwin and Sack, 1974).

If AMPT is indeed a blocker of tyrosine hydroxylase, then it should disrupt the activity of a dopaminergic system, whereas fusaric acid should not as it would only be predicted to affect the levels of noradrenaline in a catecholaminergic system. Several experiments were designed to see if these two drugs could be used to distinguish between noradrenaline and dopamine at the cockroach neuroglandular junction.

Materials and Methods.

Secretion studies were performed according to the method described in Part 2, Section 4. Glands were perfused with a colloid of reserpine (Sigma) of 50 $\mu\text{g}/\text{ml}$. As reserpine is totally insoluble in water this perfusate was prepared by dissolving

reserpine in a solution of 1% citric acid (in cockroach ringer) and diluting this 1 in 20 times in cockroach ringer. This solution was then titrated to pH 7.2 at which point an opalescent colloid of reserpine could be seen. The glands were also perfused with 20 and 200 $\mu\text{g}/\text{ml}$ solutions of alpha methyl-p-tyrosine dimethyl ester (Sigma) (MW 245, pH 7.2) and fusaric acid (Sigma) (MW 179, pH 6.9).

At the completion of such experiments, glands were fixed and prepared for electron microscopy (Part 2, Section 2). The results obtained from such experiments were inconsistent and difficult to interpret so a simpler approach was adopted where glands were incubated in solutions of the three drugs for 8 and 24 hours. These glands were then fixed and prepared for electron microscopy in the usual way (Part 2, Section 2). Control tissue was incubated in cockroach ringer for similar periods of time.

Finally, glands were also incubated in 50 $\mu\text{g}/\text{ml}$ colloids of reserpine in cockroach ringer for two hours, and were prepared for fluorescence histochemistry (Part 2, Section 3).

Results.

Fluorescence histochemistry revealed that glands incubated in reserpine colloids for two hours had very little specific fluorescence. This shows that most of the catecholamines in this tissue had been depleted. Secretion studies indicated that reserpine was having a presynaptic effect upon the neuro-

glandular synapse (figure 1a). Test doses of dopamine produced responses of the same order when given before and after incubation with reserpine (4 experiments) whereas the nerve response declined rapidly. From previous experiments on the secretory response to nerve stimulation such a decline would not have been predicted when two minutes of half-maximal stimuli, with 20 minute rest-periods between stimuli, were used (see Smith and House, 1977). Test stimuli were given at the beginning of each experiment and were used to assess the state of the preparation. It is interesting to note that the stimulus that was given after the initial test dose of dopamine in figure 1a produced a much larger response than normal. This effect has been observed several times (see also experiments with AMPT), and may be explained by an uptake of dopamine into the nerve endings. A control experiment was performed with a solution of 0.05% citric acid in cockroach ringer pH 7.2 which was applied to the gland in the usual way. This produced the surprising result that it initiated a copious flow of saliva which persisted as long as the gland was in contact with it (figure 1b). This result was not observed when the same concentration of citric acid was in solution in the reserpine colloid. After application of citrate it was also noticed that the nerve response declined. This inconsistency shall be discussed below.

Tissue that was incubated in reserpine for 8 hours was fairly well preserved, except that type A axons had vesicles of a more

circular profile (figure 5c and d) when compared to tissue that had been incubated in ringer solution for 24 hours (figure 5a and b). A log ratio distribution confirms this claim; figure 7b is a log ratio distribution of type A axon small vesicles which have been incubated in reserpine. It is organised in a cluster around zero whereas the distribution for vesicles incubated in ringer for 24 hours (figure 7a) is more spread-out and resembles the distribution that is found in freshly-prepared tissue (Part 3, Section 3, figure 5). Tissue that has been incubated in ringer solution for 8 hours also has a normal appearance.

Secretion experiments with AMPT had a similar pattern to those with reserpine but the second dopamine test dose always yielded a smaller response after the tissue had been perfused with AMPT (figure 2a). The nerve response decreased slowly during the AMPT perfusion and eventually little secretion could be elicited. The reduction of the second test dose of dopamine suggested that AMPT could be having a postsynaptic blocking effect upon the receptors that dopamine was activating. This possibility was tested with dose response curves using AMPT as an antagonist (figure 2b and c). From these curves it can be seen that AMPT seems to antagonise the secretory response to dopamine in a non-competitive manner. It was not possible to overcome this block by giving the gland larger doses of dopamine and hence to achieve a maximum secretory rate again. The non-competitive nature of the block is also illustrated in the experiments shown in figure 3. It was not possible to surmount the block and to achieve a maximal

secretory response by increasing the frequency of stimulation delivered to the salivary nerves and hence increase the transmitter output. In the experiment using the high concentration of AMPT the nerve response was not simply lowered to a different level but continued to decrease. This decrease was seen to continue after the number of stimuli was increased (figure 3a). House and Smith (1978) have shown that there is a fairly linear relationship between the number of stimuli and rate of secretion until a maximum rate is achieved. So this result may indicate that AMPT is having a presynaptic effect at the junction as well as a postsynaptic effect. In the experiment that was performed at the lower concentration (figure 3b), the rate of secretion in the presence of AMPT was reduced to a fairly uniform level and it was not possible to return the secretory rate back to its original level by increasing the number of impulses.

Tissue that was incubated in 200 $\mu\text{g/ml}$ of AMPT had type A axons with a comparatively normal appearance after eight hours but after 24 hours the small agranular vesicles of type A axons again seemed to have a circular appearance (figure 6a, b and c). The log ratio distribution confirms this claim (figure 7d).

Results obtained from secretion experiments with fusaric acid were more clear-cut than those with AMPT, although they were unexpected. Fusaric acid depressed the secretion response to nerve stimulation more or less instantly (figure 4a and b). It also greatly reduced the response to the second test dose of

dopamine. At concentrations of 200 $\mu\text{g/ml}$ fusaric acid almost completely abolished the nerve response (figure 4a). At 20 $\mu\text{g/ml}$ the response was greatly reduced. Dose/response curves revealed that fusaric acid was a non-competitive postsynaptic blocker (figure 4c and d). Again it was not possible to overcome the block by increasing the dose of dopamine.

The fine structure of tissue incubated in fusaric acid (200 $\mu\text{g/ml}$) for eight hours was normal; figure 6d illustrates a type A axon after this treatment. Tissue that was incubated for 24 hours was not normal; much of it had a granular appearance. It was possible to identify axons of type A within this tissue and these still possessed small agranular vesicles that had elliptical profiles, (figure 6e). A log/ratio distribution showed that these vesicles were distributed in a similar manner to those of tissue that was incubated for 24 hours in cockroach ringer (figure 7a and c).

Discussion.

The results obtained with the three drugs were confusing and it is difficult to come to any firm conclusions from the experiments that have been reported. Reserpine does seem to deplete axons of catecholamines as the fluorescence studies indicate. If this is the case then the gradual decline in nerve-evoked responses in the secretion studies may represent the depletion of transmitter by this drug. On the other hand citrate alone produced a decline in nerve response after the gland had been

exposed to a solution of it.

It is difficult to explain the copious flow of secretion observed after perfusing the gland with citric acid. One possibility may be that citric acid is known to be a chelating agent and that secretion may have been initiated by the movements of calcium ions. To explain the lack of copious secretion in the presence of citrate and reserpine it is necessary to speculate further. Presumably reserpine is soluble in a solution of citric acid because it is able to form some sort of bond. It may be therefore that reserpine inactivates citrate. These speculations do not help us in the original aim of testing if reserpine depletes the neuroglandular synapse of catecholamines. The evidence from fine structure suggests that incubation of the gland with reserpine may result in the depletion of transmitter. Itakura et al (1975) have claimed that reserpine depletes potassium permanganate-fixed tissue of the dense cores of vesicles, but not of the vesicles themselves. If this is the case then the change in shape of vesicles, reported above, may be a result of depletion of the transmitter substance by reserpine. It will be recalled that stimulation of the nerve resulted in similar observations (Part 6).

It was not possible to show that dopamine was present in the axons that innervate the preparation with AMPT and fusaric acid. There was a limited amount of evidence to suggest that AMPT has some presynaptic actions, but most of the observations could be explained in terms of a postsynaptic block. In the case of fusaric

acid, all of the observations could be explained this way and any presynaptic changes that may have occurred were masked by the strong postsynaptic block. Recently a paper was published by Hidaka and Asano (1976) which suggests that fusaric acid indiscriminately blocks many different receptors in the isolated rabbit artery preparation.

