# SOME ASPECTS OF THE NEUROPHYSIOLOGY OF THE COMMON WHELK, BUCCINUM UNDATUM L.

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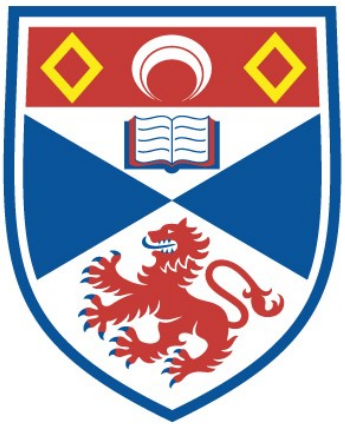
A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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# Some aspects of the neurophysiology of the common whelk, Buccinum undatum L.

by

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Thesis presented for the degree of Doctor of Philosophy in the Faculty of Science of the University of St. Andrews.

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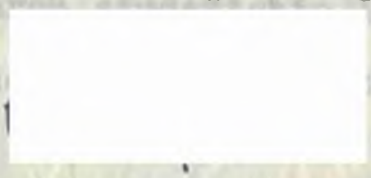
I hereby declare that the work recorded in this Thesis has been carried out by myself and that it is of my own composition. I further declare that it has not been submitted in any previous application for a higher degree.



## Research Carreer

I graduated from the University of Wales (University College of Wales, Aberystwyth) obtaining a B.Sc. degree with honours. The research work recorded in this thesis has been carried out during the nine terms between August 1961 and June 1964, during which time I held a Department of Scientific and Industrial Research studentship in the Zoology Department and Gatty Marine Laboratory of the University of St. Andrews.

## Supervisor's Certificate

I certify that Donald F. Bailey has fulfilled the conditions laid down in the regulations for a degree of Doctor of Philosophy, under ordinance 16 of the University Court of the University of St. Andrews and that he has accordingly qualified to submit this Thesis for the degree of Doctor of Philosophy.

## Acknowledgements

I hereby wish to acknowledge the help and guidance of my Supervisor, Dr. M. S. Laverack, during the work recorded in this thesis, and to thank the Department of Scientific and Industrial Research for their financial assistance. I would also like to thank both the Staff and Students of the Gatty Marine Laboratory for a great deal of useful discussion, in particular Mr. D. Ardill, B.Sc. for his assistance with the electromicroscopy and Mr. J. Stevenson for his help in producing the figures included in this thesis. Lastly I would like to thank my typist, Miss M. Thomson, for her painstaking efforts on my behalf.

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## INTRODUCTION

The Phylum Mollusca is one of the largest and most diverse in the animal kingdom. Within this Phylum the members of the Gastropoda constitute a large class, and are to be found in most types of environment. The class is divided into three orders, namely the Prosobranchiata, the Opisthobranchiata and the Pulmonata.

Anatomical descriptions of the Mollusca in general, and the Gastropoda in particular, are numerous and date back over many years. With reference to this thesis the anatomy of the nervous systems is of major interest. The general features of the nervous systems of the various Molluscan classes have been well studied and there are many papers on the detailed anatomy of individual species. The class Gastropoda has received its fair share of attention from the anatomists, whose efforts have been diverse and numerous amongst the three orders of this class. One of the most notable of the early workers was Bouvier (1886-1900) whose descriptions embraced a wide variety of the gastropod molluscs. In particular he was responsible for a detailed and comprehensive account of the general morphology, classification and the nervous anatomy of the prosobranchs (Bouvier, 1887). In the course of this work he described, in detail, the anatomy of the central nervous systems and the distribution of the peripheral nerves in many species including the subject of this thesis, Buccinum undatum L.

Organs of special sense in the Gastropoda, e.g. eyes, statocysts and osphradia, have also been well described in the course of this, and other early works, though the smaller diffuse receptors such as those in the musculature, have received less attention. Cuticular sensory endings, whilst having been described, have usually been assigned functions in an arbitrary manner without physiological evidence.

Anatomical studies of the Gastropoda, in common with all other animal classes, have undergone considerable revision following the introducation of the electron microscope. Among these studies on fine structure, have been several concerned with the nervous systems, e.g. Schlote, 1957 et seq. These observations have been invaluable in view of the considerable amount of work on the physiological properties of the neurones in certain Gastropods e.g. Helix. The Prosobranchs and Opisthobranchs have received little attention in this respect, leaving a considerable gap in the knowledge of the comparative microanatomy of the nervous systems of the Gastropoda.

Physiological aspects of the Mollusca have been studied in some detail but in relatively restricted fields. The most intensive of these is that of the nervous system, and the Molluscs have provided a disproportionate amount of the accumulated knowledge concerning both the physiology of individual neurones and their combined integrative functions. The work in this category, as well as being largely restricted to the Mollusca, has also been confined to a very few species within the group, namely

the Gastropods Helix and Aplysia, the Cephalopods Sepia, Loligo and Octopus, and, to a lesser extent the Lamellibranchs Mya and Spisula. The interest of research workers in the nervous systems of these few species is stimulated by the presence of 'giant' nerve cells and axons in the majority of these species, which constitute readily identifiable and workable physiological preparations. The relatively immense size of these few nervous elements has proved to be of great advantage in that single, identifiable neurones can be used for an extensive series of experiments. The study of the giant neurones of Helix and Aplysia in situ, and the giant axons of the cephalopods in isolation, has thus promoted a great deal of research into basic nervous physiology. This work, e.g. Tauc et al. (1955 et seq.) Arvanitaki & Chalazonitis (1955 et seq.), Arvanitaki (1939 et seq.), Bullock et al. (1948 et seq.), Hodgkin & Huxley (1952 et seq.) and Kerkut et al. (1956 et seq.) whilst providing much of our knowledge on nervous mechanisms, has, by its restricted nature, left many gaps in the knowledge of Molluscan neurophysiology.

Papers concerning the more general aspects of Molluscan neurophysiology are relatively few in number and rather scattered both in the date of their publication and in their interests. Much of the early physiological study was concentrated on the nature of the foot muscle tone, its mechanism and its nervous regulation (Jordan, 1901 et seq. and Postma, 1933 et seq.). Work employing modern recording techniques on

the nervous elements other than the giant cells and axons is very restricted, especially amongst the Gastropoda. That concerning the neurophysiology of Prosobranchs is limited to a few papers which will be discussed later.

The experimental animal.

The animal chosen for this work was the common whelk, Buccinum undatum L. This is a Prosobranch, Gastropod mollusc common round the shores of the British Isles. The reasons for this choice are, the relatively large size of the animal, which ranges from one to six inches in length, as measured from the shell; the large numbers available and their ease of collection; the layout of the nervous system, which is easy to dissect in order to expose both the central ganglia and the long nerves lying in the body cavity; and most particularly the presence of a large, bipectinate osphradium in the mantle cavity.

Detailed anatomical descriptions of the whole animal have been made by Bouvier (1887) and Dakin (1912), and these two works served as the basis for the dissection of the animal. The descriptions of the nervous system included in these dissertations proved reliable in general, except that no mention is made of the wide degree of variation which is to be found in the size, number and branching of the groups of nerves arising from any particular ganglion. The descriptions are also confined to gross anatomy with respect to the central ganglia and the nerves, no details of the microanatomy being given. The organs of special sense are described in better

detail, and drawings made from sections of such organs as the eye and osphradium are included.

Some confusion has arisen over the nomenclature applied to the nervous system in the earlier works compared with that used in more modern texts. To avoid further muddle over this feature, that used in "British Prosobranch Molluscs" (Fretter & Graham, 1962) is followed throughout this work, in preference to that of Bouvier and Dakin.

Since the descriptions of the gross nervous anatomy by the workers mentioned above, no further investigations into the nervous system of Buccinum have been carried out. There is thus no knowledge of the microanatomy either as determined by light or electron microscopy. The lack of study using the electron microscope applies to the Prosobranchs as a whole as well as Buccinum in particular, so that this species is the first representative of its order to be investigated in this way.

The behaviour of Buccinum has not been studied in any great detail. Its food consists of moribund or recently dead animals including crabs, crayfish, worms, cockles, scallops and other bivalves. The last mentioned are attacked by inserting the shell between the valves and then inserting the proboscis into the soft tissues (Fretter & Graham, 1962, Dakin, 1912 and Hancock, 1957). It is also known to attack fish in nets (Petersen, 1911) causing some damage to the plaice netted in fisheries off Denmark. Gowanloch (1927) observed that Buccinum exposed by the tide show no protective behaviour, either in re-

tracting into the shell or seeking their normal shore level. They continue to move about in a random fashion and frequently perish, either from dessication or from attack by birds after they have become moribund.

A few aspects of the physiology of Buccinum have been studied. Strunk (1935) and Needham (1935 and 1938) studied the excretory processes, the latter showing that uric acid is an excretory product.

Brock (1936) studied aspects of the digestive physiology and showed that an interesting reflex exists, whereby the valve of Leiblen is stimulated chemically by the stomach contents or secretions to contract, and so prevent regurgitation of food into the pharynx.

The work of Bacq & Coppée (1937) included experiments upon Buccinum in a study of the reactions of various invertebrate neuromuscular preparations to acetylcholine and eserine. These experiments revealed that acetylcholine produced a contraction in the isolated foot at a concentration of $5 \times 10^{-4}$ and that this effect was potentiated by the pre-addition of eserine, which also increased the sensitivity of the preparation to acetylcholine. Eserine was without effect on the neuro-muscular transmission, however, suggesting that the latter process is not cholinergic.

The most recent investigations of the physiology of Buccinum are by Welsh (1956), Fänge (1957 and 1958) and Fänge & Mattisson (1958). Welsh found that the salivary glands of the whelk

produce 5-hydroxytryptamine, which, it has been suggested, is a toxic agent used by the animal as an aid to capturing more active prey (Fänge, 1957 and 1958).

The work by Fänge & Mattison deals with the physiology of the radula muscle of Buccinum and demonstrates that there is a high concentration of mitochondria in the muscle correlated with a high oxygen consumption. The respiratory enzymes were not easily poisoned by either cyanide or carbon monoxide and would thus allow a high level of respiratory activity even in unfavourable conditions.

The experimental work upon Buccinum which has been summarised above shows that, apart from the researches of Bacq and Bacq & Coppée on the neuromuscular junction and Brock on the valve of Leiblen reflex, the neurophysiology has received no attention. Thus nothing beyond the gross anatomy described by the early workers is known concerning the nervous system of the animal.

The research topics.

The previous pages of the thesis have served to outline the state of our knowledge concerning the nervous systems of the Prosobranchs in general and of Buccinum in particular. From the review of the literature it is clear that very little work on the neurophysiology of this order has been carried out in the past so that it forms 'a subject likely to be as diverse as it is virgin' (Bullock & Horridge, in press). Topics for research into Prosobranch nervous systems are therfore present in both

anatomical and electrophysiological fields.

In the course of the work undertaken for this thesis micro-anatomical studies have been carried out on the central nervous system and the peripheral nerves, using light microscopy for the former and electron microscopy for the latter. In this way the structural organisation of the central ganglia and nerves was studied, providing details of cell body size, axon diameter, the relationship between axons and glia and the structure of the perineurium. It is thus possible to fill in some of the gap which exists in respect of Prosobranch anatomy in the comparative neuro-anatomy of the Mollusca.

Buccinum has never previously been subjected to electrophysiological investigation and is thus a completely unknown field. The experimental investigations have concentrated on the following topics:-

1) Peripheral receptors.

Peripheral receptors have been studied to the extent that afferent impulses from mechanoreceptors in the mantle region have been recorded and analysed (Laverack & Bailey, 1963). These investigations were hampered somewhat by the erratic situation regarding the presence or absence of recordable activity in the nerves. Because of this a considerable number of preparations were required in order to obtain a comprehensive analysis of the mode of reaction of the receptors.

Chemoreception is another subject of interest in relation to this animal because of the chemo- and mechanoreceptive func-

tions which have been postulated for the osphradium in the Prosobranchs as a whole. The work of Copeland (1918), Wölper (1950) and Brown & Noble (1960) all support the former theory whilst Hulbert & Yonge (1937) and Yonge (1947) put forward the latter hypothesis. In this field it was not possible to obtain records of the receptor activity, but a study of central reactions to various stimuli applied to the osphradium has revealed the function of that organ in Buccinum (Bailey & Laverack, 1963).

2) Central nervous pathways.

The functional organisation of the central nervous system in the whelk is another aspect of the animal which has received attention in the course of these experiments. With the aid of two recording channels and various lesions of connectives and commissures linking the central ganglia, it has proved possible to discover a number of central nervous pathways between afferent and efferent activity occasioned by the same stimulus.

3) Central convergence.

As a concommittant to section (2), the convergence of afferent pathways onto central neurones has been studied using both metal filled glass microelectrodes and intracellular saline pipettes.

4) Response patterns of central neurones.

The various responses of different central neurones to similar peripheral stimuli and the responses of single neurones to varied stimuli have been studied by means of intracellular saline pipettes. Using simple chemical and mechanical stimuli it has

proved possible to find a number of different types of central neurone in respect of their reactions to these stimuli.

The diversity of the results obtained from this work and the variety of techniques used in its course, have shown that standard electrophysiological methods are adequate for worthwhile studies on Prosobranch neurophysiology. Thus only further application is necessary in order to extend our knowledge of the nervous functions of this neglected group.

## MATERIALS AND METHODS

The subject of this work is the gastropod prosobrach mollusc Buccinum undatum L., the common whelk. These animals were obtained locally, mainly from the lobster fishermen of St. Andrews and Crail and occasionally from the Marine Biological Station at Millport. Considerable numbers were kept in the laboratory in order to avoid interrupting the experimentation when bad weather prevented collection of the animals. The laboratory stocks were maintained until required in tanks containing 6-8 inches of running sea water. The water flow was kept at a higher rate than is necessary for most marine animals and was well aerated at all times; these precautions were found to be essential for the survival of relatively large numbers of Buccinum in confined spaces. Interruption of either of these factors was followed by the death of many specimens. This mortality may have been due to the accumulation of large amounts of mucous which is copiously secreted by these animals.

The animals held in stock were fed occasionally with Mytilus edulis which had been removed from their shells, but they were capable of surviving without food for many weeks without any apparent adverse effects.

Under these conditions the animals remained healthy, as evidenced by the production of large numbers of egg capsules each Spring, although no specimen was kept in the laboratory for more than four months.

Dissection to expose the nervous system.

In order to expose the nervous system of Buccinum the following dissection was carried out. The shell was carefully removed by splitting it slowly in a vice until sufficient of the body was exposed to allow the columella muscle to be cut free from its insertion on the column of the shell. After this the animal could be freed from the remainder of the shell by rotation. The visceral hump and the foot were removed (see Fig. 1) and the remainder of the animal pinned to the wax floor of a perspex dish in its normal orientation. The perspex dish was then filled with sea water which acted as a physiological saline. The mantle was cut in the mid-dorsal line and deflected to either side to expose the dorsal body wall. The latter was then cut in the median line providing access to the body cavity. The large muscular proboscis and its attendent musculature were removed together with the anterior portion of the oesophagus. The later stages of the dissection were carried out under observation with a binocular microscope. A considerable intensity of illumination was required to allow easy dissection as the nervous system is of similar colour and consistency to its surroundings. Two 30 Watt focussing microscope lamps provided the illumination. Drying of the preparation by the heat from the lamps was prevented by positioning water filters in the light beams. The preparations subsequently survived for long periods in a physiologically active state.

The salivary glands were then carefully dissected free to

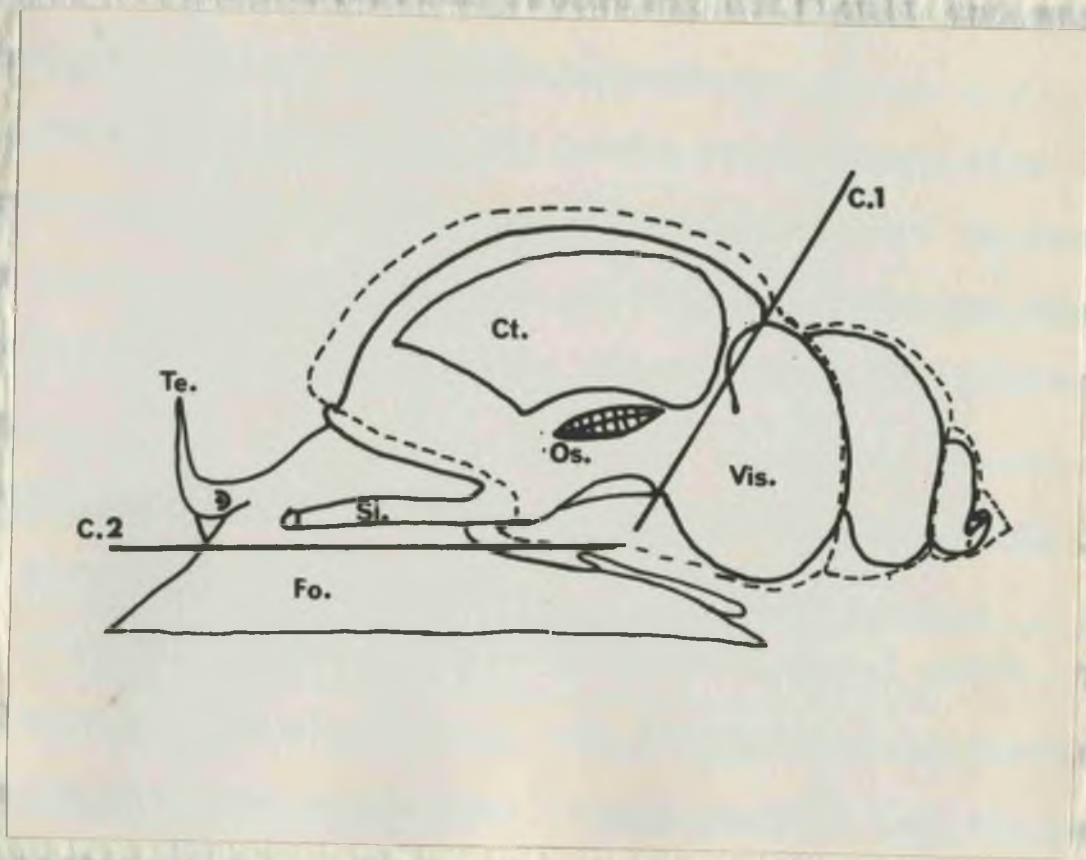



Figure 1. Diagramatic view of Buccinum from the left side. The shell is indicated by the broken line. C.1 - level of removal of the visceral hump. C.2 - level of removal of the foot. Ct., ctenidium; Fo., foot; Os., osphradium; Si., siphon; Te., tentacle; Vis., Visceral hump.

expose the central nervous system, which is composed of a number of associated ganglia (see Figs. 3 and 13). This complex lies immediately below the salivary glands around the oesophagus. The connective tissue, which invests the ganglia and nerves, made electrophysiological recording difficult and was consequently removed as a routine procedure.

This dissection was the basic preparatory step in all the neurophysiological experiments to be described in the course of this thesis. Modifications of this procedure as appropriate to particular experiments are described in the following sections.

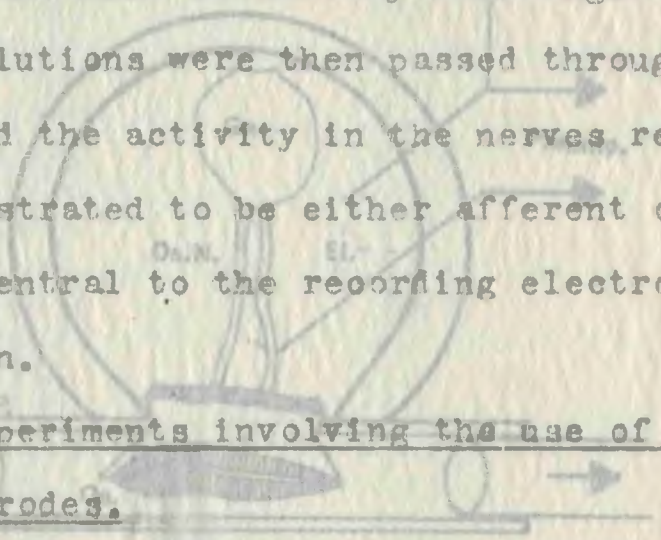
Dissection for experiments involving the use of platinum wire electrodes.

Recording nervous activity in peripheral nerves by means of platinum wire electrodes, was facilitated by further careful dissection of the connective tissue surrounding the nerves. This technique was employed in all the experiments concerning the peripheral movement receptors in the mantle, the central responses to the afferent activity of these receptors and many of the experiments carried out to investigate central nervous pathways. In these experiments the selected nerves were cut peripherally or centrally to enable afferent or efferent impulses to be recorded as required.

In the experiments concerning the central pathways the connective tissue was also removed from the central complex in order to expose the connectives and commisures of the latter, because

these were to be cut in the course of the experiments.

Attempts were made to record nervous activity in the osphradial nerves by means of the platinum electrode technique. The osphradial region, together with the osphradial nerves and the CNS, was removed to the experimental dish shown in Fig. 2. The osphradium was inserted into the polythene pipe via the slit in the side and the joint made relatively watertight with vaseline. Stimulating solutions were then passed through the tube over the osphradium and the activity in the nerves recorded. The activity was demonstrated to be either afferent or efferent by cutting the nerve central to the recording electrode and repeating the stimulation.



Dissection for experiments involving the use of metal filled glass micro-electrodes.

The use of metal filled glass microelectrodes placed in the ganglia of the central complex necessitated modifications to the dissection for two purposes. First to reduce the movements of the preparation during the experiments so that a selected recording position could be held steady in relation to the electrode tip for sufficient time for the proposed experiment to be carried out, and second to facilitate the insertion of the recording electrode.

The reduction of movement was achieved as follows. The connective tissue was dissected from the central nervous complex and many of the nerves in the body cavity. The ventral body wall and the base of the foot were then cut longitudinally in the median line, so that the preparation was almost split in two.

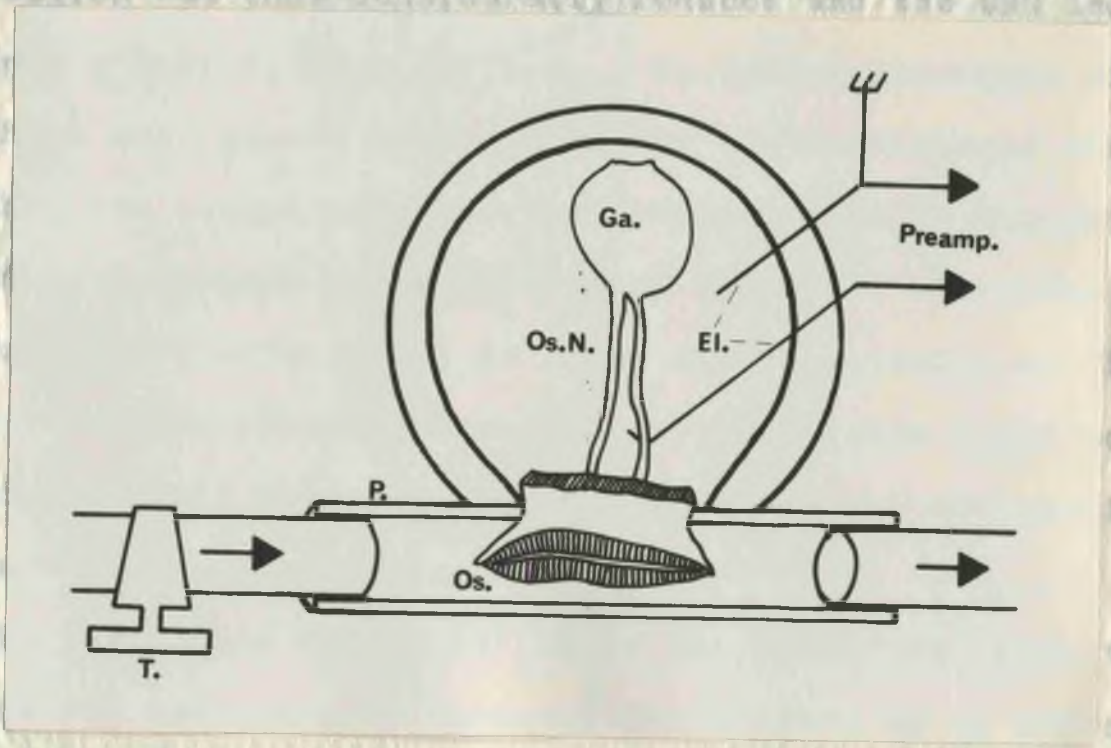



Figure 2. Experimental dish used for stimulation of the osphradium. El., electrodes; Ga., supraintestinal ganglion; Os.N., osphradial nerves; Os., osphradium; P., polythene pipe; Preamp., leads to preamplifier; T., tap; arrows indicate direction of flow of stimulus solutions. See text for further details.

A small, shaped perspex spacer was then inserted beneath the CNS into the slot created in the body wall and pinned to the wax floor of the dissecting dish (see Fig. 3). The two sides of the ventral body wall were then pressed close against the perspex and pinned firmly. The co-ordinated muscular activity of this region was thus considerably reduced and the CNS left resting upon a smooth, hard surface. To reduce movements of the CNS which were caused by the muscular contractions of the nerve sheaths, the oesophagus anterior and posterior to the CNS was pinned down through holes drilled at intervals along the perspex spacer. This arrangement is shown diagramatically in Fig. 3. Under these conditions the entire nervous system could be held relatively still under all but the most violent contractions on the part of the preparation.

To facilitate the insertion of the electrodes into the ganglia it was necessary to desheath the latter, as it proved impossible to pierce the tough outer sheath without damaging the electrode tip. Desheathing was accomplished by carefully removing all the connective tissue from around the selected ganglion and then tearing the sheath on the exposed dorsal surface with the aid of fine pointed watchmakers forceps.

The dissection outlined above was carried out in experiments in which metal filled microelectrodes were used to investigate central nervous pathways. The same recording technique was also used in experiments in which the central nervous responses of isolated ganglia to peripheral chemical stimuli were invest-

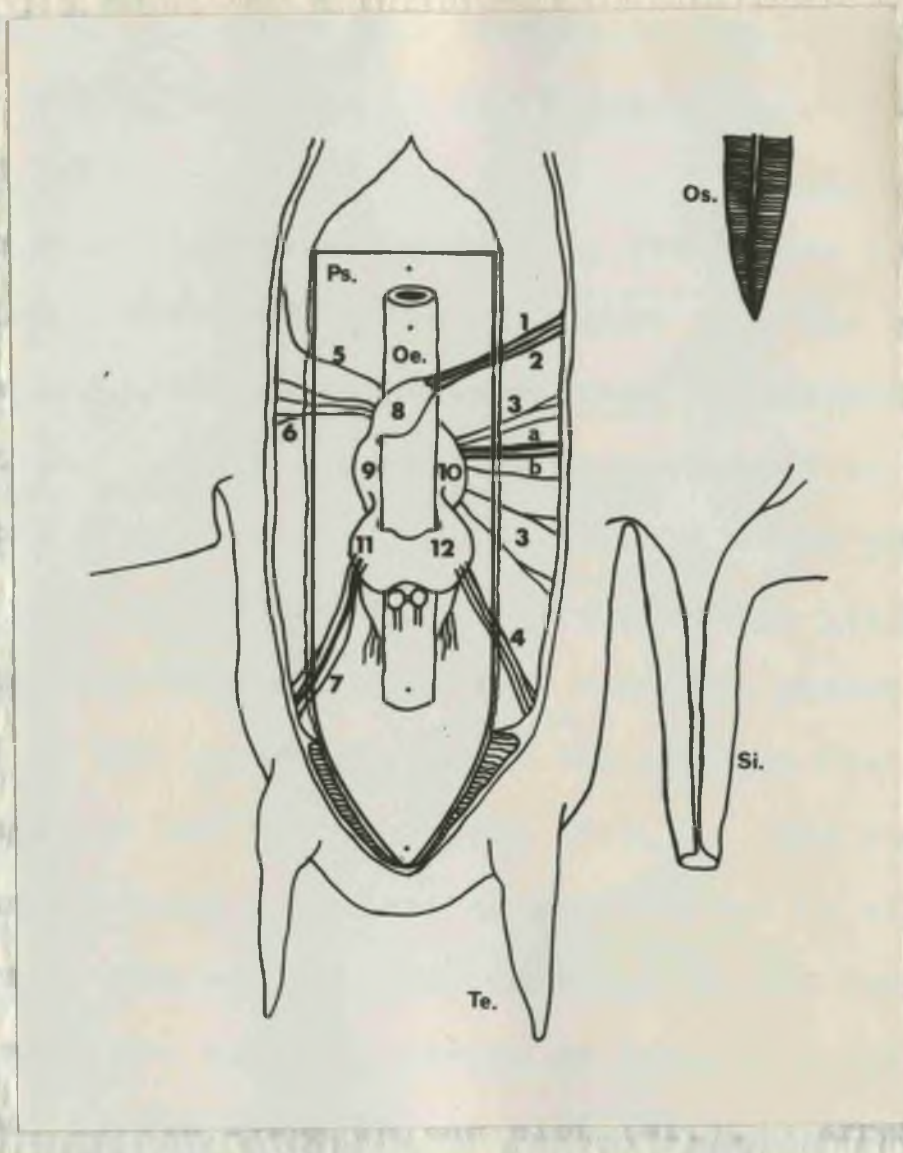



Figure 3. Diagrammatic dorsal view of the dissection for indium filled microelectrodes. Oe., oesophagus; Os., osphradium; Ps., perspex spacer; Si., siphon; Te., tentacle; 1, 2, osphradial nerves; 3., left pallial nerves; a., b., siphonal nerves; 4., left "tentacular" nerves; 5., right visceral connective; 6., right pallial nerves; 7., right "tentacular" nerves; 8., supraintestinal ganglion; 9., right pleural ganglion; 10., left pleural ganglion; 11., right cerebral ganglion; 12., left cerebral ganglion.

igated. The isolation of the selected ganglia, holding them still and the nature of the stimuli in the latter experiments all led to modifications in the dissecting technique which are outlined below.

The supra-intestinal ganglion, from which nerves run to the osphradium and ctenidium, was isolated from the remainder of the CNS together with the latter-mentioned organs and the attendent innervation. These organs were then removed to the small experimental dish shown in Fig. 4, and the whole preparation covered with sea water whilst the connective tissue was removed. The peripheral organ-bearing area was then pinned to the wax base (w) of the lower section of the dish so that the body wall rested against the dividing wall (Div.). The removal of the ctenidium obviated complicating factors due to ctenidial sensory receptors. The ganglion was arranged in the depression provided (Dep.), with the ventral, dorsal or lateral surface exposed and the intact nerves lying in the slot (Sl.). Arranging the ganglion and its nerves in this way the ganglion remained still in spite of nerve sheath contractions which, in early experiments, had caused difficulty by displacing the electrode from the recording site.

Immediately prior to the insertion of the electrode the exposed surface of the ganglion was desheathed and the lower chamber of the dish drained of fluid. Stimulating solutions could then be applied to the osphradium without risk of considerable dilution.

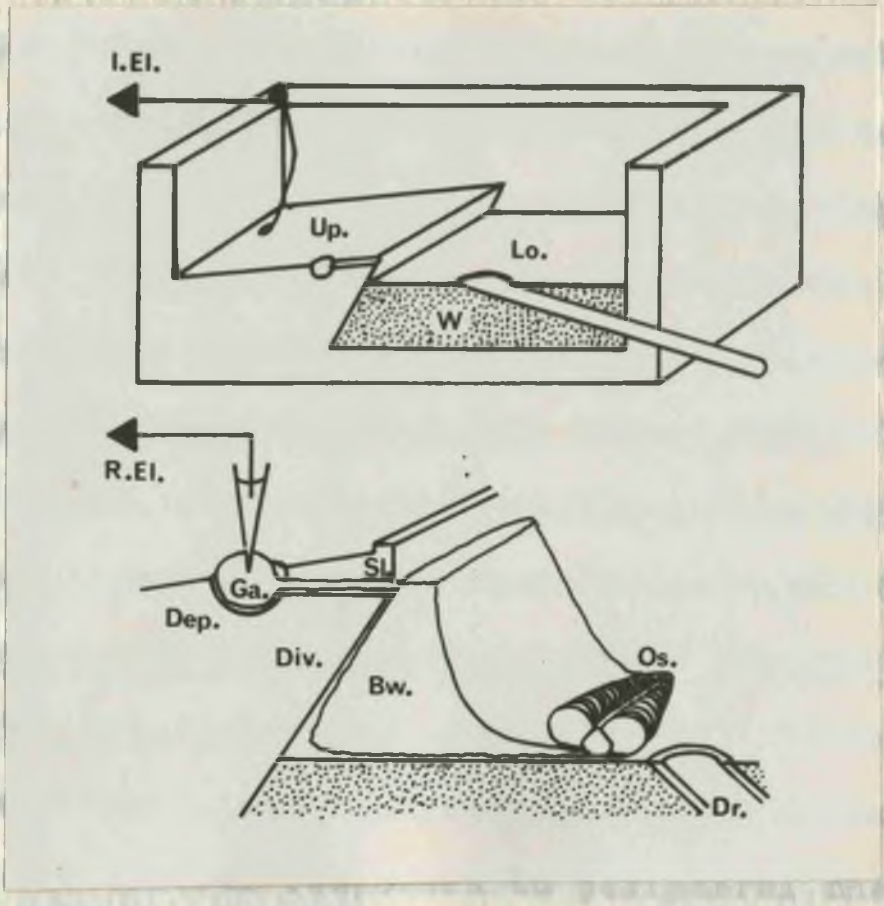



Figure 4. Experimental dish used when recording central nervous activity in response to stimulation of the osphradium, the lower figure showing preparation and recording electrode in situ. Bw., body wall; Ga., supra-intestinal ganglion; I.El., indifferent electrode; Lo., lower chamber of dish; Os., osphradium; R.El., recording electrode; Up., upper chamber of dish. See text for further details.

The preparation was maintained in a physiological state by keeping the upper chamber, containing the ganglion, filled with sea water and frequently washing the remainder of the preparation, in the lower chamber, with the same. All fluids added to the lower chamber were allowed to run out through the drain hole provided (Dr.). The gentle flow of sea water from the upper chamber to the lower along the slot, together with the immediate draining of any excess fluid in the lower chamber, made any capillary action in the reverse direction unlikely. Stimulating solutions placed in the lower chamber therefore had little chance to reach the ganglion in the upper chamber and so cause direct effects on the nervous elements recorded.

Dissection for experiments involving the use of saline filled microelectrodes.

Saline filled microelectrodes were used in experiments concerning the central responses to peripheral chemical and mechanical stimuli in order to obtain clear intra-cellular records of the activity of single cells. The dissecting technique used was that already described for the use of metal filled microelectrodes for the same type of experiments.

ANATOMY AND HISTOLOGY

The anatomical and histological studies were largely directed towards the innervation of the mantle and the osphradium because of the favourable electrophysiological results obtained from this region in the preliminary stages of this work.

Intra-vitam staining with Methylene Blue.

In order to observe the innervation of the mantle and osphradial regions the nerves were exposed and the dissection immersed in a Methylene Blue ( I C I. ZFS) solution in sea water. The course of the siphonal, pallial and osphradial nerves was followed from their exposed central roots into the musculature of the body wall and mantle, a process rendered easier by the absorbtion of the Methylene Blue by the sheaths of the nerves.

Attempts were also made to stain the sensory nervous elements in excised pieces of Buccinum mantle and body wall using the techniques of Alexandrowicz (1960). This involved the soaking of the excised regions in a 1:30,000 Methylene Blue to sea water solution. These experiments failed because, even in small pieces of teased muscle, the dye did not penetrate to any appreciable extent.

In an attempt to stain the nerve axons rather than the sheaths, Methylene Blue solutions were injected 'in vivo' into the blood system of the animal in the hope that better penetration of the stain would result by virtue of the better distribution of the stain by the circulating blood.

The animals were first anaesthetised in an aerated sea water solution containing 12 gms. of magnesium sulphate per litre. After 6-12 hours of this treatment the animals were sufficiently extended from their shells and slow in their reactions for injections to be made. Routinely, however, they were left overnight (15 hours) in the anaesthetic solution,

after which time they were devoid of tone and mobility. Various concentrations of Methylene Blue were injected by inserting the hypodermic needle into the large cephalic blood sinus near the ventral body wall. The following solutions were injected in the various experiments:-

0.5mls 0.1% Methylene Blue in 1.5% sodium chloride solution.
0.5mls 0.5% " " " distilled water.
0.5mls 1.0% " " " "
1.0mls 0.1% " " " 0.5M sucrose solution.
1.0mls 1.0% " " " "

After injection the animals were returned to fresh aerated sea water for at least 12 hours and in this period they recovered from the magnesium anaesthesia and showed normal activity. They were then dissected in a 1:30,000 Methylene Blue: sea water solution. Selected well stained regions were removed, washed in distilled water and fixed in 10% w.w. ammonium molybdate made up in 0.5M sucrose solution. After fixation they were washed, dehydrated rapidly, cleared in xylol and mounted in xylol Damar (Alexandrowicz, 1932).

Histology of the Mantle region.

The region of the mantle shown by electrophysiological experiments to contain the movement receptors (see P. 78 and Fig. 34) was removed from the experimental animal in several cases and placed immediately in Bouins fixative made up in sea water. After 24 hours fixation the specimens were washed in 70% ethyl alcohol to remove the picrate stain and then embedded in paraffin

wax. Transverse sections were cut serially at 12μ, stained in Ehrlich's Haematoxylin and Eosin and mounted in Canada Balsam.

Histology of the Central Nervous System.

The CNS was dissected as already described and removed from the body cavity together with the small section of the oesophagus around which it is arranged (see Fig. 3). The material was fixed in FAA (Formalin 2:85% alcohol 17: Glacial Acetic Acid 1) for 6 hours. Serial paraffin sections cut at 10μ were stained with Heidenhains Iron Haematoxylin (Pantin,1948).

Histology of the Peripheral Nerves.

The detailed histology of the peripheral nerves was not approachable by means of conventional light microscopy because much of the fine detail is well below the limits of resolution of the light microscope. Consequently this material was studied by means of electron microscopy. Portions of the siphonal and pallial nerves were dissected out in sea water and then fixed for 60 minutes in ice cold 1% osmium tetroxide (in veronal buffered sea water at pH 7.5) (Palade, 1952). The fixed material was embedded in an Araldite mixture (made up of equal parts of CY212 resin and HY964 hardener with a solution of equal parts of Di-n-Butyl Phthalate and DY064 accelerator in the proportion of one part of the second solution to 19 parts of the first). Sections of 250-500Å thickness were cut on a Porter-Blum ultramicrotome using glass knives. The sections were mounted on carbon coated Formvar grids and stained with lead citrate (Reynolds, 1963). The grids were treated with 0.02M sodium hydro-

xide prior to staining, to remove any grease from the surface of the sections (Ardill, 1964). The sections were examined with a Siemens Elmiskop 1 electron microscope at 80 Kv. Photographs were taken on Ilford N50 plates.

ELECTROPHYSIOLOGICAL TECHNIQUES.

Early attempts to record the nervous activity of Buccinum revealed that the extra-cellular potentials were small (less than 100μv) and of a relatively 'slow' type (time course often of some tens of milliseconds). In order to obtain the best possible recording conditions a differential, AC pre-amplifier was constructed to provide low circuit noise and high gain. By selecting the valves the noise level of the system was reduced to approximately 5μv. The gain was 1500x and the rejection ratio approximately 100;1. The circuit of this amplifier is shown in Fig. 5.

In the course of the experiments on the nervous system of Buccinum three basic recording techniques were used. These are best given short titles by refering to the electrodes which are used in each of the techniques. They will therefore be refered to in the following manner:- (1) the platinum wire electrode system, (2) the indium microelectrode system and (3) the saline microelectrode system. The type and arrangement of equipment in each of these cases varied considerably and a brief outline of the systems is given below.

The platinum wire electrode system.

Extracellular nervous potentials were recorded from nerves

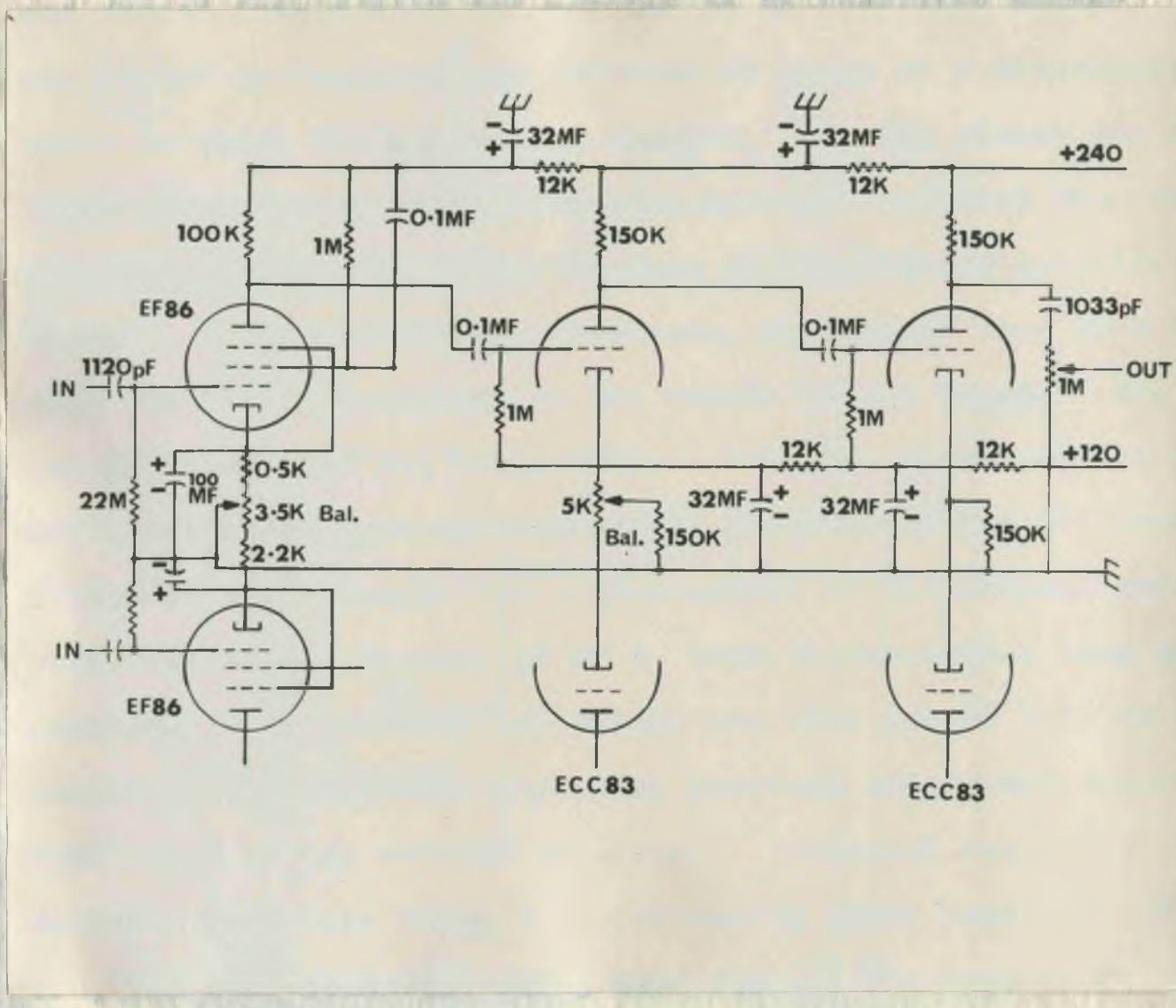



Figure 5. Circuit diagram of preamplifier. Bal., balancing potentiometers; IN., inputs from indifferent and recording electrodes; OUT., output to oscilloscope. The circuit for the lower valves is the same as that drawn for the upper valves.

by means of platinum wire electrodes. The recording electrode was shaped into a hook and mounted on an insulated holder. It was placed in the required position by means of a micromanipulator to which the holder was clamped. Having placed the electrode under the selected nerve the latter was lifted from the sea water bath into air for records of the nervous activity to be made. The indifferent electrode, a platinum wire or a steel pin, was placed elsewhere in the muscle of the preparation, which lay in an earthed sea water bath. Both the recording and indifferent electrodes were connected directly to the differential inputs of the preamplifier. The output of the preamplifier was connected to one channel of Mk 4, 1049 Cossor double beam oscilloscope. The preamplifier output was also played into an audio-amplifier and a loudspeaker which provided concurrent audio-monitoring of the nervous activity. Pictorial records of nervous activity were made using a Cossor moving paper oscilloscope camera, the same technique being used for all the experiments described in this thesis. Fig. 6 shows the above arrangements in diagrammatic form.

Sea water was used as a bathing medium during the course of the experiments. The nerves raised into air on the platinum recording electrode were kept moist by frequent applications of sea water. Under these conditions the preparations remained active for periods of 6 hours and more.