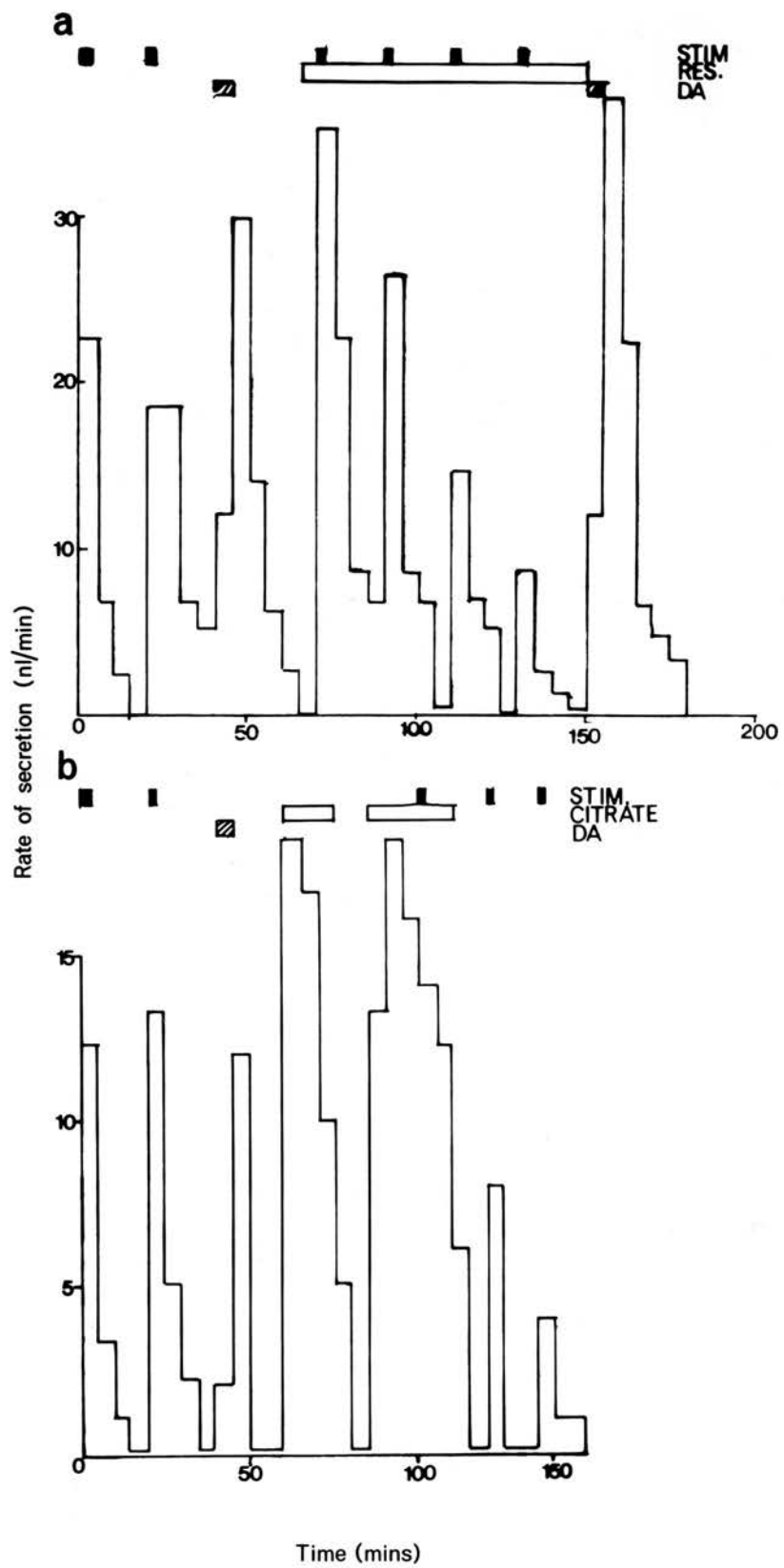
The change in shape after 24 hours incubation in AMPT of vesicles within type A axons again may indicate a depletion in transmitter although Itkura et al (1975) have claimed that AMPT actually depletes the number of vesicles in nerve endings of the rat vas deferens. In some of the terminals an aggregation of vesicles had occurred that was similar to the type produced after stimulation of the nerve (figure 6a). Van Orden et al (1966) have produced evidence that there may be two stores of catecholamines within the nerve endings of the rat vas deferens. One store can be depleted by the tyrosine hydroxylase inhibitor alpha methyl-m-tyrosine. After this drug was administered tyramine, an indirectly acting sympathomimetic, could not produce contractions of the rat vas deferens, whereas stimulation of the nerve could. If such a system also operates at the cockroach neuroglandular synapse then it would be difficult to use inhibitors of tyrosine hydroxylase as analytical tools in the identification of the transmitter.

PART 7 FIGURE 1

Reserpine

(a) In this experiment test stimuli (40V, 5Hz, for 2 minutes) and test doses of dopamine (10^{-7} M) were given in the presence and absence of reserpine (50 μ g/ml). The nerve responses declined in the presence of reserpine. A test dose of dopamine was given at the end of the experiment (STIM - electrical stimulation, RES - reserpine, DA - dopamine). Four such experiments were performed.

(b) This is a control experiment showing the effect of citrate. Doses of citrate (0.05 M) stimulated a copious flow of saliva. After these responses nerve stimulation caused smaller secretory transients (STIM - electrical stimulation, DA - dopamine).



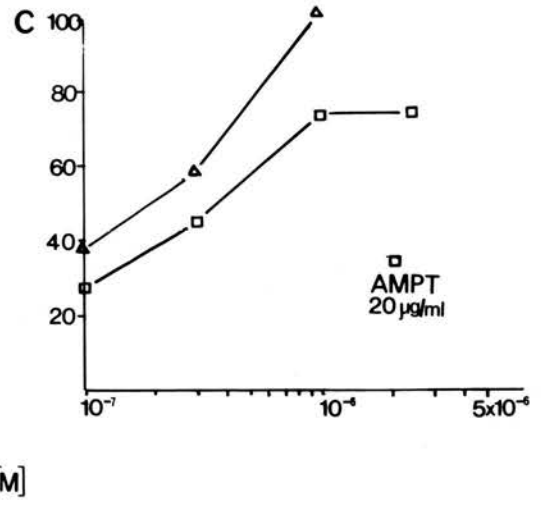
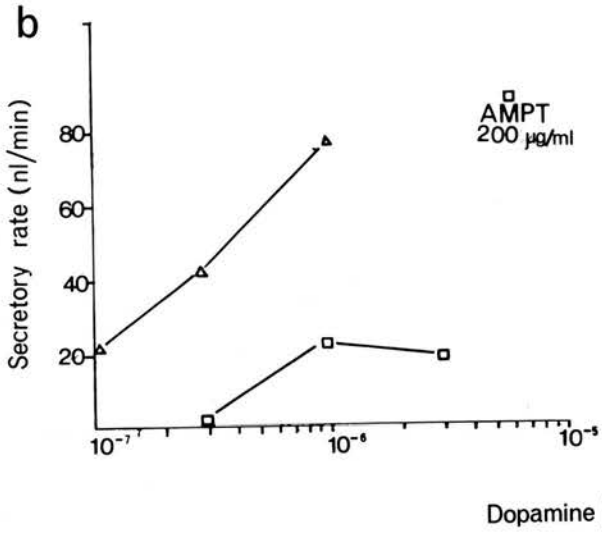
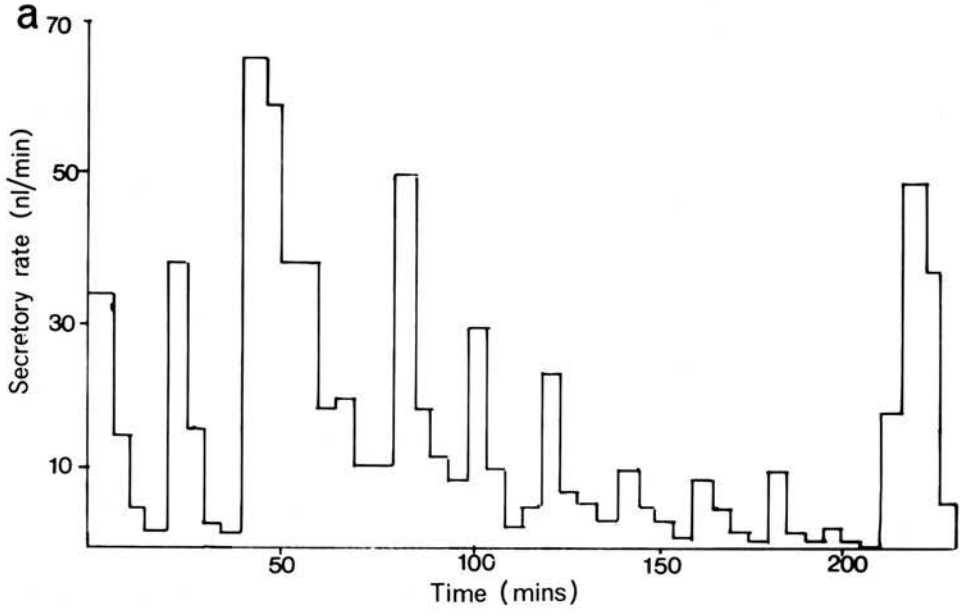
PART 7 FIGURE 2

Alpha methyl-p-tyrosine

(a) In this experiment test stimuli were given as in figure 1. The preparation was first perfused with Ringer and subsequently with Ringer containing 200 $\mu\text{g}/\text{ml}$ alpha methyl-p-tyrosine (AMPT). When the AMPT Ringer was introduced the secretory response to nerve stimulation declined slowly. However, the response to dopamine (DA) also declined. Three such experiments were performed.