The recording system described above was used for the experiments upon the movement receptors in the mantle region (see

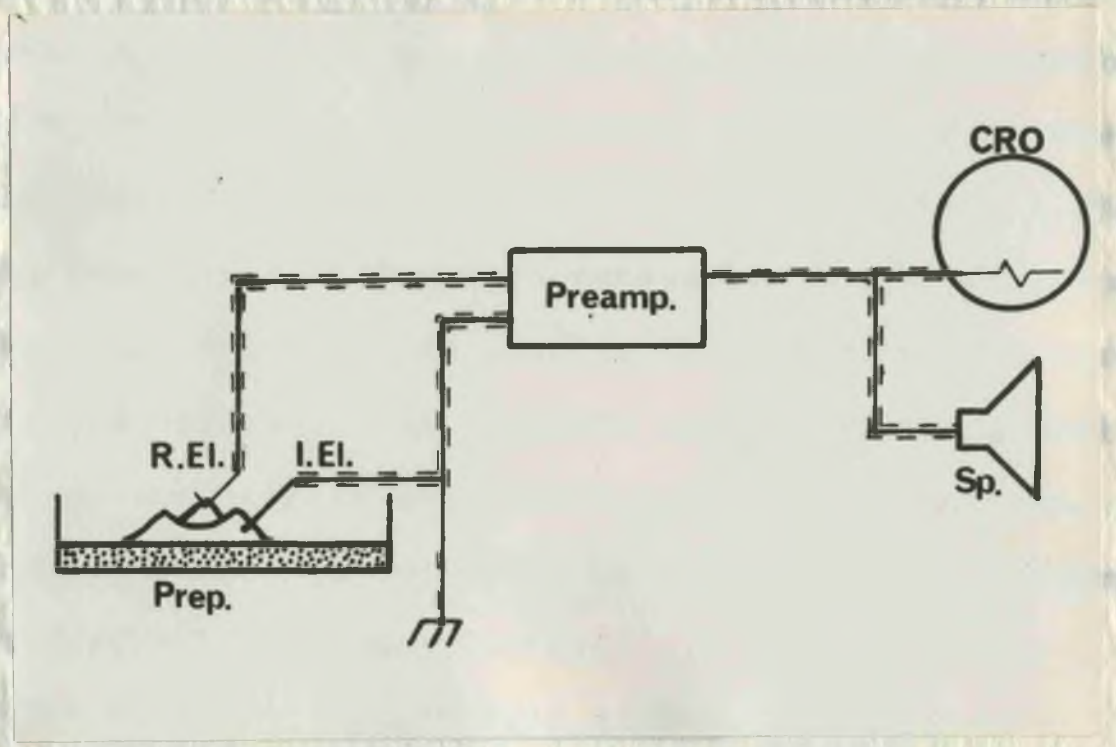



Figure 6. Diagram showing arrangements made for recording with platinum wire electrodes. CRO., oscilloscope; I.EL., indifferent electrode; Prep., experimental dish containing preparation; Preamp., preamplifier; R.EL., recording electrode; Sp., loud-speaker.

p.78). The experiments concerning central nervous pathways involved an extension of this technique in that two recording channels were used. This arrangement is shown in Fig. 7. Two sets of platinum wire electrodes were used, the recording electrode in each case being independently movable and connected to a separate preamplifier. Both the preamplifiers were built to the same circuit and were as nearly identical as possible. The indifferent platinum wire electrodes were placed in the muscle of the preparation which lay in earthed sea water. The outputs of the two preamplifiers were connected to the two separate beams of the oscilloscope. By placing the recording electrodes under identified nerves it was possible to investigate activity occuring simultaneously in two widely separated situations. Either of the recording systems could be linked to the audioamplifier for concurrent audio-monitoring.

The indium microelectrode system.

Glass microelectrode blanks were pulled from soda glass melting point tubing. They were broken off to the required tip diameter of 3-5μ, filled with an alloy composed of 30% indium and 70% woods metal and the tips plated with platinum black largely as described by Gesteland et al. 1959. The plating technique was somewhat modified in that a 1Kc/s sine wave of low amplitude was used instead of a low voltage D.C. supply (Scholes, 1963). Prior to plating the metal filling at the electrode tip was etched into a convavity using a 1:1 mixture of conc. sulphuric acid and conc. hydrogen peroxide (Scholes, 1963). Electrode

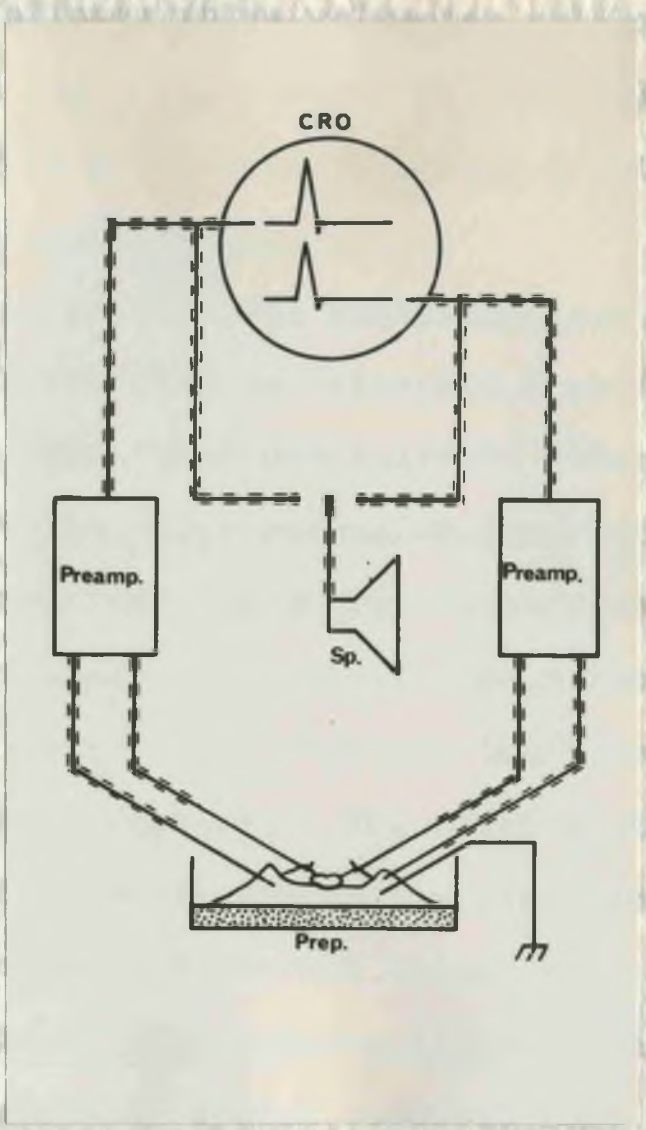



Figure 7. Diagram showing arrangements made for dual channel recording with platinum wire electrodes. CRO., oscilloscope; Preamp., preamplifier; Prep., experimental dish containing preparation. The electrodes are arranged as in Fig. 6.

resistance was monitored throughout the plating process by means of a cathode follower valve voltmeter calibrated to give a direct resistance reading and connected in parallel between the microelectrode and the indifferent platinum electrolytic electrode (Scholes, 1963).

Under these conditions, selected glass blanks would produce electrodes with resistances below 100 ohms. Electrodes of such low resistance were found necessary in order that the noise level remained at the low value essential for recording the small extracellular potentials common in the nervous system of Buccinum.

The use of indium microelectrodes which have a finite, though in this case a very low, resistance led to some modifications of the recording arrangements. The first of these was the insertion of a 'cathode follower' between the recording electrode and the preamplifier to prevent current being drawn from the preparation (Donaldson, 1958). The cathode follower increased the noise level of the recording system to about 10μv but the closer proximity of the recording electrode to the site of the nervous activity more than compensated for this initial disadvantage.

The other alterations concerned the manipulation of the electrodes during the experiments. Because several electrodes might be used in the course of one experiment, a perspex clamp which allowed for the easy insertion and removal of the electrodes was made. Indium microelectrodes necessitated more precise control of the electrode than had hitherto been the case. This was accomplished by a hydraulic advance system (Chapman, 1963 from

a system demonstrated by Prof. Schwartzkopff). This allowed for both coarse and fine movements of the electrode along its longitudinal axis. Movement of the electrode in the two horizontal planes was accomplished by mounting the hydraulic electrode advance on a Prior micromanipulator.

The recording electrode was connected to one cathode follower and the indifferent platinum wire electrode to another. The cathode follower outputs were led into the preamplifier inputs. Thereafter the recording arrangements were similar to those already described for single channel recordings using platinum wire electrodes. The indium microelectrode system is shown diagrammatically in Fig. 8.

This recording technique was used in experiments upon central nervous pathways and central responses to peripheral chemical stimuli.

The saline microelectrode system.

In some of the experiments carried out to investigate the central nervous responses to peripheral chemical stimuli saline filled micropipettes were inserted into the cell bodies of the nerve cells within the ganglia. In this way it was possible to obtain records of the intracellular potential changes.

The saline microelectrodes were prepared in the normal manner from borosilicate melting point tubes and filled with 2.9M potassium chloride solution. Chlorided silver wires were used for both recording and indifferent electrodes, the former placed in contact with the saline of the electrode and the latter lying

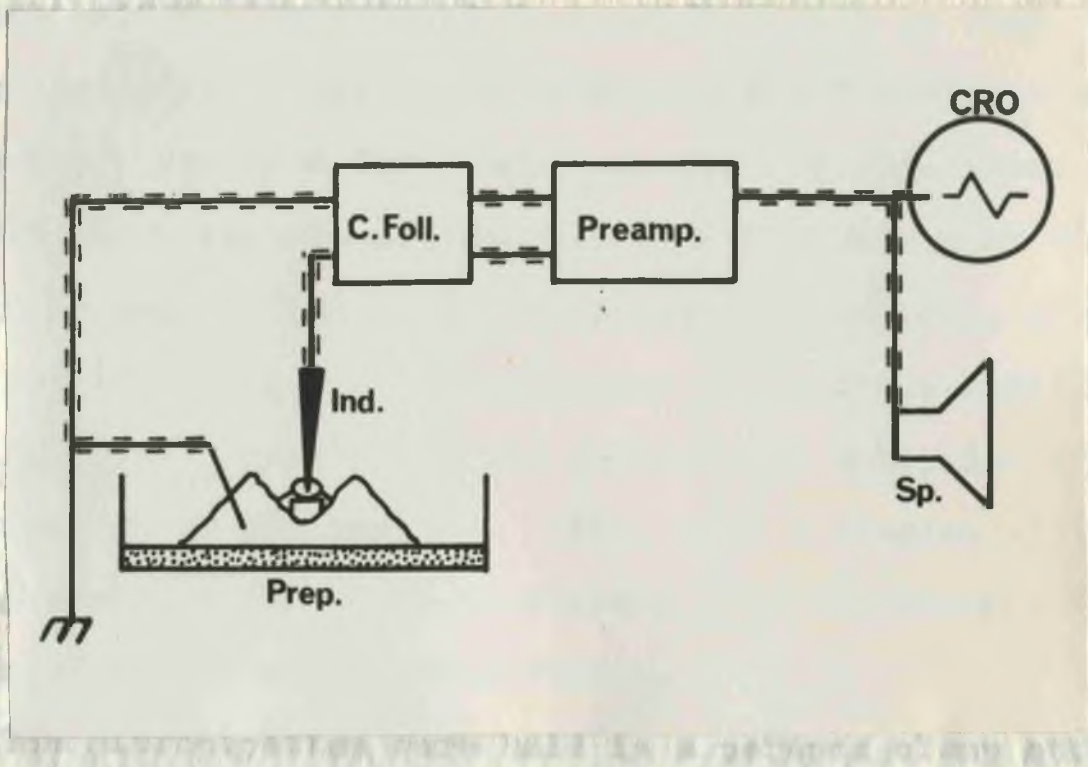



Figure 8. Diagram showing arrangements made for recording with indium filled, glass microelectrodes. C.Foll., cathode follower; Ind., indium microelectrode. Remainder as in Fig. 6.

in the sea water bathing the preparation. Only electrodes having a resistance of more than 25M ohms, as measured in potassium chloride solution, were used.

The use of micropipettes required a different recording system. The preamplifier used was a transistorised "Amatniek" amplifier manufactured by Bioelectric Instruments Inc. This is a single sided, low gain, D.C. amplifier with an input resistance in excess of $10^{11}$ ohms. Neutralised input capacity and a grid current of less than $10^{-12}$ amps are the other features of this type of instrument (Amatniek, 1958). The output of this preamplifier was fed into a Telequipment double sided, high gain, D.C. oscilloscope. The indifferent side of the oscilloscope amplifier was connected to the indifferent chlorided silver wire electrode which was earthed. Fig. 9 is a diagram of these arrangements. The audio-monitoring was dispensed with in favour of purely visual observation.

The micropipettes were held in a perspex clamp attached to a Prior micromanipulator. The latter was used to orientate the electrodes and to insert their tips into the cells. This direct mechanical manipulation system was used in preference to the hydraulic advance system used for the indium electrodes as the latter was found to lag behind the applied movements of the micrometer syringe, and was therefore found unsatisfactory for the small, quick movements needed to impale the nerve cells.

Stimuli used in the course of the experiments on Buccinum

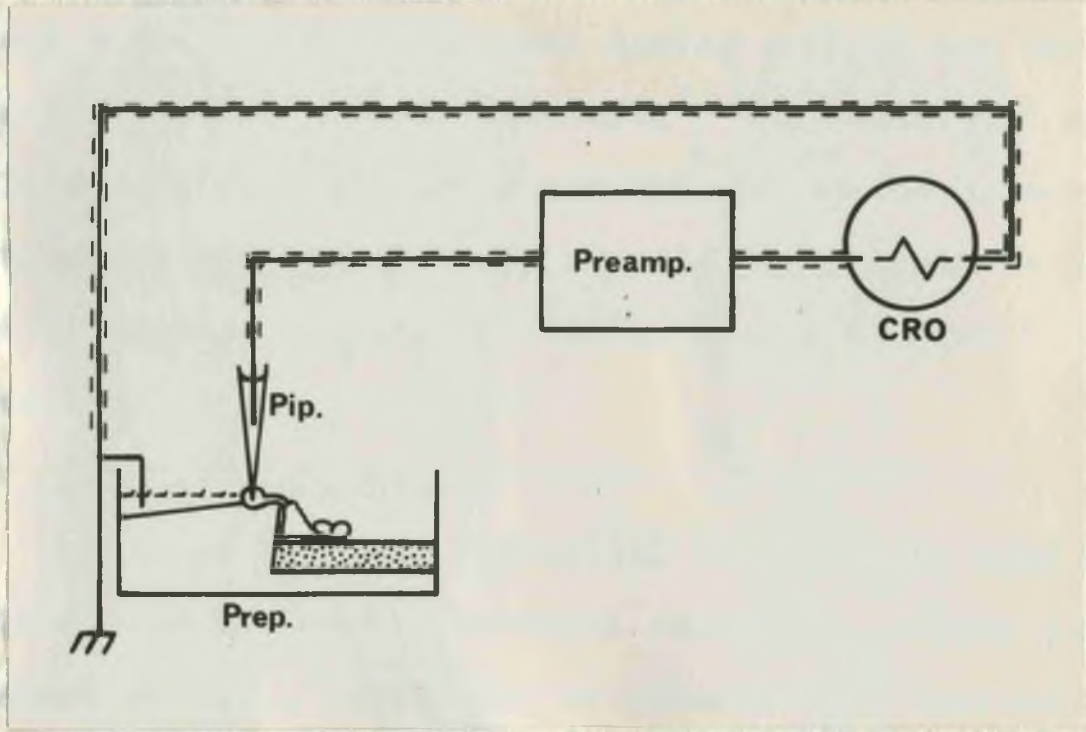



Figure 9. Diagram showing arrangements made for recording with saline filled microelectrodes. Pip., recording electrode; Preamp., preamplifier - in this case the "Amatniek". Remainder of legend as for Fig. 6.

undatum.

The stimuli used during the electrophysiological experiments upon the nervous system of Buccinum can be divided into three types, namely mechanical, electrical and chemical. The mechanical stimuli were used to investigate the properties of the mantle movement receptors and the central nervous pathways. The electrical stimuli, were also used during some of the experiments upon the central nervous pathways. The "chemical" stimuli which include solutions of varied osmotic and saline concentrations and particulate suspensions were used in experiments concerning the central nervous responses to stimuli administered to the osphradium.

Mechanical stimuli.

Two basic types of mechanical stimuli were used in the experiments on the mantle movement receptors, namely tactile and movement stimuli. The tactile stimuli were applied manually by means of a paint brush or fine glass or bristle probes according to the force required to evoke a response. The maximum force exerted by the fine glass probes was 2mgm. over an area of approximately 75 sq. μ., whilst the bristle probes exerted a maximal force of 2.5 gms. over an area of 0.125 sq. mm.

Movement stimuli were applied in four different ways during the experiments. The general plan of the means of application is shown in Fig. 10, whilst Fig. 11 gives a more detailed picture of the 'stretcher'. A small hook was attached to the mantle. The hook was moved by means of the attached rod, which, in its turn, was moved by the lever. A movement of constant velocity,

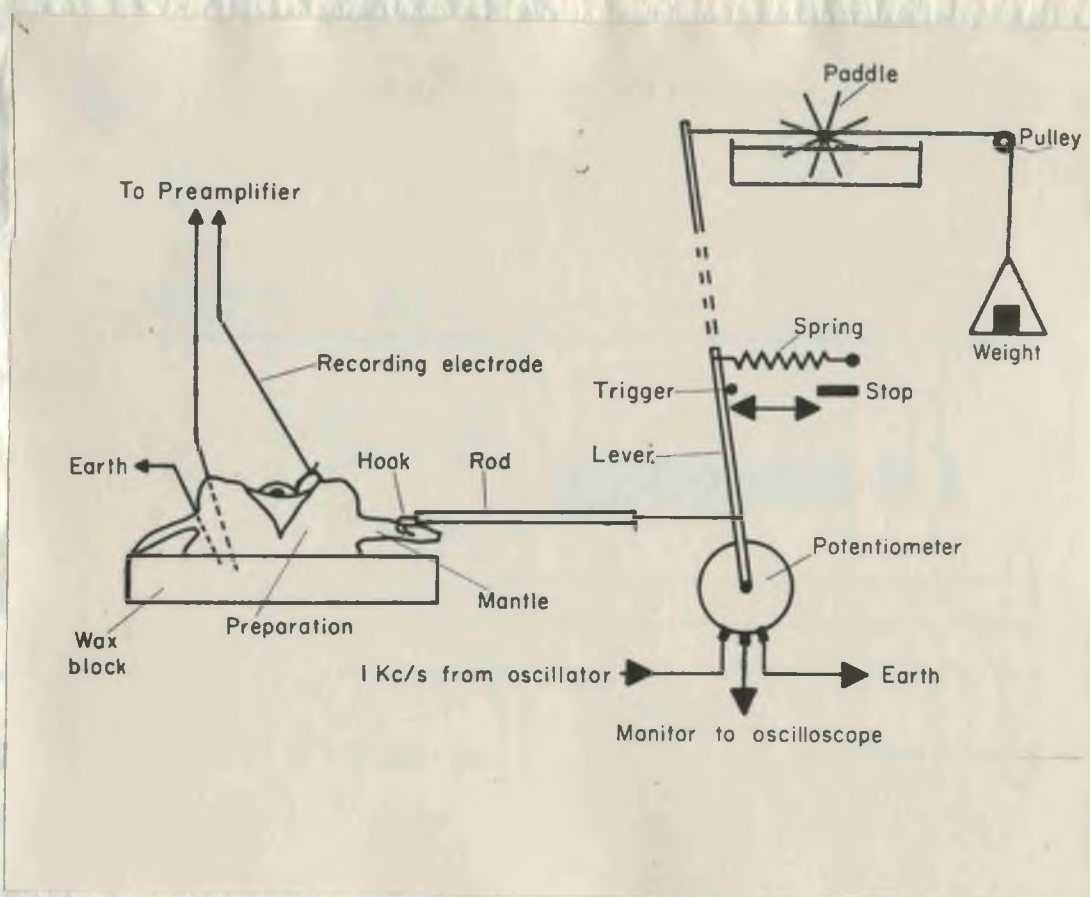



Figure 10. Diagram showing arrangements made for mechanical stretch stimulation. See text for further details.

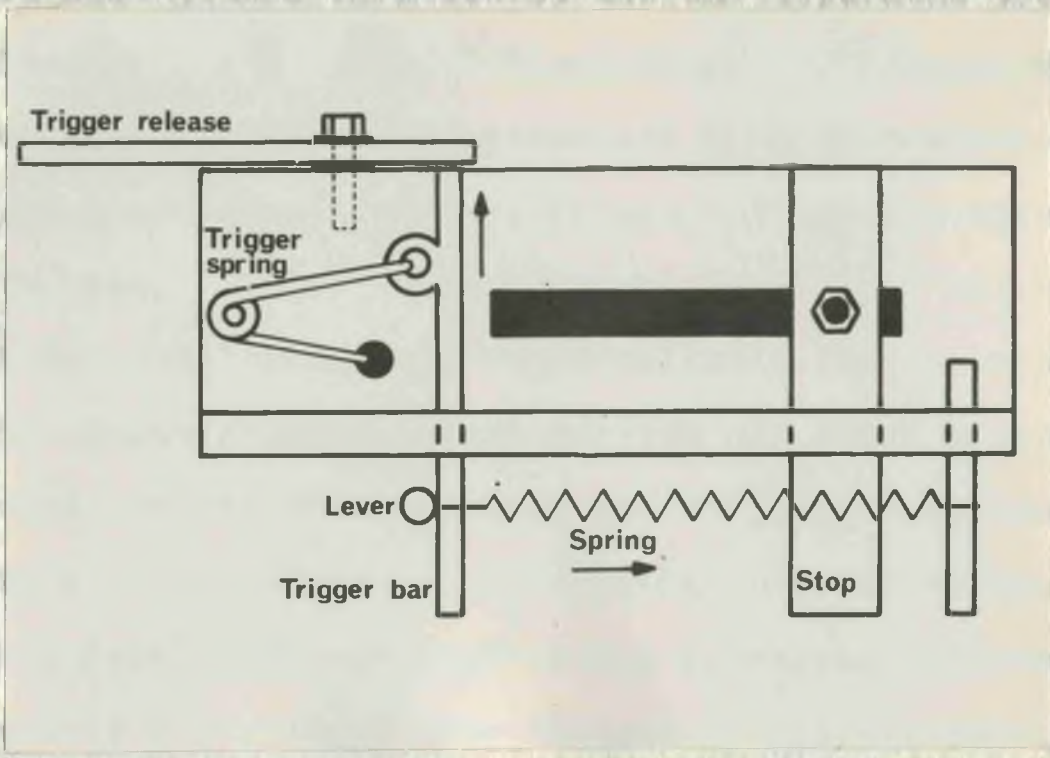



Figure 11. Diagram showing "stretcher" viewed from above. In the position indicated in the diagram the trigger spring is compressed. Depressing the trigger release allowed the trigger bar to move in the direction indicated by the upper arrow, by releasing the tension applied to the trigger spring in setting the apparatus. The retraction of the trigger bar left the lever free to move in the direction indicated by the lower arrow, until it came to rest upon the stop. The stop could be moved to known, calibrated positions along the slot indicated by the black rectangle, thus controlling the amplitude of the applied stretch.

but with a variable absolute rate, was produced by placing weights on a pan attached to the top of the lever by a thread running over a pulley. The descent of the weights caused the paddle wheel to rotate in the water-bath producing sufficient damping effects to prevent acceleration of the lever. The weights were placed in position and the apparatus set in motion by releasing the trigger. The distance of travel, the amplitude of the stimulus, was determined by a stop which could be moved to known, calibrated positions and against which the lever came to rest.

More rapid movements, in which the stimulation was completed in approximately 2msec., were carried out using a spring to pull the lever towards the stop when the trigger was released. The amplitude of the movement, or stretch, applied to the preparation could be varied between 1 and 5 mm. by moving the stop. Stimulation in one mm. steps over the same range was carried out by hand, as was rapid repetitive stimulation involving many cycles of stretch and relaxation. In all these methods the movements of the lever caused the spindle of a potentiometer to rotate. A 1Kc/s constant voltage sine wave was applied across the potentiometer and the variations in the centre-tap readings, after the output sine wave had been rectified, provided a stimulus monitoring deflection of the lower beam of the oscilloscope.

Electrical stimuli.

In a number of the experiments on central nervous pathways electrical stimuli were applied directly to the nerves in place

of the peripheral mechanical stimulation. Electrical stimuli were produced by discharging a diode in the circuit shown in Fig. 12 (Horridge, 1963. pers. comm.). The amplitude and the frequency of the shocks could be varied at will. The stimuli were applied to the selected nerves through a low resistance, one to one transformer in order to reduce the stimulus artifact. Records of the efferent nervous activity in other nerves caused by such stimuli were made in the manner already described.

Chemical stimuli.

The stimuli used in experiments on central responses to peripheral chemical stimulation were all dissolved in filtered sea water. The test stimulus applied in all cases was obtained by crushing 8-12gms. of whole Mytilus in 50mls of sea water and allowing the resultant mixture to stand for at least 30 minutes. The turbid supernatant solution was then drawn off as required and used as a stimulus. Solutions of various synthetic chemicals (B.D.H.) were made up at $10^{-1}$ or $10^{-2}$M concentrations for stock solutions and kept in a refrigerator until required. Solutions for use as stimuli in experiments were made up from stock by dilution with sea water. Where necessary these solutions were neutralised with dilute hydrochloric acid in sea water or sodium carbonate in sea water to pH 7.5 using B.D.H. universal indicator paper as a means of testing pH. Thus the stimulating solutions were of the same order of alkalinity as the sea water.

Neutral chemical test solutions were applied to the osphradium by means of glass dropping pipettes holding between 1 and 2 mls., and in all cases it was attempted to spread the solution

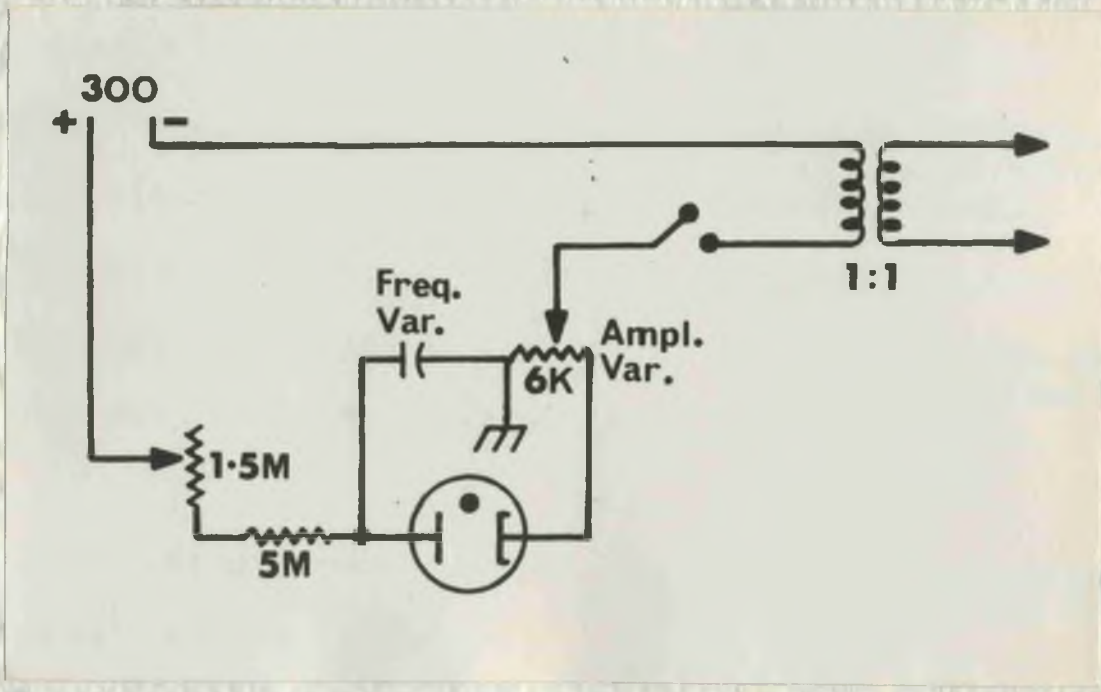



Figure 12. Diagram of circuit used for electrical stimuli. Ampl.Var., potentiometer used to vary amplitude of pulse; Freq.Var., capacitor, changed to alter the frequency of pulses. Arrows indicate leads to stimulating electrodes.

relatively evenly over the whole osphradium. The chemicals used as stimuli in the course of the experiments were as follows:-

Adipic acid
β-Alanine
l-aspartic acid
Betaine hydrochloride
l-cysteine hydrochloride
Gelatine
Glucose
l-glutamic acid
l-glutamine
Glutaric acid
Glutathione (saturated solution)
l-glycine
Glycogen
Indole
Lactic acid
Malonic acid
l-proline
Quinine hydrochloride (saturated solution)
Succinic acid
Sucrose
Trimethylamine oxide hydrochloride
l-tryptophan
l-tyrosine

Other stimuli used to test the sensitivity spectrum of the osphradium were as follows:-

(1) Touch - using a paint brush.

(2) Carborundum particles of the following sizes in sea water, less than 20μ, 20-200μ, and greater than 200μ. The graded particle sizes were obtained by sieving carborundum powders through 80 and 200μ mesh sieves.

(3) Sea water adjusted to pH values of 10.05, 9.4, 8.5, 7.4, 6.8, 4.9, 2.3 and 1.4. These solutions were prepared by adding dilute hydrochloric acid or sodium hydroxide to sea water and testing the pH of small samples with a Pye 'Dynacap' pH meter.

(4) Sea water adjusted to saline concentrations equivalent to

200%, 160%, 133%, 115%, 75%, 50%, 25% sea water and also distilled water, were used as test solutions. The saline solutions were prepared from known volumes of sea water by evaporation or by diluting with distilled water as necessary.

(5) Sea water to which sufficient Mannitol had been added to produce a solution with an osmotic pressure approximately equivalent to 150% sea water. The amount of Mannitol needed was derived in the following way:-

The average chlorinity of sea water is 18.98‰ giving an osmotic pressure of sea water of 22 atmospheres at 10°C. (Sverdrup, Johnson and Fleming, 1942). Therefore 150% sea water osmotic pressure = 33 atmos.

$$W = \frac{xVxM}{RxT}$$

Where W = the weight of solute of molecular weight M necessary to produce a solution of volume V and osmotic pressure at the absolute temperature T when R is the gas constant. (Maron and Prutton, 1958).

When V = 1 litre and = 11 atmospheres at 10°C. then by substituting figures in the above equation:-

$$W = 86.28 \text{ gms/l}$$

i.e. by adding 86.28 gms of Mannitol to 1 litre of sea water the resultant solution is therefore osmotically equivalent to 150% sea water.

The onset and duration of application of stimuli were in-

dicated by means of a manually operated switch circuit producing a small D.C. shift in the lower beam of the oscilloscope. A time marker pulse was also fed into the lower beam amplifier. In the case of all the stimuli applied in sea water the active application of the stimulus was marked on the records and the preparation left for some seconds or, if a response was observed, until the response ceased. The stimulating solution was then washed away with filtered sea water. The washing was marked in the same way as the stimulus and besides restoring the preparation to normal it served as a usefuly control in cases where the osphradium proved sensitive to mechanical distortion as well as to the chemical stimuli.

## RESULTS

### ANATOMY AND HISTOLOGY.

#### General Anatomy of the Nervous System.

Many dissections of the anterior central nervous complex, situated in the cephalic region, showed that the details of the main central complex as figured by Bouvier (1887) and Nakin (1912) are correct (Fig. 13). The anterior complex consists of 10 ganglia which lie close together and have very short commisures and connectives between them. The ganglia comprise paired pedal, cerebral, buccal and pleural ganglia and unpaired sub - and supraintestinal ganglia. The pedal and cerebral ganglia form a circumoesophageal ring being connected by commisures between the pairs of ganglia and connectives between the pedal and cerebral ganglia on each side. Anterior to the cerebral ganglia are the two buccal ganglia which are connected to the cerebrals by connectives and to each other by a supra-oesophageal commisure. Torsion in the embyonic developement of Buccinum has produced the effect of a second virtual circumoesophageal ring in that the paired pleural ganglia and the subintestinal ganglion form a semicircular loop ventral to the gut, and the supraintestinal ganglion, connected to the right pleural by the supraintestino-pleural connective, overlies the gut and gives off nerves to the left side of the body. The supraintestinal ganglion also gives rise to the left visceral connective. The right visceral connective arises from the subintestinal ganglion.

The visceral connectives, sometimes called the visceral

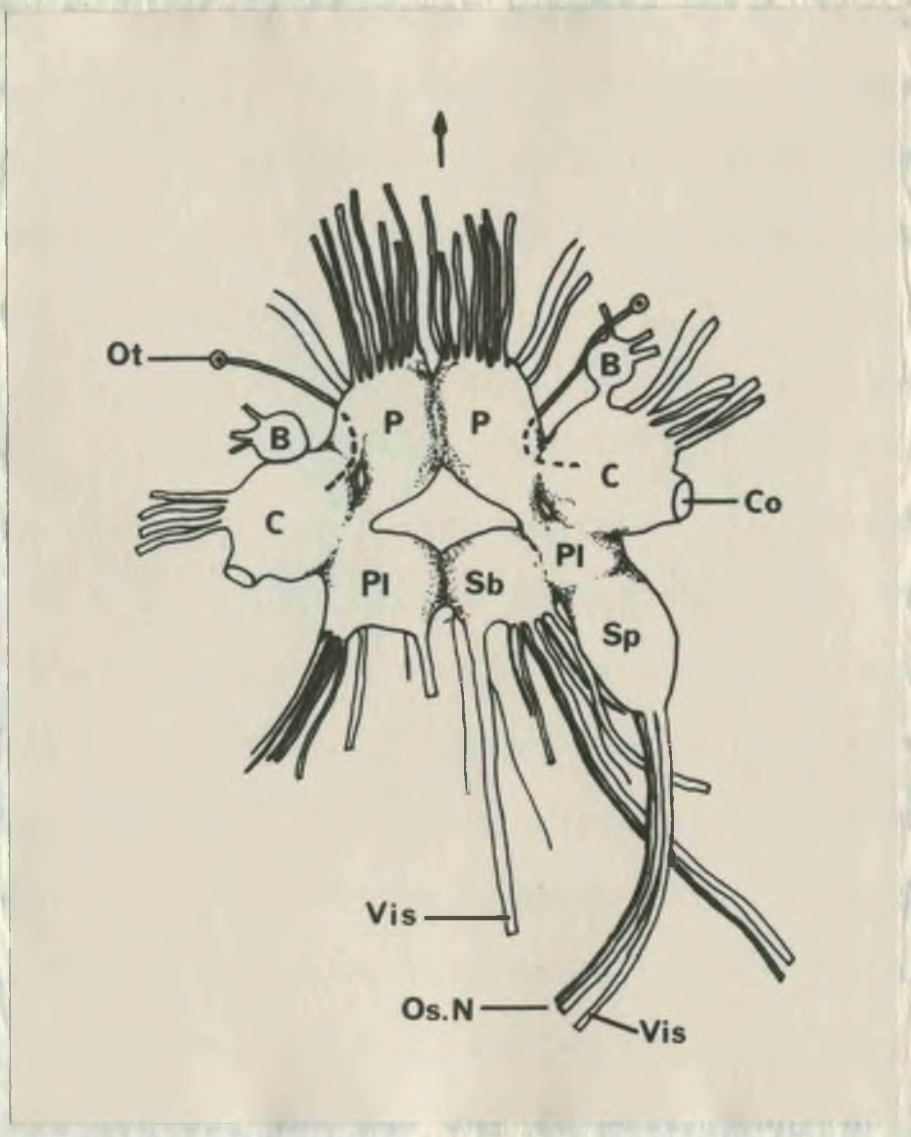



Figure 13. The anterior central nervous complex of Buccinum, viewed from the dorsal side. Modified from Bouvier (1887). B., buccal ganglion; C., cerebral ganglion; Co., cerebral commissure - cut in the mid-dorsal line to allow for removal of the oesophagus; Os.N., osphradial nerves; Ot., otocyst; Pl., pleural ganglion; Sb., subintestinal ganglion; Sp., supraintestinal ganglion; Vis., visceral connective.

commisures (Dakin, 1912), connect the anterior central nervous complex to the paired visceral ganglia which lie just posterior to the body cavity. The latter ganglia, with their commisure and the visceral connectives thus complete the visceral loop which, together with the anterior complex, makes up the complete central nervous system.

The nerves arising from the ganglia are by no means as regular and precise as the arrangement of the ganglia themselves. In this respect the older descriptions leave something to be desired in that they make no mention of the great variations that occur in the organisation of the nerves. On the left side of the animal, to which the most detailed attention was directed during the electrophysiological experiments, it was found that the number of nerves arising from any of the ganglia was not constant, though the area innervated by any given set of nerves appeared to remain much the same in the different animals. The size of the nerves, and the way in which many of them branched whilst still in the body cavity, was also extremely variable. Fig. 14 gives some idea of the extreme cases of this variation found in the tentacular, pallial, siphonal and osphradial nerves, which originate from the cerebral, pleural and supraintestinal ganglia. The most consistent feature of the innervation of the left side of the body was the occurence of two large nerves running to the siphon, but even in this respect there was variation since, occasionally, only one nerve was present. The osphradial nerves were also more constant in their organisation

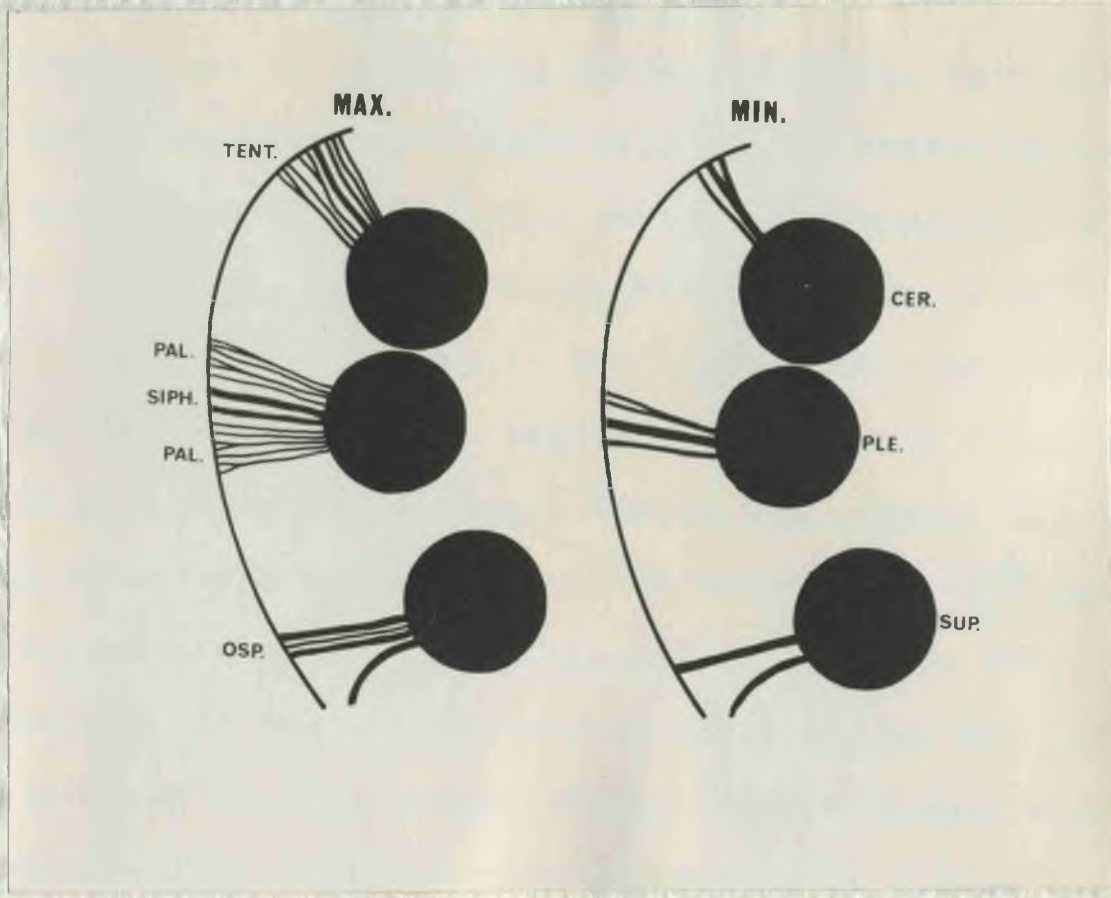



Figure 14. Diagrammatic representation of the extremes of the variation found in the innervation of the left side of the body. CER., cerebral ganglion; PLE., pleural ganglion; SUP., supra-intestinal ganglion; OSP., osphradial nerves; PAL., pallial nerves; SIPH., siphonal nerves; TENT., nerves to tentacle and surrounding region.

since there were generally two present, but in some specimens a small third nerve was found and rarely there was only one osphradial nerve.

Distribution of nerves in the mantle.

Dissections of the nerves arising from the left pleural ganglion and supplying the left side of the mantle showed that the siphonal nerve(s) gengrally pass to the base of the mantle without branching. On reaching this region three main branches arise: the anterior passes to the siphon, the lateral to the anterior edge of the mantle and the posterior to the zygoneuric connection. When two siphonal nerves are present the anterior usually innervates the siphon region and the lateral and posterior tracts are derived from the posterior nerve (Fig. 15). The remainder of the pallial nerves arising from the left pleural ganglion break up into progressively finer branches soon after reaching the body wall and were impossible to follow for any great distance thereafter.

The osphradial nerves, which arise from the supraintestinal ganglion, resemble the siphonal nerves in that they pass through the mantle for a distance of several millimetres without branching (Fig. 15). Before reaching the osphradium, however, they diverge, turning anteriorly and posteriorly, and give rise to a series of branches running to the osphradial ganglion in the axis of the osphradium. The anterior branch of the nerve, or part of the anterior nerve when two osphradial nerves are present, passes forward to complete the zygoneuric connection with the

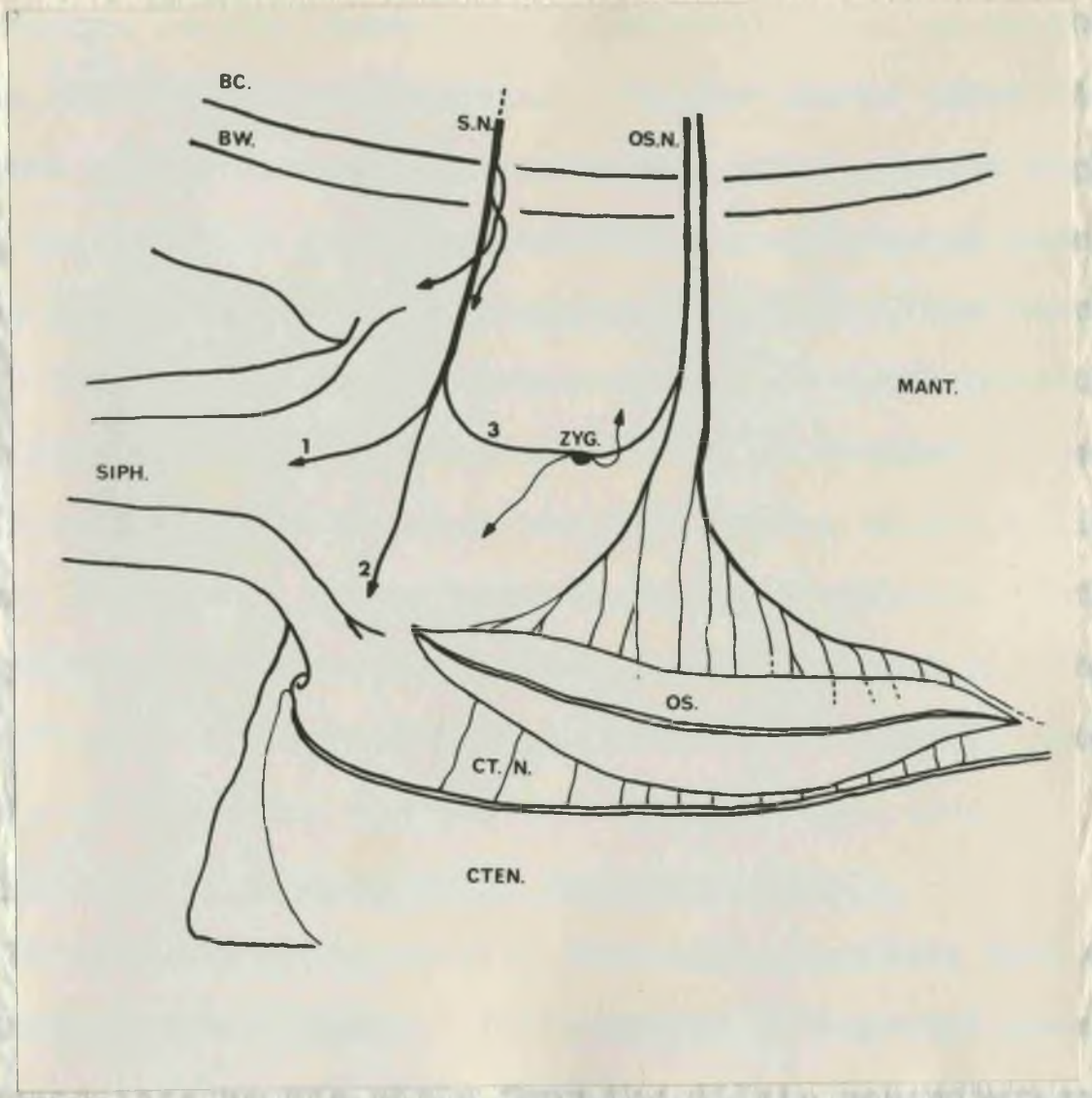



Figure 15. The distribution of the nerves in the mantle region. BC., body cavity; BW., body wall; CT.N., ctemidial nerves; CTEN., ctenidium; MANT., mantle; OS., osphradium; OS.N., osphradial nerves; SIPH., siphon; S.N., siphonal nerve; ZYG., zygoneuric connection; 1., anterior, 2., lateral and 3., posterior branches of the siphonal nerve.

posterior branch of siphonal nerve(s).

Nerves passing beyond the osphradial ganglion to the base of the ctenidium were observed. Whether these arise directly from the osphradial nerve branches and run dorsal to osphradial ganglion, or arise from the osphradial ganglion and pass thence to the ctenidium was not clear from the dissections carried out.

The remainder of the nerves arising from the anterior central nervous complex were not dissected or studied in such great detail as to be able to give the full extent of the variations present elsewhere, mainly because the main emphasis of the electrophysiological experiments was directed towards the nerves of the left side of the mantle and the anterior central complex itself.

Intravitam staining with Methylene Blue.

The results obtained with this technique were most disappointing in all cases. Small pieces of material generally failed to take up the stain from the dilute sea water solution, even when teased out to a considerable extent.

The results from the injected specimens were only slightly better than those from excised pieces. Solutions of Methylene Blue in sea water, saline or distilled water all failed to stain any of the nervous tissues when injected into the animal in very dilute solutions. When the concentrations were increased the dye tended to crystallise out in the body fluid of the animal, and remain there in the solid state until the animals were dissected. This problem was overcome by making up the stronger Methylene Blue solutions in hemimolar sucrose solution which

effectively stopped the crystallisation of the dye. However, even with this technique the results were poor and only rarely was it possible to observe stained axons within the nerves of the body cavity. The stain failed to penetrate into either the central ganglia or the peripheral regions of the nerves embedded in the musculature. This general pattern was reversed on only one or two occasions when some staining of the body walls was visible. In one of these cases it was possible to make out a single multipolar nerve cell situated in the body wall musculature. Fig. 16 is a drawing of the nerve cell made from this specimen after fixation and mounting. All the mounted preparations faded in 2-3 weeks, suggesting that the method of fixation used for these studies is not wholly satisfactory.

Microscopical anatomy of the mantle region.

Transverse serial sections through the mantle revealed nothing concerning the nervous system that had not already been discovered by dissection. The nerves were visible in the sections but little or nothing could be seen of the innervation of the complex muscle sheets which exist in the mantle and careful observation failed to bring to light any possible sensory structures. It seems likely therefore, that these receptors are of small size and may only be revealed in detail by successful intravitam staining followed by electron microscopy.

Microscopical anatomy of the central nervous system.

The general structure of the central ganglia conforms to the typical gastropod pattern. Beneath the soft investing connective tissue the ganglia, commisures and connectives are covered

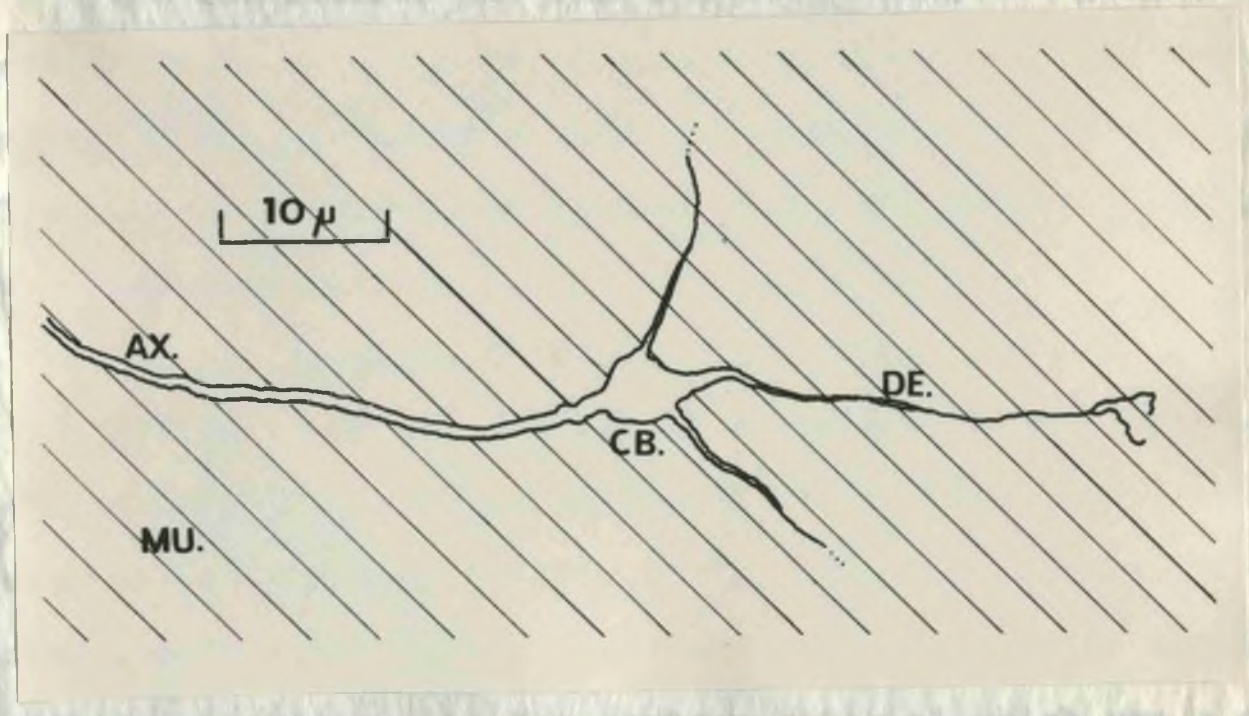



Figure 16. Multipolar cell in body wall musculature. AX., axon; CB., cell body; DE., dendrite; MU., muscle fibres.

by a tough, fibrous sheath or perineurium. Within this sheath lies the nervous tissue which forms two distinct regions; the outer region composed of a rind of ganglion cell bodies, and the inner region in which fibre tracts and the tangled neuropile can be seen (Fig. 17).

The commissures and connectives linking the ganglia are covered with a sheath continuous with that investing the ganglia, within which lie dense columns of approximately parallel axons. The last arise from, and connect with, the neuropile regions of the various ganglia.

The perineurium.

The perineurium consists of a dense, fibrous layer which stains deeply with haematoxylin. It is about 20μ thick and encompasses the ganglia (Fig. 17). The inner border of the sheath is clearly defined and does not give rise to any of the radial incursions into the nervous material which are a consistent feature of the nerve sheaths.

The fibres which make up the bulk of the perineurium are of variable diameter and appear in the sections as dense bands 0.5-2μ thick. The fibres are randomly orientated and single fibres of over 100μ length were measured from the sections, though it is probable that these measured lengths are by no means the full length of any given fibre (Fig. 18).

The ganglionic nerve cell bodies.

The nerve cell bodies, which range in a size from less than 1μ to more than 50μ, lie in a peripheral rind immediately below the perineurium, through which they can be clearly seen in the

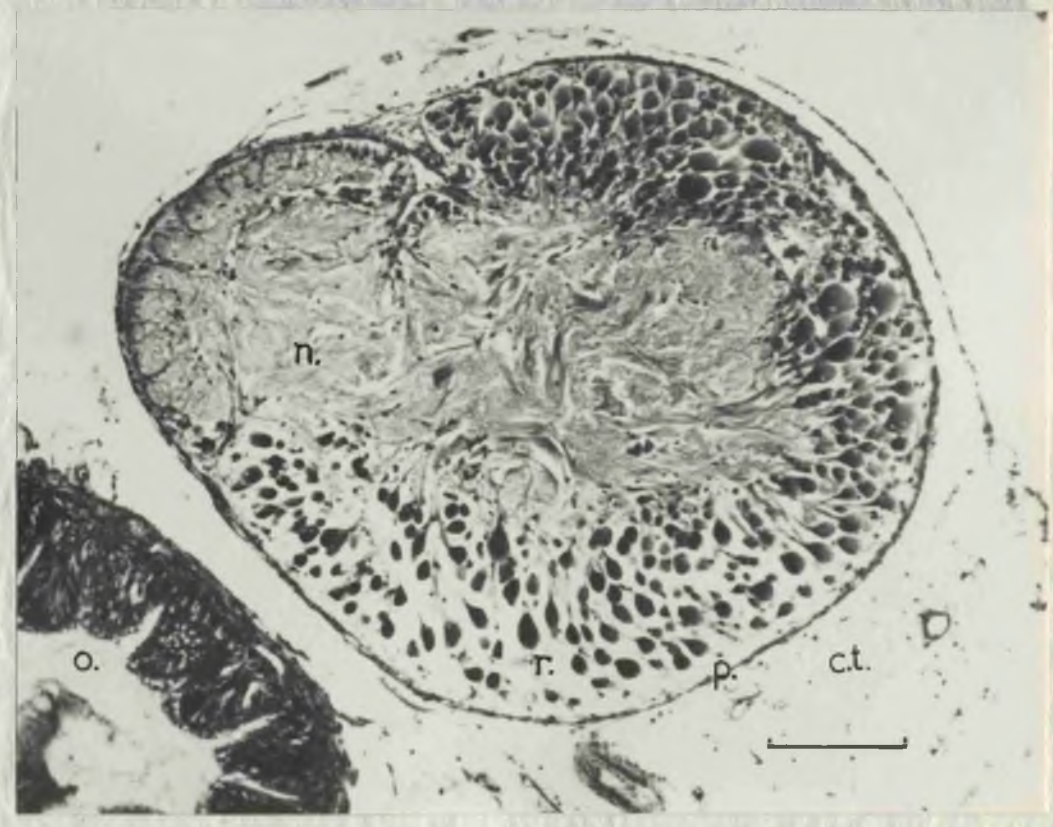



Figure 17. Transverse section of the supra-intestinal ganglion. c.t., connective tissue; n., neuropile; o., oesophagus; p., perineurium; r., rind of nerve cell bodies. Scale mark, 200u.

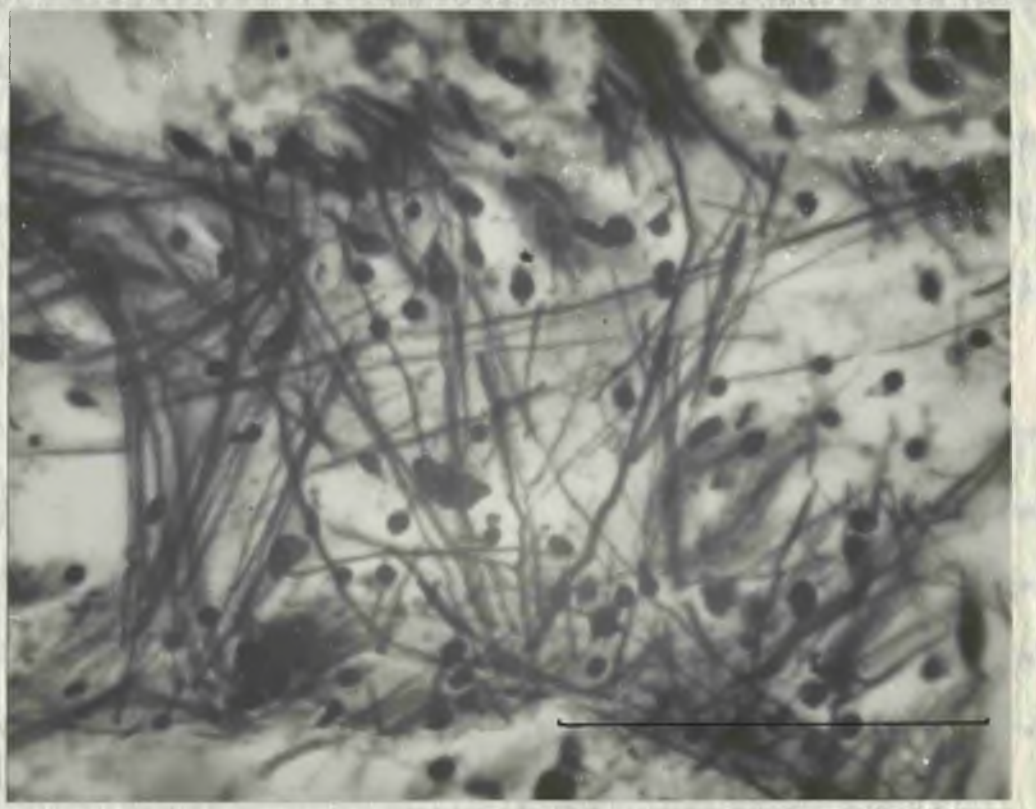


Figure 18. Transverse section through the subintestinal (lower) and right pleural (upper) ganglia, showing the fibrous nature of the perineurium. Scale mark, 100u.

dissected specimen as a dark yellow mass. The colour is due to a pigment, probably a carotenoid, contained within the cell bodies in granules or vacuoles readily visible in fresh squash preparations of isolated ganglia, and in the sections. From the squash preparations it was possible to measure the diameters of the cell bodies and obtain some idea of the cell body size range in individual ganglia. This data is summarised below and compared with values for the same ganglia obtained from the sections of the central nervous system (in brackets). The cell sizes formed a continuous range and the divisions below are arbitrary.

| | Less than 1μ-5u | 6-10μ | 10-20μ | 20-40μ | 60-70μ |
|---|---|---|---|---|---|
| Pedal ganglion | Maj. (50.3%) | M (21.4%) | M (23.9%) | M (4.4%) | F (0) |
| Cerebral " | Maj. (62.7%) | M (27.6%) | F ( 8.3%) | F (2.2%) | 0 |
| Pleural " | Maj. (61.4%) | M (22.2%) | M (13.4%) | F (3.0%) | 0 |
| Subintestinal ganglion | Maj. (58.2%) | M (27.0%) | M (11.0%) | F (3.7%) | 0 |
| Supraintestinal ganglion | Maj. (44.4%) | M (17.4%) | M (29.7%) | M (7.1%) | 0 |

Maj. - Majority, M - Many, F - Few, 0 - no cells of the sizes placed at the tops of the columns. The percentages in the second list of figures were calculated from counts of approx. 1000 cells made from sections of each of the various ganglia.

In general the two lists agree in the relative proportions of the various cell body sizes present. No cells greater than 50μ in diameter were seen in any of the sections. This might be due to shrinkage during fixation of the sectioned material. The percentages given are in all probability only very approximate due to the difficulty of counting cells in the size range <1μ.

The figures for the range <1μ - 5μ are consequently low and the remaining figures correspondingly too high. From these figures however it is obvious that there are no 'giant' cells present in the anterior central nervous ganglia of Buccinum.

The large number of unipolar nerve cells contained in the ganglia give rise to large processes which extend into the central regions of the ganglia. The processes from groups of adjacent nerve cells cross the peripheral rind as bundles forming small, radial, intraganglionic tracts which break up on entering the core of the ganglion (Fig. 19).

The structure of most of the cell bodies is largely obscured in the sections because of the dark staining pigment vacuoles, but in some cases it was possible to see lightly stained nuclei within the cells (See Fig. 20). The pigment granules or vacuoles appear to fill much of the remainder of the cell body forming a dense layer between the nucleus and the cell wall.

The fibrous ganglionic core.

The dense neuropile shown in Fig. 21 occupies much of the central core of all the ganglia and although many tracts of nerve fibres may be seen in different regions of the nervous system little idea can be gained at this level of the true anatomy of the core because it is impossible to distinguish between the fibre tracts which may pass through the ganglia and those arising within the ganglia themselves.

The neuropile is made up of vast numbers of tangled dendritic fibres. Each ganglionic nerve cell fibre, on reaching the cen-

Figure 19. Transverse section through the left cerebral ganglion showing "intraganglionic" tract of nerve cell processes. Scale mark, 200u.

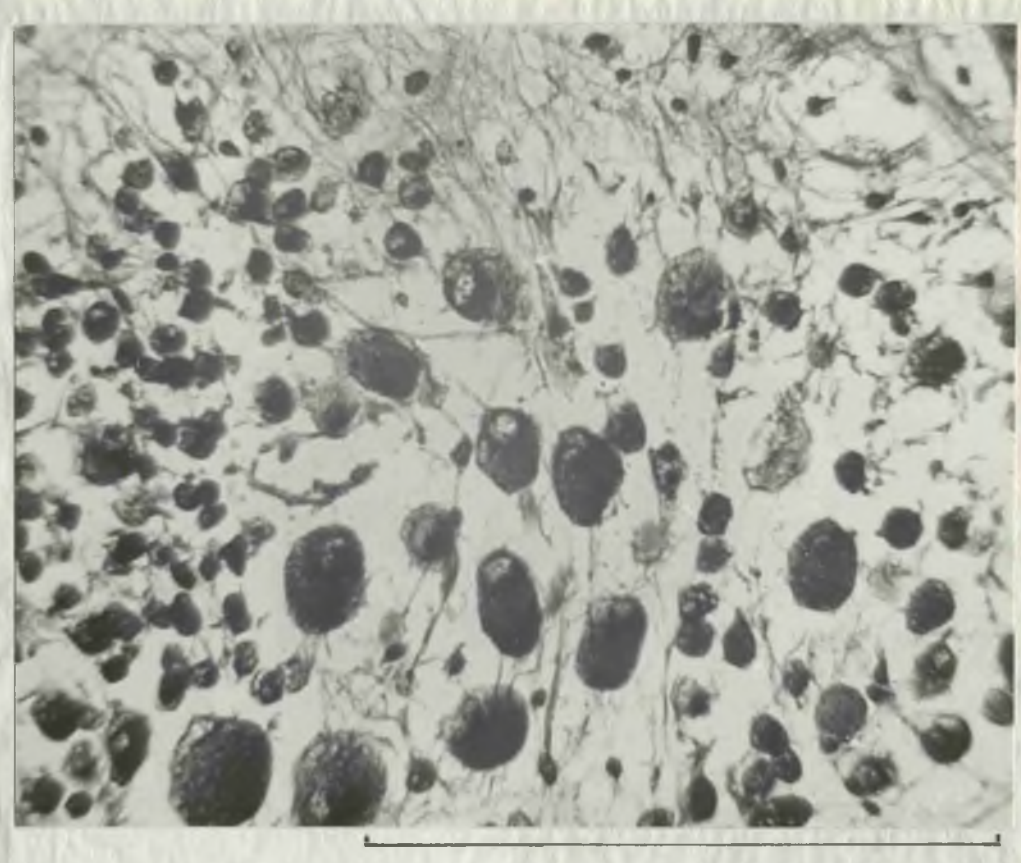


Figure 20. Transverse section through the sub-intestinal ganglion showing nerve cell bodies and their nuclei. Scale mark, 200u.

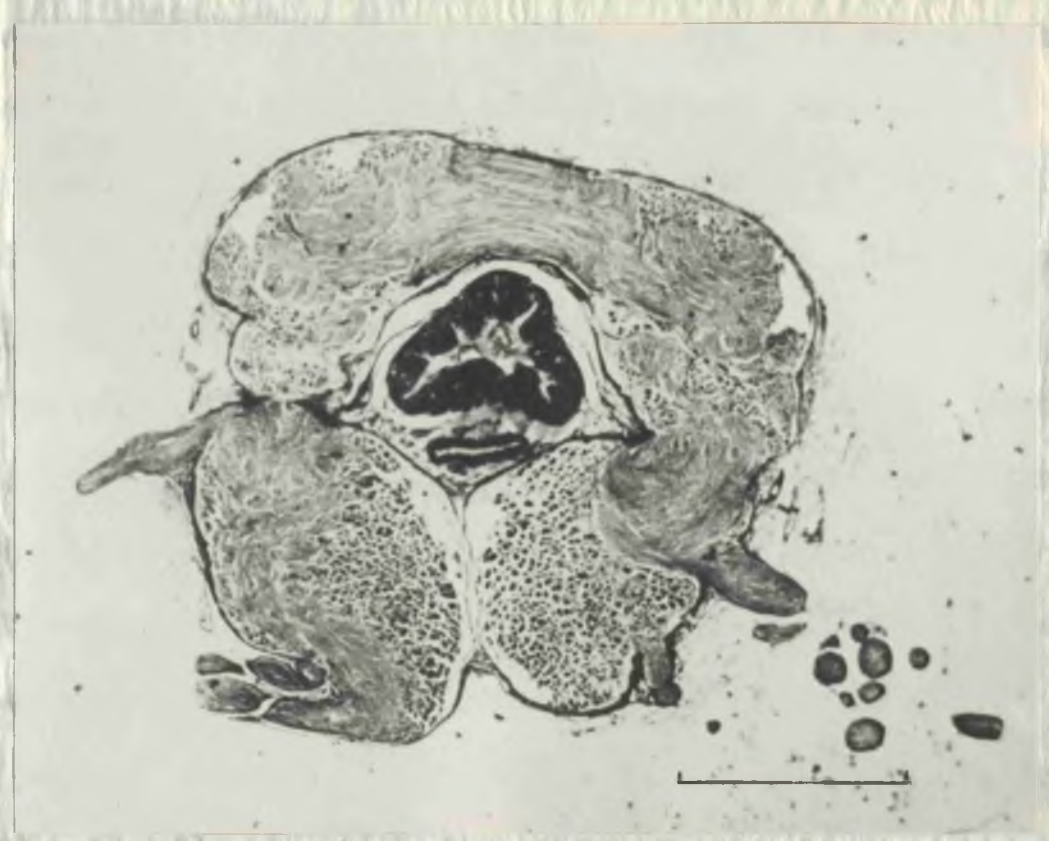


Figure 21. Transverse section through cerebral and pedal ganglia showing extensive neuropile regions, the cerebral commissure (top) and the origin of one of the pedal nerves (bottom left). Scale mark, 1mm.

tral core, breaks up into a number of fine dendrites which mingle with those of the other ganglion cells, the afferent and efferent nerve fibre dendrites, and those formed by fibres lying in the connectives and commissures. Thus the axons which pass into the nerves, connectives and commissures are linked up in the central cores of the ganglia. Fig. 21 shows both the central origin of a nerve and the cerebral commissure. In both these cases it is evident that the tracts of nerve fibres quickly spread into the neuropile near their entry into the ganglia.

Commissures and Connectives.

Excepting the visceral connectives, all the commissures and connectives of Buccinum are short due to the concentration of the ganglia. The nerve processes which pass through the cylindrical perineuria form a dense mass of parallel fibres. Interspersed amongst the axons are cell bodies containing nuclei, and wedge shaped groups of satellite cells. The last are derived from the sheaths in much the same manner as in nerves. Fig. 22 shows a region of the cerebral commissure in which all these features are visible.

The structure of the peripheral nerves.

The nerves are approximately circular in transverse section and the nerve fibres within them are not divided into bundles. The sheath investing the nerves appears thinner than that around the ganglia and does not stain as densely with Haematoxylin. Fig. 23, a T.S. of the osphradial nerve, shows the above features and also the characteristic wedge shaped projections of the sheath

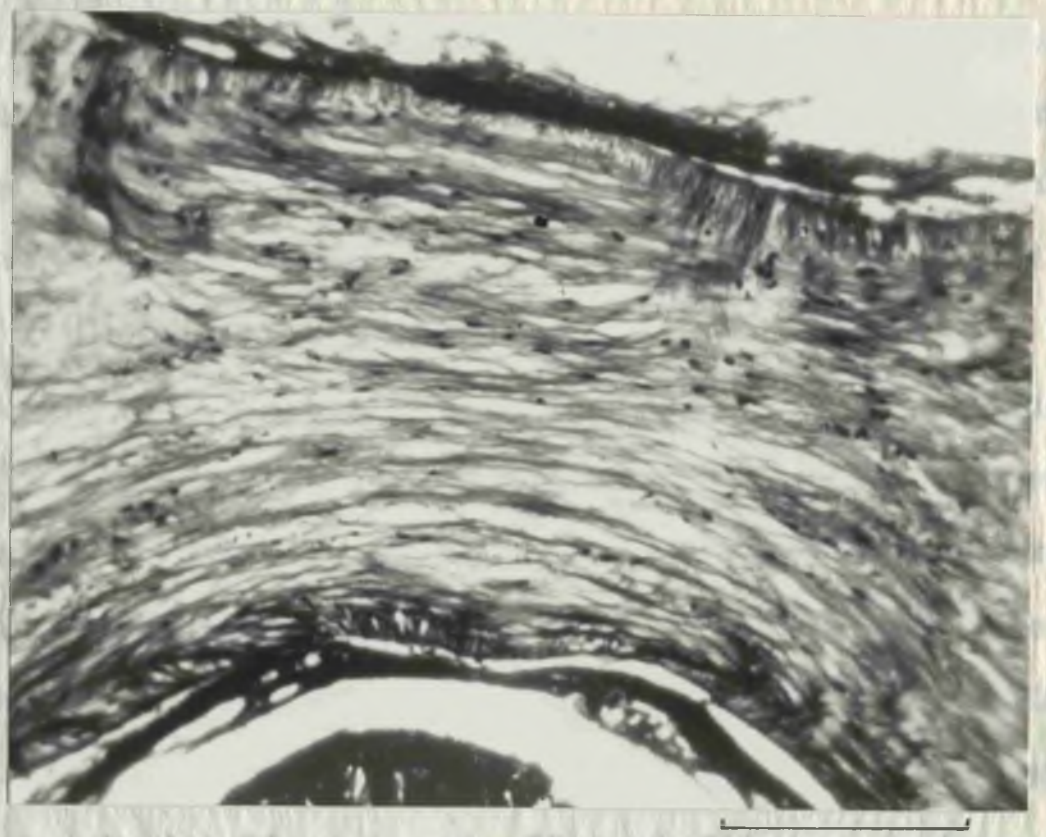


Figure 22. Transverse section of the cerebral commissure. (See text). Note glial nuclei and the wedge shaped group of satellite cells, top right. Scale mark, 200u.

into the nervous material. The latter are shown in greater detail in Fig. 24, at a higher magnification, where the feathery appearance of the central ends is more distinct.

From the photomicrographs shown in Figs. 23 and 24 it is obvious that a great deal of the fine detail of the nervous material is not visible by means of these techniques. In order to investigate the micro-anatomy of the nerves it was necessary to prepare electronmicrographs. For this purpose the pallial and siphonal nerves were chosen because they had been used in the course of electrophysiological experiments as well as being convenient representatives of the peripheral nerves. During the course of the experiments it had proved possible to record nervous activity from the pallial, but not from the thicker siphonal nerves. Any structural differences between them were thus of interest in that they might help to explain this result. The pallial nerves were also known to be of the "mixed" type, i.e. containing both afferent and efferent fibres, another feature on which the anatomical study might throw some light.

The nerve sheath.

The nerve sheath is 8-10μ thick in the pallial nerve and 12-15μ thick in the siphonal nerve. In both cases the sheath is made up of a finely fibrous ground substance, much of which appears to be of an extracellular nature since no fibroblast membranes are visible over large areas of the sheath (Fig. 25), and the outer sheath limiting membrane is by no means distinct. Within the sheath ground substance there are many smooth muscle fibres and a smaller number of fibroblast cells. These are both

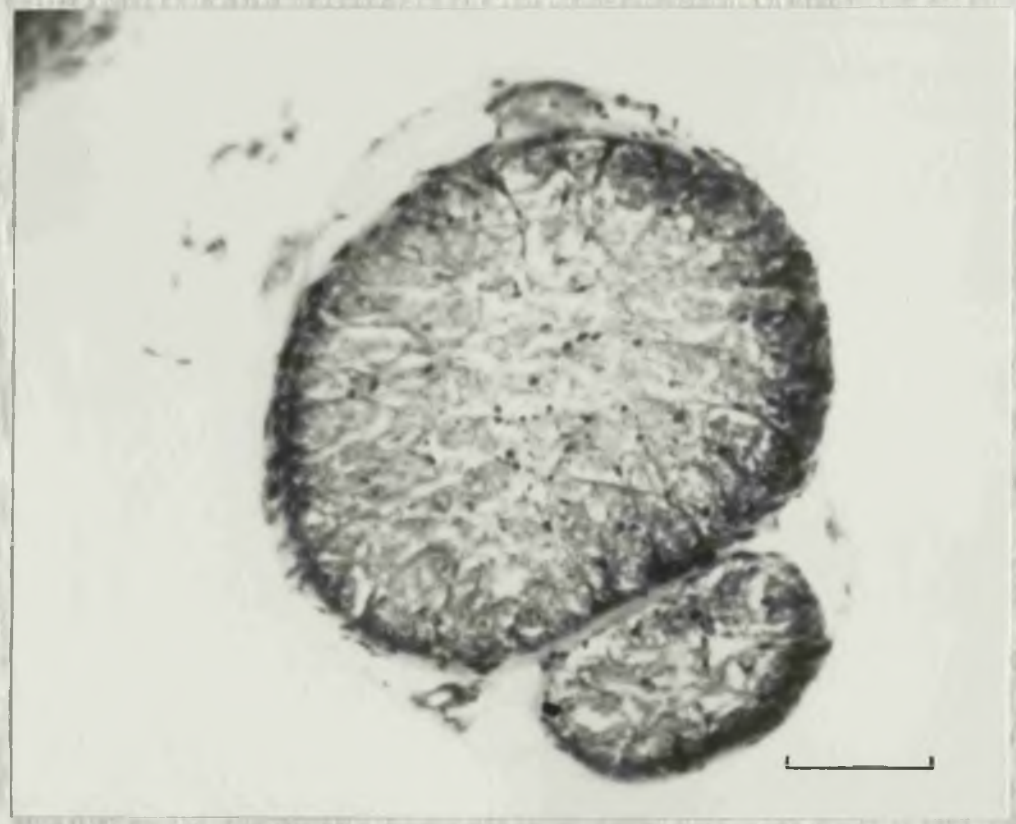


Figure 23. Transverse section of an osphradial nerve. (See text). Scale mark, 200u.

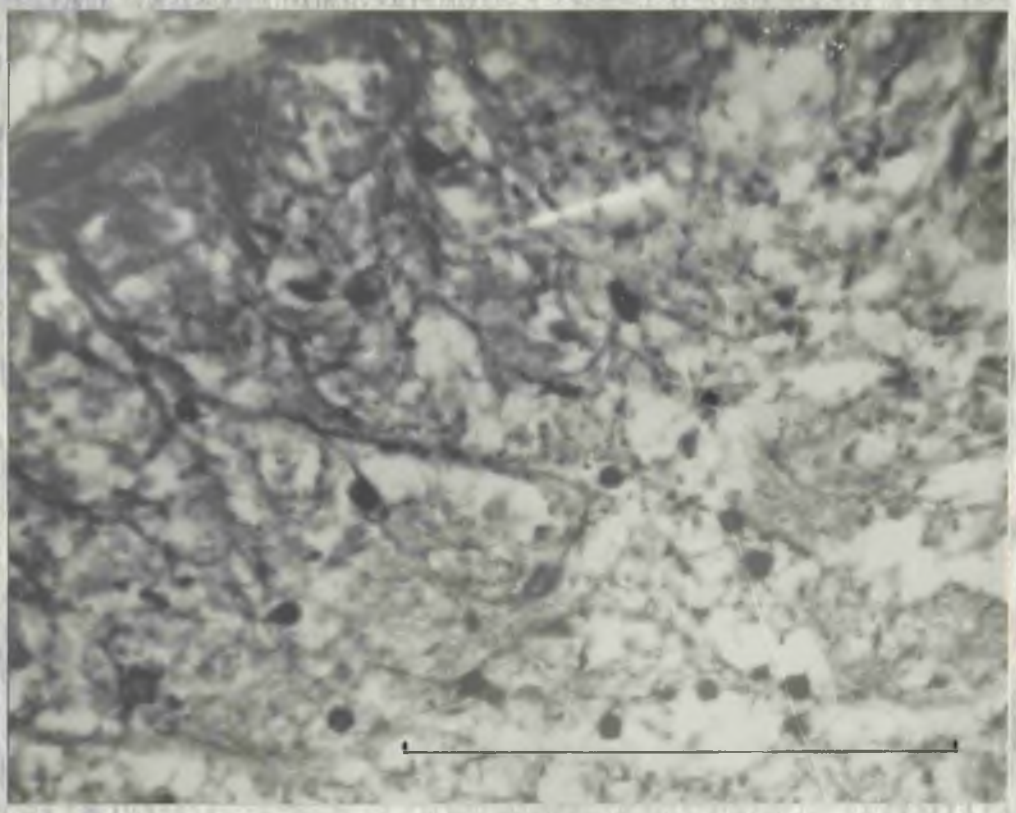


Figure 24. Transverse section of an osphradial nerve at higher magnification than Fig. 23. (See text). Scale mark, 200u.

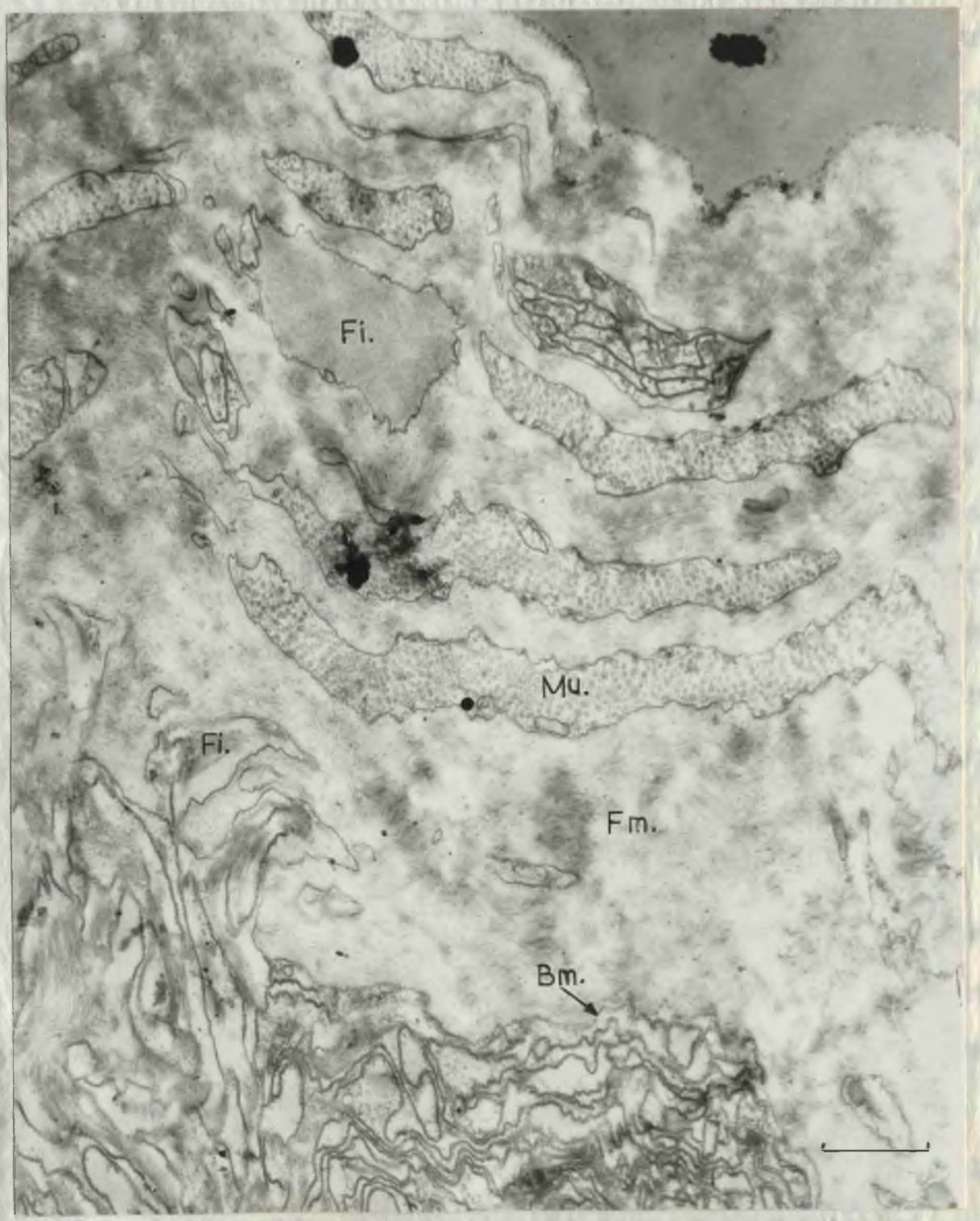



Figure 25. Electronmicrograph of a transverse section of a pallial nerve showing the sheath. Bm., basement membrane of sheath; Fi., fibroblast cell; Fm., fibrous matrix of sheath; Mu., muscle fibre. Note the interruption of the basement membrane by the sheath projection at bottom left of figure. Scale mark, 1u. (x 15,000).

separated from the ground substance by well defined cell membranes. The fibroblasts are filled with the fine fibres which characterise them, whilst the smooth muscle cells contain large numbers of discrete myofibrils. The muscle cells are radially flattened and tend to follow the form of the outer surface of the sheath by bending into u- and v- shapes.

The inner limit of the sheath is irregular but a distinct limiting, or basement, membrane is visible in many places. The latter is interrupted at the regions of the sheath invasions of the nervous elements and in such places it is possible to see many more fibroblast cell membranes (Fig. 25). The fibroblasts, which therefore appear to be concentrated in the sheath radial to the projections, thus invade the nerve cortex giving rise to the wedge shaped divisions so characteristic of the nerves in transverse section (Fig. 26).

The satellite or glial cells.

The fibroblast cells described above can also be identified as the satellite cells, which are interspersed with the nervous elements, by the presence of the characteristic fibrils which they contain (Figs. 26 and 27). Thus, in Buccinum, the glial cells would appear to be derived from the perineurium cells.

The glial cells are extremely numerous within the nervous material and lie closely apposed to the nerve fibres. Most of the glial cells appear as thin elongate radial projections in T.S., especially close to the sheath projections. Individual glial cells do not surround, and therefore isolate, single nerve

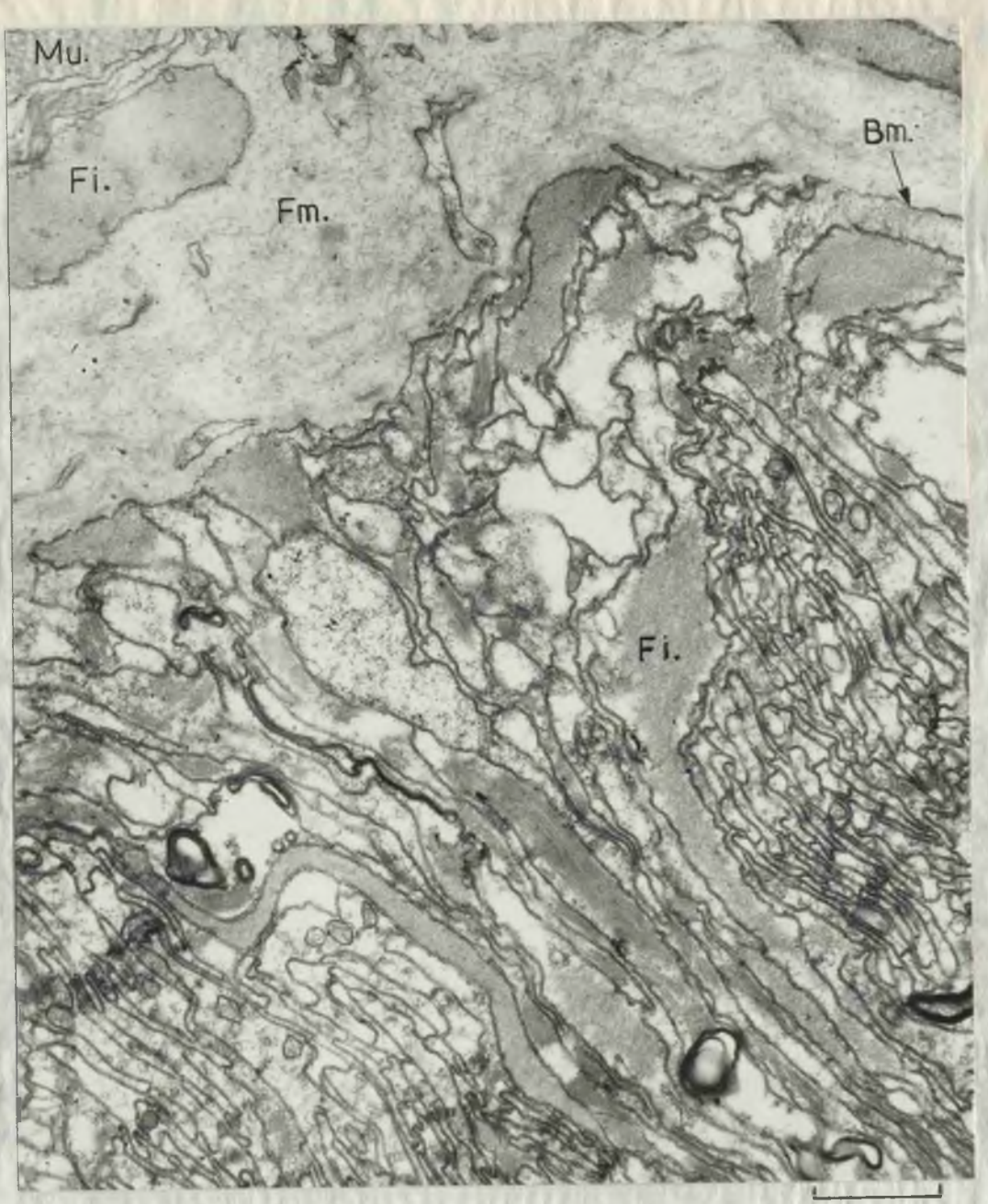



Figure 26. Electronmicrograph of transverse section of a siphon nerve in the region of a sheath projection. Legend as for Fig. 25. Scale mark, 1u. (x 15,000).

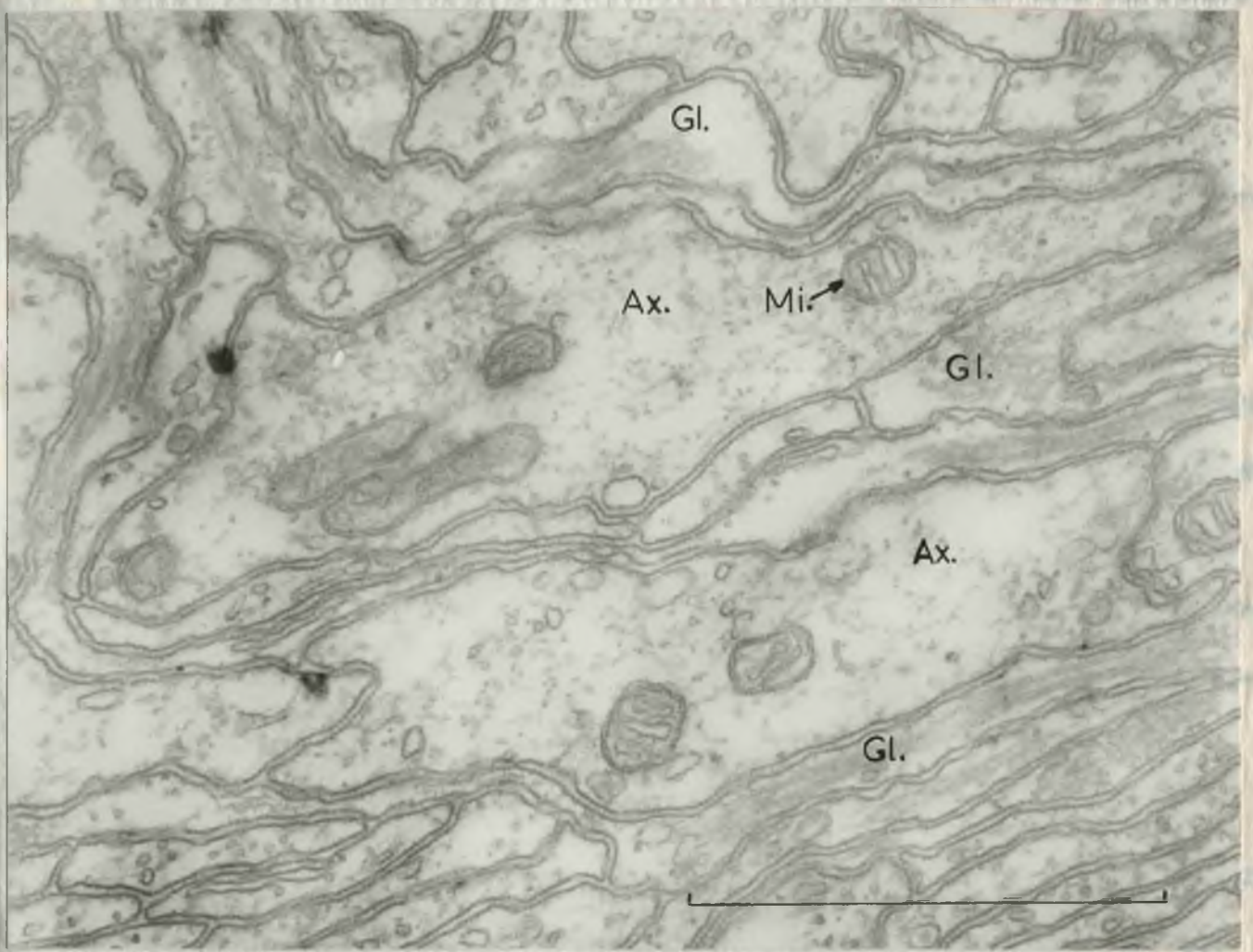



Figure 27. Electronmicrograph of transverse section of a pallial nerve. Ax., axon; Gl., glial cell; Mi., mitochondrion. Scale mark, 1u. (x 60,000).

fibres but rather tend to form a multicellular layer which surrounds a small group of axons (Fig. 27). Within the glial cells the bulk of the cytoplasm appears to be fibrillar and mitochondria and other inclusions, are not numerous.