(b) This is a log dose/response curve for dopamine stimulation which shows that 200 $\mu\text{g}/\text{ml}$ of AMPT reduces the secretory response to dopamine and seems to do so in a non-competitive way.

(c) Doses of 20 $\mu\text{g}/\text{ml}$ AMPT also reduce the response to dopamine in a non-competitive manner.

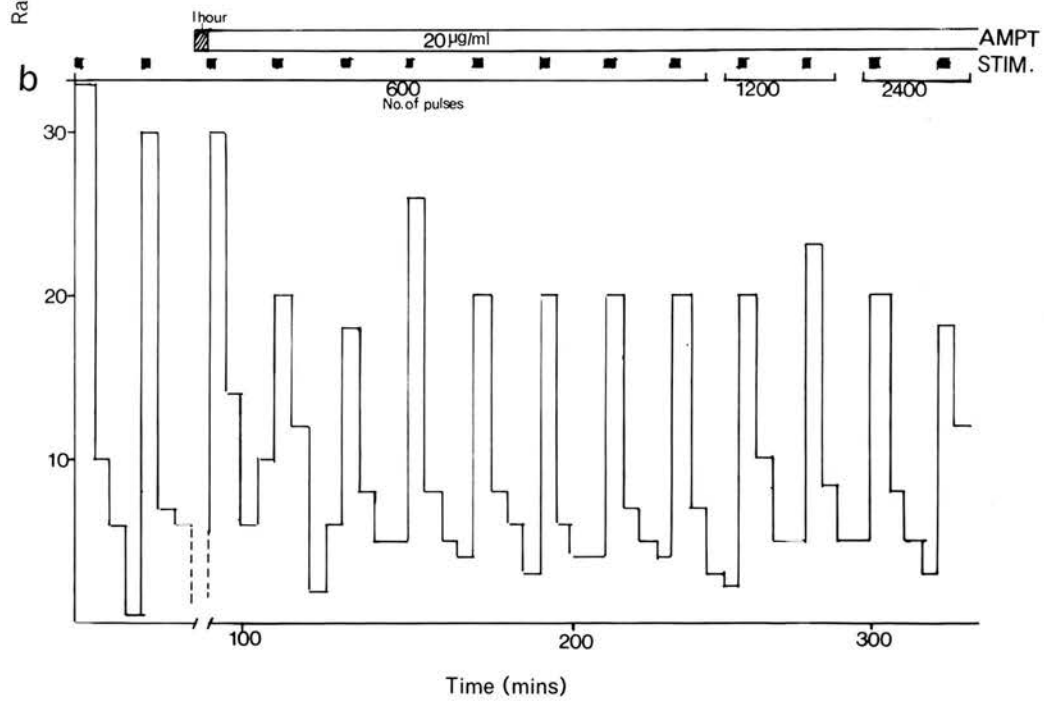
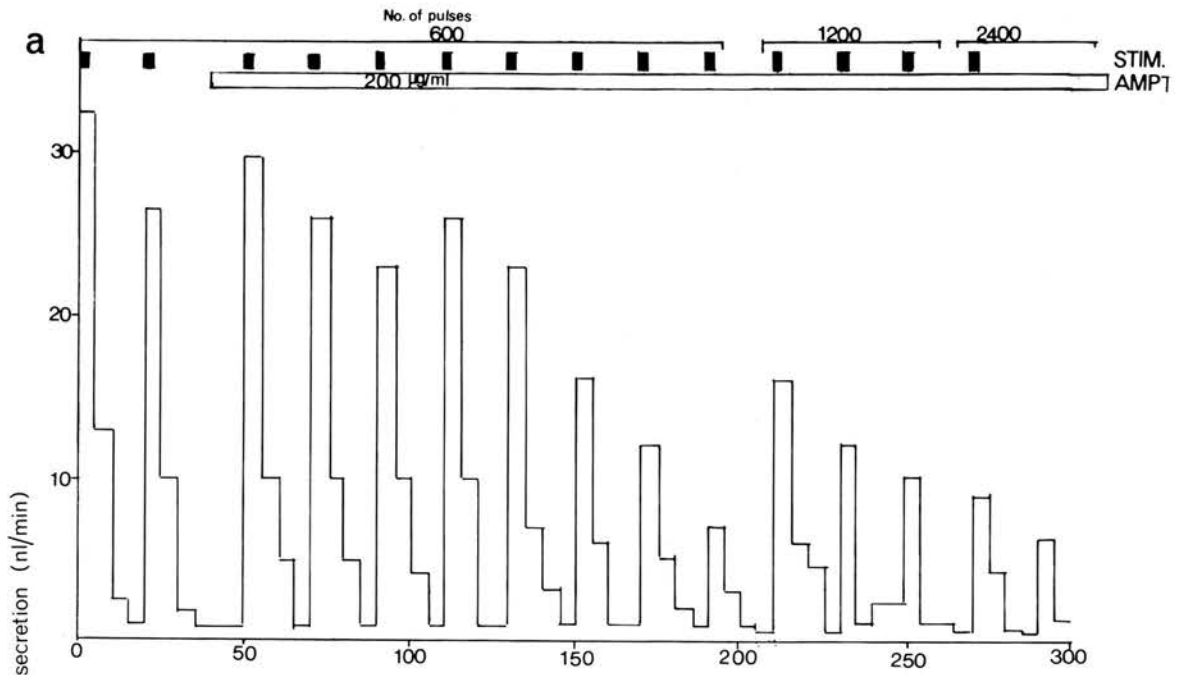


PART 7 FIGURE 3

Alpha methyl-p-tyrosine

(a) In this experiment an attempt was made to overcome the postsynaptic block of transmitter receptors of AMPT by increasing the number of stimuli entering the nerve net and hence increasing output of transmitter. This was achieved by increasing the frequency of stimulation (2 minute pulses at 40V, 5, 10 and 20 Hz). It can be seen from the graph that the secretory response to nerve stimulation decreased slowly in the presence of 200 $\mu\text{g}/\text{ml}$ AMPT. There was limited success in overcoming the block by increasing the number of stimuli.

(b) This figure shows a similar experiment as that in figure (a) except that 20 $\mu\text{g}/\text{ml}$ AMPT was used and the gland was perfused for an hour with AMPT Ringer before stimulation.



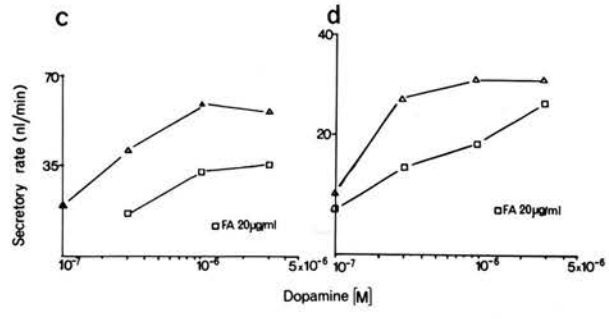
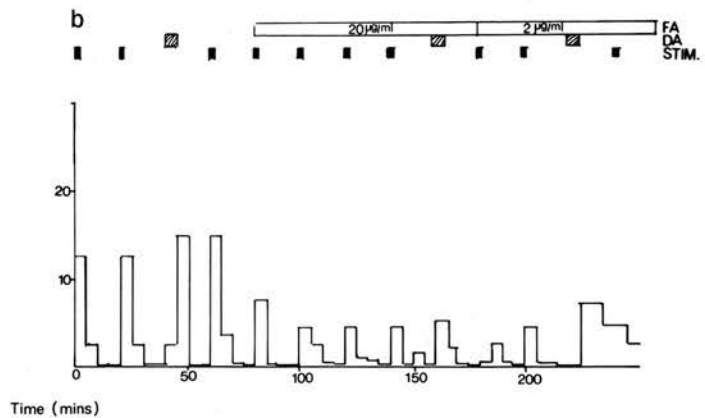
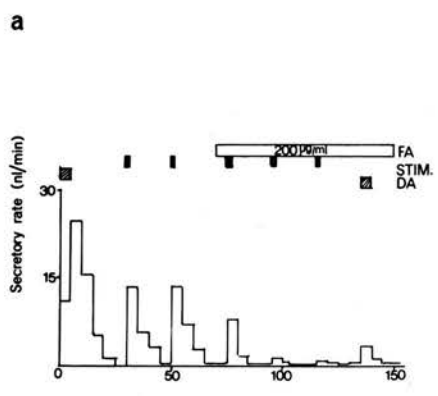
PART 7 FIGURE 4

Fusaric acid

(a) In this experiment test stimuli were given in the presence of fusaric acid (200 $\mu\text{g}/\text{ml}$). It may be seen from the graph that the secretory responses to nerve stimulation and to dopamine were reduced to nearly zero in the presence of fusaric acid (FA - fusaric acid, STIM - nerve stimuli (2 minute pulses, 40V, 5 Hz), DA - dopamine).

(b) This is an experiment similar to that shown in figure (a) except that fusaric acid at 20 $\mu\text{g}/\text{ml}$ and 2 $\mu\text{g}/\text{ml}$ was used. Again, 20 $\mu\text{g}/\text{ml}$ doses of fusaric acid decreased responses to both the stimuli. In the presence of 2 $\mu\text{g}/\text{ml}$ fusaric acid it was only possible to increase the response to dopamine slightly.

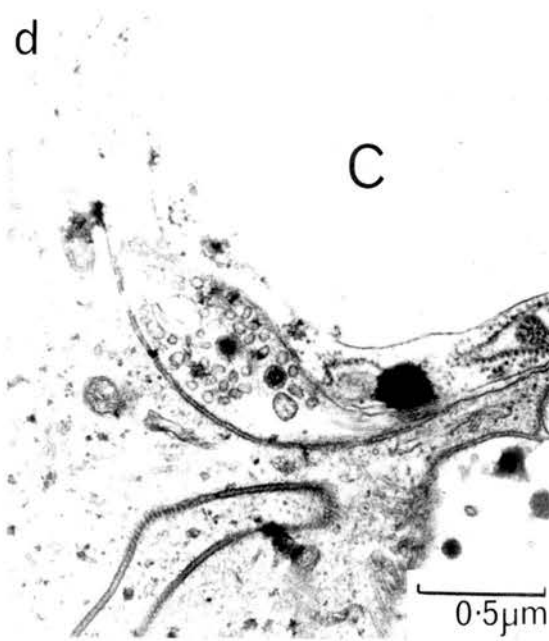
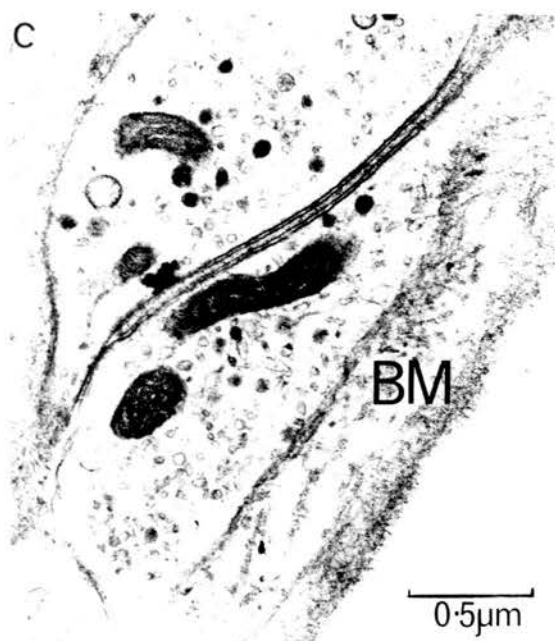
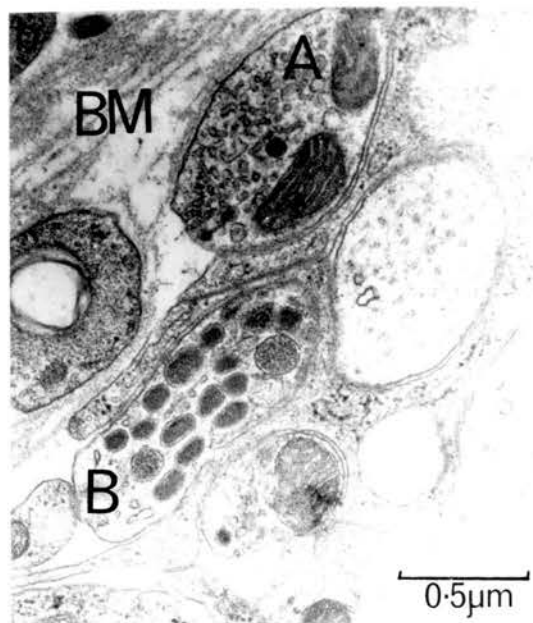
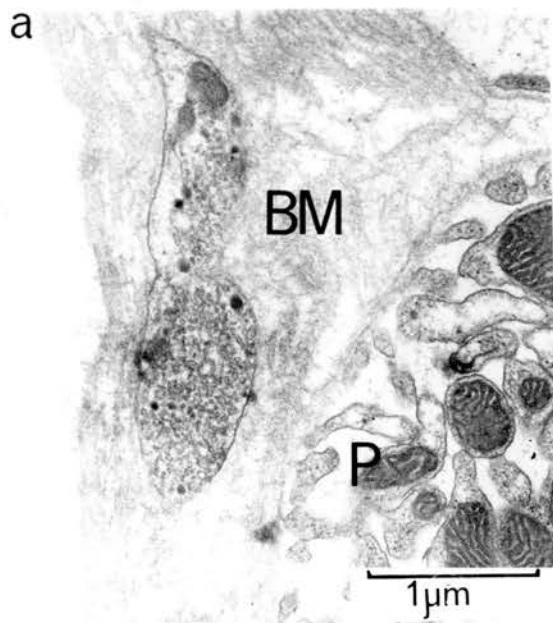
(c) and (d) These are log dose/response curves for dopamine in the presence of 20 $\mu\text{g}/\text{ml}$ fusaric acid. Like AMPT, fusaric acid seems to block receptors in a non-competitive manner.



PART 7 FIGURE 5

(a) and (b) Tissue that was incubated in cockroach Ringer for 24 hours. Note that type A axons still have elliptical vesicles (BM - basement membrane, P - peripheral cell, B - type B axon).

(c) and (d) Tissue that was incubated in reserpine (50 $\mu\text{g/ml}$) for 8 hours. Note that type A axons have circular vesicles (BM - basement membrane, C - central cell).

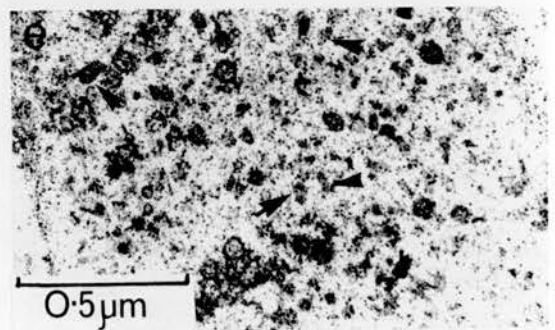
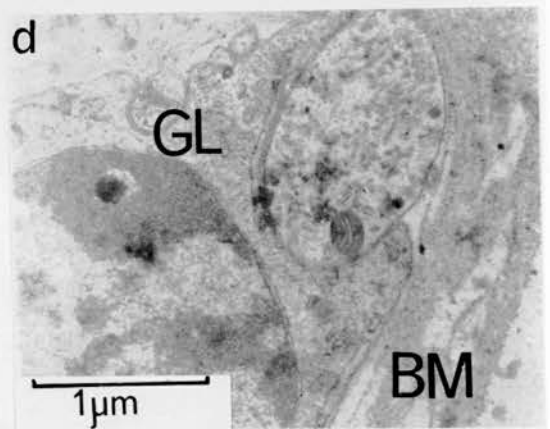
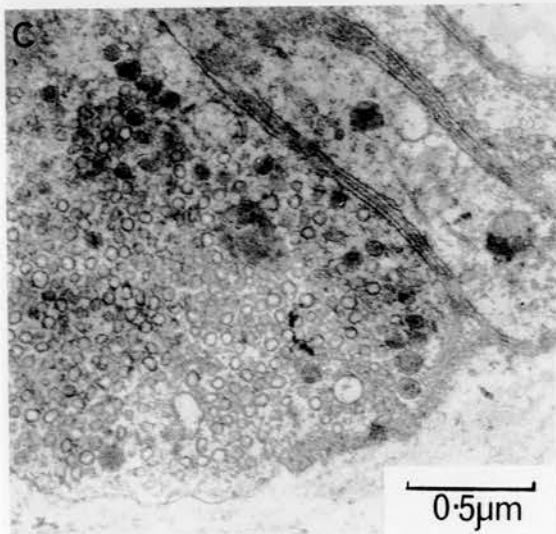
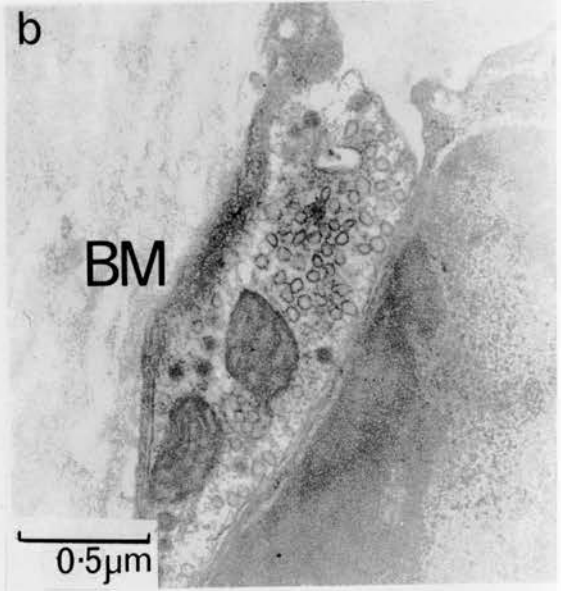
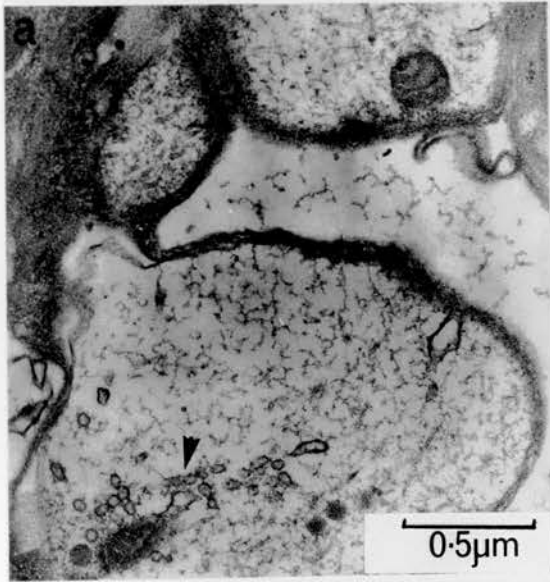