The nerve fibres.

The nerve fibres are generally small in the Buccinum nerves which have been sectioned, and the electrophysiological results suggest that this is true for all the nerves from which activity has been recorded. The largest axons seen in the nerves were only 0.5-1μ across the shortest axes of their irregular shaped cross sections (Fig.27). In spite of their irregular shape the nerve fibres had no deep, narrow invaginations in the membranes into which the glial cells penetrated, as have been reported for Helix (Schlote, 1957).

The axons are usually arranged in small groups in such a way that each axon makes contact with several other nerve fibres and/or several glial cells. The intercellular distances in each of these two cases is approximately 150Å.

Within the cytoplasm of the axons mitochondria, vacuoles and neurotubules can be observed. The mitochondria are between 1000 and 1600Å in diameter. The vacuoles are of rather irregular shape and between 400 and 600Å in diameter. The neurotubules seen in the axons are relatively few in number, regular in shape and approximately 150Å in diameter. On these criteria it is possible to distinguish the larger axons from the glial cells which usually display finely fibrous cytoplasm (Fig. 28).

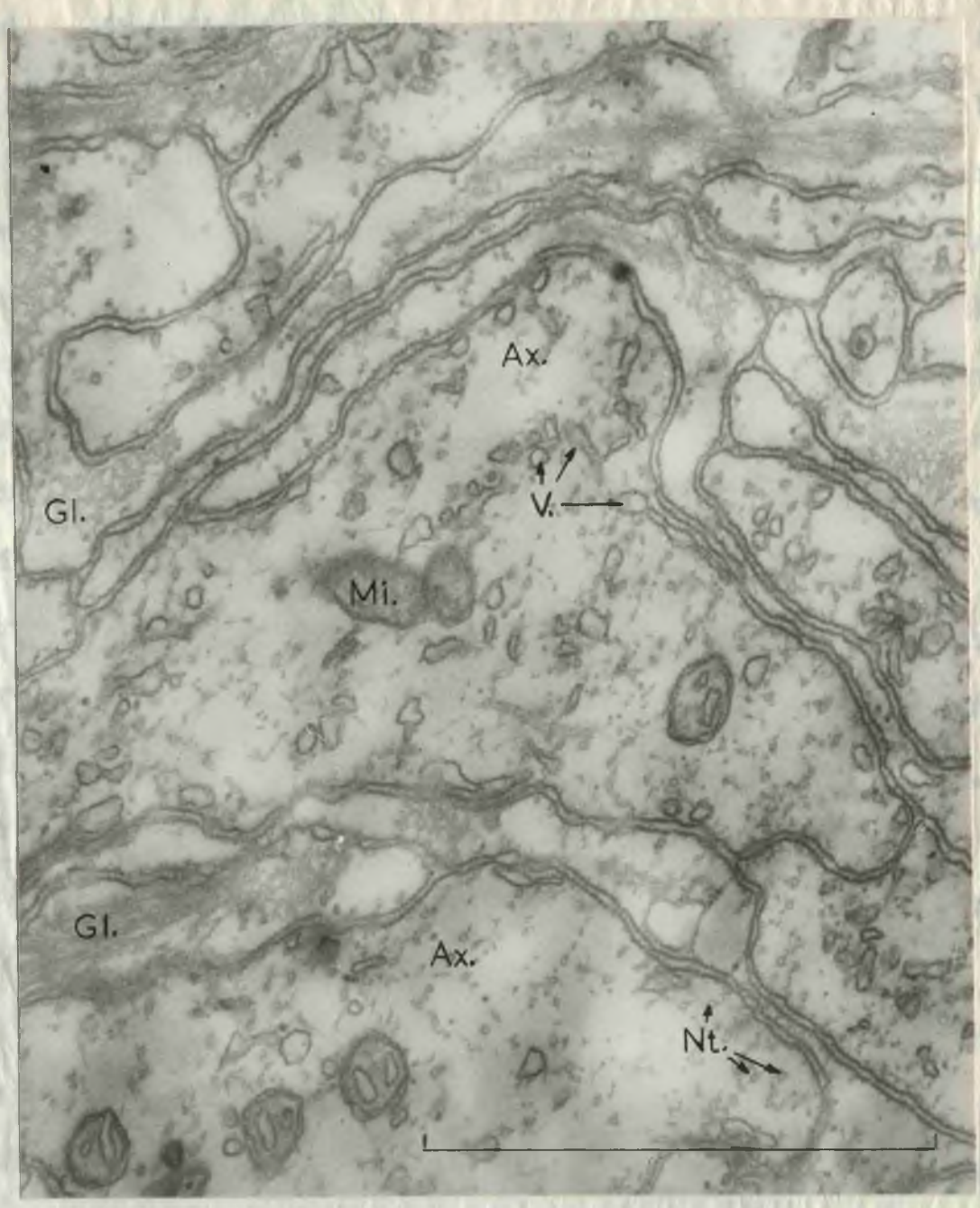



Figure 28. Electronmicrograph of transverse section of a pallial nerve. Nt., neurotubules; V., vesicles; remainder of legend as for Figs. 25 and 27. Scale mark, lu. (x 60,000).

Within the axons two unusual types of inclusion have been found. The first of these is the regular, electron dense granules which may be seen in Fig. 29. These are ovoid bodies, about 1100 by 1500Å in size, bounded by a darker staining membrane. The second unusual feature was found in certain of the nerve cells and takes the form of an intracellular tubule or vacuole as shown in Fig. 30A. These structures are made up by several rings of dense membrane about 50Å in thickness which are not in a spiral formation. The number of membranes varies in the different "tubules" found in various cells. The myelin figure membranes show certain differences from the normal cell membrane (see Fig. 30B), being composed of a central dark staining layer bounded on either side by less dense layers. This structure is typical of myelin membranes (see Finean, 1961). Within the membrane structure there appears to be little or no cytoplasm.

There are two other features of the micro-anatomy of the nerve which cannot be classified under the heading of nerve cell or glial cell inclusions on the evidence so far obtained. In the midst of the nerve cortex, cells containing large irregular nuclei can be observed. Fig. 31 shows one of these nuclei in association, in this case apparently within the same cell, with the other noteworthy inclusion. The latter, which is shown in Fig. 32 at a higher magnification, can be seen to be made up of a large number of very thin membranes (approx. 25Å). In cross section these bodies, which were 1-2μ in diameter, displayed close packed membranes running in different directions in various

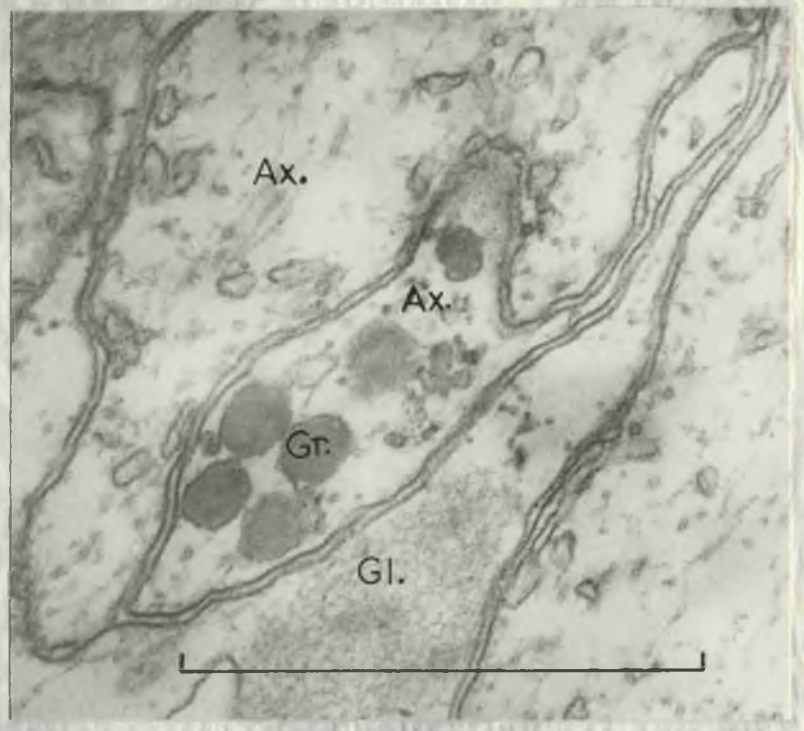



Figure 29. Electronmicrograph of transverse section of a pallial nerve. Gr., dense granules; remainder of legend as for Figs. 25 and 27. Scale mark, 1u. (x 60,000).

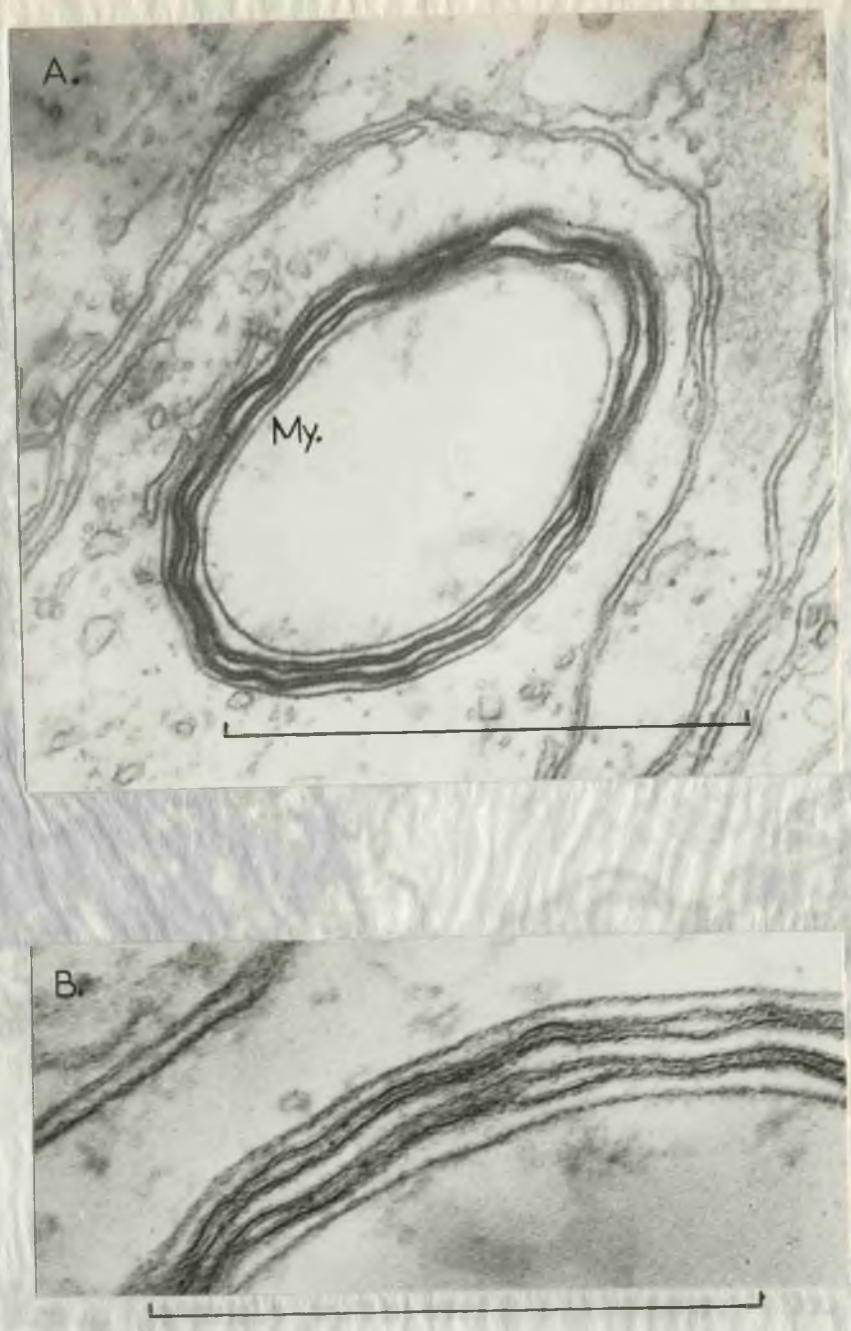



Figure 30. A. Electronmicrograph of transverse section of a pallial nerve. My., myelin figure within an axon. Scale mark, 1u. (x 60,000). B. Myelin figure membranes at a higher magnification compared with normal cell membranes (on left). Scale mark, 0.5u. (x 140,000).

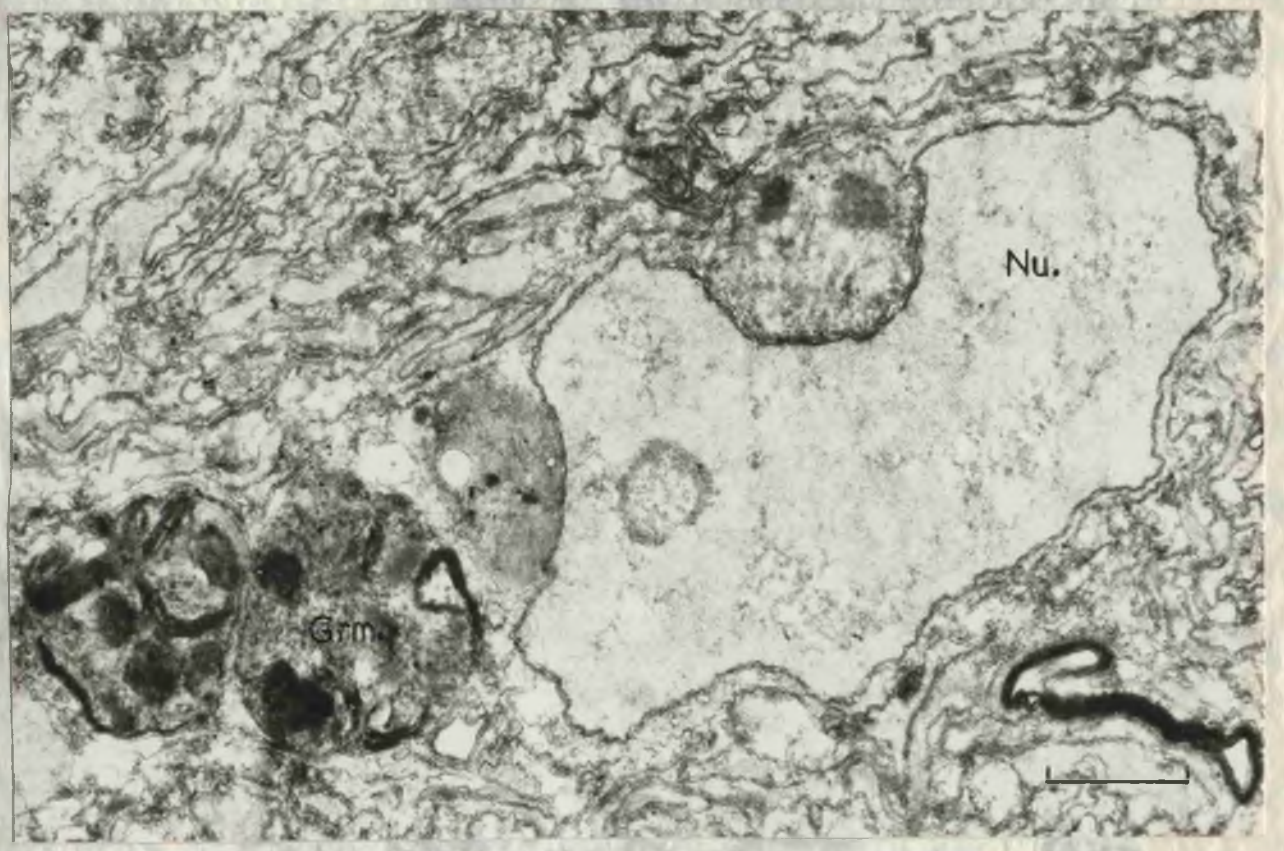



Figure 31. Electronmicrograph of transverse section of a siphon nerve. Grm., granule with endomembranes; Nu., nucleus. Scale mark, 1u. (x 15,000).

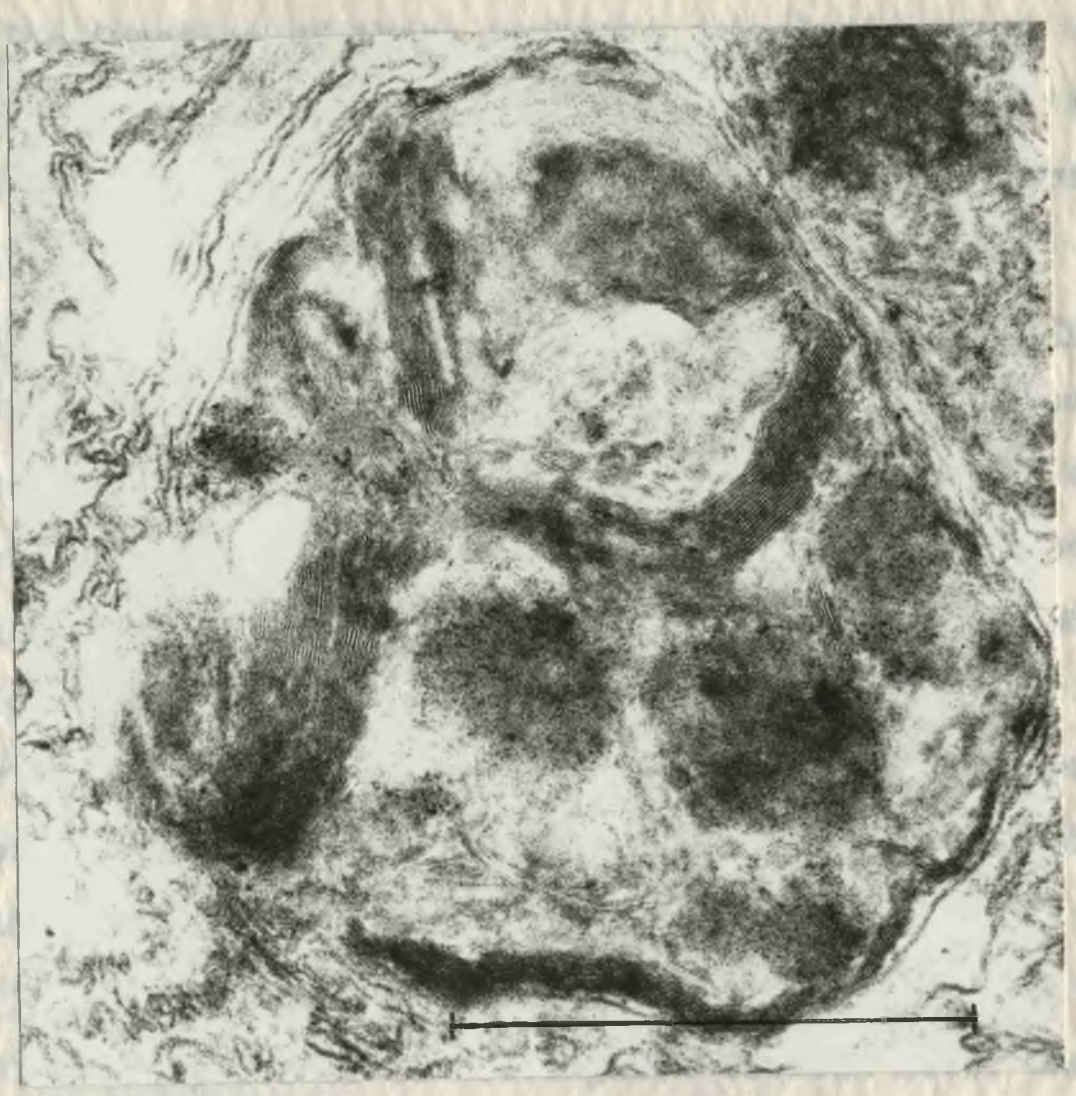


Figure 32. Granule with endomembranes from Fig. 31 at a higher magnification, showing clearly the membranous structure. Scale mark, 1u. (x 60,000).

regions of the structure (c.f. Schlote & Hanneforth, 1963).

The function of these membranous inclusions and the intracellular tubules previously described is unknown and at present there is not sufficient knowledge of the complete structure for any useful hypothesis to be formed.

Differences between the pallial and siphonal nerves.

There are no basic differences between the two nerves which can be seen from the electronmicrographs. The only observable difference is that the thicker siphonal nerve has a somewhat thicker sheath. Visual observations of the nerves whilst the sections were in the electron microscope suggest that there were fewer of the larger diameter axons in the siphonal nerve. These factors may have been responsible for the electrophysiological results mentioned earlier.

Differences between afferent and efferent axons.

It is impossible to distinguish between motor and sensory fibres in any way. There is no sharp division in fibre size which might provide some anatomical distinction, although this could hardly have been expected from the electrophysiological results which showed no such differentiation in the size of the recorded nervous potentials.

In conclusion it is most remarkable that recordings of nervous activity were at all feasable, by means of the platinum wire electrode system, in view of the small size of the axons in the nerves, and the thickness of the glia and perineurium which surround them.

MECHANO-RECEPTORS IN THE MANTLE.

Peripheral responses in nerves cut centrally to the electrode were obtained to both tactile and movement (stretch) stimuli by means of the platinum wire electrode technique. These responses differed in sensitivity to deformation and also with regard to the area served by one receptor unit.

Tactile Receptors.

The surface of the body wall, mantle and siphon was explored with a fine glass probe, which could exert a maximum force of 2 mgm. over an area of about 75μ (cf. Murray, 1960). Action potentials were observed originating from very discrete areas of about 1sq. mm. on the body surface. These units were scattered in various places, and it is reasonable to assume that only a small fraction of the total population has been monitored. The majority of the active units recorded were found in the area where the siphon and the side of the head meet. The response to a light touch was rapidly adapting and often showed active phases at the start and finish of a period of stimulation (Fig.33).

Movement Receptors.

Tactile receptors are highly sensitive and strictly limited in their locus of activity. By using a bristle probe which exerted a greater force (2.5 gm. over an area of 0.125 sq. mm.) some further units could be induced to fire. These were the movement receptors, and firing was probably due to deformation of the stimulated area rather than to a simple touch stimulus because probing over all or most of the area shaded in Fig. 34 produced a response in these units.

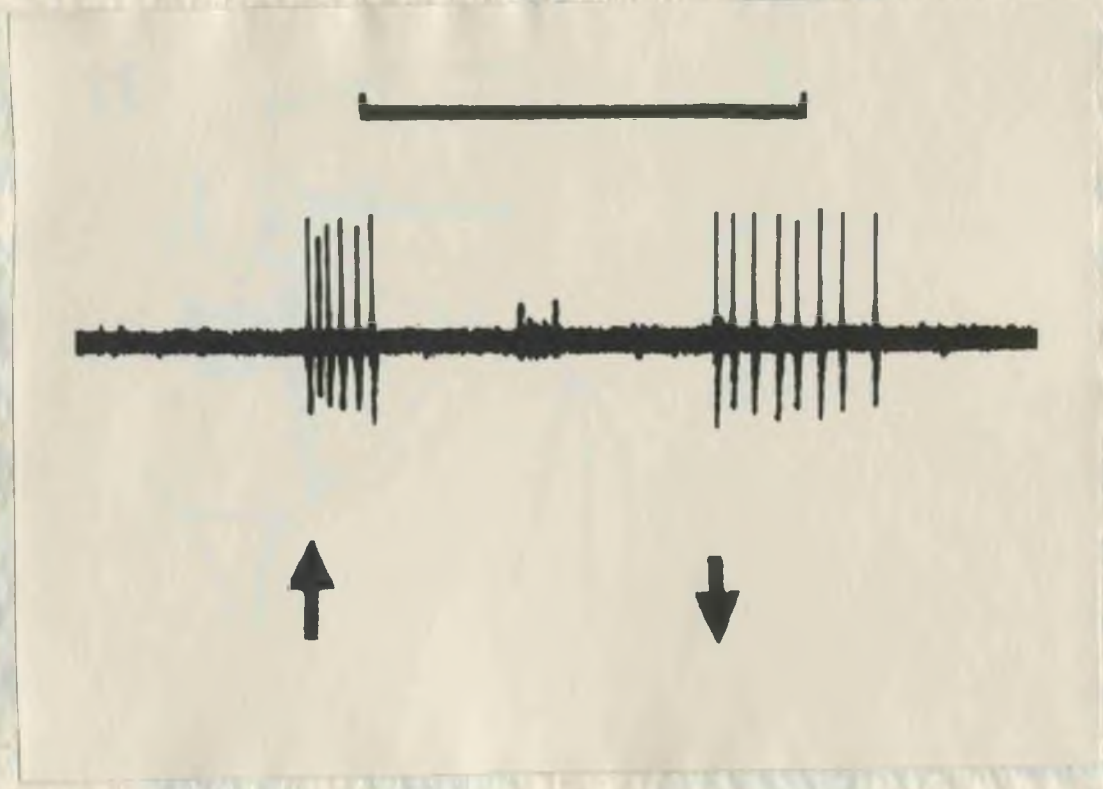


Figure 33. Response of a tactile receptor to stimulation with a fine glass probe. Arrows indicate the beginning and end of stimulation. Time marker - 0.5 secs.

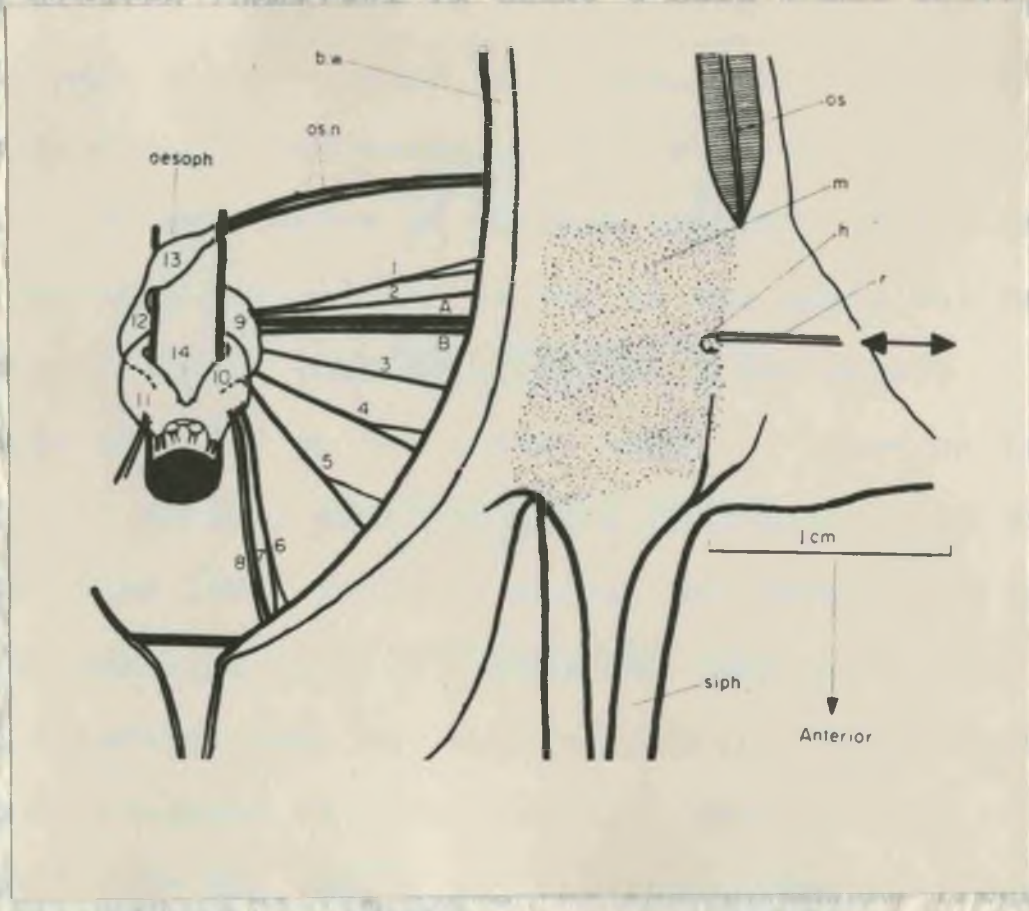



Figure 34. Diagram of the left pallial innervation of Buccinum. b.w. body wall; h., hook; m., mantle; oesoph., oesophagus; os., osphradium; os., osphradial nerves; r., rod; siph., siphon; 1,2,3,4 and 5, pallial nerves; A and B, siphon nerves; 6, 7 and 8, tentacular nerves; 9, left pleural ganglion; 10, left cerebral ganglion; 11, right cerebral ganglion; 12, right pleural ganglion; 13, supra-intestinal ganglion; 14, buccal ganglion. The stippled area is that within which a bristle probe could elicit a movement redeptor discharge. The scale represents 1 cm for a relatively large specimen. (Laverack & Bailey, 1963).

Three types of movement receptors have been seen in the course of these experiments.

Type 1.

This type gave what may be termed an 'on' response. When rapid stretch (complete in about 2 msec.) was applied to the mantle edge a short burst of spikes occurred, which had a duration of approximately 200 msecs., and the pattern of the volley was similar for any degree of stretch between 1 and 5 mms. (Fig. 35). This activity was not due solely to one unit, but resulted from a number of units each contributing a few spikes to the total (usually only one or two spikes each). These units are all phasic and rapidly adapting since continuation of the stimulus at its final level fails to elicit any further activity.

Stimulation by slow extensions varying in rate from 1mm. per 1.8 secs to 1mm. per 0.36 secs over a total distance of 5 mm, invoked responses in these units. The activity of one such unit is plotted in Fig. 36, using the instantaneous frequency method (Pringle and Wilson, 1952). It will be seen that the first spike occurs after approximately the same degree of stretch in each case. Frequency then reaches a maximum before falling again even during active stretching. The burst was usually complete before stimulation had ceased. After this no activity was seen, as previously. The actual number of spikes that occur during each slow extension is surprisingly constant.

When stimulation occurred in the form of discrete, rapid, one millimeter steps, a small burst occurred with each small increase in tension (Fig. 37A). Similarly a burst of potentials

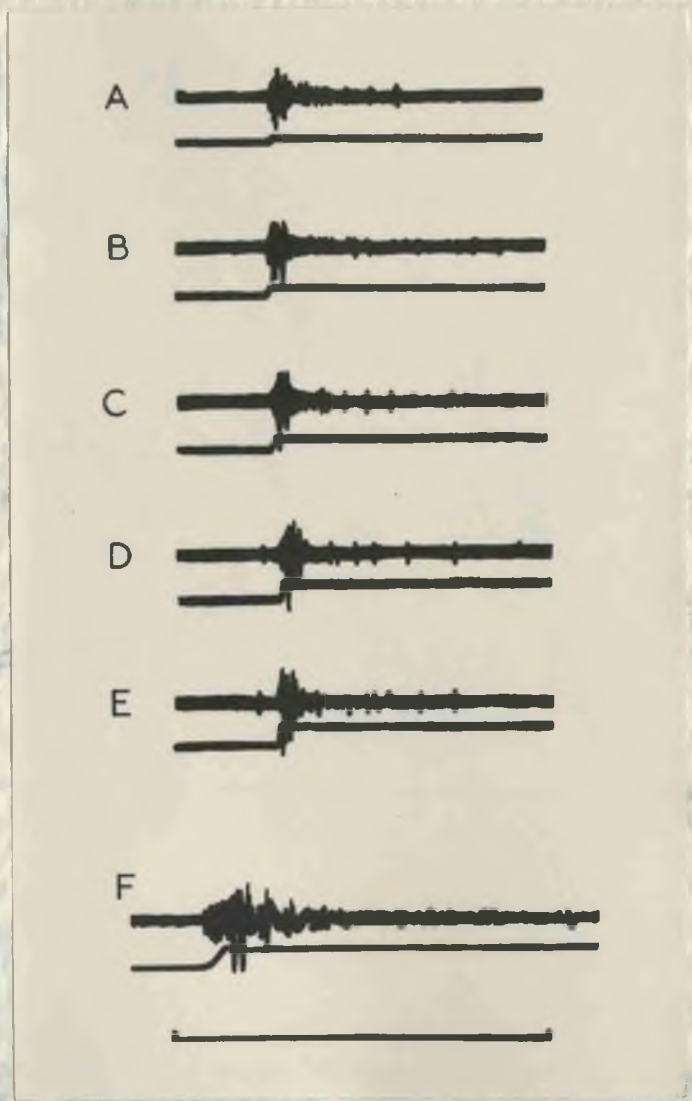



Figure 35. Type one movement receptor response to rapid extension of the mantle. The lower beam indicates the time course and extent of the movement, upward deflection indicating an extension. A,B,C,D and E are successive stimuli increasing by one mm. steps from one to five mm. total extension of the mantle. The time mark represents 1 second for A - E.

F is a repeat of stimulus E, i.e. 0-5 mm. extension, filmed at a faster speed. The time mark in this case represents 0.25 seconds.

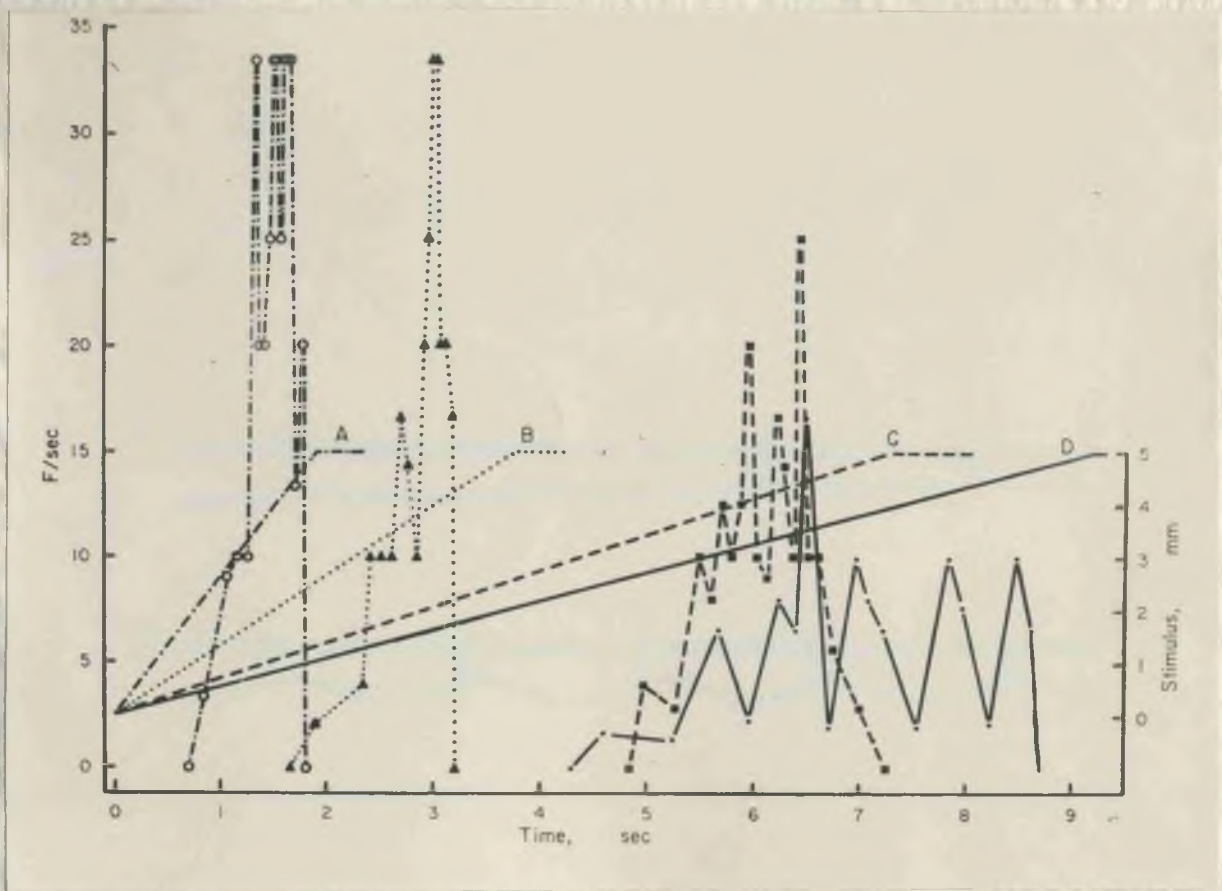



Figure 36. Graph of the response of a type one movement receptor when stimulation is applied over the same distance (5mm) at four different rates. Frequency is the instantaneous frequency calculated by the method of Pringle & Wilson (1952), i.e. the reciprocal of the interval between successive spikes plotted at the mid-point between them. The responses and the stimuli are drawn in the same style for each rate of extension. A( ), 1mm/1.85 sec; B( ), 1mm/1.45 sec; C( ), 1mm/0.75 sec; D( ), 1mm/0.36 sec. (Laverack & Bailey, 1963).

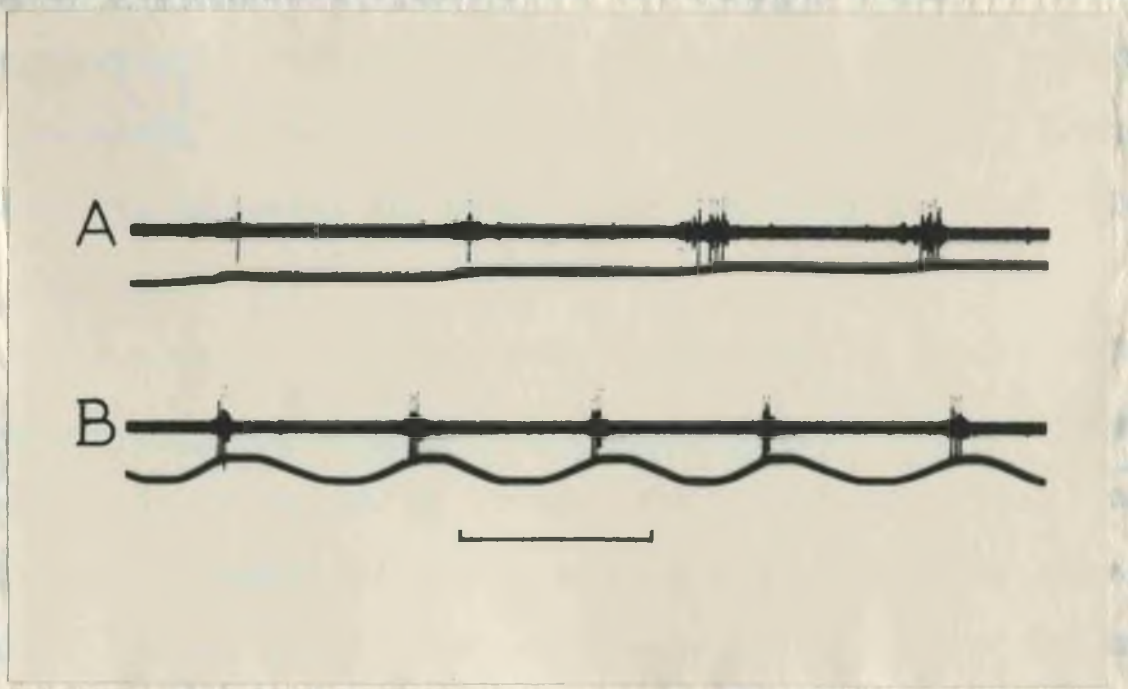



Figure 37. A. Response of type one movement receptors to stepwise extension of mantle in successive steps from 0-5mm. B. Response of same receptor to repeated sinusoidal stimulation over the same range of extension. Time mark represents 1 second.

occurred at the same point of the cycle when a repeated rhythm of stretch and relaxation is employed as stimulus (Fig. 37B).

Type 2.

The second type of receptor showed small bursts of potentials at both extension and relaxation. There was no long lasting activity. This phasic pattern of response was seen whether the stimulus was applied rapidly or slowly, in large or small steps, or rhythmically repeated. In all cases a volley occurred with each increase of tension and with each relaxation, (Fig. 38 illustrates the response to small stepwise tensions and relaxations).

Type 3.

This type of receptor was spontaneously active before stimulation at a frequency of approximately 1 impulse per two seconds. Responses to stimulation were superimposed upon this background discharge. Rapid extension caused a small volley of spikes to occur. This was composed of two or three potentials at a frequency of between 10 and 20/sec., followed by a period of adaptation, during which the frequency fell almost back to the basal level within 1 - 1.5 secs. If the tension was maintained, however, the frequency remained above the spontaneous rate for many seconds and adaptation took place very slowly. When the stimulus was released, a further short burst of impulses occurred followed by another period of slow adaptation.

The responses of this type of unit are plotted in Fig. 39. The maximal frequency at the onset of stimulation rose with the increase in intensity of stimulation i.e. with increasing distance of movement at fast rates. The volley which followed relaxation

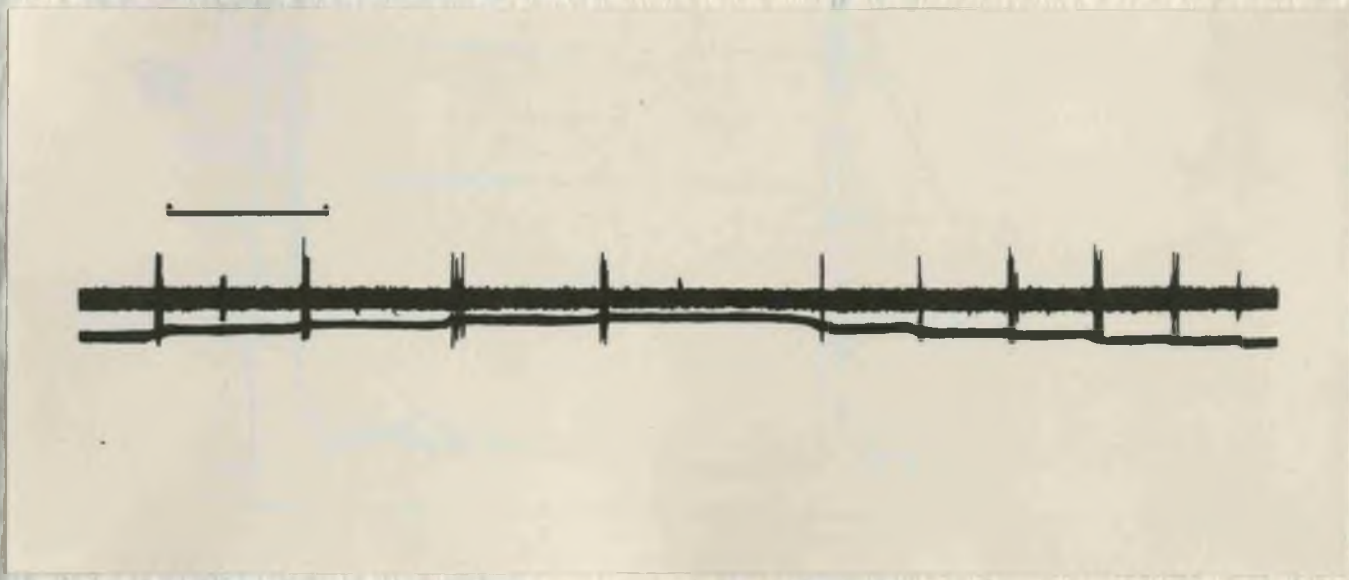


Figure 38. Type two movement receptor discharge in response to "on" and "off" phases of stimulation applied in a stepwise manner. Total extension is 5mm. Time mark represents 1 second.