PART 7 FIGURE 6

(a), (b) and (c) Tissue that was incubated in 200 $\mu\text{g}/\text{ml}$ alpha methyl-p-tyrosine for 24 hours. Note that the vesicles are circular in profile and that in figure (a) they seem to be aggregating (arrow) (BM - basement membrane).

(d) Tissue that was incubated in 200 $\mu\text{g}/\text{ml}$ fusaric acid for 8 hours. The vesicles of type A axons are still elliptical (GL - glial cell, BM - basement membrane).

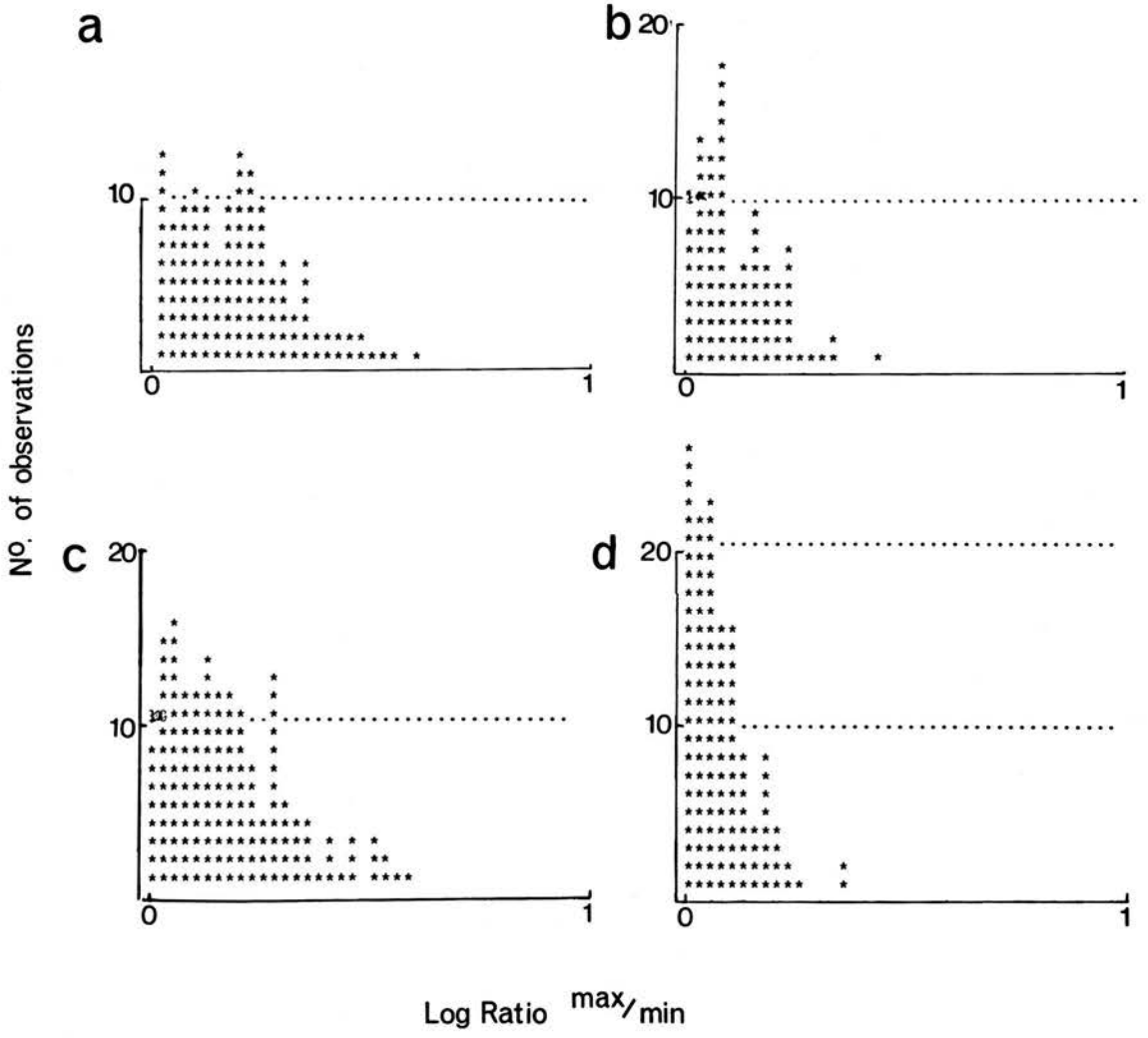
(e) Tissue that was incubated in fusaric acid (200 $\mu\text{g}/\text{ml}$) for 24 hours. Note the poor quality of fixation of this type A axon. Some elliptical vesicles are still identifiable (arrows).



PART 7 FIGURE 7

Log/ratio distributions of agranular vesicles from type A axons.

- (a) 24 hour incubation in cockroach Ringer.
- (b) 8 hour incubation in reserpine.
- (c) 24 hour incubation in fusaric acid.
- (d) 24 hour incubation in alpha methyl-p-tyrosine.



PART 8 : GENERAL DISCUSSION AND CONCLUSIONS

The general ultrastructure of the cells and ducts of the cockroach salivary apparatus resembles that of many other transporting epithelial systems. Such systems have been reviewed by Berridge and Oschman (1972). The control and function of central cells is still not fully understood and little can be added to the conclusions of Bland and House (1971). It is also only possible to conjecture what the function of the secretory ducts are; some clues may be found in the ultrastructure of the cells. Smith and House (1979) have discussed the possibility that the acinar ducts may be involved in modifying the ionic composition of saliva. The secretory duct cells are thought to provide the mucous component of saliva (Bland and House, 1971). Peripheral cells seem to cause movement of water into the acini and hence initiate the flow of saliva. As for the reservoirs they may be some form of storage organ for water.

The cells of the acini are electrically coupled and possess many gap junctions. This coupling may be responsible for the spread of hyperpolarising current through the acinus and hence the synchronisation of secretion. It may also be that gap junctions allow the cells of the acini to communicate with each other and exchange various substances; Procion dye passes easily from cell to cell.

Many axons were found to be connected with the salivary apparatus. The largest of these were found in the salivary nerves

TREATMENT	PRESENCE OF CATECHOLAMINES	METHOD
permanganate fixation	yes	U
dichromate fixation	yes	U
5-hydroxydopamine	yes	U
6-hydroxydopamine	yes	U and F
reserpine	yes	F (and U?)

Evidence for the presence of catecholamine in cockroach salivary gland axons.

U = ultrastructural histochemistry

F = fluorescence histochemistry

TABLE 1

which arise from the suboesophageal ganglion. When these nerves were cut many degenerating axon profiles were found among the smaller axons that innervate the salivary gland. This was not observed when the stomatogastric nerve was cut.