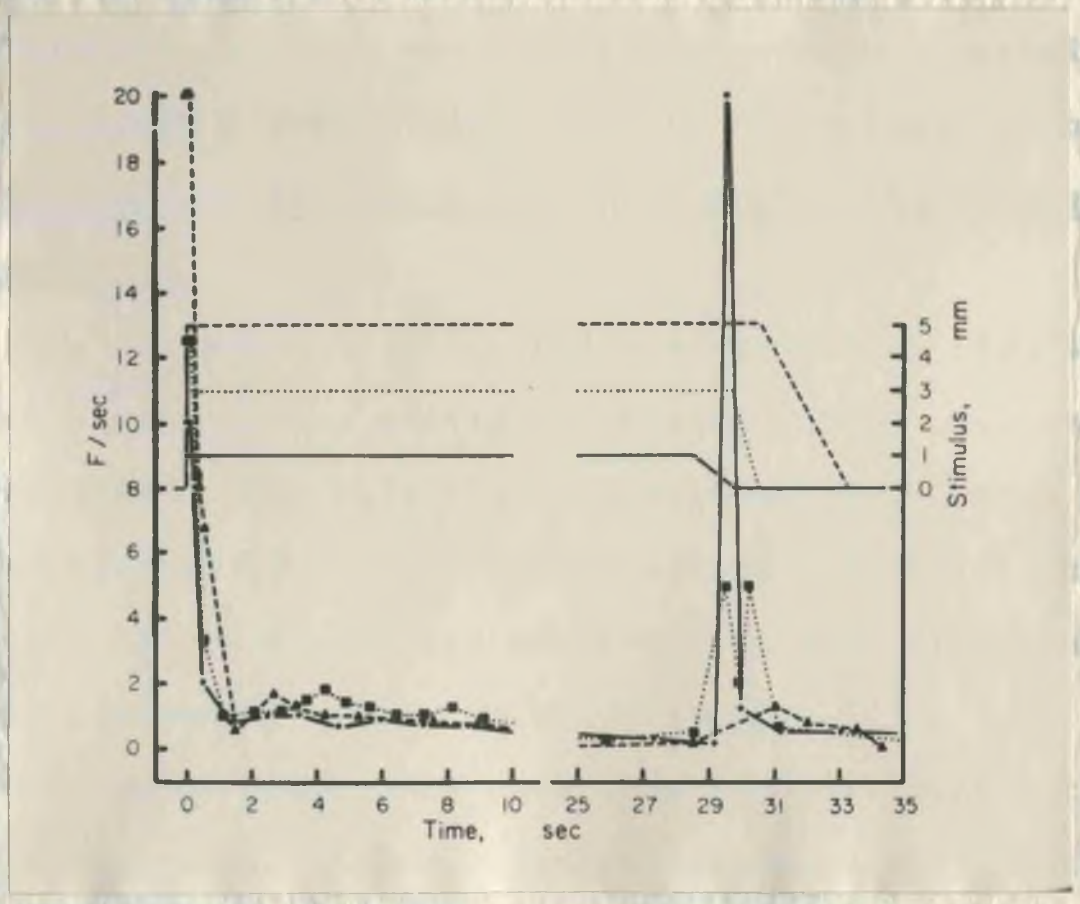



Figure 39. Graph of type three movement receptor discharge in response to rapid stimuli of increasing intensity. The frequency is determined as in Fig. 36. The zero of the graph corresponds to the onset of stimulation in each case. Prior to this point the frequency is of the order of 1 impulse/2 secs. in each case. There is an interruption of the graph between 10 and 25 secs. after stimulation, during which period the frequency fell slowly. (Laverack & Bailey, 1963).

decreased in size over the same range i.e. it diminished in frequency and number of impulses with increasing distance of relaxation. No distinctive or distinguishable activity was invoked in such a unit by stepwise or regularly repeated stimulation.

This type of unit was rare in occurrence compared with types 1 and 2, being observed twice only in over ninety preparations.

Central activity in response to mantle mechanoreceptor stimulation.

In a few cases activity has been monitored from the central root of a cut pallial nerve, as opposed to the peripheral recording reported above. In these experiments peripheral sensory input is injected into the central nervous system via the remaining uncut pallial nerves. The activity recorded from central stumps is thus presumably reflex motor in origin.

Only rapid extensions gave rise to repetitive activity in these experiments. Slow movements produced very little effect. Central units were inactive before stimulation, but gave repetitive, adapting discharges when the preparation was stretched, and generally gave some impulses when again relaxed (see Fig. 40).

The probable reflex nature of these units can be seen from the time course of the response. The latency of peripheral units in response to stimulation is less than 2 msecs. (see Fig. 35), but the central unit activity is consistently 60-80 msecs. behind the completion of the stimulus. A small volley of potentials of similar time course to receptor activity can sometimes be noted from central stumps as is shown in Fig. 41 and may well

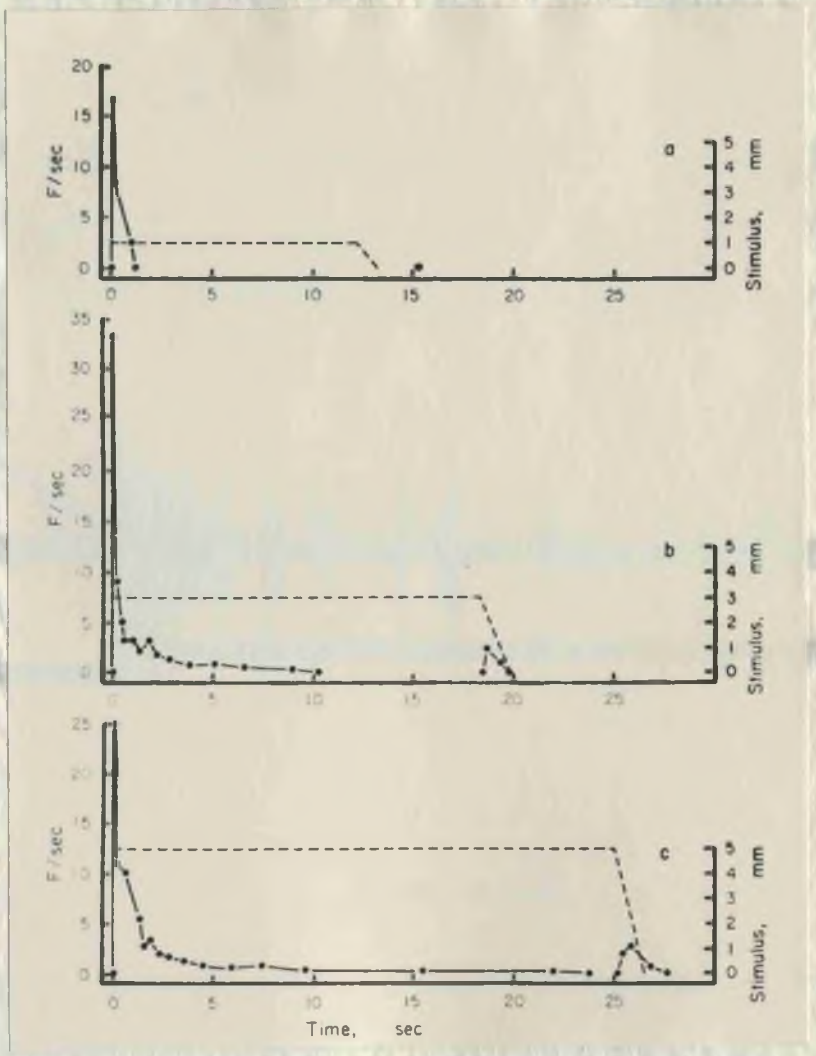



Figure 40. Graph of the discharge of a central unit in response to rapid movement stimuli of increasing intensity. The frequency is calculated as in Fig. 36. a, 1mm extension; b, 3mm extension; c, 5mm extension; the stimulus in each case being indicated by the broken line (Laverack & Bailey, 1963).

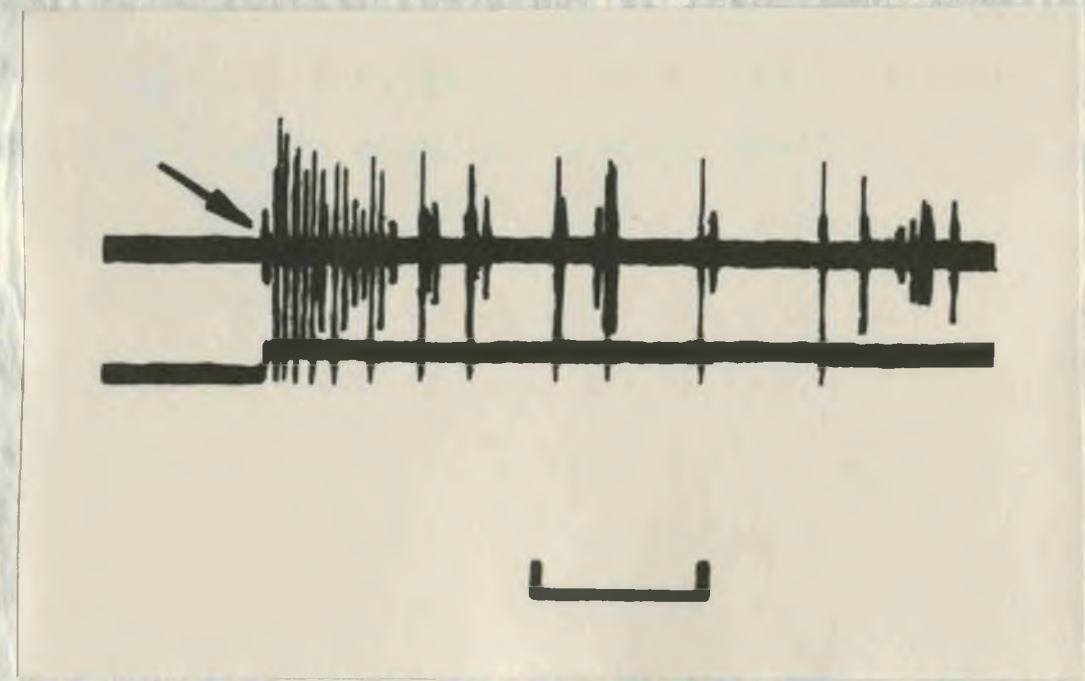


Figure 41. Central reflex discharge in response to a rapid extension of 3mm. Note the small burst at the onset of stimulation (marked by an arrow), which probably represents sensory input to the left pleural ganglion. Time mark represents 1 sec.

be the activity of the primary receptors, picked up by reason of their proximity or geometry relative to the recording electrode, and illustrating very well the magnitude of the latency of the reflex motor activity.

Activity within these reflex motor neurones shows a delay of 60-80 msec. after the completion of the stimulus, then a volley at an initial frequency of 16-33/sec. adapting to zero within a time proportional to intensity of stimulus. Usually a small burst also occurred with relaxation of the mantle.

REFLEX PATHWAYS IN THE CENTRAL NERVOUS SYSTEM.

(1) Reflex pathways related to mechanical stimulation of the left mantle region.

The last results of the previous section dealt with reflex efferent activity in the pallial nerves arising in the central complex in response to peripheral movement stimuli. By means of a dual channel recording set-up (see p. 26) it was possible to monitor concurrent activity in two of the pallial nerves. One nerve was left intact and carried afferent activity into the central complex as well as the reflex efferent activity, whilst the second nerve was cut peripheral to the recording electrode so that only afferent activity was recorded from this nerve. The remainder of the nerves were cut to isolate the CNS from any other afferent activity. Touch and movement stimuli were then applied to the mantle region before and after the various commissures and connectives were cut. In this way it was possible to note the diminution or loss of the reflex activity and so to trace the reflex pathways through the ganglia.

Fig. 42 is a diagrammatic representation of the eight major ganglia of the anterior central nervous complex. The cerebral commissure and the buccal ganglia are left out for the sake of clarity. Superimposed on this outline are some of the overall pathways which are involved in the reflex activity. These pathways are drawn as continuous lines since no valid indication of the position or number of synapses involved in any reflex could be deduced using the described experimental methods. It proved impossible to relate individual afferent and efferent units in

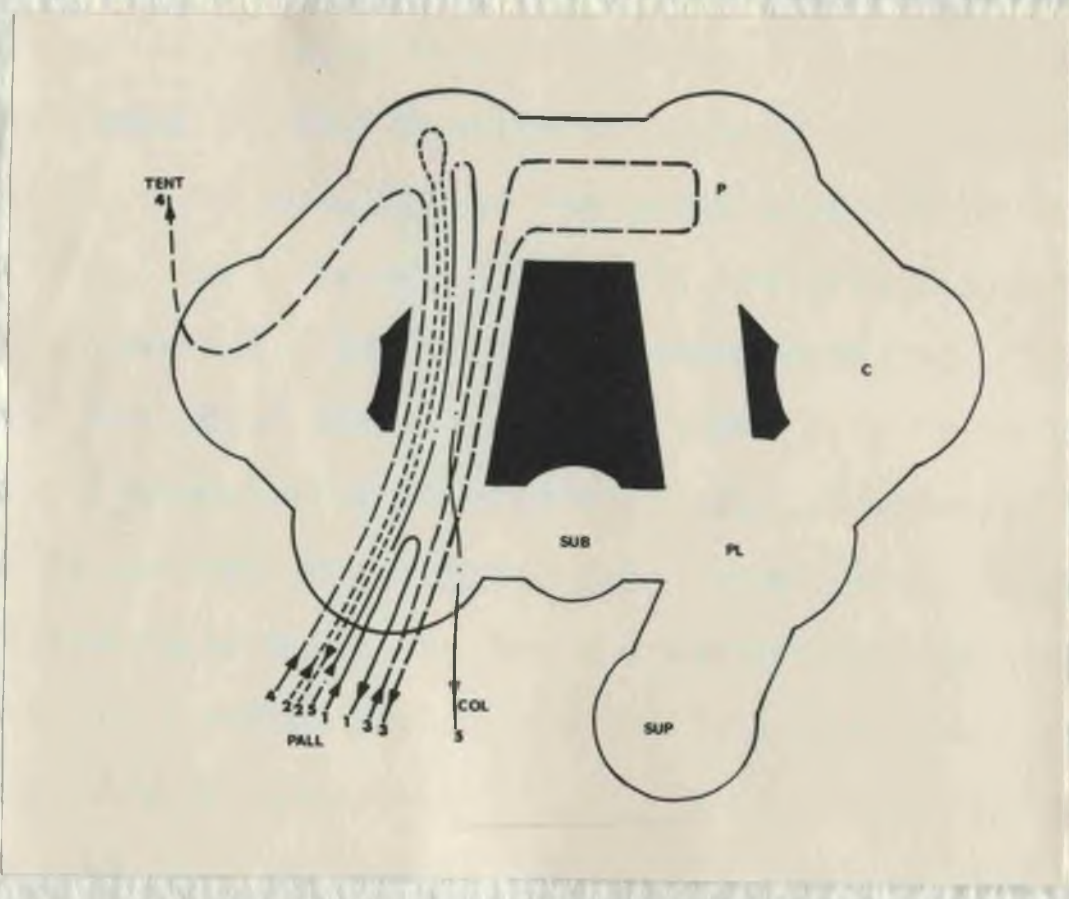



Figure 42. Reflex pathways relating to mechanical stimulation of the mantle region. C., cerebral ganglion; COL., columella nerve; P., pedal ganglion; PALL., pallial nerves; PL., pleural ganglion; SUB., sub-intestinal ganglion; SUP., supra-intestinal ganglion; TENT., tentacular nerve; 1-5., pathways.

any of the experiments because of the small size and multiplicity of potentials in the bursts of activity which were recorded.

The pathways numbered 1, 2 and 3 in Fig. 42 relate to reflex afferent activity passing down the pallial nerves. Pathway 1 demonstrates that the reflex activity may arise in the isolated pleural ganglion without reference to any other ganglionic interaction. Pathway 2 shows a longer loop which passes beyond the left pleural ganglion to the pedal ganglion of the same side. The afferent and efferent paths both traverse the left pleuropedal connective. Pathway 3 is longer than the other two and involves the pedal ganglion of the right side in the reflex activity passing out of the left pleural ganglion. The pathway passes in and out of the right pedal ganglion by means of the pedal commissure demonstrating an interesting contralateral involvment of a reflex pathway having both origin and effect in the left side of the body.

Reflex activity can be initiated in nerves other than the pallial nerves of the left side, as shown by pathways 4 and 5. Pathway 4 shows that activity initiated in the left mantle region passes into the cerebral ganglion of the same side, having passed through the left pedal ganglion. Efferent activity can thus be observed in the left tentacular nerves as a result of afferent activity passing into the left pleural ganglion.

Pathway 5 demonstrates that reflex activity passes to the posterior regions of the body under the same conditions, since the efferent burst was recorded in the columellar nerve. This reflex also passed through both the left pleural and left pedal

ganglia before emerging from the former.

Mechanical stimulation of the left mantle thus causes reflex activity on at least three levels since one, two or three ganglia may be involved in the reflex arcs. It seems reasonable to assume that many of the looped reflex arcs have at least one synapse in each ganglion through which they pass. Since simple bipolar interneurones appear to be lacking in the ganglia, synapses onto the existing unipolar cells will affect a relatively large number of other synapses which are common to that particular interneurone in the ganglionic neuropile. It is therefore possible to envisage a widespread effect in the various ganglia resulting from afferent activity passing into one ganglion from a relatively small area of the body. The greatest effect in the case of the "Pallial reflexes" would appear to be in the pedal ganglia since this is the common path of the majority of the pathways demonstrated in these experiments.

(2) Reflex pathways associated with the retraction response.

The protective retraction response is a general feature of molluscs. Strong mechanical stimulation of the head region of Buccinum will produce such a response and the efferent activity occasioned by such stimuli was investigated using similar techniques to those described in section 1.

The tentacular nerves of the left side were left intact whilst the remainder of the nerves were cut to isolate the CNS. The nervous activity in the tentacular nerves and various others was then monitored concurrently. Reflex pathways were esta-

blished by cutting the commissure and connectives.

Fig. 43 shows only the complete pathways which were established by these means. As in section (1), reflexes at three levels could be observed and in one case (pathway 5) five ganglia were involved in the reflex - the left pedal ganglion doing duty twice over. Mechanical stimulation of the head region thus has an even more widespread effect in the CNS than similar stimulation of the mantle.

Some of the pathways shown to be involved in the retraction response are more direct than most of those involved in the mantle movement reflexes e.g. pathways 1-3 in Fig. 43 (cf. Fig. 42) and it is possible that these are related to the initial rapid movement which precedes the full retraction. The initial response consists of the contraction of the tentacles, siphon and the head region only. This contraction can be elicited in the whole animal by relatively light mechanical stimuli which, if unrepeated, will not cause the full retraction. Repeated light stimulation or a single strong stimulus produce the initial fast response which then grades into the full retraction response whereby the shell is lifted over the whole head region leaving only the foot extended. Further stimulation will then cause the retraction of the foot. The reflex pathways passing through the pedal ganglia, Nos. 4-6 in Fig. 43 are therefore most likely to be concerned in the full retraction response.

Pathway 7 indicates that as in the pleural ganglion some of the reflex arcs are completed within the ganglion to which the

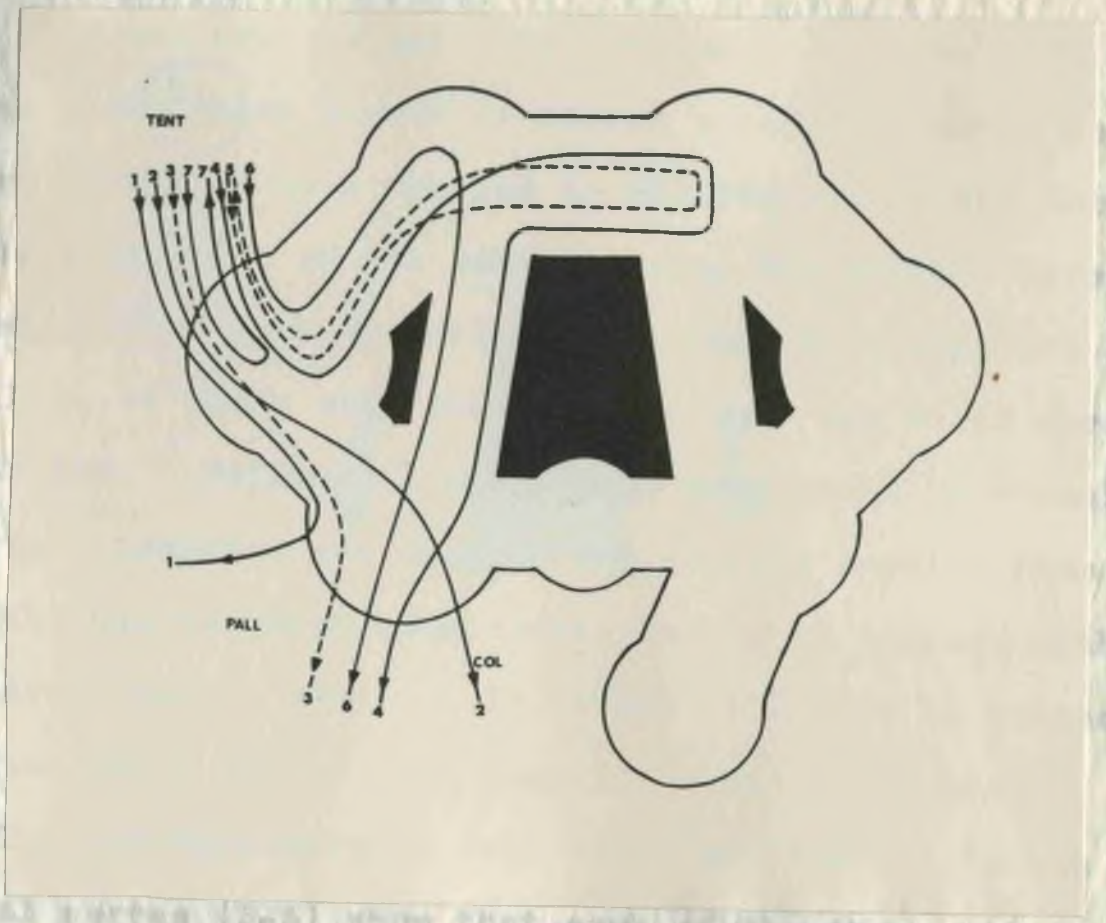



Figure 43. Reflex pathways relating to the retraction response. Legend as for Fig. 42. (see text).

afferent pathways run. This type of reflex is probably concerned with the retraction of the tentacle itself.

(3) Central nervous pathways relating to contralateral coordination.

Efferent nervous activity monitored from the left pallial nerves showed that bursts of action potentials could be elicited by mechanical stimuli applied to the tentacular and mantle regions of the right side of the body. Using the methods already described, reflex pathways relating to this activity were deduced.

Fig. 44 shows only the complete pathways which were demonstrated. Pathways 1 and 2 were occasioned by stimulating the right tentacle and crossed the central complex from right to left via the cerebral commissure, though by analogy with the pathways described in section (1), there will also be connections with the pedal and pleural ganglia of the right side.

The pathways arising from afferent impulses in the right pallial nerves (3-5) show that each of the three commissures linking the paired ganglia of the anterior complex carries reflex arcs between receptors in the right mantle and motor axons sending efferent impulses to the left mantle.

The pathways described in this section again demonstrate that afferent activity arising from relatively small areas of the body produces widespread effects on reaching the ganglia of the central complex.

Central effects of afferent activity.

The insertion of indium microelectrodes into selected ganglia confirmed the pathways which have already been described, since

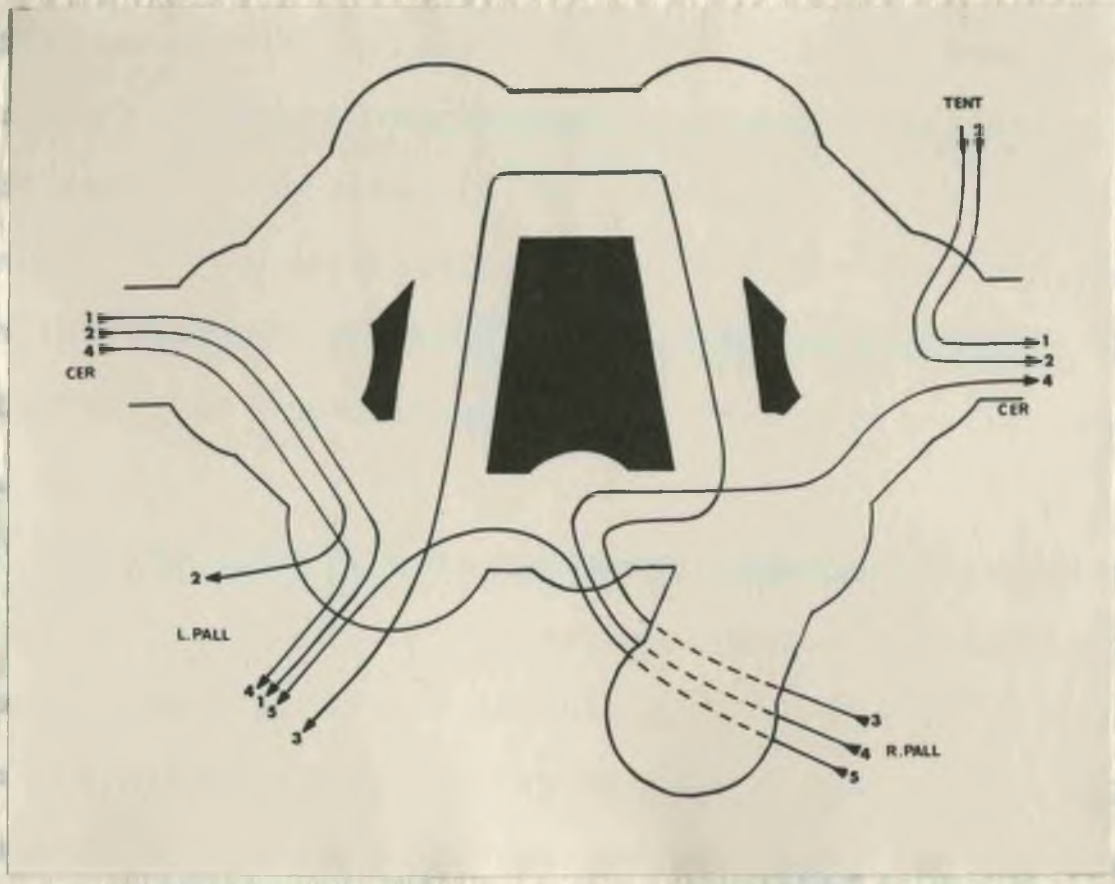



Figure 44. Reflex pathways relating to contralateral coordination. CER., cerebral commissure - through which pathways 1, 2 and 4 pass from the right to the left cerebral ganglion; L. PALL., left pallial nerves; R. PALL., right pallial nerves. Remainder as in Fig. 42.

the recorded activity in any ganglion could be influenced in some way by the stimulation of afferent pathways in the nerves which had been used to elucidate reflex arcs, passing through that ganglion, in the experiments described above.

The use of indium electrodes made it possible to detect both excitatory and inhibitory effects of afferent impulses on the neurones of the central nervous complex. Excitatory effects could cause an increase in spontaneous activity, or elicit action potentials from previously silent neurones (see Fig. 45, A and B). With this technique inhibitory effects were noted by the reduction of the impulse frequencies of spontaneously active neurones (see Fig. 45, C).

In the course of the indium microelectrode experiments it was usual to find groups of central neurones reacting to the same stimuli (see Fig. 45). Isolation of single units was difficult with the relatively large tip diameters used. The multiplicity of units affected by a peripheral stimulus, and the absence of specific central areas of peripheral representation which was noted, both confirm the diffuse nature of the central reactions to peripheral stimuli.

Convergence of afferent pathways in the central nervous complex.

Using the indium electrodes it was possible to observe the effects of stimulation of various regions of the body on the same group of central neurones. Fig. 46 illustrates four cases of convergence of afferent pathways onto neurones in various ganglia. In each case the ganglionic neurones are affected by at least

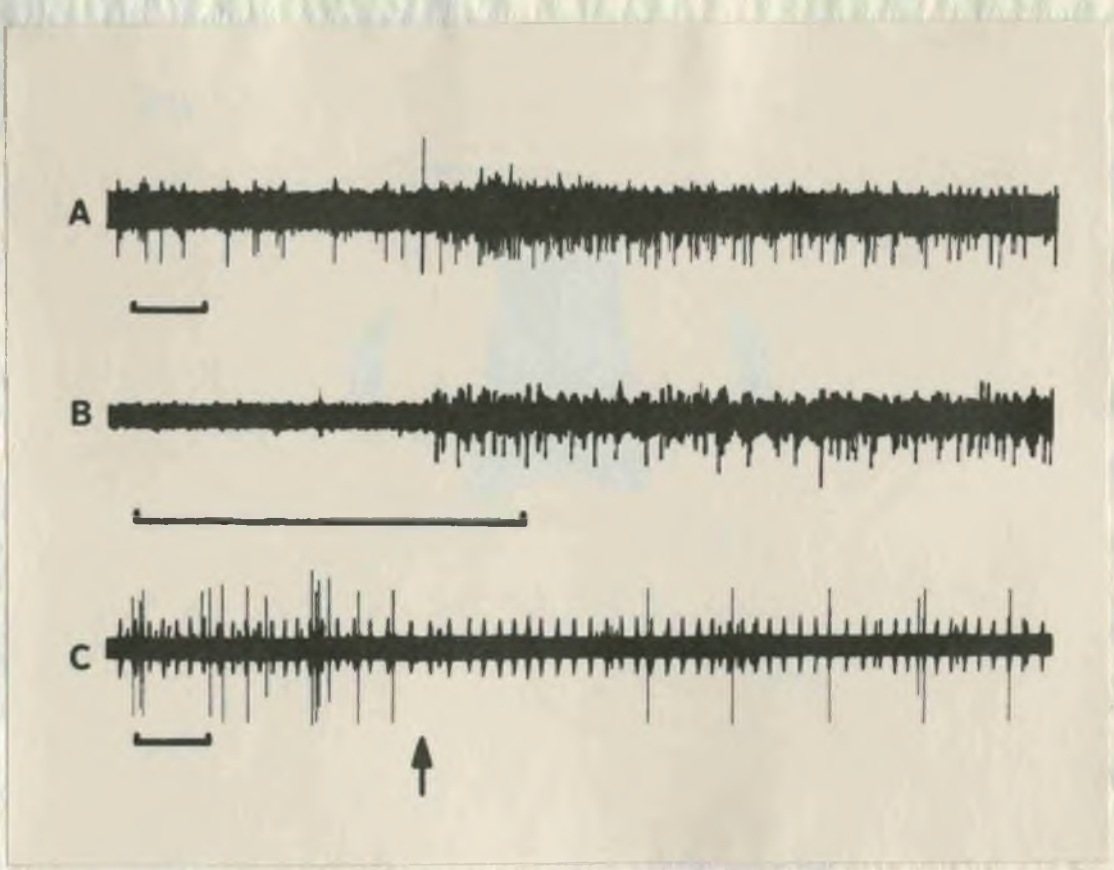



Figure 45. Responses of central neurones. A, increase in activity in pedal ganglion in response to touching the head. B, response of "silent" neurones in the supra-intestinal ganglion elicited by acid stimulus applied to the osphradium. C, spontaneously active neurone in the pedal ganglion inhibited by touching the tentacle of the same side. Time marks represent 1 second in each case. Arrow represents onset of stimulus in each case.

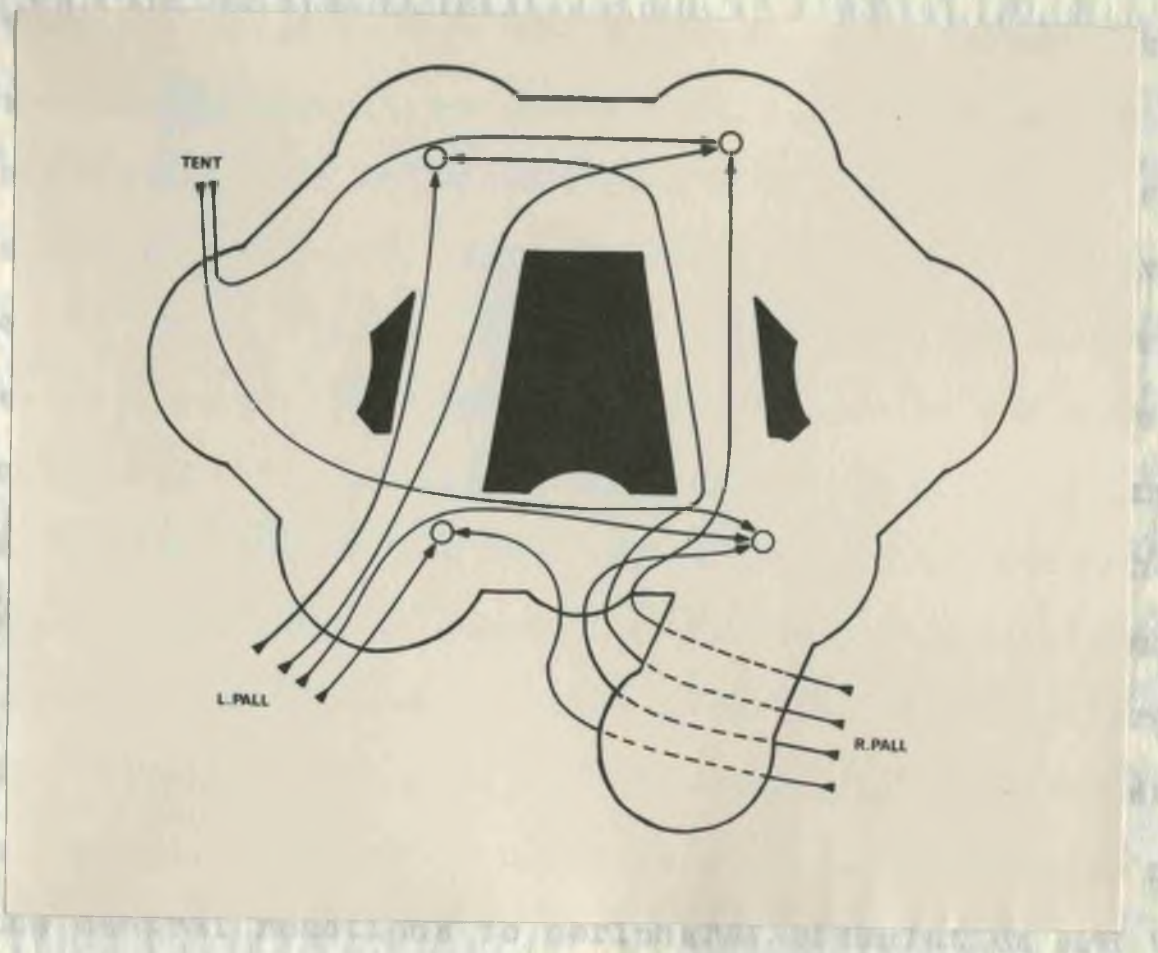



Figure 46. Convergence of afferent pathways upon central neurones. Circles represent site of recording electrode. Remainder of legend as in Fig. 44 (also see text).

two pathways which converge from opposite sides of the body. Mechanical stimulation of the various body regions produced similar effects on the central neurones which in most cases was an excitation. The pathways derived from these experimental results are assumed to take the shortest route between the afferent nerve and the central recording site because it proved impossible to cut commissures and connectives with the recording electrode in situ and retain the desired recording site. The exact route taken by any nervous activity therefore remained unknown in these cases and the pathways drawn in Fig. 46 merely indicate the known convergence. The points of convergence of afferent impulses from the same regions of the body are dispersed throughout the ganglia of the central complex. In order to reach such different localities afferent impulses must take widely divergent routes through the ganglia. The results of these experiments upon central convergence therefore confirm the diffuse nature of the central reactions to peripheral stimulation and the absence of specific central representation of the various body regions.

The converging pathways demonstrated by these results are all of a similar afferent origin i.e. all arise in response to some form of mechanical stimulation. In a later section of the results (see p. 138) it will be seen that afferent pathways from different types of receptors also converge onto central neurones and can have similar or opposite effects upon their activity.

Efferent responses to preganglionic electrical stimulation.

The effect of preganglionic shocks on the postganglionic

efferent activity has been studied in the clam (Mya) by Horridge (1958 and 1961). In these experiments a cerebral ganglion was isolated, and the afferent and efferent activity caused by electrical stimulation monitored from the viscero-cerebral connective and the efferent nerves respectively. In the ganglion thus isolated it was found that impulses in the viscero-cerebral connective would initiate patterned trains of motor impulses in the efferent nerves. In view of the demonstrated dispersion of sensory input on reaching the central nervous system in Buccinum, a few similar experiments were carried out on this animal with the CNS and the majority of the nerves intact in order to determine whether similar patterned responses could be evoked from the complete CNS.

Single maximal electrical shocks were applied preganglionically and the evoked activity monitored from the efferent nerves. Fig. 47 shows the result of one such experiment. From this it may be seen that similar patterns of impulses were evoked by each of the four consecutive electrical stimuli. These are comparable to those recorded by Horridge (1961). The intensity of the response increases a little and the central delay shortens slightly with each successive stimulus, but the overall pattern of the efferent response remains much the same.

Since such patterns may result from a single ganglion or from the entire CNS (Mya and Buccinum respectively) it would appear that the diffuse nature of the central pathways has much less effect upon the final motor output to any region than might be expected, and may therefore be utilised more for the long term,

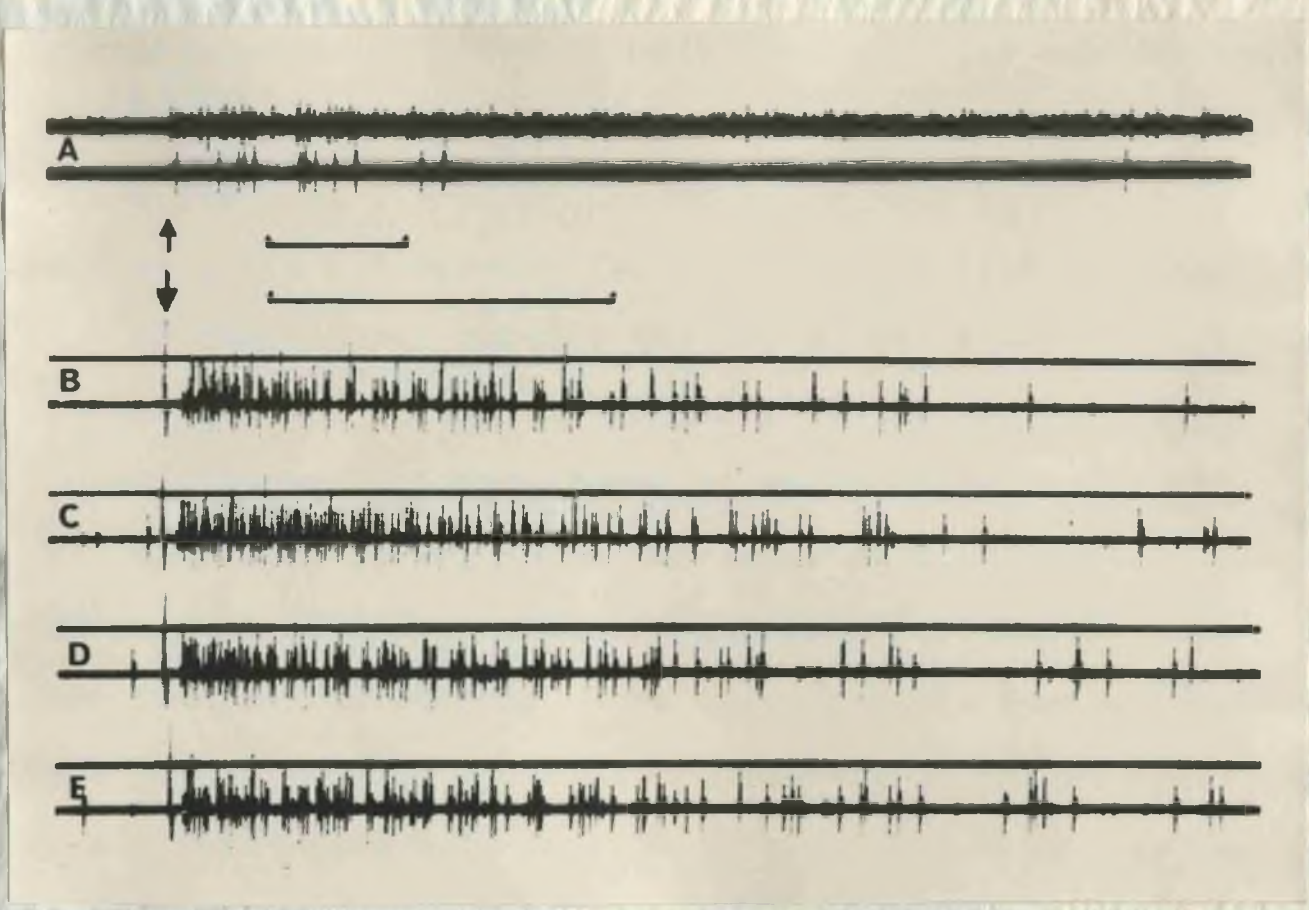



Figure 47. Patterned efferent activity evoked in a pallial nerve (lower trace) in response to preganglionic electrical stimuli applied to a tentacular nerve of the same side. A, response in both nerves elicited by touching the head. B, C, D and E, responses in the pallial nerve to successive shocks applied to the tentacular nerve at intervals of 4.5 secs. Arrows indicate onset of stimulus. Time marks represent 1 second, upper for A and lower for B - E.

overall co-ordination of the various body regions than for the modification of the immediate motor output to any particular region. Thus the dispersion of the reflex pathways in the CNS may be compensated by their ultimate convergence onto a relatively small number of efferent motor neurones for any region. For example, in the retraction response, if the first afferent impulses reaching the motor neurones are able to set up the motor impulse pattern necessary for the quick initial response, regardless of their route through the CNS or their point of origin, the animal will be capable of fast, co-ordinated, protective responses without the involvement of monosynaptic pathways. Afferent impulses arriving later may re-inforce the initial responses and so give rise to the complete retraction, but are not necessary for its initiation. The fact that the various pathways have passed through a number of synapses in the various ganglia will however have made available more information for longer term co-ordination.

CENTRAL NERVOUS RESPONSES TO STIMULATION OF THE OSPHRADIUM.

In the course of these experiments all the three basic recording techniques were used. Each produced some results but those from the experiments using microelectrodes were by far the most informative.

Efferent activity in the osphradial nerves.

The preparations were set up as described on p. 12. Platinum wire electrodes were placed under the osphradial nerves and then raised to lift the nerve into air for recording purposes. In the majority of preparations spontaneous activity was seen in the osphradial nerves (see Fig. 48A). In each preparation attempts were made to record nervous responses to applications of the following stimuli to the osphradium:-

1) Touch
2) Sea water currents
3) Particles of carborundum in sea water
4) Chemicals in sea water solution e.g. betaine hydrochloride, l-glutamic acid, l-glycine, indole, glucose, sucrose, l-glutamine, glutathione, quinine hydrochloride and extracts of Mytilus prepared by the method described on p. 39.

All attempts to record afferent activity in the nerves in response to the above stimuli failed. The spontaneous activity described above and shown in Fig. 48A was found to be entirely of an efferent nature, since cutting the osphradial nerves proximal to the recording electrodes abolished the activity in all cases, whilst cutting the nerves peripheral to the electrodes left the activity undiminished.

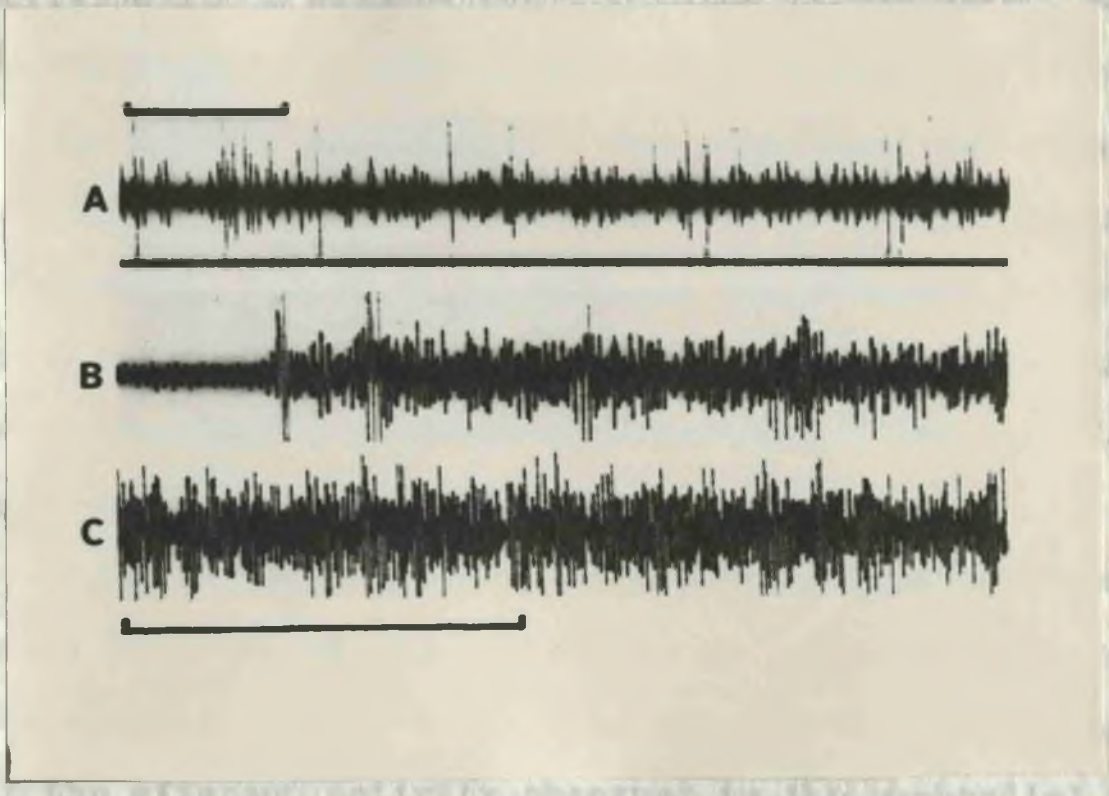



Figure 48. Efferent activity recorded from the osphradial nerves: A, spontaneous activity; B and C, 'acid' response recorded from a previously 'silent' nerve, the traces are continuous. Time marks represent 1 second, upper for A only and lower for B and C.

In some cases the efferent activity in the intact nerves could be seen to increase, or, less frequently activity could be invoked in a "silent" nerve, when certain stimuli were applied to the osphradium. In all cases the stimuli which elicited such responses were of an extremely acidic nature, e.g. unbuffered $10^{-2}$M betaine hydrochloride in sea water, and the response lasted for several minutes. A response of this type is shown in Fig. 48 B and C. The experiment was repeatable if the stimulating solution was washed off the osphradium within 10 seconds. Prolonged exposure to the stimulus led eventually to a cessation of the response and in this case the experiment was not repeatable as fresh applications of the stimulus no longer caused efferent activity.

Experiments upon the whole animals showed that the acidic stimuli produced strong retraction responses when they were introduced into the sea water near the animal. This suggests that the efferent activity observed in the osphradial nerves in vitro was either of a protective motor nature or, more probably was produced as a result of the arrival of injury discharges in the afferent sensory neurones at the ganglionic synapses.

The origins of the reflex efferent activity described above appear to reside in the supraintestinal ganglion, since the removal of the remainder of the CNS failed to abolish, or even cause a visible reduction in the observed response.

The experiments utilising platinum wire electrodes therefore failed to show any sensory activity in the osphradial nerves of

a sufficient size to be recorded by external electrodes. Afferent fibres must be present in the nerves however, since reflex efferent activity could be observed in response to noxious stimuli when all other afferent routes were eliminated. These results show that the osphradial nerves are of the 'mixed' type and suggest that the afferent pathways are axons of small diameter.

In the hope of obtaining records of the afferent activity from these small fibres, attempts were made to split the osphradial nerves into smaller bundles for further experiments with platinum wire electrodes. This technique failed because once the nerve sheath had been cut the nervous elements within disintegrated at the slightest touch of the dissecting instruments, suggesting that the structure of the nerves must therefore be of a similar nature to that of the pallial and siphonal nerves as described on pps. 61-72. Axons of such an unmyelinated form can have very little inherent mechanical strength and must depend on the nerve sheath for this property.

Because of the difficulties of recording the afferent sensory activity from the osphradial receptors no direct knowledge of their responses is available. At a later date, however, when the use of indium filled microelectrodes had been established as a successful technique on Buccinum, experiments were carried out first in an attempt to record afferent impulses from the osphradial ganglion, which lies in the axis of the organ, and second, in order to investigate the demonstrated central effects

of stimulation of the osphradium which occur in the supraintestinal ganglion. The former experiments met with no success but the latter provided the data to be described in the following section.

Spontaneous activity of neurones in the supraintestinal ganglion.

Penetrations of the supraintestinal ganglion with microelectrodes established that spontaneously active neurones were common in the isolated ganglion. In the majority of cases the spontaneous discharge was of a relatively regular frequency (see Fig. 49A). Such activity was frequently modifiable in response to stimulation of the osphradium as will be shown below.

In a small number of preparations spontaneous activity of a different type was observed. This took the form of periodic bursts of activity in small groups of neurones without the preparation being stimulated. This type of result is shown in Fig. 49B. The similarity of the patterns of activity and their regularity of occurrence suggest that some pacemaker neurones are involved. In two of the preparations exhibiting such activity it was possible to modify the bursts by stimulating the osphradium (see Fig. 63 below). In the remaining preparation the periodicity and pattern remained unchanged in spite of mechanical and chemical stimuli applied to the osphradium. Only when an acid solution of pH2.3 was placed on the osphradium was there any change in the activity. A large burst of action potentials was evoked followed by a contraction of the preparation which moved the ganglion relative to the electrode and thus dis-

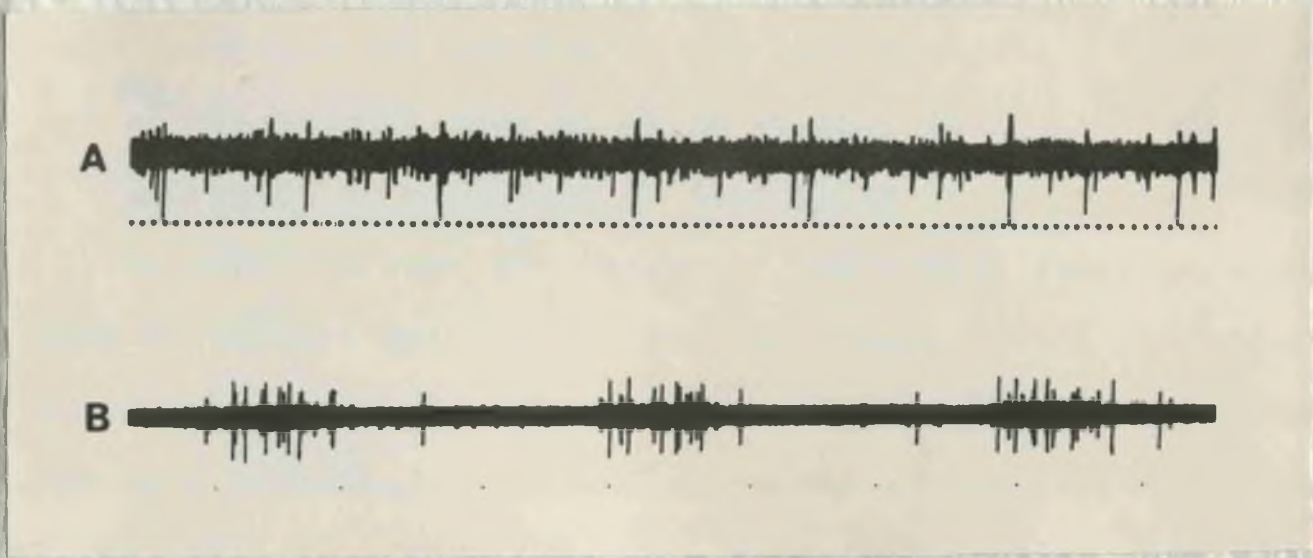



Figure 49. Spontaneous activity of neurones in the supra-intestinal ganglion. A, regular spontaneous discharge from several neurones. Time marker frequency is 10/sec. B, periodic bursts of spontaneous activity from several neurones. Time marker frequency is 1/sec.

turbed the recording conditions.

Central nervous responses in the supraintestinal ganglion.

The results in this section concern the activity of single, or more commonly, small groups of neurones in the isolated supraintestinal ganglion in response to stimulation of the osphradium. The experimental preparation was set up as described on pps. 17-19 and observations of the nervous activity were made in order to obtain information concerning the sensory modalities whose receptors are located in the latter organ. A complete list of the stimuli used is given on pps. 39-43.

1) Responses to particles in suspension in sea water.

In order to test the theory of osphradial function proposed by Hulbert and Yonge (1937) and Yonge (1947) sea water containing suspended carborundum particles of known size was used as a stimulus. Three size ranges of particles were employed, viz: less than 20μ, 20 - 200μ, and greater than 200μ. The smallest particles in the first range would remain in suspension for many minutes but the largest settled out after 5 - 30 seconds. Similarly, the smallest particles of the middle range remained in suspension for some few seconds whilst the larger particles settled immediately. The particles of the last range all settled immediately, the largest particles being of a similar size to large sand grains.

Stimulation of the osphradium with any of the above failed to elicit a central response on any occasion. The smallest and middle range particles are of a size which could easily be swept

into suspension by wave action, whilst the largest particles should represent a considerably supramaximal stimulus in view of their rapid settling properties. It is therefore possible to rule out sensitivity to sediments in suspension as a sensory modality of the receptors of the osphradium of Buccinum.

2) Responses to variations in pH.

Sea water adjusted to various known pH values between 1.4 and 10.05 were used to stimulate the osphradium on a number of occasions. Central responses to such stimuli were only elicited by those solutions having pH values of less than 4.0 or greater than 9.0. The normal pH of sea water is in the region of 7.8 and the buffering effects of the bicarbonate are well known. Local pH variations, e.g. near decomposing organic material in still water, are feasible however, but it seems unlikely that such high and low values as those necessary to elicit responses experimentally would be achieved. The responses obtained from the animal in these electrophysiological experiments closely resembled that illustrated in Fig. 48 B and C for the 'acid' response in the osphradial nerves and repeated or prolonged stimulation with such solutions again caused an irreversible diminution or loss of the central responses.

The osphradium would thus appear to be insensitive to variations of pH over a wide range of values above and below that of normal sea water. The observed responses to extreme values of pH are most probably caused only by death or injury discharges in the osphradial receptors when the pH of the sea water stimulus is such that physical damage results from its application to the

osphradium.

3) Responses to ionic and/or osmotic concentration.

To test the osphradium for sensitivity to ionic or osmotic variations in the surrounding medium, sea water was concentrated or diluted to produce a range of solutions from 200% sea water to distilled water. Central responses to the applications of such solutions to the osphradium were limited in much the same way as those described above for pH. Responses were only seen in cases where 200% sea water or dilutions of 50% and greater were used. Both these values can be considered as unlikely in the normal environment, though the latter might be achieved in estuarine regions. Generally, however, Buccinum lives below E.L.W.M. and would not be expected to encounter such dilutions. The osphradial receptors do not appear to be sensitive to an increase in the osmotic concentration alone since a stimulus solution of mannitol in sea water osmotically equivalent to 150% sea water also failed to elicit any central response any more than they were affected by the simultaneous increase in both the ionic and osmotic concentrations of 150% sea water.

Small variations in the overall ionic and osmotic concentrations of the environment will not therefore cause any afferent activity in the osphradial receptors. There remains a possibility that the animal can detect areas where the sea water has been greatly diluted, because central nervous responses were elicited experimentally using such stimuli. Whether such activity is meaningful to the animal or merely represents injury discharges in the receptors is difficult to determine by electro-

physiological experiments. Some marine molluscs are, however, tolerant to fresh water to a remarkable degree and fresh water turgor has been used as an aid to dissection in some cases (Krijgsman and Brown, 1960). This would suggest that the effects of dilute sea water or fresh water are not very harmful to such animals. This tolerance and the fact that the experimentally induced responses could be repeated or prolonged without any adverse effects or diminution of the response suggest that the nervous responses to such stimuli were not occasioned by injury discharges in the receptors and thus may have some significance to the animal and therefore be a means by which estuarine regions can be detected. Behavioural experiments using choice chamber techniques might throw some light on this problem since the overt responses of the animal to such factors could then be studied.

4) Responses to tactile and chemical stimuli.

(1) Responses to touch and 'extracts'.

The only stimuli which consistently elicited central responses in the supraintestinal ganglion when they were applied to the osphradium were tactile and chemical. The former were of sufficient force to move and distort the osphradium and were therefore much stronger than the forces exerted by the suspended particles, already described, which failed to invoke any central responses. The effective chemical stimuli comprised sea water extracts of Mytilus (see p.39), similar extracts of the visceral hump of Buccinum and neutral sea water solutions of eight synthetic chemicals.

The Mytilus extract was tested on whole Buccinum in the

tanks and proved sufficiently attractive to cause the proboscis extension response which is typical of the observed feeding behaviour of this species. This extract was therefore used as a test stimulus in all the following electrophysiological experiments, in order that, what may be considered as "a normal food response" might be compared with the effects of the various synthetic chemicals.

Typical records of the nervous responses to the tactile and 'extract' stimuli described above are shown in Fig. 50, though in this case a single unit is clearly visible, a situation not encountered in the majority of the recordings made with indium microelectrodes. The most striking difference between the two responses is their time course. Fig. 51 shows time/frequency graphs of responses shown in Fig. 50 with which many of the following results may be compared. The response to touch was short and did not outlast the active application of the stimulus to any great extent. The 'Mytilus response' on the other hand had a long time course, often in excess of 30 seconds, but adapted eventually even when the stimulus remained in contact with the osphradium. Both these responses can be compared with a control application of sea water which elicited no response and shows that water currents over the osphradium are not a natural stimulus to osphradial receptors. Jets of water of sufficient force to distort the osphradium could however elicit a brief response, but, by exercising some care during 'washing' these responses could be eliminated.

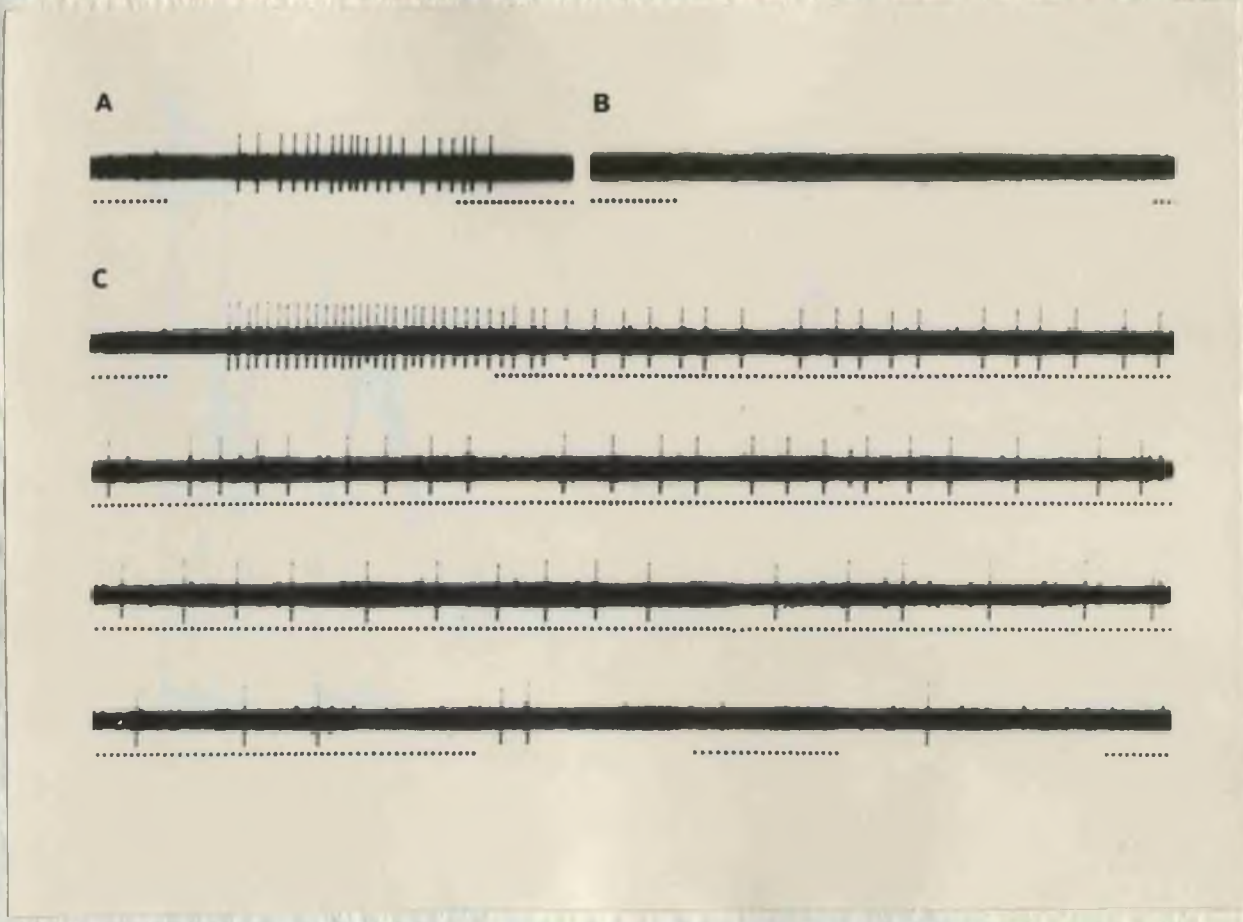



Figure 50. Responses of a central neurone in the supra-intestinal ganglion. A, response to touching osphradium; B, control application of sea water to the osphradium showing absence of response to water flow; C, prolonged response to '*Mytilus* extract' applied to the osphradium, the traces are consecutive. The time marker frequency is 10/sec. The interruptions in the time marker indicate the duration of the stimulus in A and B. In C the same feature at the beginning represents the active application of the extract, whilst the gaps at the end of the response indicate washing the osphradium with sea water.

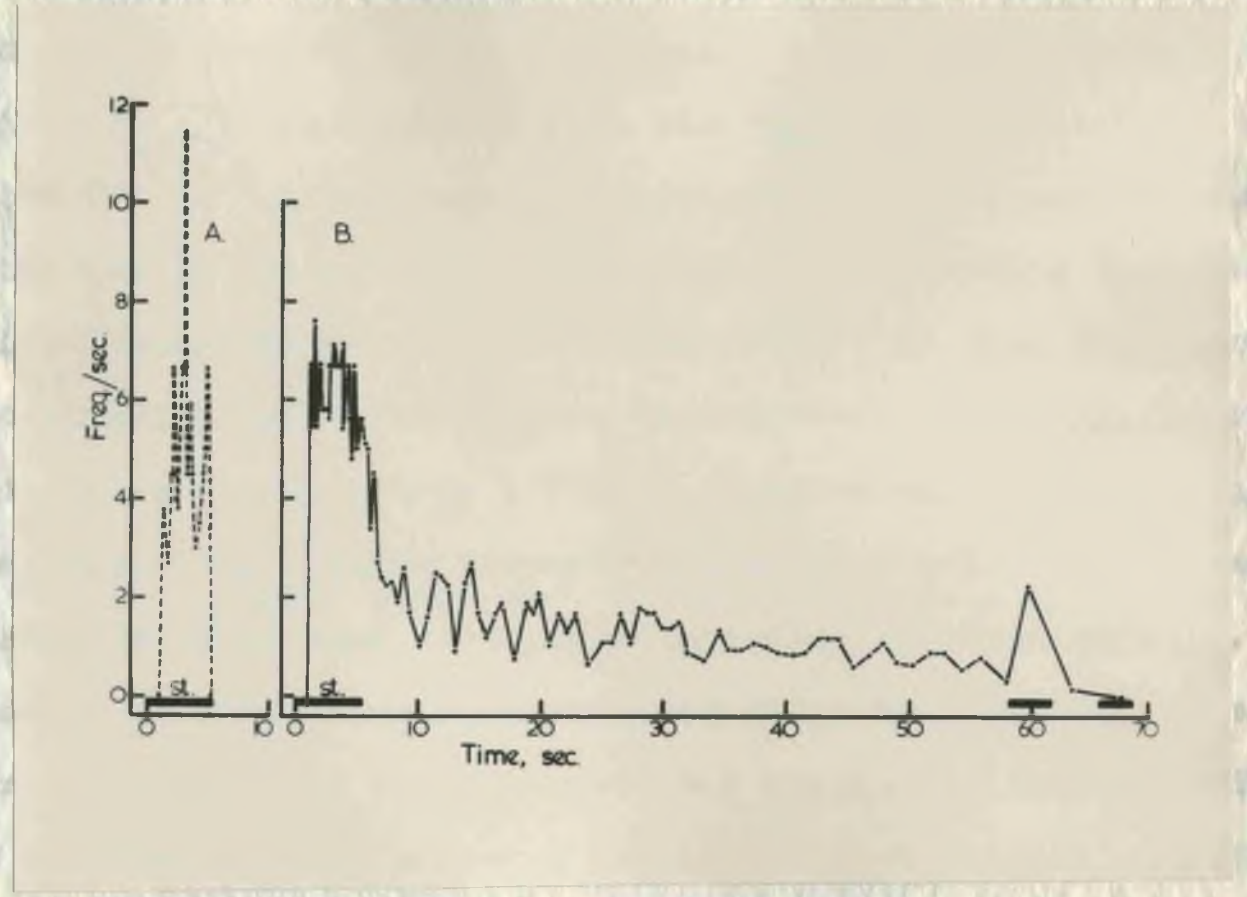



Figure 51. Graphs of the activity of the single unit illustrated in Fig. 50. The points are plotted in the same manner as in Fig. 36. A, response to touching the osphradium; B, response to '*Mytilus* extract'.

The extended time course of the '*Mytilus* response' was confirmed by intracellular recording from central neurone cell bodies in the supraintestinal ganglion. Fig. 52 is a typical result obtained by this technique and shows the general depolarisation of the cell which initiates a train of impulses. In both this and the previous case the '*Mytilus* response' was elicited from a 'silent cell'. Frequently however, spontaneously active cells produced similar results by increasing their impulse frequencies. Intracellular recordings from the supraintestinal ganglion cells also demonstrated that '*Mytilus* extract' could have an inhibitory effect on spontaneously active neurones (see Fig. 66). Touch responses in central cells also showed variation since some cells were inhibited by these stimuli. An example of this type of response is shown in Fig. 66. The various effects of different stimuli applied to the osphradium on the same central neurone, and of the same stimuli on different cells are, however, dealt with in more detail in a later section.

Nervous activity similar to the '*Mytilus* response' could be elicited from central neurones by stimulation using sea water extracts of the visceral hump of both male and female *Buccinum* (see Fig. 53 A and B). No difference between the responses to extracts from male and female was discernable, suggesting that the osphradium is not sensitive to any male or female 'scents' which might conceivably be produced in this region.

The origin of the afferent activity leading to the observed

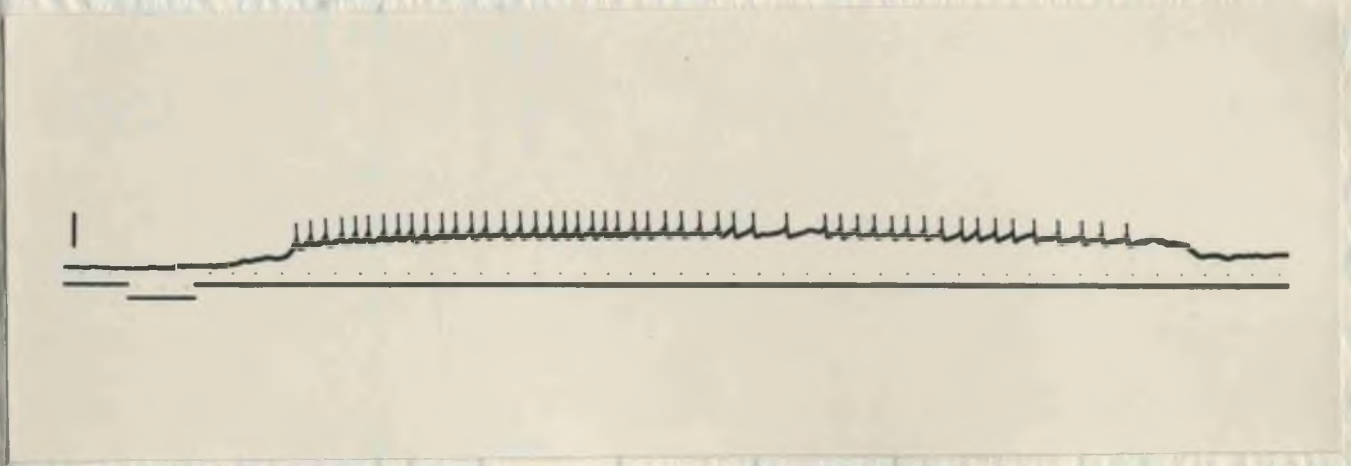


Figure 52. Intracellular recording of the '*Mytilus* extract' response from a single supra-intestinal ganglion neurone. The depression in the lower beam indicates the active application of the stimulus. Time marker frequency 1/sec. The response was allowed to adapt out naturally. Resting potential 35 mv; vertical bar represents 20mv.

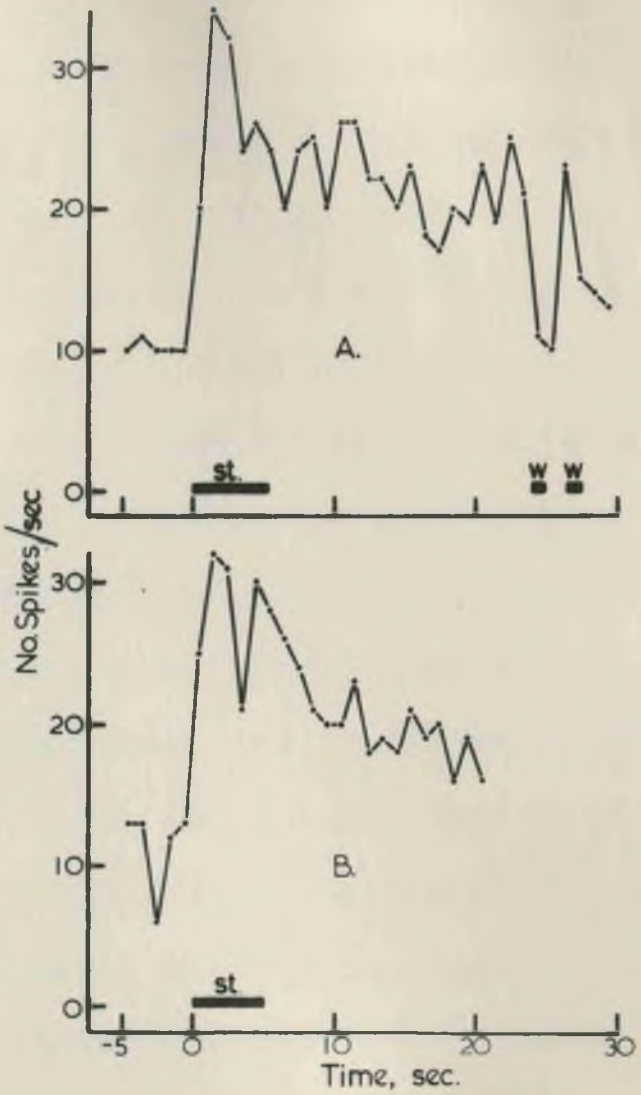



Figure 53. Graphs of multi-unit responses from supra-intestinal ganglion neurones elicited by extracts of the visceral hump of Buccinum placed on the osphradium. Total number of impulses for each second are plotted against time and the stimuli and washing indicated by the solid bars at the beginning and end of the graph respectively. A, response to 'visceral hump extract' of the same sex as the experimental animal; B, response to similar extract of the opposite sex (female).

central responses to touch and the various extracts appears to differ. In several experiments the osphradium was carefully removed from the mantle after the various stimuli had been applied and the results recorded. The stimuli were then re-applied and whilst the responses to extracts and synthetic chemicals were abolished, the response to touch was generally unchanged. It would appear therefore that the majority of the mechanoreceptors are situated in the mantle musculature underlying the osphradium. Because of their position it is likely that they comprise the same types of receptor as have already been described electrophysiologically in an earlier section of the results (see p. 78). The central responses are therefore of interest in that they may well represent the central expression of afferent activity which has already been studied in some detail. The central responses were not divisible into three types corresponding to the three types of afferent activity but on the other hand it is possible that one or other of the types of sensory input is responsible for the inhibitory influence on some of the central neurones.

(ii) Responses to synthetic chemicals.

Neurones in the supraintestinal ganglion were found to respond to stimuli comprising neutral sea water solutions of the following chemicals:-

$10^{-3}$M l-glutamic acid

$10^{-3}$M l-aspartic acid

$10^{-3}$M adipic acid

$10^{-2}$M glutaric acid

$10^{-2}$M succinic acid

$10^{-2}$M malonic acid

$10^{-2}$M betaine hydrochloride

$10^{-2}$M trimethylamine oxide hydrochloride

Unconfirmed, weak responses where also obtained using the following chemicals:-

$10^{-3}$M l-glutamine

saturated solution of gluthathione.

Each type of response, excepting the last two, was confirmed several times in each preparation in which it was located and proved to be repeatable in different preparations. Single unit preparations of these responses were not obtained in the experiments where indium microelectrodes were used. Because of this poor localisation, the large number of units responding, and the inherent variation of the size of the impulses from single units it was found impossible to plot time/frequency graphs of the responses of single neurones to the above stimuli. The graphs of the nervous activity which are included below are therefore graphs of the total nervous activity involved in each response. The thresholds of the various stimuli were also difficult to determine accurately, mainly because of the lengthy experimental procedure. A central response, once found, had to be subjected to at least thirty consecutive stimuli if strictly comparable results and threshold values were to be obtained. This large number of stimuli, together with the time taken up by washing stimuli away from the osphradium and allowing time for recovery after

each stimulus, led to a total experimental time of at least two hours. During this time the electrode had to remain in the same position within the ganglion. Such prolonged conditions of stability were seldom achieved due to the movements of the animal which might arise for a variety of reasons. On two occasions, however, virtually complete sets of data were obtained and on the basis of these, and the confirmations obtained from the many other, more fragmentary results, it is possible to give a reasonably precise account of the various excitatory responses of the supraintestinal ganglion cells.

The glutamic acid response.

Figs. 54 and 55 show central responses to $10^{-3}$M l-glutamic acid placed on the osphradium, compared with the 'Mytilus extract' responses from the same preparations. Both the pattern and the time course of the responses are very similar in both cases. The glutamic acid response closely mimics that to 'Mytilus extract' and would appear to be its synthetic equivalent for all intents and purposes. The threshold for the central response to glutamic acid is approximately $5 \times 10^{-5}$M.

The aspartic acid response.

This type of response is shown in Fig. 56 where a $10^{-3}$M solution was used. The time course of the response is similar to that for 'Mytilus extract' but the build up to a lower maximal frequency is slower. The threshold for the response would appear to be even lower than that for glutamic acid however, as a $10^{-6}$M solution produced a just visible increase in activity, whilst a

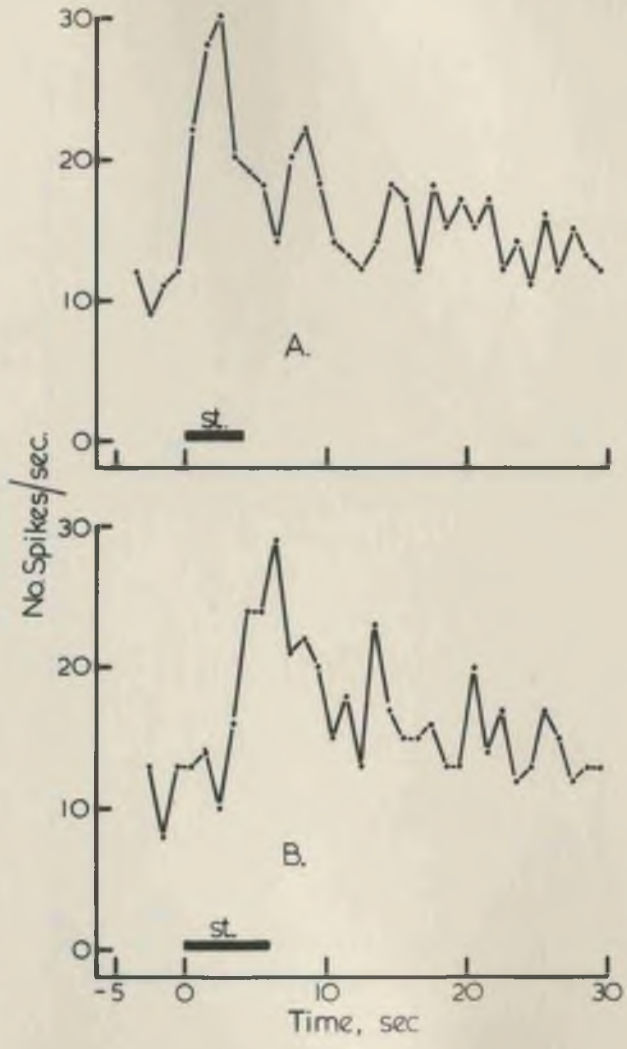



Figure 54. Graphs of multi-unit responses from supra-intestinal ganglion neurones elicited by A, 'Mytilus extract' and B, glutamic acid. A and B are taken from the different responses of the same units. The graphs are plotted as in Fig. 53.

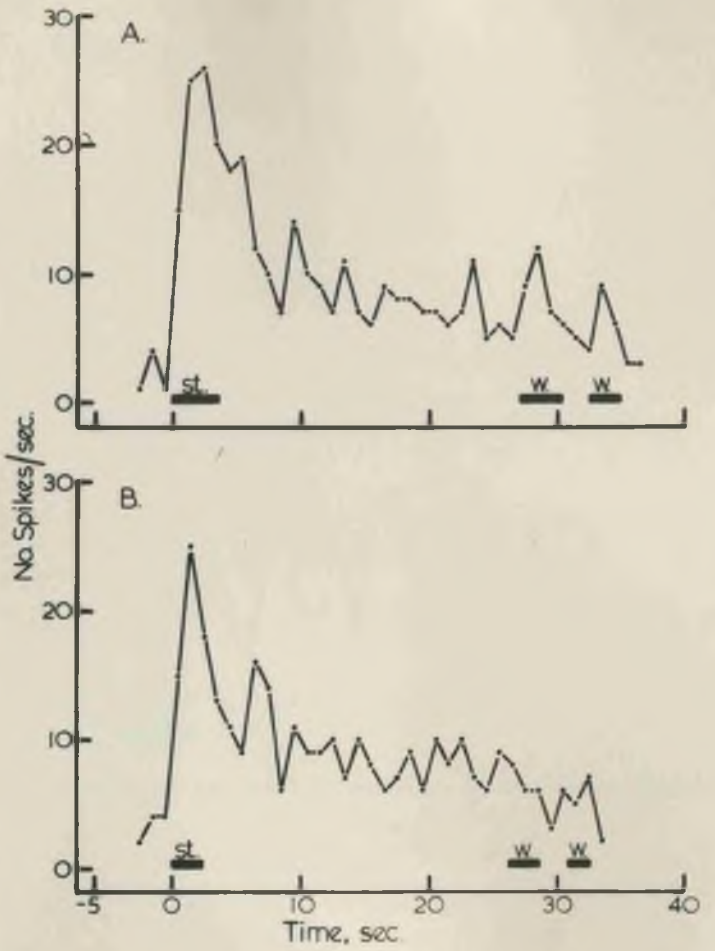



Figure 55. Graphs as for Fig. 54, but from a different preparation. A, '*Mytilus* extract response'; B, glutamic acid response.

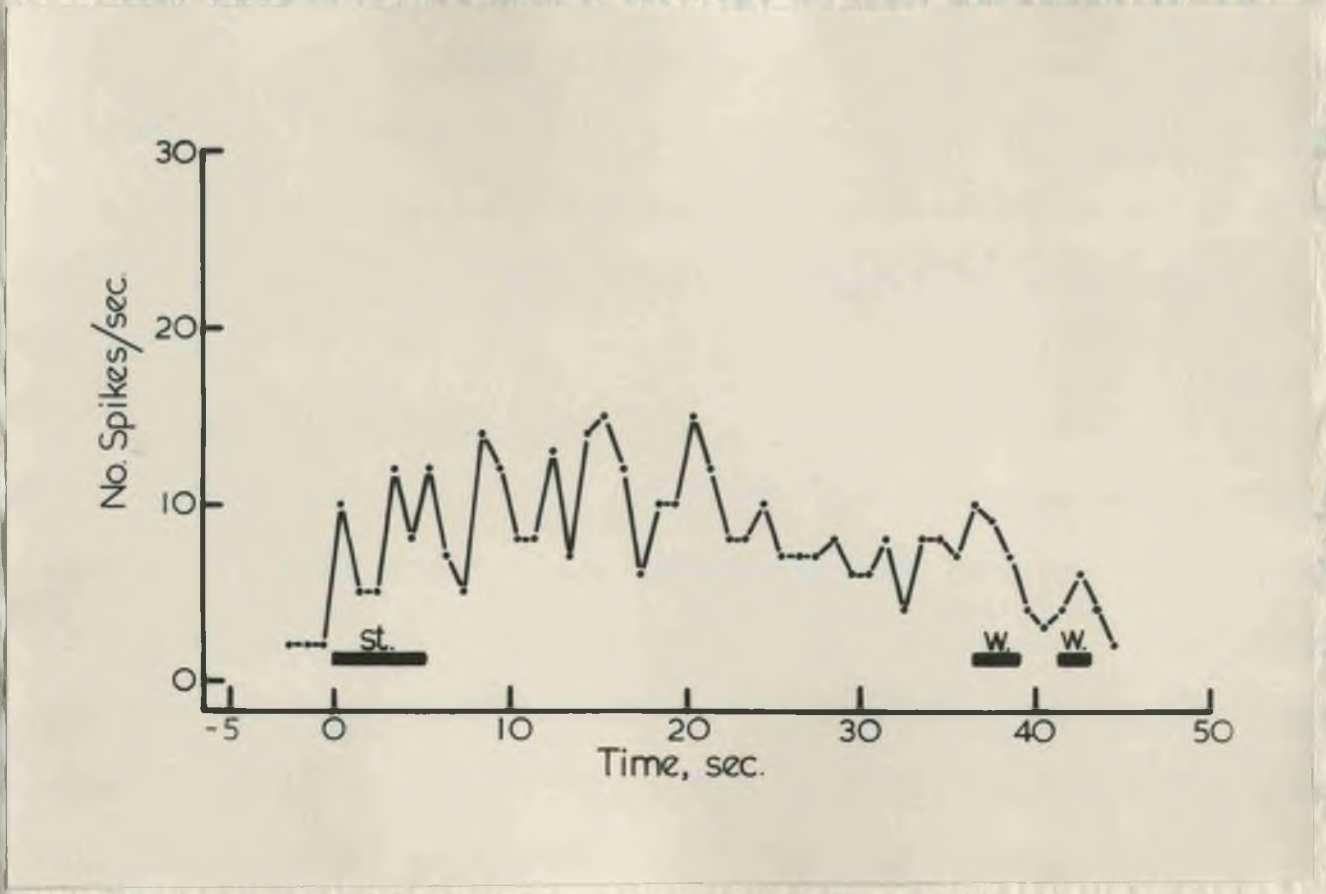



Figure 56. Graph of 'Aspartic acid response'. Preparation as in Fig. 55; method of plotting as in Fig. 53.

$10^{-7}$M stimulus did not. The threshold would appear therefore to be approximately $10^{-6}$M.

The adipic acid response.

The response as shown in Fig. 57 was elicited by $10^{-3}$M solutions. The maximal frequency of the response is lower and achieved more slowly than in the response to '*Mytilus* extract'. The adipic acid response is also of a noticeably shorter duration. The threshold for the adipic acid response is approximately $5 \times 10^{-4}$M.

The glutaric acid response.

This response differs somewhat from the comparable '*Mytilus* response', being shorter and having a very much higher threshold concentration. The maximal frequency achieved is also lower than for the '*Mytilus* response'. The concentration of the stimulating solution was $10^{-2}$M in this case because a $10^{-3}$M concentration proved to be subthreshold for the central response. This response is illustrated in Fig. 58.

The succinic acid response.

This response, illustrated in Fig. 59 and elicited by $10^{-2}$M solution also had the properties of being of lower maximal frequency, achieving the same at a slower rate and lasting for a shorter period than the equivalent '*Mytilus* response'. The threshold concentration was also higher, being greater than $10^{-3}$M.

The malonic acid response.

A $10^{-2}$M solution of malonic acid was an effective stimulus in the case shown in Fig. 60, but was ineffective in two other experiments. In the response illustrated a lower maximal fre-

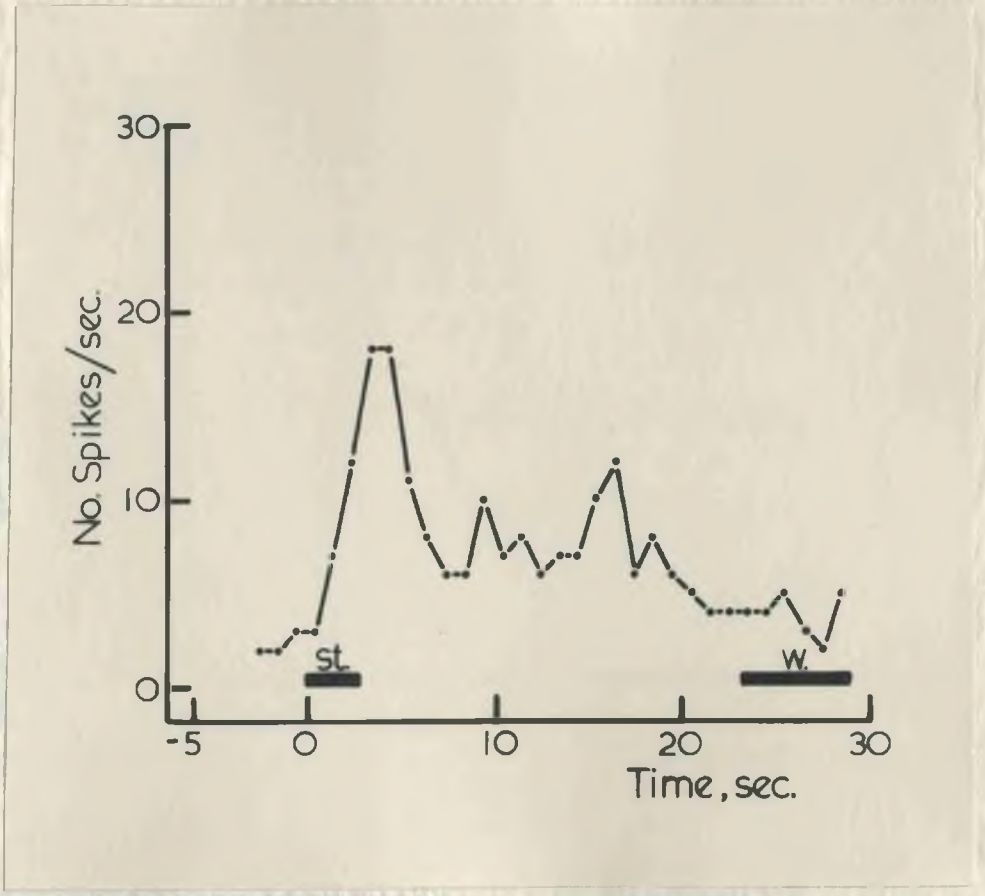



Figure 57. Graph of 'Adipic acid response'. Preparation as in Fig. 55: method of plotting as in Fig. 53.

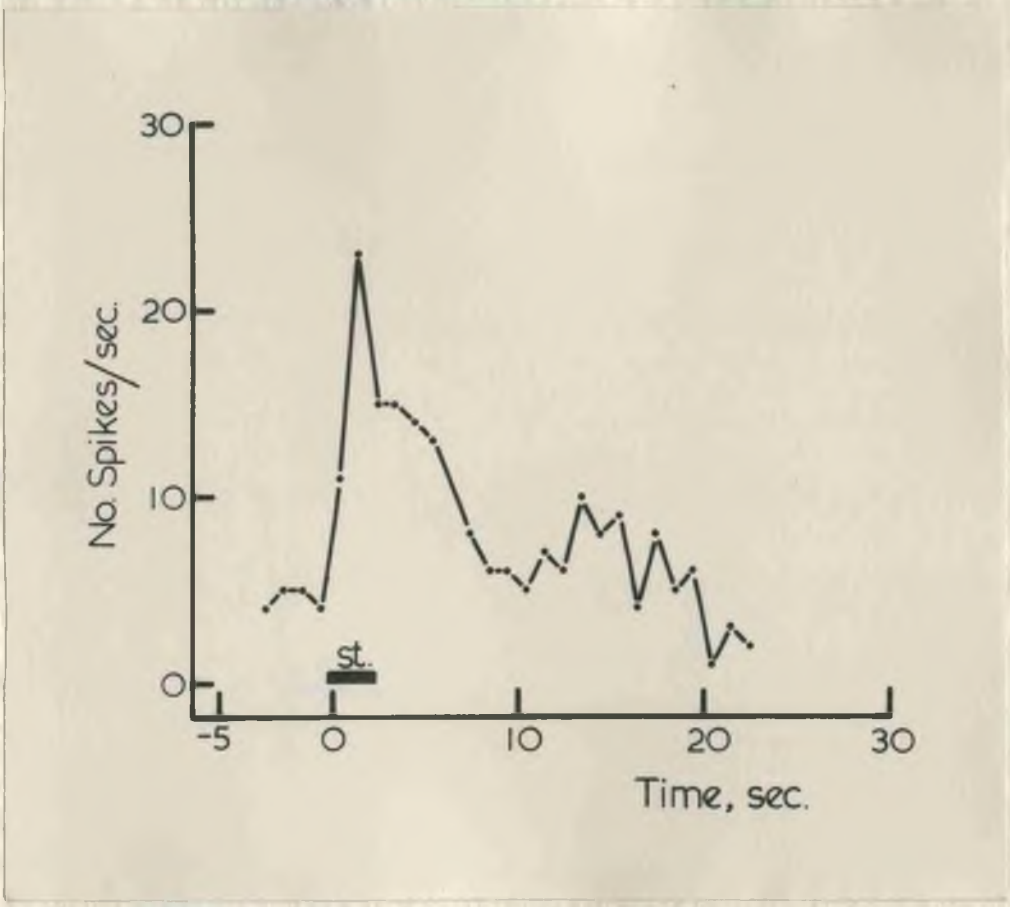



Figure 58. Graph of 'Glutaric acid response'. Preparation as in Fig. 55; method of plotting as in Fig. 53.