The small axons that innervate the gland were classified into two types: type A and type B. Type B axons were thought to be neurosecretory cells, whereas type A axons were thought to contain catecholamines. Evidence to support the latter idea has accumulated throughout this study and is summarised in Table 1. It was found that axons were not associated with any particular type of cell, but were distributed apparently in a random manner round the glands. It was not possible to achieve one of the original aims of this study which was to demonstrate the presence of dopamine within the nerve endings.

The mechanism of transmitter release from type A axons is not fully understood. Several type A axons were seen to possess dark objects on their cell membranes which were surrounded with vesicles. Such objects were seen more frequently after the nerves were electrically stimulated. These presynaptic specialisations did not seem to be associated with axonal swellings. No postsynaptic specialisations were found even though many axons were situated between acinar cell membranes and were devoid of any glial coat. The long latency of the secretory potential cannot be explained in terms of the distance of axons from the cells. No information is available on the position of the

receptors of these cells. Ginsborg, House and Mitchell (1978) have even suggested that they may be inside the cells.

Stimulation of the salivary nerves resulted in a change in shape of vesicles which could not simply be dismissed as an artefact as this did not occur in parts of the nerve that were not stimulated and yet were fixed at the same time. Similar changes were also found after the glands were incubated in reserpine or alpha methyl-p-tyrosine, two drugs that should reduce the levels of catecholamines within the nerve cells. The latter results do not have the same convenient experimental control of the stimulation experiments and therefore could be criticised and dismissed as fixation artefact. It was seen that permanganate fixative also changed the shapes of the small agranular vesicles of type A axons. If these observations are not artefactual then it may be that a change in shape of vesicles occurs after the transmitter has been released.

Some of the results presented in this thesis represent preliminary studies of the many interesting phenomena that the salivary apparatus of the cockroach presents. It seems highly probable that this preparation will eventually contribute much to our knowledge, not only of salivary glands and the production of secretion but also of dopaminergic systems and perhaps even the formation of tumours.

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APPENDIX

Some of the work reported in this thesis has been published in *Tissue and Cell* (1978), Vol 10, pp 699-706, and was communicated at the York meeting of the Society for Experimental Biology, April 1979.

DAVID J. MAXWELL

FINE STRUCTURE OF AXONS ASSOCIATED WITH THE SALIVARY APPARATUS OF THE COCKROACH, *NAUPHOETA CINEREA*

ABSTRACT. Previous studies have shown that the neurotransmitter at the salivary gland of the cockroach is probably dopamine. An ultrastructural study of the innervation of these glands was made. It was concluded that there are two types of axon present designated type A and type B. Type A axons possess small agranular elliptical vesicles and large granular vesicles, whereas type B possess only large granular vesicles of larger diameter than those found in type A. The relationship of these types of axon to the cells of the acini is discussed. It is concluded that type A axons are catecholaminergic.

Introduction

THE salivary gland of the cockroach, *Nauphoeta cinerea* (Oliv.), is a bilaterally symmetrical structure, which consists of acini and ducts (Bland and House, 1971), as is the case with several other species of cockroach (e.g. Lebedev, 1899; Whitehead, 1971). Caudal to the acini is a pair of reservoirs each of which gives rise to a duct. These reservoir ducts form a final common duct with a pair of secretory ducts which run from the acini. Two nerves, one associated with each reservoir duct, go to the acini, forming a dense network over the surface of the gland (Bowser-Riley, 1978).

Fluorescence studies, employing the techniques of Falck and Hillarp (1962), have also revealed a dense network of fluorescing axons investing the acini. Microspectrofluorimetry has indicated that this fluorescence is produced by a catecholamine (Bland *et al.*, 1973). Electrophysiological methods (Bowser-Riley and House, 1976) and experiments involving the collection of secretion from isolated

glands (Smith, 1977; Smith and House, 1977), indicate that dopamine may be the neurotransmitter of the salivary nerves. It therefore became apparent that there was a need to perform an ultrastructural study of the innervation of these glands. Only one previous attempt to study the fine structure of nerves involved with the cockroach salivary gland has been made and this on a different species (Whitehead, 1971). An electron microscopic investigation of the acini of *Nauphoeta* was made by Bland and House (1971). Their nomenclature shall be employed in this account.

Materials and Methods

Whole glands of adult cockroaches (*Nauphoeta cinerea*), were dissected out under cockroach Ringer (Smith and House, 1977), and pinned out in glass Petri dishes, which contained a layer of Sylgard 184 (Dow-Corning Inc.), in preparation for fixation. The fixative was adjusted to a theoretical osmolarity of 370 mOsm according to the method of Weakley (1972). Cockroach ringer was found to have an osmolarity of 349 mOsm when measured by depression of freezing point. The fixative was adjusted to 370 mOsm as it is desirable that it should be hypertonic compared to the tissue. The fixative consisted of 4% glutaraldehyde in 0.05 M sodium

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cacodylate buffer with 2.8% sucrose at pH 7.2. Glands were fixed in this for 1 hr at 4°C. The glands were then washed at 4°C in buffer (pH 7.2) which consisted of 0.05 M sodium cacodylate adjusted to an osmolarity of 349 mOsm with 8.3% sucrose. Post-osmication was achieved by immersing the glands in a solution of 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2, at 4°C, for 1 hr. The glands were washed again, dehydrated in alcohol, cleared in propylene oxide and embedded in Araldite.

One micron thick reference sections were cut using a Porter-Blum MTL microtome and were stained with toluidine blue. Ultrathin sections (pale silver) were cut on a Reichart OMU 3 ultramicrotome with the aid of a diamond knife. Sections were then mounted on grids, stained with uranyl acetate and lead citrate (Reynolds, 1963) and viewed with an AEI EM6B electron microscope.

Data were collected from electron micrographs by a machine, which was recently developed in this laboratory (Clark, Ensor and Maxwell, unpublished). This machine, which consists of a size-to-voltage transducer arranged like a vernier caliper, enables the user to store linear measurements directly in

computer files. In this case a PDP-12 computer was used to perform several statistical tests upon the data, including a *t*-test for unpaired samples.

Results

Tissue

The acini of the cockroach were found to consist of two main types of cell, peripheral and central cells (Fig. 1) thus confirming the observations of Bland and House (1971). Peripheral cells were often found in pairs, linked together by a septate desmosome. These surround a single lumen which possesses a brush-border of microvilli. A notable feature of peripheral cells is the high density of mitochondria which are wrapped in a highly invaginated membrane. Central cells consist mainly of large secretory granules and endoplasmic reticulum. In addition to these cells, duct cells were also observed; these have a luminal layer of chitin. The entire acinus is surrounded by a basement membrane (see Fig. 9 and also e.g. Fig. 2).

Types of axon observed

Axons were classified according to the vesi-

Abbreviations for figures

A	type A axon	Gl	glial cell
B	type B axon	LGV	large granular vesicle
BM	basement membrane	MV	microvilli
C	central cell	P	peripheral cell
CA	clear axon	Tr	tracheole
D	duct		