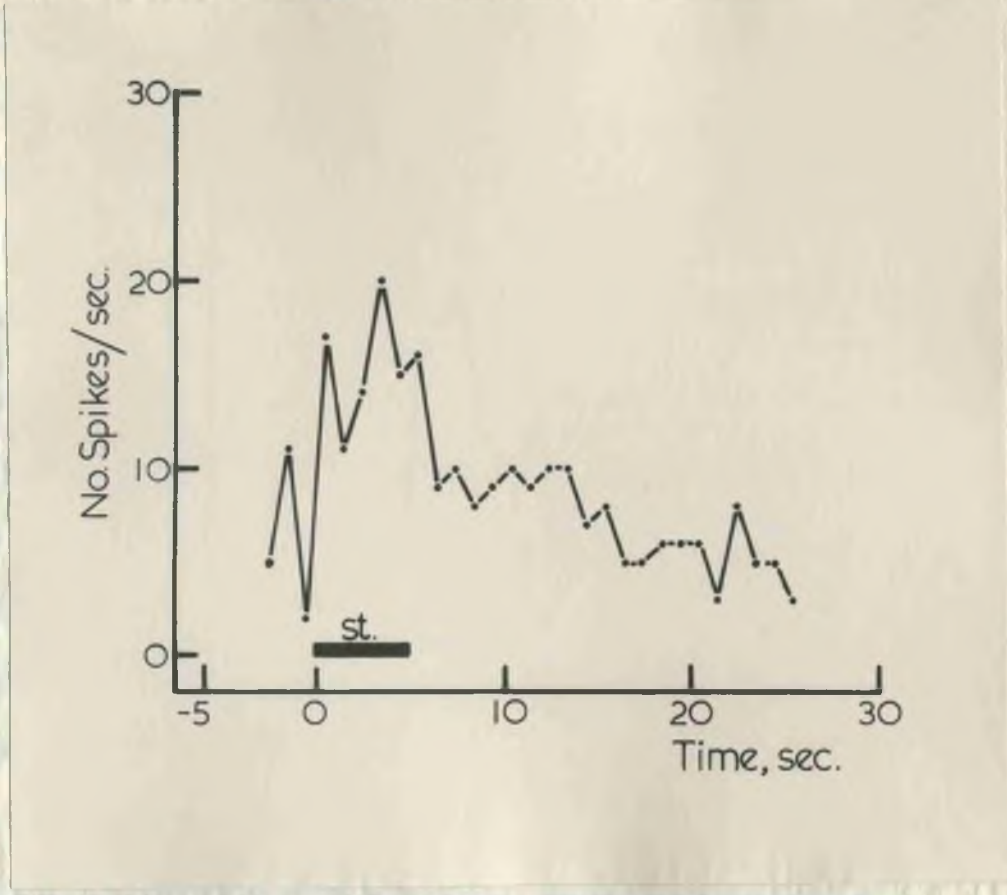



Figure 59. Graph of 'Succinic acid response'. Preparation as in Fig. 55; method of plotting as in Fig. 53.

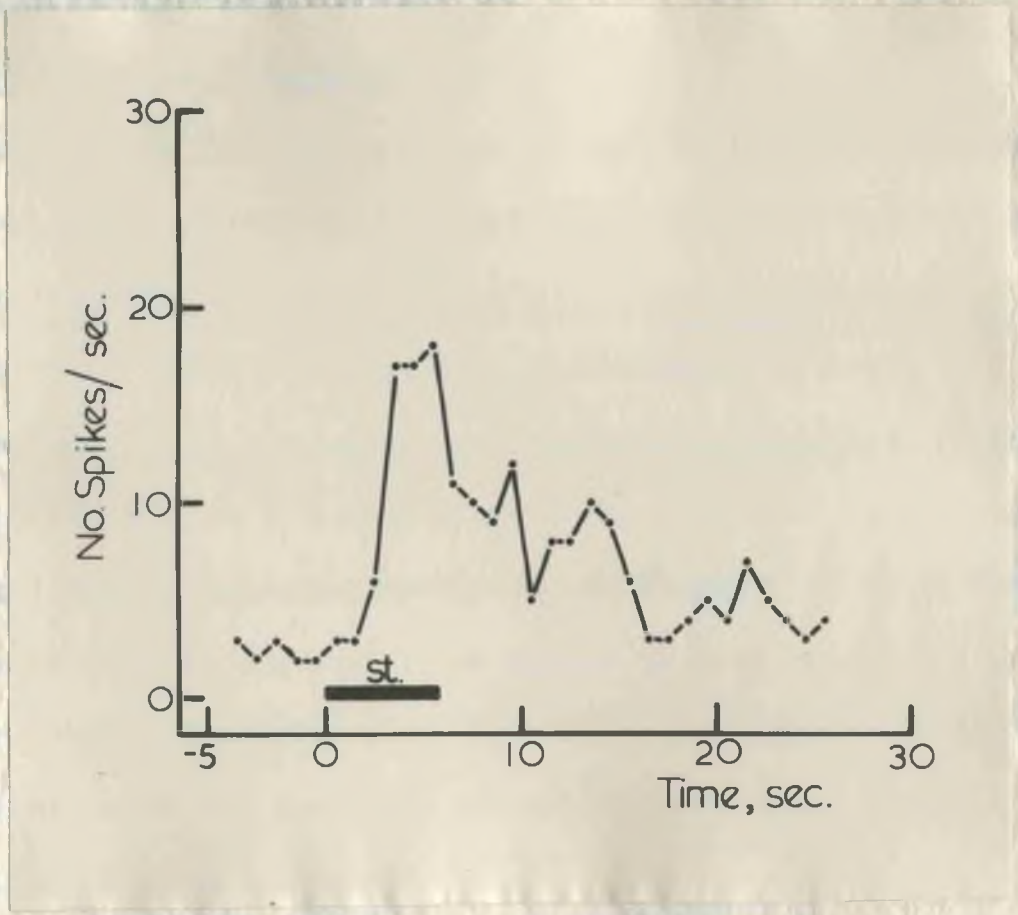



Figure 60. Graph of 'Malonic acid response'. Preparation as in Fig. 55; method of plotting as in Fig. 53.

quency was achieved at a slower rate of rise than the comparable '*Mytilus* response'. The threshold concentration for this single response was again high compared to that for glutamic acid since it lies at approximately $10^{-2}$M.

The betaine response.

Central nervous responses to $10^{-3}$M betaine hydrochloride in sea water were observed in four experiments, but the same solutions proved to be non stimulatory in an equal number of cases where central responses to other chemical stimuli had been seen. When present, the response was generally similar to that for '*Mytilus* extract' or glutamic acid, though the peak frequency was lower and the time course somewhat shorter. Fig. 61 shows one example of a betaine response where a single unit was recorded. The threshold concentration for betaine was below $10^{-3}$M, the concentration used in the experiment illustrated in Fig. 61 but the lower limits were not defined.

The trimethylamine oxide response.

The central responses to $10^{-2}$M T.M.O. were very weak in several cases but in others produced a result very similar to that of betaine. The response was more consistent in its occurrence than that for betaine and only on one occasion was there no visible response in a preparation which responded to '*Mytilus* extract' and glutamic acid. The T.M.O. threshold concentration was between $10^{-2}$ and $10^{-3}$ Molar.

The l-glutamine and glutathione responses.

A solution of $10^{-3}$M l-glutamine proved an effective stimulus

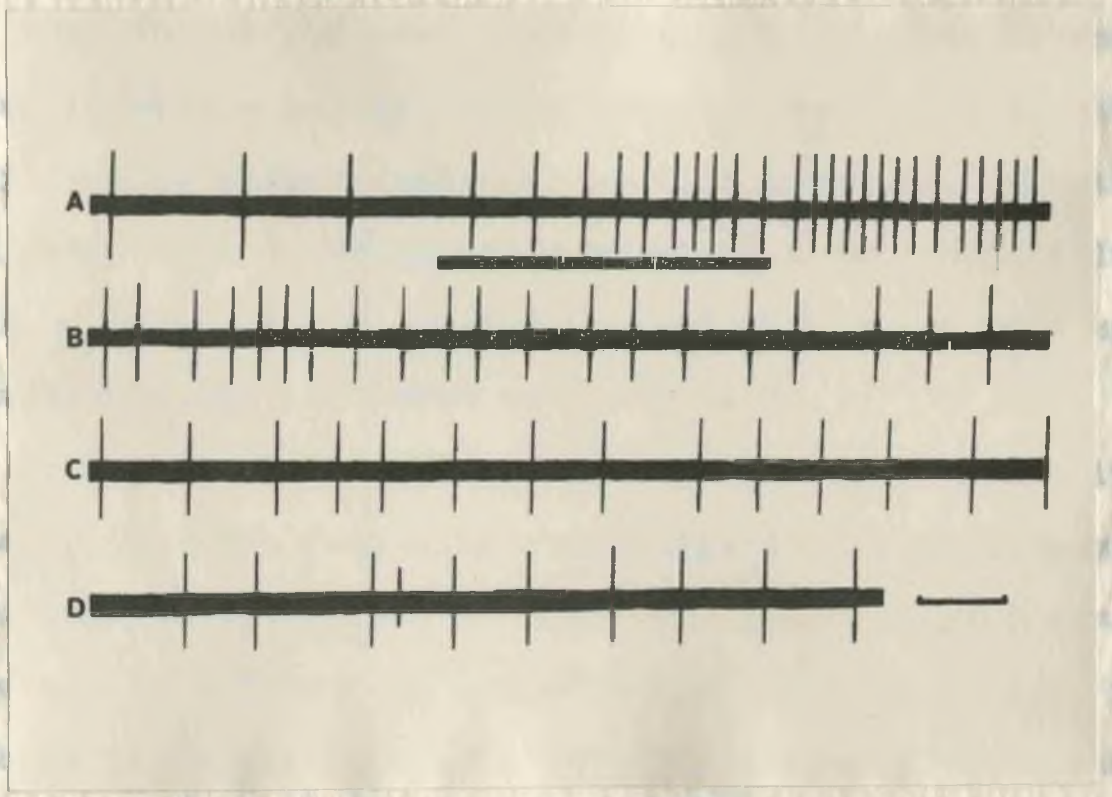



Figure 61. Response of a single supra-intestinal ganglion neurone to a betaine stimulus applied to the osphradium. The bar in A indicates the application of the stimulus. Time mark represents 1 sec.

on only one occasion, in spite of frequent trials in the course of experiments upon preparations which responded to other chemical stimuli. It would therefore appear to be a much poorer stimulus than the other chemicals which gave the results described above.

Glutathione was used only once as a stimulus since it was almost insoluble in sea water, though a saturated solution did elicit a weak central response on this occasion. Because of the weak response and the unlikely event of a saturated solution occuring in the normal environment of the animal this substance was subsequently discarded as a stimulus.

From the results illustrated and described above it is possible to draw the following conclusions:- (1) Chemoreception is undoubtedly the main sensory function of the osphradium. (2) The chemoreceptive functions are of a food finding nature, the 'normal' response being elicited by a '*Mytilus* extract' which was an effective feeding stimulus in the whole animal. (3) The synthetic chemicals which mimic the '*Mytilus* response' most closely are l-glutamic and l-aspartic acids, which are effective at threshold values compatible with normal environmental stimulation. (4) The dicarboxylic nature of the effective amino acids appears to play an important part in the stimulatory process.

The stimuli employed in testing osphradial receptor modality and the results obtained are summarised in diagrammatic form in Fig. 62.

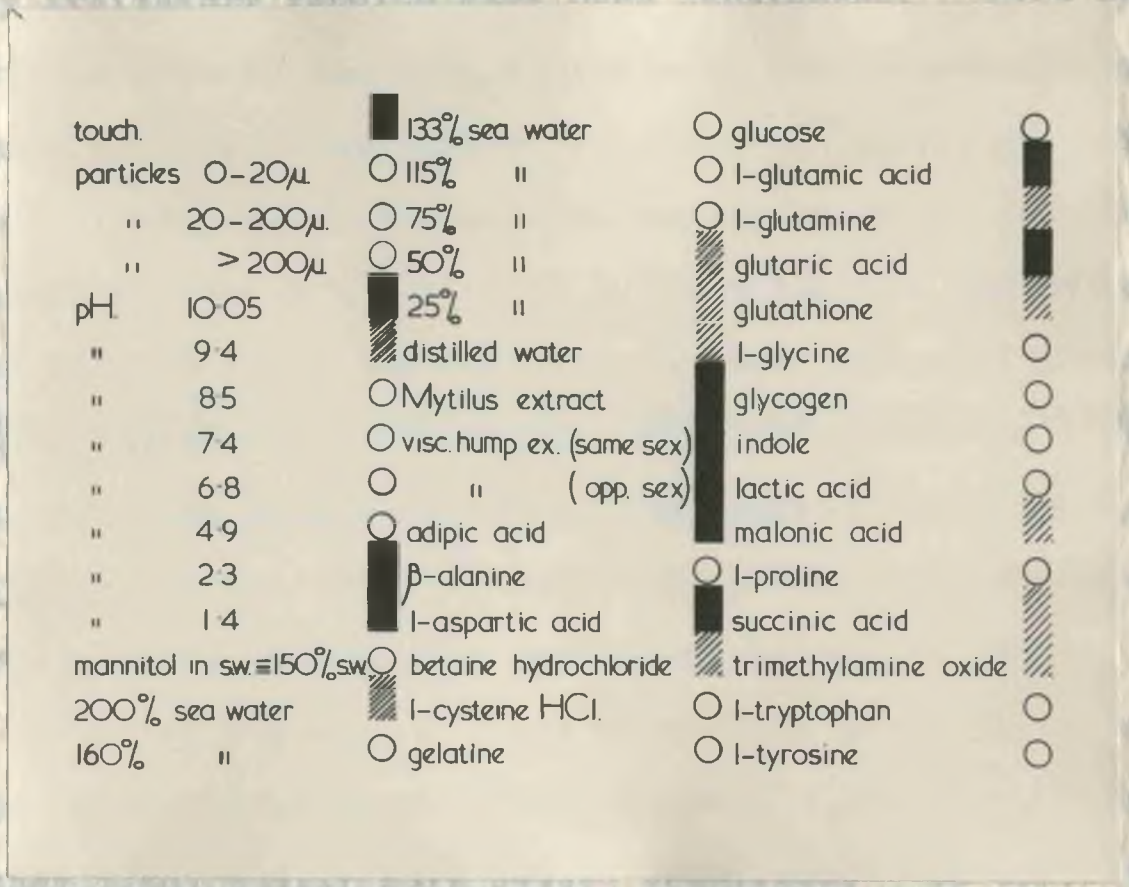



Figure 62. Diagram summarising the effects upon the central neurones of the various stimuli applied to the osphradium. Solid squares indicate an effective stimulus; hatched squares, a partially effective stimulus i.e. producing a response only on some occasions or producing a response only at high or extreme concentrations; O, indicates a totally ineffective stimulus.

The interaction of afferent pathways on neurones in the supra-intestinal ganglion.

In the previous section of the results dealing with the responses of central neurones to stimulation of the osphradium, only excitatory results have been considered. This type of result was seen in the vast majority of the experiments using indium microelectrodes, but a few cases of inhibitory effects were noted. Fig. 63 shows one such response, in which periodic spontaneous bursts were inhibited by touching the osphradium (see also Fig. 49 B). The inhibition, which affected several units, lasted for a considerable time after the cessation of the stimulus. It was also observed on one occasion that touch and '*Mytilus* extract' stimuli had respectively excitatory and inhibitory effects on the same spontaneously active units. Because of the large numbers of units which were generally recorded in this type of experiment a few neurones showing inhibitory responses might easily have passed unnoticed. In order to overcome the inherent difficulties of the indium electrodes used, i.e. poor single unit isolation, further experiments of a similar type were carried out using intracellular microelectrodes. This enabled the various responses of single central neurones to be studied in greater detail.

Using the technique described on pps. 31-33 it was quickly found that one cell could often show different types of response to osphradial stimulation by different modalities of stimulus, and that the same stimuli could produce opposite results in

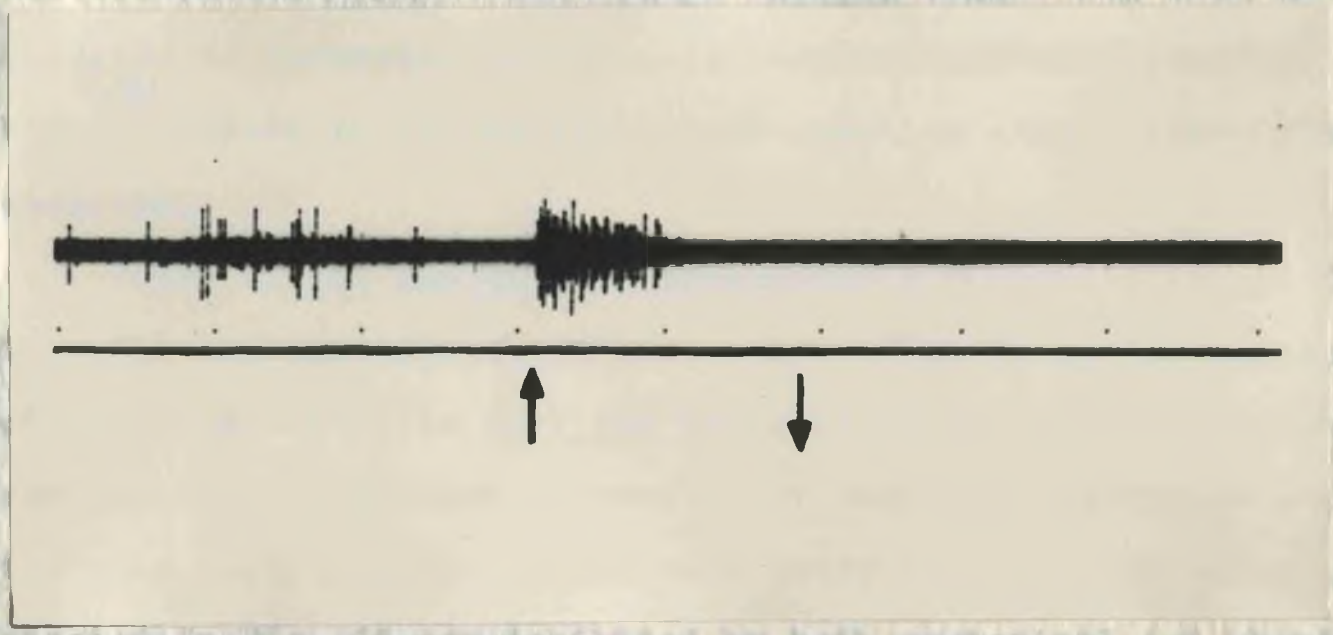


Figure 63. Record showing the inhibition of the spontaneous bursts of activity, shown in Fig. 49 B., occasioned by touching the osphradium. Arrows indicate the onset and end of stimulation. The inhibition lasted for between 20 and 30 seconds after the termination of the stimulation. Time marker frequency is 1/sec.

different central neurones. Fig. 64 is a diagrammatic representation of the various types of central neurone which can exist in respect of mechanical and chemical stimuli. The diagram also shows those types of neurones which have been observed in the course of the limited number of experiments which have been carried out using intracellular electrodes. Thus six of the eleven cell types have been observed, suggesting that the experiments involving indium electrodes presented a somewhat biased picture since the inhibitory reaction passed largely unobserved.

Figs. 65, 66 and 67 show photographic records obtained from three of the six cell types observed. Fig. 65 is the response of a 'silent' cell to '*Mytilus* extract' stimulation of the osphradium. This cell was unaffected by mechanical stimulation of the same region. The spontaneous activity of the neurone illustrated in Fig. 66 was inhibited by both mechanical and chemical stimulation of the osphradium, whilst that shown in Fig. 67 was affected in opposite ways by the same two stimuli i.e. inhibited by mechanical and excited by chemical stimulation.

The reactions of the central neurones of the supraintestinal ganglion to osphradial stimulation are thus diverse and complex since the majority of the possible responses to the two forms of stimulation have been shown to occur in the course of a few experiments. Thus groups of similar afferent codes from both the mechanoreceptors and chemoreceptors in the mantle region undergo considerable integration in the supra-intestinal ganglion. The

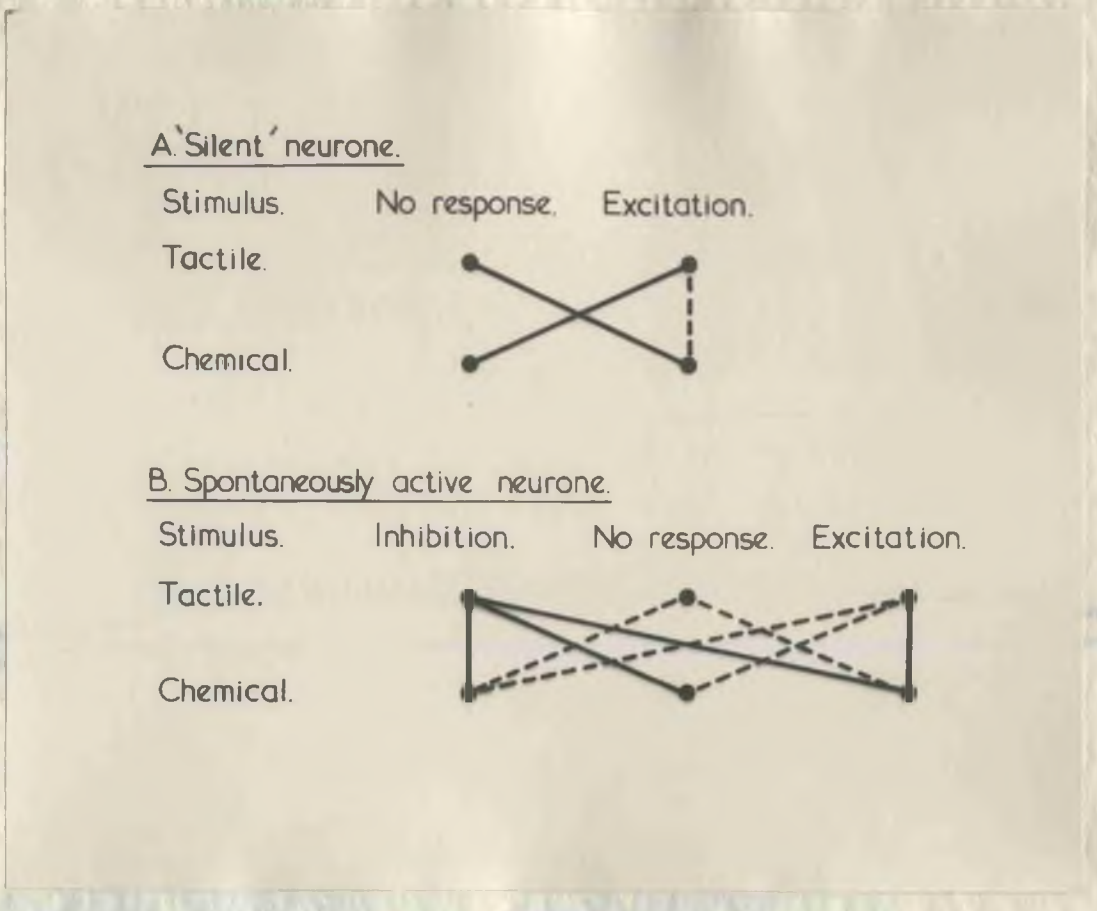



Figure 64. Diagrams to show predicted types of central neurone in respect of their response patterns to tactile and chemical stimulation of the osphradium. A, possible responses from an initially silent neurone; B, possible responses from an initially spontaneously active neurone. The solid lines represent cell types found experimentally and the broken lines those which were predicted but not demonstrated.

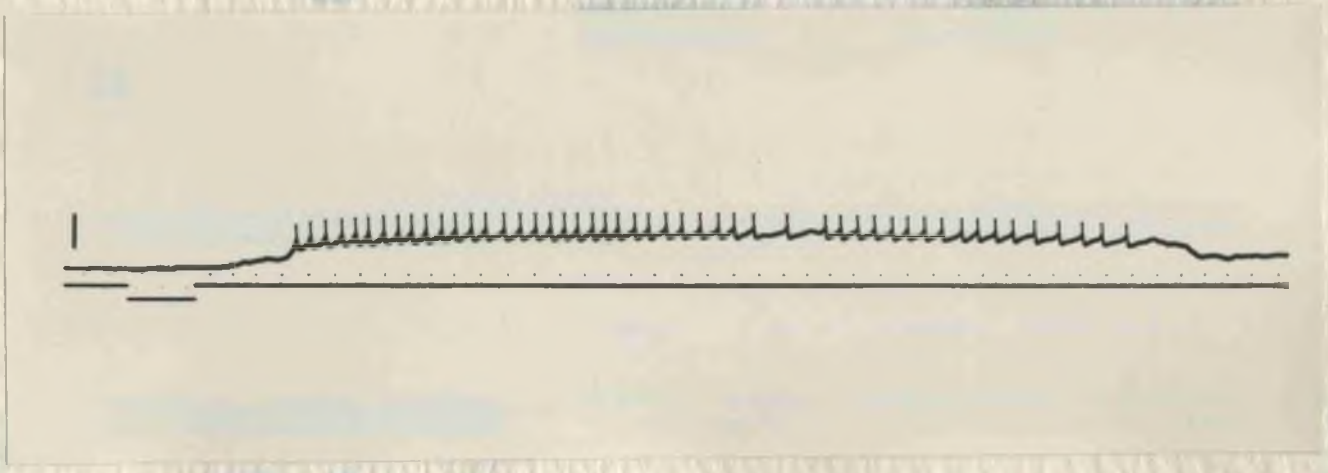


Figure 65. Repeat of Fig. 52. This neurone, initially silent, responded to '*Mytilus* extract' but not to tactile stimulation of the osphradium. Resting potential, 35mv; vertical bar represents 20mv. The depression of the lower beam indicates the active application of the stimulus. Time marker frequency is 1/sec.

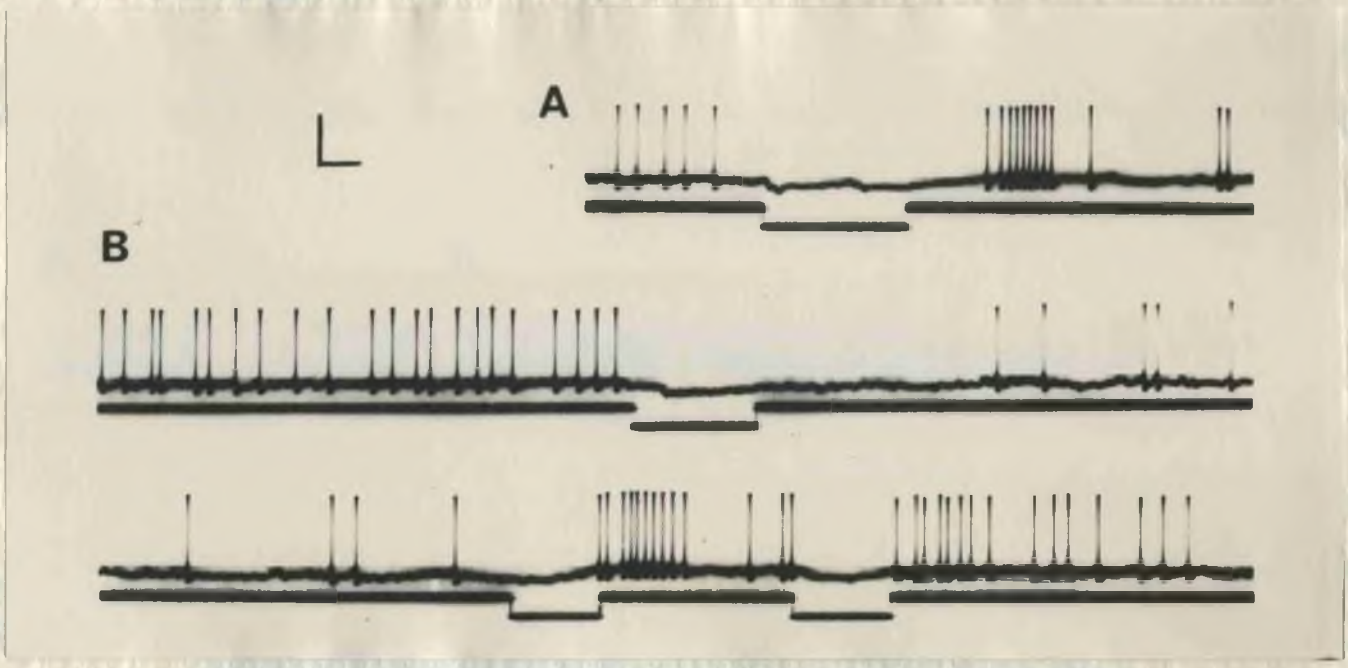



Figure 66. Intracellular recording from supra-intestinal ganglion cell whose spontaneous activity was inhibited by both tactile (A) and chemical (B, '*Mytilus* extract') stimulation of the osphradium. The vertical bar represents 20mv and the horizontal bar 1 second. The two parts of the trace in B are consecutive. Resting potential, 50mv. The lower beam depressions represent the duration of the stimulus in A, and both stimulus (first) and washing (second and third) in B.

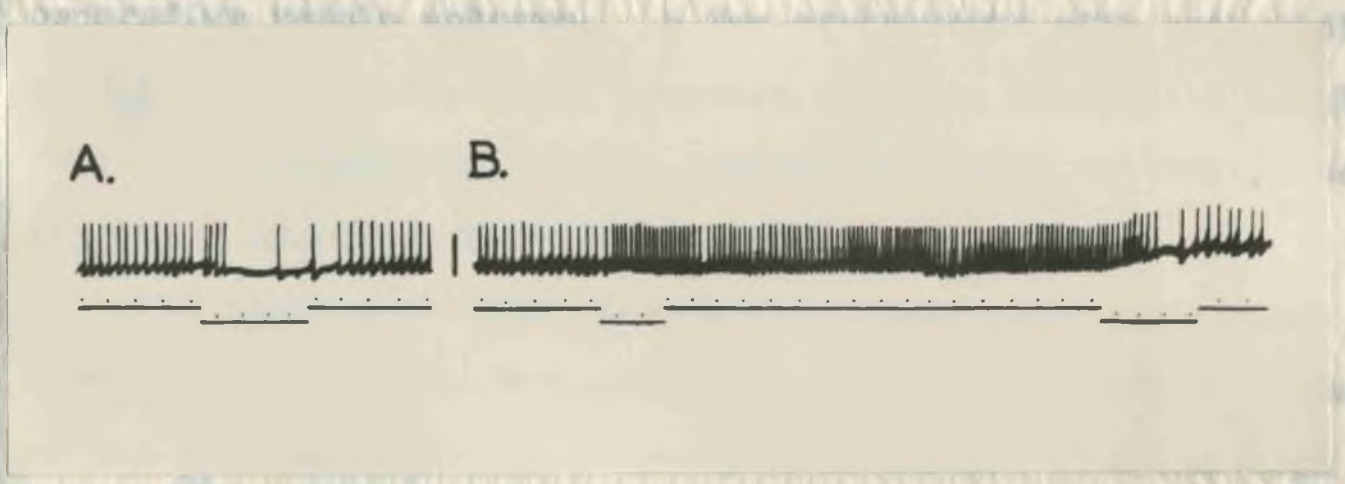



Figure 67. Intracellular recording of from a spontaneously active supra-intestinal ganglion cell. A, shows the inhibition caused by tactile stimulation of the osphradium; B, shows the excitation caused by '*Mytilus* extract' applied to the same organ. Resting potential, 30mv. Lower beam depressions, as in Fig. 66; vertical bar represents 10mv; time marker frequency is 1/sec.

activity of the mechanoreceptors, which is known to be mainly of an excitatory nature (see pps. 78-88), is transformed into both excitatory and inhibitory central codes. However, without a far greater number of results on this topic, it is impossible to show whether there is any relationship between any one type of peripheral mechanoreceptor and a particular type of central reaction.

The afferent impulse patterns of the chemoreceptors of the osphradium remain unknown. A few experiments with both indium and saline microelectrodes inserted into the osphradial ganglion failed to produce any visible nervous activity, so that these receptors could not be investigated.

The records obtained from a relatively small number of single cells has thus exposed a greater diversity in the nature of the central responses to peripheral stimuli than had been observed in experiments using the previously described techniques.

Branching axons in the central nervous system.

In the course of the intracellular recordings a number of the impaled neurones gave clear evidence for the branching of their axons. Fig. 68 shows three examples of such evidence. In A, the intracellular record of a spontaneously active unit is shown in which a number of spikes of differing amplitude can be seen. Thus several zones of the axonal membrane are active at the same time in the unstimulated preparation. The record shown in Fig. 68 B shows that two distinct propagated spike trains can also arise in one cell in the unstimulated preparation. Both

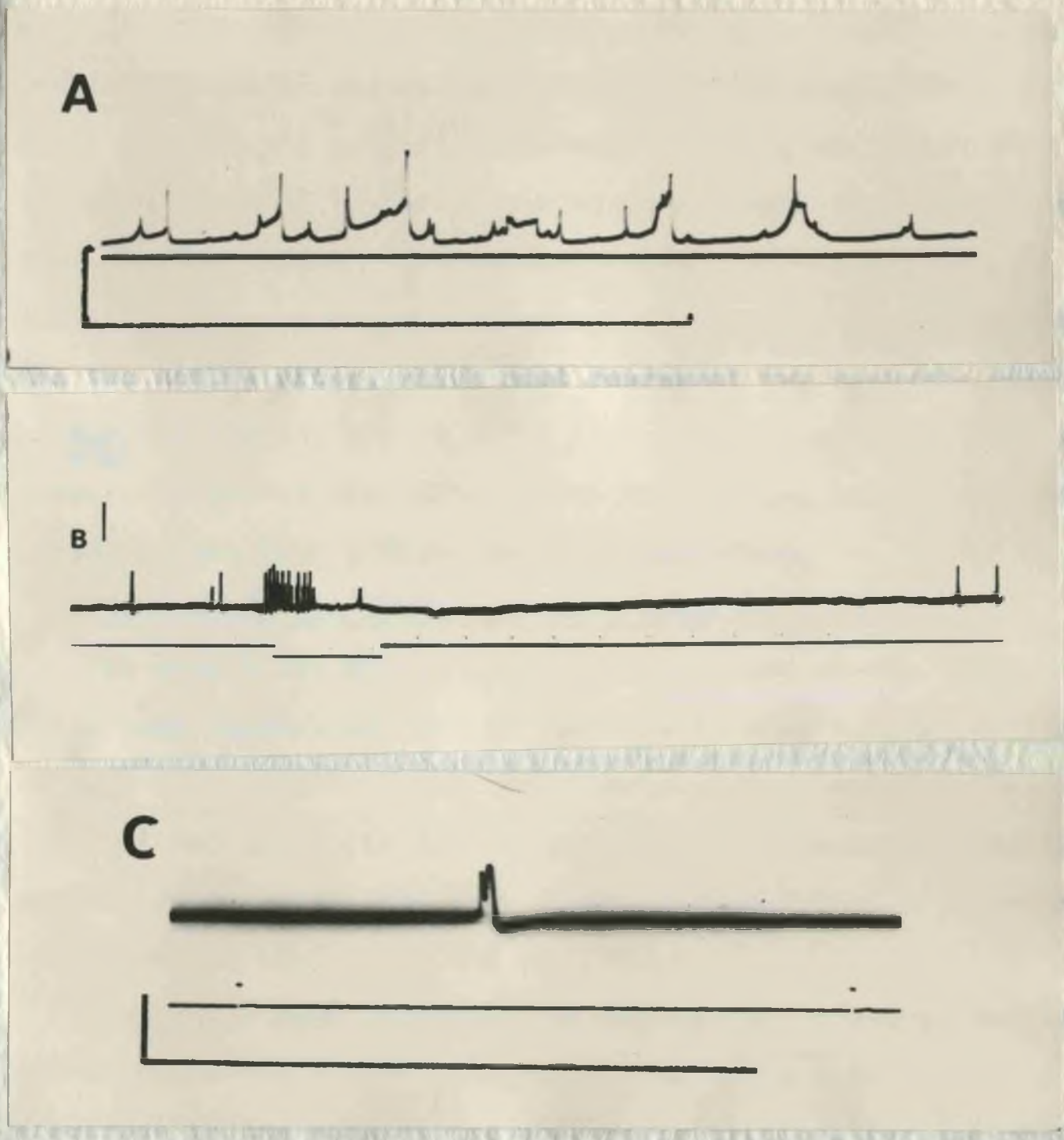



Figure 68. The interaction of concurrent activity in branched dendrites. A, the interaction of several active zones as recorded in the cell soma; resting potential, 60mv; vertical bars represents 50mv; horizontal bar represents 1 second. B, two distinct trains of impulses excited initially by tactile stimulation of the osphradium and then passing into post excitatory depression; resting potential, 15mv; vertical bar represents 5mv; time marker frequency is 1/sec. C, the double spike effect (see text); resting potential, 30mv; vertical bar represents 20mv; horizontal bar represents 1 second.

spikes are affected in a similar manner by the same stimulus which resulted in the excitation of both pathways. Fig. 68 C shows the 'double spike' phenomenon. The spike illustrated is one of a train of impulses arising in a single cell of an unstimulated preparation. The impulses were observed for several minutes during which time the shape of each remained constant. The two active sites, which must represent two separate axonal branches since two active sites in one branch would lead to a cancelling out of the spikes, were thus firing at the same time intervals and with a fixed delay between them.

Excitatory and Inhibitory post synaptic potentials.

In only a few of the preparations, where single ganglion cells were penetrated, was it possible to observe either IPSP's or EPSP's. Fig. 69 shows one example of each type of post synaptic potential. The EPSP's in B are spontaneous, but mainly sub-threshold whilst the IPSP's in A were elicited by a mechanical stimulus applied to the osphradium.

The infrequent occurrence of records of this type suggests either, that the actual position of the tip of the recording electrode in the soma of the neurone is critical for the observation of the post synaptic potentials, or, that in some cases only are the cell bodies invaded by such potentials, i.e. when the latter arise at synapses close to, or on, the soma.

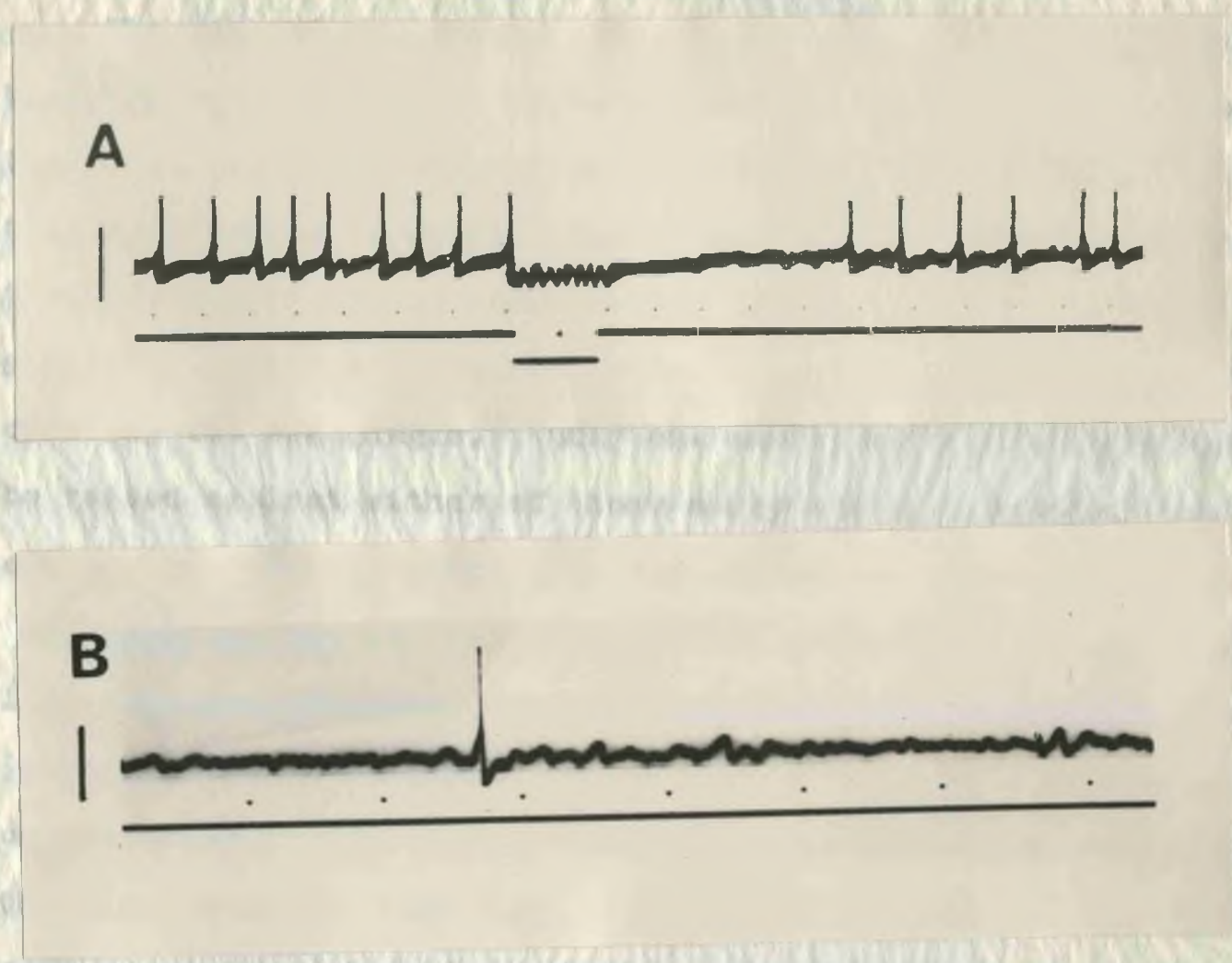



Figure 69. Records of post synaptic potentials from supra-intestinal ganglion neurones. A, IPSP's elicited in a spontaneously active neurone by tactile stimulus applied to the osphradium; resting potential, 50mv; vertical bar represents 40mv; time marker frequency is 1/sec; B, spontaneous EPSP's; resting potential, 50mv; vertical bar represents 20mv; time marker frequency is 1/second.

DISCUSSION

General anatomy of the nervous system.

The general anatomy of the nervous system of Buccinum has been described in detail by both Bouvier (1887) and Dakin (1912). The large number of dissections carried out in the course of this work served to confirm these descriptions with regard to the arrangement of the concentrated anterior central nervous ganglia i.e. those contained in the anterior body cavity and encircling the oesophagus. Only one small point of dissent can be raised against either of these early works. Bouvier described the cerebral ganglia as "equal in size but appreciably asymmetrical due to the fact that the posterior region of the left ganglion possesses narrow connections with the pallio-pedal (pleuro-pedal) connective, while nothing comparable is present on the right". The connections described are not included in the figures of the 1887 work, and in that by Dakin no mention of such connections is made. In the present work, neither dissection nor examination of serial sections of the nervous centres revealed any such connections. The assymetry of the cerebral ganglia is therefore in doubt, since it has not been observed by earlier workers on Buccinum such as Jhering (1877), by later workers such as Dakin (1912), by the reviewers Fretter & Graham (1962) or in the course of the present work.

Regarding the number of nerves which arise from any one ganglion, the early descriptions, cited above, leave something to be desired. Bouvier is specific in most cases as to the number,

distribution and sometimes even the branching of the nerves arising from a specified ganglion. With reference to the nerves of the left pallial (pleural) ganglion, he states that "there are four nerves to the body wall". Dakin is less specific and specifically describes only the more important nerves, mentioning groups or bundles of others. Over three hundred dissections, carried out as a necessary part of this work, have revealed that the number and branching patterns of nerves from particular ganglia vary to a considerable degree and in a completely unpredictable manner, a feature which neither of the above authors has mentioned.

Microanatomy of the CNS.

Studies of the general internal structure of the central ganglia are relatively few in the Gastropoda especially when compared to the much more extensive works upon the giant cells and axons. The Prosobranchs, lacking giant cells, have been particularly neglected in this respect and the only works on record appear to be those of Bernard (1888) on the Monotocardian Valvata piscinalis and Haller (1884, 1886) on the Diotocardians Turbo, Fisurella and Haliotis. These works are, however, sufficient to demonstrate that the structure of the ganglia of Prosobranchs is very similar to that of other Gastropods. The ganglia are thus composed of a peripheral layer of nerve cell bodies which surrounds the inner fibrous neuropile region.

The studies made upon Buccinum have confirmed this general pattern in respect of this species, as may be seen in Figs. 17

and 21. As in the other Prosobranch species examined no giant cells, such as those in Helix, are present in Buccinum. The range of ganglion cell body diameters is however quite large, ranging from less than 1μ to between 40 and 50μ or 60-70μ depending on whether the measurements are taken from fresh squash preparations or from slides prepared in the manner described on p.22. Bernard (1888) gives the corresponding size range in Valvata as 4-20μ, whilst Tauc (Bullock & Horridge, in press) gives the value of 90μ for the largest cells present in Haliotis. The cells are thus considerably smaller than the 'giants' of the pulmonates but occupy a similar range to the "small to large" cells contained within the ganglia of the latter (Bullock & Horridge, in press).

The pigment vacuoles seen within the nerve cell bodies are a common feature of the Gastropods. In Buccinum the colour is a deep yellow which appears to be similar to that in Fissurella (Haller, 1886). In general the pigment is largely contained within the soma of the cells and little or none is visible in the axons, thus conforming to the general pattern which appears to exist amongst the Gastropods.

The unipolar nature of the ganglion cell bodies of Buccinum is consistent with the other descriptions of similar cells in the Prosobranchiata. Haller (1886) illustrates nerve cell bodies whose stem processes apparently fuse and connect two cell bodies. No evidence of this sort of structure was found in the ganglia of Buccinum.