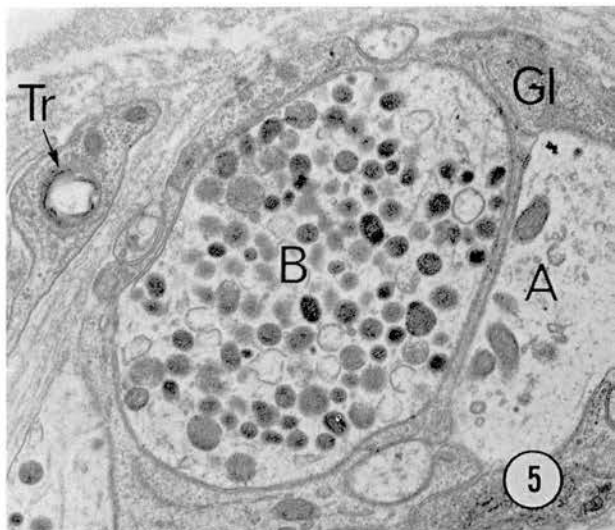
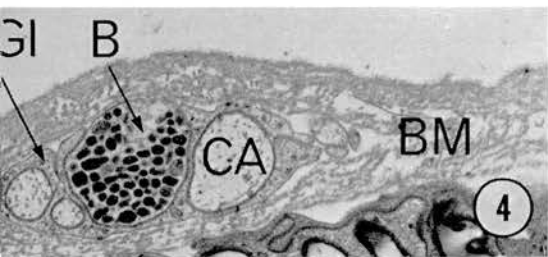
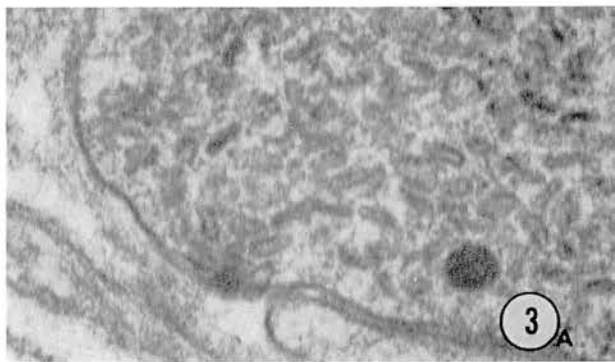
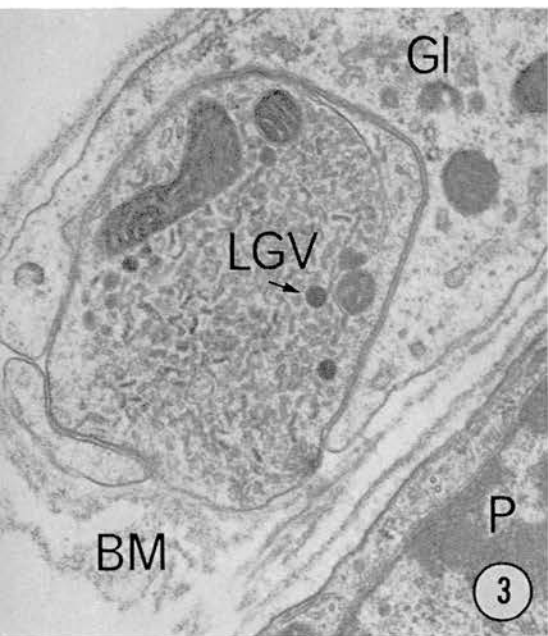
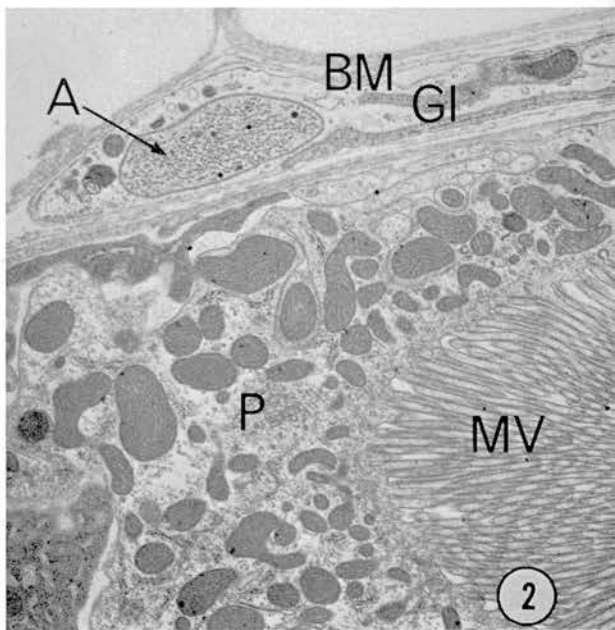
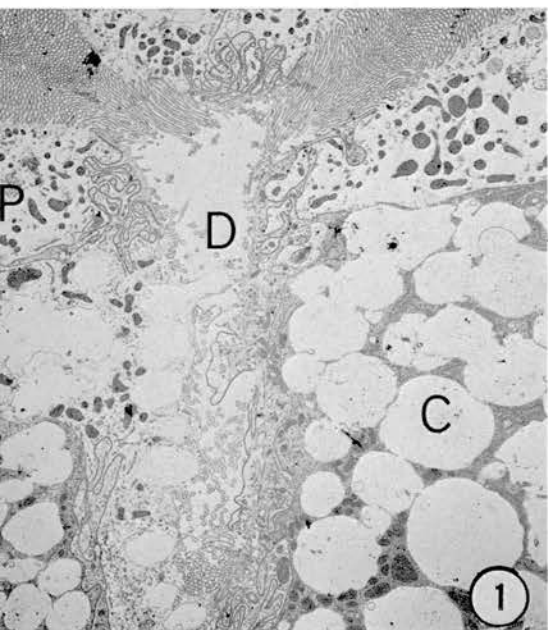
Fig. 1. A section through an acinus showing peripheral cells, central cells and a duct. $\times 3000$.

Fig. 2. A type A axon on the edge of a peripheral cell. Note that the axon is surrounded by a glial cell which in turn is surrounded by basement membrane. $\times 10,000$.

Fig. 3. Another type A cell displaying the agranular elliptical vesicles and the large granular vesicles. Note that the glial cell does not completely surround the axon. $\times 30,000$. Fig. 3a is an enlargement of Fig. 3, illustrating a structure which may be a releasing site. $\times 90,000$.

Fig. 4. A typical type B axon in the basement membrane. The large granular vesicles are prominent. This axon is surrounded by glial cells and is accompanied by some clear axons. $\times 10,000$.

Fig. 5. A part of a nerve running between acini. Note the A and B axons and a tracheole. $\times 20,000$.



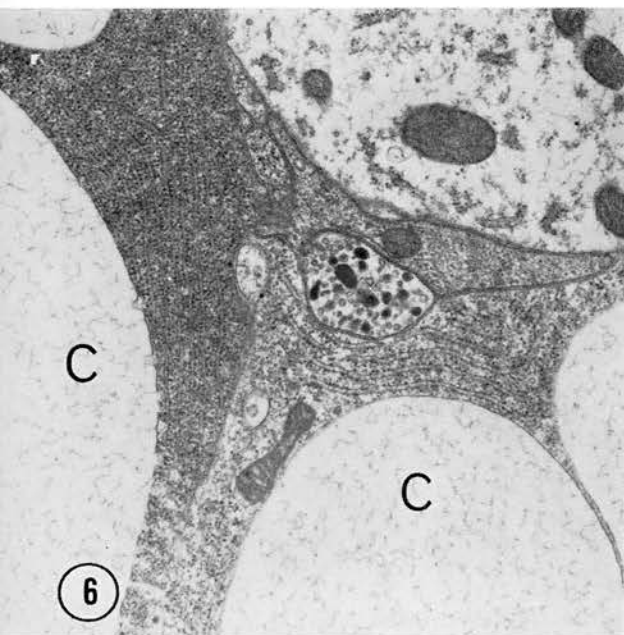


Fig. 6. A type A axon completely surrounded by central cells. $\times 20,000$.

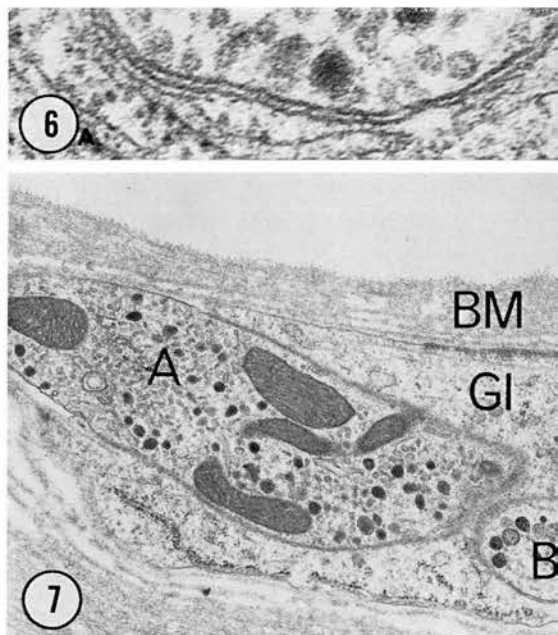


Fig. 6a. Shows that the distance between the axon and central cell membrane is approximately 200 \AA . $\times 100,000$.

Fig. 7. Types A and B axons in close apposition at the basement membrane of an acinus. $\times 15,000$.

cular structures present in them. It was decided that there were two types which have been called 'A' and 'B'. Type A was found to be five times more numerous than type B. In addition to these, axons possessing no vesicular structures were also seen; these shall be referred to as clear axons.

Characteristics of type A (Fig. 2)

Type A axons contain large dense vesicles (osmiophilic) which have a mean diameter of 920 \AA . A distribution of these diameters is shown in Fig. 8a. They also possess agranular vesicular profiles, which are not circular and have a mean dimension of 440 \AA (Fig. 3). Stereological analysis of these was performed by plotting the ratio of the maximum dimension to the minimum dimension of each vesicle against percentage cumulative frequency and comparing this with the curves produced by Elias *et al.* (1970). The curve derived by this means corresponded most closely to that of a prolate rotary ellipsoid (Fig. 8c).

Characteristics of type B (Fig. 4)

Type B axons possess large osmiophilic vesicles which are usually densely packed. A distribution of their diameters is shown in Fig. 8b. The mean diameter of these is 1380 \AA which is significantly different from the diameters of type A large granular vesicles. A *t*-test confirms this claim ($P < 0.0005$).

Relationships between type A and B axons and the cells of the acinus

Fig. 9 illustrates the major features of the innervation of the gland. Type A axons were found in the basement membrane of the acinus (Figs. 2, 9a). These axons were invariably in conjunction with glial cells; on some occasions the glial cell was seen not to surround the axon completely (Fig. 3). The axon in Fig. 3 is not only free of a glial surrounding in one corner, but also possesses a conglomeration of vesicles suggesting the presence of a releasing site. The vesicles are aggregated

round a dense object on the axon membrane; this could be a presynaptic rodlet (see Osborne, 1977).

Type A axons were also found at several other sites; Fig. 6 shows an axon running between several central cells (Fig. 9b). The extracellular space between these axons and the acinar cells was usually not more than 200 Å wide. Axons in this position are in-

frequently associated with glial cells, as is also the case with *Periplaneta* (Whitehead, 1971). Axons, like those between central cells, may also be found between peripheral and central cells (Fig. 9c), and between central and duct cells (Fig. 9d).

Type B axons are also prominent in the basement membrane of the acini (Figs. 4, 9f), on the surface of duct cells (Fig. 9g) and in

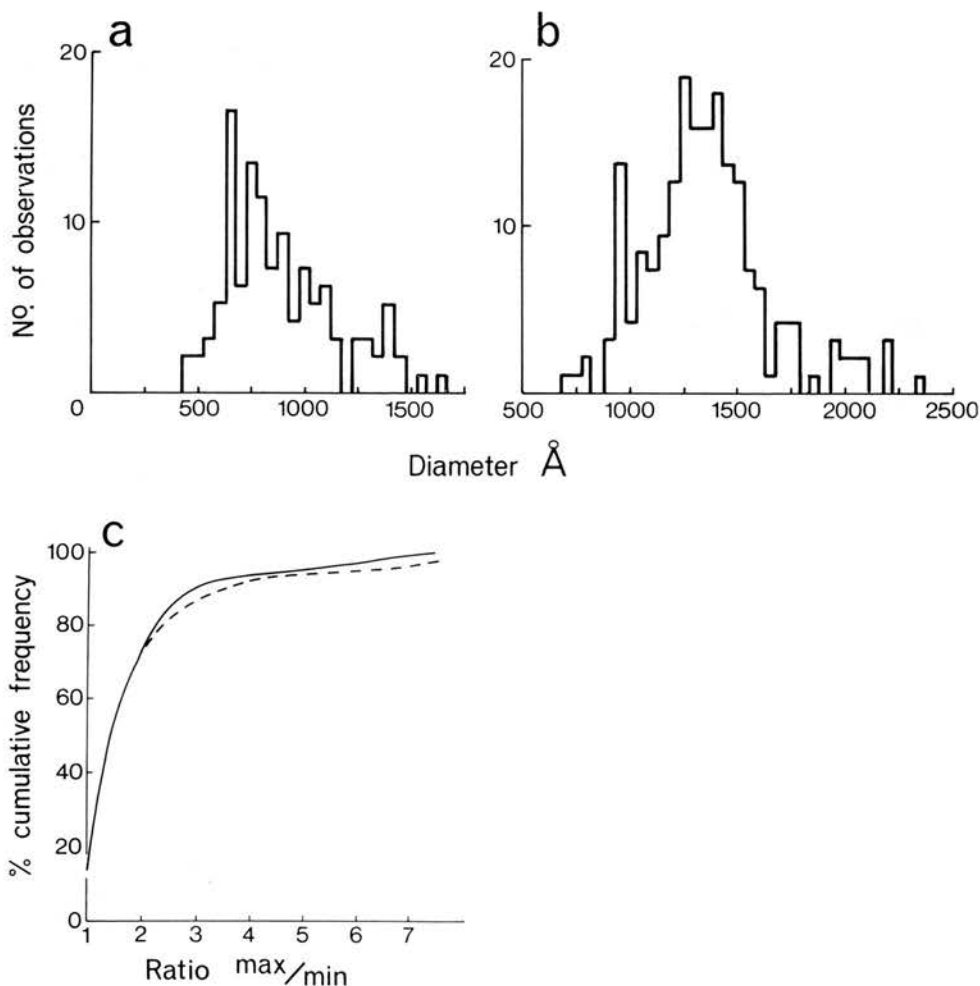


Fig. 8a. A histogram of the diameters of 116 large granular vesicles as measured from several type A axons.

Fig. 8b. A histogram of the diameters of 187 large granular vesicles measured from several type B axons.

Fig. 8c. A plot of cumulative frequency against the ratio of the maximum to the minimum dimension of the agranular vesicles of type A axons. The dotted line represents a curve produced by Elias *et al.* (1970) for a prolate rotary ellipsoid.

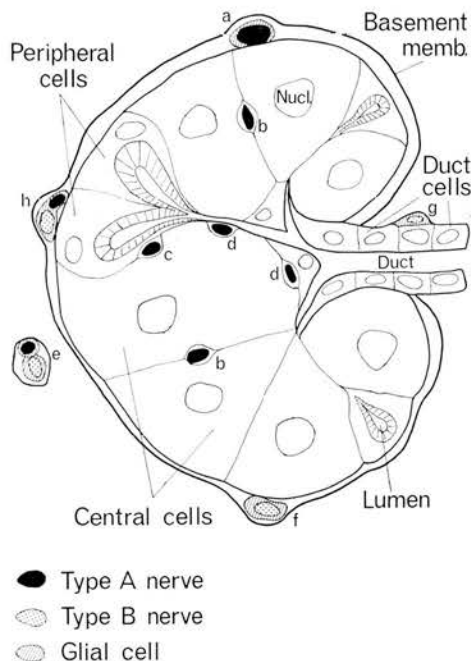


Fig. 9. A schematic diagram (not to scale) showing the main features of a cockroach acinus with special reference to the relationships of types A and B axons to the cells of the gland (see text for full description).

the large nerves which traverse the acini (Figs. 5, 9e). They are usually associated with glial cells, are never found deep in the core of acini (i.e. Fig. 9b-d), and do not possess any obvious form of releasing site.

Bundles of several axons are often seen running over the surface of acini in the basement membrane. These may consist of any combination of types A, B or clear axons. Fig. 7 illustrates one of these bundles consisting of types A and B axons. As yet no functional synaptic connections have been demonstrated between the axons associated with the basement membrane.

Discussion

The ultimate aim of this study was to determine the types of axon present in the salivary apparatus of the cockroach and to correlate this with what is already known about the innervation of the gland from studies involving electrophysiology and fluorescence microscopy. Condensation with paraformaldehyde gas (Bland *et al.*, 1973) has

demonstrated the presence of catecholamines in the salivary nerves. Electrophysiological (Bowser-Riley and House, 1976) and secretion studies (Smith, 1977; Smith and House, 1977) have suggested that dopamine is the neurotransmitter in these glands. The question must now be answered: which type of axon, A or B, employs dopamine as a neurotransmitter?

Previous ultrastructural studies on nerves that are known to contain catecholamines show that they are not unlike type A (Bloom, 1970; Bennet, 1972). Type A axons are also known to react with permanganate fixative (D.J.M., unpublished observation), which according to Hökfelt and Jonsson (1968) reacts specifically with catecholamines to form an electron-dense precipitate.

There is no evidence to indicate that type B axons are in any way associated with catecholamines, indeed they appear to resemble the neurosecretory type cells which have been reported in insects by many authors (e.g. Mancini and Frontali, 1967; Miller, 1975). The role of the clear axons is unknown, these may be a separate category in their own right, or more probably, as adjacent sections show clear areas in axons of types A and B, may be sections through these areas which are free of vesicular profiles.

It is possible that the elliptical vesicles observed in type A axons are a fixation artifact (Gray, 1977). However some authors have noted elliptical and circular vesicular profiles in the same section but in different nerves (Wickelgren, 1977). This observation reduces the likelihood of their being artifactual.

Intracellular recording from acinar cells in the cockroach salivary gland has shown that a hyperpolarizing potential is evoked by stimulation of the salivary nerves (Ginsborg and House, 1976; Ginsborg *et al.*, 1976). This response has a latency of 1 sec. This latency may be reduced to not less than 380 msec when dopamine is ionophoresed on to the surface of the acini, rather than the nerve being stimulated (Ginsborg and House, unpublished observations). If one considers that the shortest distance that transmitter may have to diffuse is 200 Å (Fig. 6) until it encounters the membranes of the acinar cells, then this excessively long latency cannot be explained in conventional terms of diffusion. Even the proposed

releasing site of a type A axon found within the basement membrane (Fig. 3) should yield a theoretical latency of less than 1 msec, assuming that the transmitter must travel $1\ \mu\text{m}$ to receptor sites on the acinar cell membranes and that the basement membrane does not impede its journey. These observations and calculations may be consistent with the apparent absence of any postsynaptic specializations in the vicinity of axons containing vesicles. It may be that in many systems, including the cockroach salivary gland, that employ catecholamines as neurotransmitters they are released from varicosities (Elfvin, 1963; Bennet, 1972;

Bowser-Riley, 1978) and have to travel a considerable distance before coming in contact with their receptor sites.

Studies are now in progress to determine the histochemical properties of types A and B axons.

Acknowledgements

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