In recent years the more general study of Molluscan ganglion

structure has been extended by means of the electron microscope. The subjects of the research are scattered amongst the Molluscan classes and include Aplysia (Rosenbluth, 1963), Octopus and Eledone (Lilly, Gray & Yong, 1963), Unio (Fahrman, 1961) and Archachatina (Amoroso et al., 1964). Whilst none of this research deals with prosobranchs in particular certain features of interest emerge especially from the papers dealing with the gastropods Aplysia and Archachatina. One of these is the cytological distinction between large and small nerve cells i.e. those greater than 60μ and those less than 60μ. The former are deeply penetrated by the surrounding glial cells and have irregular nuclei, whilst the latter are not penetrated and have regular ovoid nuclei.

The ganglionic nerve cells of Buccinum, whilst verging on the apparently critical size of 60μ, have not been observed to show any 'giant' cell features in the course of the light microscopical investigations carried out. The nuclei observed were all of regular shape and the cell bodies never appeared to be penetrated by glia.

The second point of interest is the density of the cytoplasm and the low chromatin content of the nuclei contained in the cell bodies. The optical sections prepared from Buccinum are far to obscured by the contained pigment vacuoles to compare with the former but the nuclei, appearing as light masses in the sections, (see fig. 20), with few dark enclosed areas would suggest a similarity in the latter feature.

Electronmicrographs of Aplysia ganglion nerve cells (Rosenbluth, 1963) show that even the smaller examples have rather irregular outer surfaces which lie immediately outside a layer of non-pigmented, non-basophilic ectoplasm. Thus in light micrographs the edge of the pigmented area of the cell body may easily be taken for the edge of the cell. This fact may easily help to explain the apparent isolation of the somata in the ganglia of Buccinum and the fact that measurements of the largest cells from sections and fresh preparations do not entirely aggree.

Study of ganglionic structure, as described by the electronmicroscopists, reveals that there is very little extracellular space within the ganglion, nerve cells and glial cells being packed tightly together throughout. In the light micrographs of Buccinum the glial cells are conspicuous by their absence in the peripheral rind and besides the nerve cell bodies very little detail can be made out. This appears to be similar to the case found in Fissurella by Haller (1886) since little detail of such structure is included, though small glial nuclei could be seen in the neuropile regions, as may be seen in Figs. 17 and 19 for Buccinum. Dakin (1910) managed to observe more detail of the glial cells in the visceral ganglion of Pecten since the glial sheath round the cell bodies and axons is described. The ganglion sheath ground substance was also observed to give off extensions which passed between the ganglion cells. No such extensions were seen in Buccinum, but, assuming that glial cells are present in abundance but have not been stained by the technique employed, electron microscopy might easily reveal such

structures. It is worth noting however, that the perineurial invasions of the nervous material of the commissural regions are clearly visible (see Fig. 22) so that it is equally possible that such features are lacking in Buccinum ganglia.

The neuropile regions of the ganglia of Buccinum, beyond revealing the typical tangled network of fibrils interspersed with nerve fibre tracts, are not visible in any great detail using light microscopy. The glial nuclei are visible in these regions but precise descriptions of the architecture and cytology of the glia must await electron microscopy.

Microanatomy of peripheral nerves, connectives and commissures.

Studies of the structure of nerves, connectives and commissures in Molluscs have been scattered throughout the various classes, as have those relating to the central nervous system. The Gastropoda, have, once again, remained rather neglected and observations of limited extent have been carried out in recent years by Ramsay (1940), Arvanitaki & Cardot (1941 a and b), Turner & Nevius (1951), Turner (1951 and 1953), Schlote (1955) Nisbet (1956) and Batham (1961). The most revealing studies, however, have been those of Schlote (1957) and Schlote & Hanneforth (1963) employing electron microscopical techniques on Helix nerves.

The normal light microscopical techniques reveal little concerning the fine structure of nerves and commissures. This may be seen from Figs. 22, 23 and 24. These results do, however, serve to confirm the general layout of these structures. Each is surrounded by a tough perineurium which stains deeply with

haematoxylin. Within this outer sheath lie large numbers of parallel axons which are interspersed with glial nuclei. Characteristic of nerves, connectives and commissures are the feathery radial projections of the perineurium into the nervous material (cf. Turner & Nevius, 1951, Schlote, 1957, Nakajima, 1961 and Fährmann, 1961).

The perineurium, which has received little attention, is composed of connective tissue cells and longitudinal muscle cells lying in a fibrous ground substance. All these features appear in the electronmicrographs of Buccinum nerves shown in Figs. 25, 26. The sheath appears to be composed mainly of the extracellular fibrous matrix in which the fibroblast and muscle cells are situated. The fibroblast cells contain fibres of similar appearance to those in the ground substance and it seems feasable that the latter have in some way been derived from the former. Besides this hypothetical function the fibroblast cells are also responsible, in Buccinum at least, for the radial incursions of the sheath into the centre of the nerve. Within the nerve the glial cells are also characterised by their inclusion of fibres so that there appears little doubt that the perineurium cells also provide the nerve cells with their investing glia. In this case, therefore, the glial cells would appear indistinguishable from the connective tissue of the sheath (cf. Bullock & Horridge, p. 1303, in press).

The muscle cells of the Molluscan perineurium have been studied in detail only by Rosenbluth (1963) in Aplysia, though

the presence of such muscle cells is reported in Helix by Schlote (1957) and Archachatina by Nisbet (1961). The long dark fibres seen in Fig. 18 are thus most probably muscle cells in the ganglion sheath. The large cells visible in the nerve sheath (Figs. 25,26) are similar muscle cells in cross section at a much greater magnification, in which the discrete myofibrils can be clearly seen. The irregular nature of the cell membranes and the distortion of the cell into shapes corresponding to the sheath shape most probably represents the result of contraction during the dissection or fixing procedure. The functions of such muscle fibres are by no means certain. They could certainly help to reduce the length of the nerves during contraction and so adjust the nerve lengths to fit the state of extension of the animal. In this respect similar muscle cells are known to exist in an earthworm (Havet, 1916) and in a leech (Rohde, 1891). Both animals considerably vary their dimensions during locomotion. It has also been suggested (Eales, 1921) that in Aplysia they are concerned with circulating blood through the central nervous system via the sinuses which underly the perineurium. In Buccinum nerves however, blood sinuses immediately below the perineurium are not visible, so that the first hypothesis is more apposite in this region.

The work of Rosenbluth (1963) also demonstrates the fibrous nature of the extracellular perineurial matrix, present in the ganglionic regions, which appears very similar to that found in Buccinum nerve. The structure of Gastropod perineurium thus

appears to be of a similar nature in both peripheral and central regions, the only difference lying in the fact that the sheath covering the central structures is somewhat thicker than that investing the peripheral nerves.

The glial cells of Buccinum nerves do not invest individual axons as in vertebrates but conform to the general pattern which is emerging for Gastropod nerves, connectives and commissures (Schlote, 1957, Schlote & Hanneforth, 1963, Nakajima, 1961). In these regions small groups of axons are invested by processes from a number of glial cells so that any given glial or nerve cell membrane is flanked by several other glial or nerve cell membranes (see Figs. 27 and 28). The arborisations of the glial cells do not, however, serve to divide the nerves into fascicles.

The inclusions contained within the irregularly shaped glial cells in other molluscs are numerous and include lipids, fine fibrils and small mitochondria. In the sections of Buccinum nerve examined, the glial cells contained nothing besides the fine fibrils and a few small, rather irregular vacuoles. The characteristic fibrils closely resemble those illustrated by Schlote (1957), Schlote and Hanneforth (1963) and Nakajima (1963). In Buccinum, however, they also resemble those found in the fibroblast cells and the ground substance of the perineurium, a fact which has lead to the hypothesis that the glial cells are derived from, or at least of the same type as, the fibroblasts in the perineurium. Schlote (1957) has suggested that the glial cells are of connective tissue type and hence the same re-

lationship may apply in Helix.

The axons found in Buccinum nerves are very similar to the smaller axons found in Helix (Schlote, 1957 et seq), except that their cross sectional shapes are irregular whereas those in Helix nerves are ovoid. The axon diamters range from much less than 1μ to 1μ, and their common inclusions include neurotubules, mitochondria and small irregular vacuoles. The size and form of these basic features are all similar to those seen in Helix and Cristaria. A further feature of similarity with the axons of Helix is the presence of dark staining, homogeneous granules bounded by a distinct membrane (see Fig. 29). Granular inclusions of similar dimensions and structure have been reported by Schlote (1957) and by Nakajima (1961).

Besides the inclusions described above there are two further types which have been seen in cross sections of Buccinum nerves. Both are relatively large and, whilst one was clearly contained within an axon, the other so filled the cell in the sections, that the type of cell in which it was included was not distinguishable. Both types of inclusion have previously been noted in Helix nerves (Schlote & Hanneforth, 1963) and described as 'myelin figures' and Granules with endomembranes' respectively. The myelin figures (see Fig. 30) are composed of a number of approximately concentric membranes which differ from the cell membrane in certain respects (see p. 72) and enclose part of the axonal cytoplasm. The function of these Gastropod myelin figures remain unknown, however, and as yet there is insufficient

knowledge upon which to form any hypothesis.

Whilst various forms of myelin figures are known in other animals e.g. degenerating human cutaneous nerve (Finean, 1961) the 'granules with endomembranes' appear to be unique to the gastropods and have previously only been described in Helix glial and muscle cells (Schlote & Hanneforth, 1963). Those found in Buccinum, (see Figs. 31 and 32), whilst being of similar structure, are approximately four or five times the size of those from Helix. The functions of the endomembranous granules, as with the myelin figures, remains unknown.

Axonal-glial relationships in the Molluscs appear to vary to a certain extent. A consistent feature of the larger axons of other Gastropods is the intucking of their membranes to form deep channels filled with protrusions from the surrounding glial cells. In Aplysia (Batham, 1961) fibres of 3-4μ diameter had several deep infoldings whilst those of 2-3μ diameter had one or two less extensive intuckings. Axons of lesser diameters were without such modifications. In Helix only fibres of less than 0.5μ diameter were completely free from invaginations (Schlote, 1957). In Cristaria, on the other hand (Nakajima, 1961), even the largest axons of the cerebro-visceral connective, which were approximately 6μ in diameter, were not invaginated in this way even though the membranes of the fibres were "sinuous" in cross section.

The axons seen in Buccinum nerves showed none of the invaginations, though the outlines might well be described as

"sinuous", in spite of the largest fibres reaching 1μ in diameter. There would therefore appear to be specific variation in respect of this feature amongst the Mollusca, such that the increased surface area resulting from such differentiation, whether necessary for increased conduction velocity or increased metabolite transfer, may be of use to only some species. The critical size for axon invagination and its advantages may vary in relation to the environment and habits of the species.

Mechanoreceptors.

Little work has been directed towards the analysis of the sensory armament of the molluscs, although Kennedy (1960) has demonstrated the spectral sensitivity of photoreceptor elements of Spisula solidissima, and Hughes & Tauc (1962) have shown central activity in response to tactile stimuli in Aplysia. Tactile responses have also been recorded from the pallial nerves of Archachatina by Nisbet (1961).

Turner & Nevius (1951) were the first to report mechanoreceptor activity in Gastropods. In a study of the organisation of Ariolimax central nervous system they state that, "stretching the foot muscle set up repetitive, synchronised bursts of large amplitude. The end organs in the muscle appear to accomodate rapidly to stretch, but upon release of tension there is another burst of impulses". The similarity of this report, short though it is, with the work reported on p. 85 is evident.

Gray (1960) has shown that mechanoreceptor units are also to be found in the mantle of Octopus. He described two types

distinguishable by their activity patterns. Within the skin (epithelium) phasic units were found that showed rapid adaptation. Other units located within the muscle showed tonic activity, slowly declining in frequency but active throughout the period of stimulation. The receptive field of the latter units was of the order of 0.5 - 1.0 cm. in diameter. Mechanoreceptors similar to both these types have been found in Buccinum.

Tactile receptors showing rapidly adapting responses to the onset of stimulation, lacking responses to prolonged stimuli and often giving an "off response" are to be found in other groups of animals. In particular those demonstrated in toad skin (Bufo bufo) by Höglund & Lindblom (1961) are closely comparable to those found in Buccinum. A further point of similarity between the receptors of these vastly different species is the irregularity of the impulse frequencies. Höglund & Lindblom applied strictly linear stimuli and still obtained an irregular discharge, confined in the majority of cases to the active phase of stimulation. In Buccinum the touch stimuli were not as controlled as those employed by the above authors; the movement stimuli were, however, applied in a strictly linear manner and the irregularity of the individual trains of impulses continued to be a feature of the responses from the different mechanoreceptor units involved. Similarly irregular spacing of impulses has been noted in cat cutaneous sense organs by Iggo & Muir (1962). This irregularity of impulse discharge in cutaneous mechanoreceptors continues to be a puzzling feature.

Iggo & Muir have established that their receptors were profusely branched at their extremities. Although the receptors in this case were slowly adapting, it is possible that the branched nature of such receptors may be responsible for the irregularity of impulse frequency due to the different levels of depolarisation which could occur at the diffuse receptor sites. The summation of such depolarisations to produce impulses at the spike generating region could conceivably lead to the threshold value for this process occurring at irregular intervals, thus being responsible for the typically irregular discharge patterns.

Whilst the structure of the touch receptor endings of Buccinum remain unknown, it is worth noting that branching sensory cells have been found in the epithelial layers of Helix pomatia (Schulz, 1938) and Limax agrestis (Retzius, 1892). These endings were interpreted as being touch sensitive on a purely structural basis. It would now seem likely that this classification is correct and further investigation might prove it to be so.

The movement receptors demonstrated in the mantle regions of Buccinum fall into three types. Types 1 and 2 were both phasic and differed only in that they gave "on" responses and "on - off" responses respectively. The third type of movement receptor was infrequently found and was tonic, the response being superimposed on a spontaneous pattern of impulses. The last type of receptor is similar to those demonstrated by Gray (1960) in that the response is tonic, but the long lasting activity in

Octopus is invoked solely by the stimulus, the unit being silent beforehand.

The Type 1 receptors of Buccinum (phasic, "on" response only) have similar response patterns to those of flexion and extension sensitive movement receptors of Cancer (Cohen, 1963) and Carcinus (Wiersma & Boettiger, 1959). Thus the train of impulses occasioned by movement stimuli is restricted to the active phase of the stimulus. The impulse frequency in all cases is proportional to the rate of movement, and there is no response to movement in the reverse direction. The Type 2 receptors are similar in many ways to the above but lack the directional sensitivity of the Type 1 receptors and those in the Crustaces. A similar type of response pattern has been noted, however, from some of the parapodial mechanoreceptors of the Polychaete Harmothoë (Horridge, 1963).

The Type 3 receptors in Buccinum, besides their similarity to those described by Gray for Octopus, also have certain features in common with certain of the pharyngeal proprioceptors of the dogfish (Satchell & Way, 1962). The latter receptors were silent in the unstimulated preparation but fired a burst of impulse when movement stimuli were applied. Maintained distortion was accompanied by a slowly declining response frequency and relaxation led to a second burst of impulses, whose frequency decreased fairly slowly.

The movement receptors found in Buccinum thus reveal characteristics similar to several other known forms of mechanoreceptor.

The different features from other forms are, however, combined in one receptor unit type in some cases. The range of receptor types in the whelk should however be sufficient to provide an accurate position and movement sense for the animal. The mechanoreceptors have been studied only in the mantle region at the base of the siphon (see Fig. 34), but are probably to be found elsewhere in the body. Besides the function postulated above it may also be suggested that these receptors are concerned in the rapid contraction movements of the animal in view of the reflex efferent activity which is fed back into the stimulated area. This idea is strengthened by the demonstrated central nervous pathways which extend from the receptors of the mantle region to the head and columella muscle, indicating that the afferent activity of these end organs has a widespread effect in the rest of the nervous system (see Fig. 42).

The end organs mediating the movement responses are not known with any certainty. Alexandrowicz (1960) has given a description of the anatomy of muscle receptor organs in Eledone cirrhosa. These were found as a discrete substellar body near the stellate ganglion. Their nervous elements have their dendrites ending on muscle fibres and up to fifty separate fibres are involved. Sections of the siphon base area of Buccinum have revealed no structures that might be construed as mechanoreceptors. Intravitam staining with methylene blue met with slightly greater success in that multipolar cells were found on one or two occasions (see Fig. 16). These cells are of similar

external appearance to those described by Alexandrowicz but are only about one tenth the size. It is possible, therefore, that similar types of end organ mediate movement responses in both the Cephalopod, Eledone and the Gastropod, Buccinum. A similar basic pattern of structure of muscle receptors has also been found in insects (Finlayson & Lowenstein, 1955; Slifer & Finlayson, 1956). This type of receptor cell-muscle relationship is therefore by no means unique and may well be common type of receptor in a wide variety of animals.

Reflex pathways in the central nervous system.

Little work has been carried out upon the nervous activity of gastopod molluscs in relation to their normal behaviour. In the past a few workers have studied the effects of electrical stimuli, applied to nerves, on movements of various regions of the animal. In this way ten Cate (1928) studied the protective movements of the 'wings' of Aplysia. The few recent researches include work by Turner & Nevius (1951) on Ariolimax, Nisbet (1961) on Archachatina, Hughes & Tauc (1961) on the giant axons of Aplysia, and Hughes & Tauc (1962) on further aspects of central organisation in the same animal.

The work of ten Cate (1928) demonstrated that stimulation of the wing nerves of one side of the animal brought about responses in regions innervated by other ipsilateral nerves and also those innervated by the three contralateral nerves. Similar responses also resulted when the pleuro-visceral connectives were stimulated. By removing the other ganglia it was

established that the ipsilateral effects were mediated by the pedal ganglion of that side whilst the contralateral effects were mediated by the pedal ganglion of the opposite side. Contralateral transmission for this particular effect being carried by axons in the pedal commissure. Similar dispersion of efferent activity caused by localised afferent input has been seen in Buccinum (see Figs. 42 to 44).

Turner & Nevius (1951) demonstrated the convergence of afferent pathways onto motor neurones in the pedal ganglia, viz "Impulses may be recorded in the pedal nerves following stimulation of almost any nerve leading to the central nervous system". Pathways deduced from experiments upon the pedal and cerebral ganglia of Ariolimax reveal two other points of interest in discussing the work carried out on Buccinum. Firstly there is the convergence of afferent pathways from both sides of the body onto efferent neurones in the pedal ganglia. Secondly, it was found that both pedal and cerebral commissures were involved in contralateral conduction, and that in this way long "neurone loops" were formed linking afferent activity passing into the pedal ganglion with efferent activity leaving the same ganglion.

The more extensive work of Hughes & Tauc on Aplysia central nervous system, besides plotting the paths of the giant axons has extended the knowledge concerning the effects of mechanical stimulation, the patterns of axonal branching and synaptic connexions and neuronal pathways. In the course of these experiments

stimulation "of the sensory pathways via the normal receptor mechanisms" was employed to a considerable extent. This was also done in the experiments on Buccinum for the same reason, namely "to obtain information of greater physiological significance in respect of the normal functions and behaviour of the animal".

The work of Kerkut & Walker (1962) is of interest in relation to the central organisation of the gastropod CNS. One section of their results is devoted to the mapping of cell connections in various ganglia. In this way they established a number of orthodromic and antidromic pathways related to the activity of single cells in the supra- and sub-oesophageal ganglia of Helix.

The work upon the CNS is thus rather fragmentary, though the general picture which is emerging is one involving a fair degree of complexity. Bearing these features in mind the experiments upon Buccinum CNS were carried out with four subjects in view. The central reactions to mechanical stimulation of the mantle had already aroused interest and these were investigated in more detail. The reflexes involved in the distinctive retraction response formed a second obvious subject for investigation. Because electrical stimuli can throw little light upon normal behavioural pathways, the more natural mechanical stimuli were employed to investigate pathways which pass into one side of the CNS and emerge from the other i.e. contralateral coordination. Finally, whilst Kerkut & Walker (1962) have investigated convergent pathways by means of electrical stimuli, the

convergence of the mechanoreceptor pathways in various ganglia on both ipsi- and contra-lateral central neurones was also studied in Buccinum to obtain some idea of the true extent of the convergence involved.

The results from experiments upon the "mantle reflexes", (see Fig. 42), yield several points of interest. First, there is the activity in the pleural ganglion itself. The afferent axons are thus capable of activating adjacent motor neurones directly. This is similar to the case in Octopus (Gray, 1960), where mantle mechanoreceptor axons synapse with motor units in the stellate ganglion. The significance of the latter reflexes is not understood but those in Buccinum may well be concerned in the retraction responses, especially considering that the siphonal afferent pathways pass into the same ganglion and the mechanoreceptors of the musculature of its basal region could well be excited by the retraction of the siphon. This hypothesis is the more likely when the reflex pathways passing to both the tentacular and columellar regions are considered. Sudden movements of the mantle cause efferent activity to all the regions most concerned in the retraction response. Slow movements are largely without effect in eliciting central activity.

The significance of the longer pathways is less easy to understand. It is possible that these feed information into other neurone networks along their paths and thus cause efferent activity in further regions, besides returning motor impulses to the mantle region, in which case a considerable afferent input

from the mantle passes to both the pedal ganglia. The foot however is the last region to be retracted in a protective response and it would seem likely that afferent impulses from a much wider field, or repeated stimulation of one region, would be necessary to cause effective efferent activity in the pedal nerves. Observations on the intact animal tend to confirm this view since considerable stimulation is needed to obtain a complete retraction. This hypothesis is born out by the work of Turner & Nevius (1951) who found that the largest axons in the pedal nerve require the summation of impulses from several presynaptic regions to initiate post-synaptic responses.

The results summarised in Fig. 43 with the retraction response specifically in mind, show many points of similarity to those concerning the mantle reflexes. Reflex activity arises in the cerebral ganglia in a similar manner to that in the pleural (cf. pathway 1 in Fig. 42 with pathway 7 in Fig. 43). It is possible that this type of activity is involved in the initial quick responses of the tentacles. However, it should be noted that the initial rapid contraction is retained when the tentacular nerves are severed. The change in behaviour resulting from such a lesion is not in the contraction itself but appears to lie in its prolongation. Light tactile stimulation of a tentacle with its innervation intact leads to a contraction which lasts for several seconds before the tentacle is again extended. When the tentacle is isolated the contraction is relaxed almost immediately. Cutting the nerves also results in a maintained

partial contraction of the tentacles. It would appear, therefore, that the complete innervation is unnecessary for the initial rapid contraction but is essential for both maintained contraction and full extension of the tentacles.

Besides the local reflex discussed above, tentacular afferent activity also results in efferent bursts in the pallial and columellar nerves. Some of the pathways take the shortest possible route through the ganglia whilst others pass through one or both pedal ganglia. It would seem, therefore, that the tentacular, mantle and columellar regions are closely integrated in their responses to mechanical stimulation of the exposed regions of the animal. There is thus a high degree of co-ordination in the execution of the retraction response.

Pathway 5 in Fig. 43 is very similar to pathway 3 in Fig. 42. Both pass through both pedal ganglia but result in efferent activity passing to the stimulated area. Bearing in mind the involvement of the pedal ganglia in many of the other pathways it is clear that the pedal ganglia must exert a major influence on movements of almost all regions of the body. The fact that some pathways pass directly from cerebral to pleural ganglia and thence to the mantle and columellar muscle suggests that the initiation of the retraction is direct and the longer pathways involving the pedal ganglia may prolong, continue or increase the strength of the contraction.

The pathways resulting in contra-lateral efferent activity, summarised in Fig. 44, show that both commissures and the sub-

intestinal - pleural connective of Buccinum carry contra-lateral reflex pathways from the right to left mantle regions (see pathways 3-5). Contra-lateral pathways from the right tentacular region to the left mantle also pass through the cerebral commissure and fail to pass through the pedal ganglia in at least come cases. Portions of pathways which might easily resemble those in Buccinum have been found in Aplysia (Hughes & Tauc, 1962) and Helix (Kerkut & Walker, 1962) but unfortunately the full extent of these reflex paths has not been worked out in these other species, so that a close comparison is not possible. The work of Hughes & Tauc does, however, show that there are a considerable number of pathways passing through the pedal ganglia from both the pleural and cerebral ganglia. These are a mixture of direct and synaptic types of pathway, and the former demonstrate that impulses may pass straight through a ganglion without passing across a synapse. Branches of the axon might easily occur however, since other axons have been shown to branch (Hughes & Tauc, 1961, and Turner & Nevius, 1951). By means of such branches the information in the through conducting pathway might be relayed to inter-neurones in the ganglia, though the latter would be unable to modify the activity in the through conducting pathway.

The dispersion of information throughout the various ganglia and the presence of both ipsi- and contra-lateral afferent pathways passing along all the available anatomical connections, all tend to make unlikely the existence of specific areas of peri-

pheral representation in the CNS.

The convergence of afferent pathways upon interneurones or motor neurones in various molluscan ganglia has been demonstrated by Turner & Nevius, (1951) and Kerkut & Walker, (1962). The former authors demonstrated that post-synaptic potentials in pedal motor axons had the same form when any of three afferent paths were stimulated electrically. Kerkut & Walker demonstrated the orthodromic and antidromic connexions of a number of cells in the supra- and sub-oesophageal ganglia by similar means. In this way they demonstrated convergent orthodromic pathways arising in widely separated nerves.

The convergent pathways demonstrated in Buccinum were deduced from extracellular recordings and thus the details of the antidromic pathways were not available. Also it was usual to record the activity from a number of neurones so that the patterns of impulses from single cells were seldom distinguishable. A better situation applies to the results obtained from the supra-intestinal ganglion in response to osphradial stimulation which will be discussed later. The convergent pathways summarised in Fig. 46 do show, however, that groups of neurones at the sites indicated would respond to peripheral stimuli passing into the CNS from widely dispersed regions. All the illustrated cases of convergence in Buccinum had both ipsi-lateral and contra-lateral connexions. As in Helix the maximum number of paths found to converge was three. These were using only mechanical stimuli, however, and a wider range of stimuli might have shown

a larger number of converging pathways since it has been shown that single central cells will respond to peripheral stimuli of different modalities (see p. 138). A second feature in common with Helix was the common occurrence of crossed (contra-lateral) afferent pathways, which in Helix are in contrast to the uncrossed nature of the efferent pathways. This latter feature contrasts with the arrangement in annelids and vertebrates where crossed motor axons are commonly found (Kerkut & Walker, 1962).

The patterned efferent activity induced by single preganglionic shocks has been described in Mya (Horridge, 1961) whence it arose from a single isolated ganglion. Similar experiments carried out on Buccinum revealed that similar responses resulted from the entire CNS when a single nerve was maximally stimulated.

Naturally occuring, centrally determined patterns of motor impulses are by no means uncommon in invertebrates. The patterned bursts of impulses from the crustacean heart ganglion (Maynard, 1955) and those from isolated ganglia to the sound-producing tymbral muscle of the cicada (Hagiwara & Watanabe, 1956) are but two examples. Two hypotheses concerning the origins of these bursts of activity have been proposed. The first is that of Maynard (1955), who suggested that the final motor output is the result of a series of inter-neurones each in turn exciting the next in the chain. The periodicity of the impulse patterns being determined by closed loops of neurones within the series. The second theory is due to Horridge, (1961 and 1964).

In this theory the concept of actual anatomical connections, i.e. a circuit diagram, is replaced by a chemical specificity in the relations between one group of neurones and another. The tangled and random nature of synaptic connections in the neuropile is therefore of lesser importance so long as the chemical sensitivity of the neurones and the transmitters which they produce are genetically fixed. Thus incoming afferent neurones would release a specific transmitter into the neuropile and this would then selectively excite some fraction of the motor units.

These two theories are difficult to separate experimentally and more recent evidence from vertebrate neurophysiological studies suggest that features of both may in fact be combined in certain cases. Fisher & Coury (1964) have shown in the rat brain that neural pathways of considerable extent can be selectively stimulated by the injection of cholinergic drugs into a variety of brain structures. Thus tracts of fibres with at least a partially ordered histological pattern, a specific chemical sensitivity an a predicatable behavioural effect are known to exist in the vertebrates. It is possible that similar systems exist in the invertebrates, causing patterns of efferent impulses which result in definite behavioural reflex activity on the part of the animal.

<u>The structure and function of the osphradium</u>.

The function of the osphradium, a structure found in the mantle cavity of many molluscs, has been the subject of some

speculation. It is generally accepted to be a sense organ. This conclusion is based upon its constancy of position in the line of the inhalent current (Yonge, 1947) and the histological studies of such workers as Bernard (1890) and Stork (1935). The extensive research of Bernard showed that, besides ciliated and mucous cells, the epithelium of the "organe de Spengel" (osphradium) in a wide variety of molluscs contains large numbers of neuro-epithelial cells. These cells are linked by multipolar nerve cells which themselves connect at various levels, until small nerves running to the branchial or osphradial ganglion are formed. This description is confirmed by Stork (1935) in a number of different species. In the cases investigated by these, and other, workers there does not appear to be a specific "sensory" region of the osphradium. In Buccinum, however, Dakin (1912) states that "the osphradium .....attains a degree of complexity which is probably never exceeded in the Mollusca", and recognised three specialised regions of the osphradial leaflets. These are sensory, glandular and ciliated, the first being the most extensive and occupying "the greater part of the free lateral surface of the leaflets". Dakin also saw "free nerve-endings" in the osphradial epithelium and considered these to be "without doubt the important sensory structure in the organ". Such controversy over the exact nature of the sensory endings has little bearing upon the general conclusion of sensory function for the organ but might form the background for an interesting electron microscopic study, (Anderson, 1963).

Having been assigned a sensory function, the osphradium remained a subject of interest since speculation arose as to the modality of stimulus to which it is sensitive. Copeland (1918) on the basis of excising the osphradia of Busycon, and observing the food finding ability of the experimental and control animals, decided that the olfactory sense was located in the osphradium. Henschel (1932) and Brock (1936) also came to the same conclusion for Nassa and Buccinum respectively. Hulbert & Yonge (1937) considered this theory unsatisfactory since herbivorous and plankton feeding gastropods also had well developed osphradia in a large number of cases. They postulated therefore, that the osphradium acted as a sensory organ "concerned with the estimation of the amount of sediment carried into the mantle cavity by the water currents created by the lateral cilia on the gills". Yonge (1947) reiterated this theory after an extensive survey of the pallial organs of the Gastropods.

Subsequent workers on the subject, namely Wölper (1950) on Paludina, Brown & Noble (1960) on Bullia and Michelson (1960) on Australorbis, have favoured the chemoreceptive theory. In a recent review of chemoreception in gastropods Kohn (1961) states that Yonge now considers that a chemosensory role of the osphradium may have evolved secondarily from the original particle detecting mechanism. Kohn was also responsible for the only recorded attempt at electrophysiological investigation of activity in the osphradial nerves. In the course of this he failed to record any afferent activity in the nerves of Busycon, as

similar experiments on Buccinum also failed.

Investigation of central nervous activity in the supra-intestinal ganglion of Buccinum has demonstrated conclusively the chemoreceptive function of the osphradium in this species, and the absence of any central activity caused by mechanoreceptors of a sufficient sensitivity for the monitoring of inhaled sediments. During the course of the experiments it has also been noted that copious production of mucous occurs from the hypobranchial gland, osphradium and mantle surface. This must severely impede the entry of particles into the mantle cavity in the natural situation and their contact with any part of the mantle surface.

Central nervous responses to stimulation of the osphradium.

All attempts to record afferent nervous impulses in the osphradial nerves failed. The experiments did however, show that efferent bursts of activity could be initiated by noxious stimuli (see Fig. 48). This reflex activity could be produced by the isolated supraintestinal ganglion, demonstrating once again the type of reflex pathway shown in Figs. 42 and 43 whereby reflex efferent impulses are directed back to the stimulated area through the ganglion which receives the afferent impulses. The prolonged nature of the efferent bursts and their irreversable loss with prolonged stimulation suggest that this activity was caused by injury discharges in the osphradial receptors. These results indicate that there are motor neurones passing from the C.N.S to the osphradium and thus this organ must also

be considered as an effector of some kind. The function of the motor activity is by no means clear but observations of the osphradium during mechanical stimulation showed that local withdrawal responses took place. Noxious stimuli also caused contractions in the mantle region and it is probable that these resulted from the observed reflex efferent activity in the osphradial nerves and constitute protective responses.

Indium and intracellular microelectrodes inserted into the supra-intestinal ganglion showed that the central neurones responded in various ways to both chemical and mechanical stimulation of the osphradium. Before discussing these responses however, it is of some advantage to consider the spontaneous activity of neurones in the supra-intestinal ganglion.

A large fraction of the neurones in the isolated supra-intestinal ganglion were spontaneously active. The majority showed trains of impulses of a relatively regular nature. In a few cases, however, intermittant bursts of activity in a group of neurones was noted (see Figs. 49 and 63). The frequency of the example shown in these figures was approximately 15/min. Similar activity has been noted in the parapodial nerves of Aplysia (Hughes & Tauc, 1962), where they were frequently accompanied by rhythmic movements of the animal, especially the parapodia. No such movements were noted in Buccinum but it must be remembered that the supra-intestinal ganglion is connected to the visceral CNS by the left visceral connective. It is possible that the rhythmic discharges, which in some cases could be

modified by peripheral stimulation, have some bearing upon the activity of the organs in the visceral hump.

The results of the experiments carried out on central responses to varied stimulation of the osphradium are such that they represent the first successful electrophysiological investigation of osphradial function and gastropod chemoreception. The results have the disadvantage that they are not the responses of the receptors or their afferent activity but the information which has been obtained is, nevertheless, useful in filling the considerable gap in our knowledge of these subjects (see Kohn, 1961).

The chemosensory abilities of gastropods, especially the marine forms, have recently been reviewed by Kohn (1961) and behavioural responses to sea water and inorganic ions, predators and distant food are well documented. Mating and homing are further aspects in which chemoreception may have some part to play. The importance of this type of reception amongst the gastropods is therefore obvious. The only previous attempt to obtain electrophysiological results concerning gastropod chemoreception would appear to be that of Kohn & Tateda (see Kohn, 1961), though other unsuccessful attempts may not have been published. (Duncan, pers. comm.).

Experiments upon the central nervous responses to various stimuli applied to the osphradium of Buccinum e.g. particles, pH, ionic concentrations and "food extracts", have shown that this organ, for this species at least, is chemoreceptive.

Since the concealed position of the osphradium rules out a contact chemoreceptive function, the organ must be concerned with chemoreception at a distance, i.e. the location of food. The results do not justify the elimination of other sites as distance chemoreceptors but the extensive development and sensitivity of the osphradium indicate at the very least that it is an important organ in this respect.

Results showing that the "natural responses" of central neurones could be invoked by stimulus solutions of certain synthetic chemicals are revealing in two respects. Firstly they indicate the probable nature of the effective constituent of the food extract stimuli, though this can only be confirmed by chromatographic fractionation of the latter and subsequent testing on both the whole animal and the osphradial preparation. Secondly, the results indicate a distinct relationship between chemical structure and effective stimulation of the osphradial chemoreceptors.

The most effective chemical stimuli were glutamic and aspartic acids. Both were capable of eliciting responses at very low concentrations, which are compatible with the theory of distance chemoreception. Responses to amino acids have been noted in a number of marine animals, e.g. Carcinides (Case & Gwilliam, 1961, 1963, Case, Gwilliam & Hanson, 1960), Nereis (Case, 1963 and Case & Gwilliam, 1963), Limulus (Barber, 1956, 1963), Nassarius (Henschel, 1932) and Panulirus (Laverack, 1964). Thus amino acid chemoreceptors may be a common feature amongst marine inver-

tebrates. It is also worth noting in respect of Buccinum's sensitivity to "Mytilus extract" that Mytilus muscle has been shown to contain both glutamic and aspartic acids in free form (Potts, 1958). The occurrence of these chemicals in the fresh extract is thus virtually certain.

The relationship between chemical structure and effective stimulation.

From the results of the experiments upon central nervous responses to osphradial stimulation it is clear that glutamic and aspartic acids are the synthetic compounds which mimic most closely the more natural responses to 'Mytilus extract'. The threshold for these chemicals ($5x10^{-5}$ and $10^{-6}$M) are also compatible with environmental stimulation. The dicarboxylic acids on the other hand have higher thresholds ($5x10^{-4}$ to $10^{-2}$M), though the majority were found to be effective stimuli. Adipic and glutaric acids were those which were most effective and also those which are structurally most similar to glutamic and aspartic acids. Figure 70 illustrates the structural formulae of those chemicals found to be effective or partially effective in stimulating the osphradial chemoreceptors.

From the results it is clear that glutamine was virtually ineffective as a stimulus whilst the dicarboxyl amino acids and several of the dicarboxylic acids were effective. It may therefore be postulated that the criteria for stimulation of the osphradial chemoreceptors are a straight chain compound of four to six carbon atoms, bearing a carboxyl radical at either end.

| | |
|---|---|
| l-glutamine | $H_3N.CO.CH_2.CH_2.CH(NH_3).COOH$ |
| l-glutamic acid | $HOOC.CH(NH_3).CH_2.CH_2.COOH$ |
| l-aspartic acid | $HOOC.CH_2.CH(NH_3).COOH$ |
| adipic acid | $HOOC.CH_2.CH_2.CH_2.CH_2.COOH$ |
| glutaric acid | $HOOC.CH_2.CH_2.CH_2.COOH$ |
| succinic acid | $HOOC.CH_2.CH_2.COOH$ |
| malonic acid | $HOOC.CH_2.COOH$ |
| glutathione | $HOOC.CH(NH_2).(CH_2)_2.CO\text{-}NH.CH(CH_2SH).CO\text{-}NH.CH_2.COOH$ |
| betaine | $(CH_3)_3.N.CH_2.COOH$ |
| trimethylamine oxide | $(CH_3)_3.NOH$ (OH) |

Figure 70. Structural chemical formulae of the effective and partially effective synthetic chemical atimuli employed during the experiments upon osphradial sensitivity.

The smaller dicarboxylic acids and the long chain of glutathione are very much less effective as stimuli and diverge from the criteria stated above.

The positive results obtained using betaine hydrochloride, trimethylamine oxide hydrochloride and the single positive result using glutamine cannot be explained on the basis of the hypothesis stated above. It is, however, possible that more than one type of chemoreceptor is present in the osphradial epithelium and, whilst those responding to the dicarboxylic amino acids are much more common, there are others which respond less specifically to organic molecules with an acidic nature. The high thresholds for these three compounds, which are of a similar order to those for the partially effective short chain dicarboxylic acids, would however, indicate a lack of sensitivity in the latter receptors.

Until further experiments show whether these results are consistent and therefore more than one type of receptor is likely, it seems reasonable to postulate one type of receptor with a specificity directed towards the criteria stated above. These receptors are, however, incapable of absolute rejection of such highly acidic radicles as betaine and TMO and weakly acidic, "close fitting" radicals such as glutamine, when any of these is present in high concentrations.

Olfactory specificity based in some way upon the structural chemistry of the stimulating molecule is a relatively frequent occurrence in the animal kingdom. Various theories

concerning the mechanism of this specificity have been proposed and have been recently reviewed by Ottoson (1963). These theories all postulate that a certain physical or chemical feature of the molecule is responsible for the stimulation of the cell. Thus degree of unsaturation, functional groups, complementary configuration to membrane receptor sites, shape of the molecule and molecular vibrations, i.e. "osmic frequencies" have all been suggested as the "effective characteristic" of odorous molecules (Ottoson, 1963).

Just as diverse as theories concerning effective characteristics are those concerning the initial stages in receptor activation. Membrane shrinkage, pore membranes leading to "puncture" of the membrane by odourous molecules which in turn cause permeability changes, and enzyme activation are three arrangements which have been proposed (Ottoson, 1963) and, as for the different "effective characteristic" theories, all are difficult to distinguish experimentally and most of the results must be considered as being rather ambiguous.

One feature of olfaction now generally accepted is that the initial event in excitation involves the adsorbtion of the stimulatory molecule onto the receptor membrane. Specificity arises in this process in so far as the stimulating molecule has to fit into certain "sites" on the membrane. The theory of site specificity has been extended by Amoore (1962, 1963), for human olfaction, to the extent of recognising seven basic types of olfactory site, each having a specific shape in three

dimensions and/or certain bonds or charges in specified positions. This theory at least has the advantage that predictions as to a subjective odour analysis can be made and verified experimentally.

The experimental results obtained from Buccinum can yield little of value in respect of evidence favouring any of the above theories. The electrophysiological recording site being remote from the receptor or its afferent impulse train and the problem as to whether one or more receptor types are present, the results give no information concerning receptor activity. Suffice it to say that the recorded activity demonstrated that a specificity existed favouring relatively small, dicarboxylic organic radicals, in which the reactive groups are terminal. Also the normal mammalian gustatory stimuli e.g. Hydrochloric acid, quinine, sucrose, were without effect.

The interaction of afferent pathways on neurones in the supra-intestinal ganglion.

The intracellular recording of the activity of neurones in the supra-intestinal ganglion confirmed the results obtained with metal filled microelectrodes with respect to the sensitivity of the osphradial region to mechanical and olfactory stimulation. It also revealed a greater diversity of neurone types than had previously been noted in so far as six of eleven predictable cell types were demonstrated. The convergence of afferent pathways onto single central neurones was thus confirmed. Many of the pathways resemble those demonstrated in Helix sub-oesophageal ganglia by Kerkut & Walker (1962) except that the orthodromic

pathways all ran along the osphradial nerves, thus giving no opportunity for "crossed" pathways. The advantage of the results obtained from Buccinum lies in the fact that receptors were stimulated, showing the frequent convergence of pathways from different identifiable types of receptor.

The results indicating the branching nature of the dendrites of central cells may be compared with those of Tauc & Hughes (1963), showing that this feature is common to both Aplysia and Buccinum and probably throughout the Gastropods. Similarly the observation of IPSP's and EPSP's closely resembling those illustrated by Tauc & Gerschenfeld (1962) and Hughes & Tauc (1962) in Aplysia and by Kerkut, (1963) and Kerkut & Thomas, (1964) in Helix indicates that the relationships between the soma and many of the synaptic regions are similar in those three species, and thus may represent a characteristic feature of the gastropod central nervous system.

Aspects of the organisation of the nervous system.

The results obtained from the experiments carried out upon Buccinum demonstrate certain features of the overall organisation of the nervous system of this animal. The stimulation of receptor afferents and the resultant efferent activity can be related to certain parts of the normal behaviour of the animal. The receptors stimulated, namely mechano- and chemoreceptors obviously play a considerable role in monitoring environmental factors in respect to the animal and the effects of their afferent input are widespread both within the nervous system itself and

the animal as a whole.

The afferent activity of the mechanoreceptors, which has been investigated in some detail as a separate subject, is closely related to important natural reflexes carried out by the animal. Some aspects of this which have been demonstrated are the 'local' mantle reflexes, the far more extensive retraction responses and their involvement in contra-lateral coordination.

The afferent activity of the chemoreceptors of the osphradium is involved in the vital process of food location and also probably with 'local' protective responses of the mantle region.

The afferent activity of both these types of receptor initiates extensive efferent activity from the central nervous system which results in important and complex behavioural responses. The importance of this relation between nervous input and output is obvious and the intermediate stages between these activities is of considerable interest. The experiments carried out upon Buccinum throw some light on these processes and the way in which this, and possibly, other nervous systems work. In this respect three features of the results obtained may be discussed further.

The results demonstrate a dispersion of afferent activity from restricted regions of the body to widely separated parts of the central complex. Whilst largely precluding the specific central representation of peripheral regions this aspect of the

organisation suggests a complex involvement of many central regions in behavioural responses, in that the sensory input is spread out immediately on reaching the central complex. This dispersive stage would appear to be followed by a converging and integrative process. The activity in interneurones, initiated by the afferent discharges of the receptors, converges on further interneurones in such a way that the latter's activity becomes modified by afferent activity from different regions of the body and by activity arising in receptors sensitive to different modalities of stimulus. This co-ordinating and integrative stage is followed by a further convergent phase by which the smaller number of motor neurones activating the effector organs are stimulated, so producing the observed behaviour of the animal.

Individually all the processes described above have been demonstrated to occur; the dispersive and convergent integrative phases in Buccinum and the final convergent phase in other molluscs (Turner and Nevius, 1951 and Kerkut and Walker, 1962).

The complexity and extent of these processes are predictably vast and form a topic of considerable interest and increasing complexity as further types of receptor afferent activity are considered. The work recorded in this thesis has, however, given some idea of the varied nervous activity at all stages of the input to output cycle.

## SUMMARY AND CONCLUSIONS.

Various aspects of the nervous system of Buccinum undatum L. have been studied. The general anatomy has been observed by dissection and histological techniques. The microanatomy of both central and peripheral nervous systems has been investigated by means of light and electron microscopy. A variety of electrophysiological techniques have been used to record the activity of neurones within both peripheral and central nervous systems.

From the results obtained the following conclusions may be drawn:-

(1) The general anatomy of the central nervous system conforms closely to the descriptions of earlier workers, but there is considerable variation in the peripheral nervous system.

(2) The microanatomy of the CNS and peripheral nerves closely resembles that observed in other gastropods and shows certain features in common with other Molluscan classes.

(3) Four types of mechanoreceptor have been shown to exist in the mantle region. These comprise a rapidly adapting touch receptor, probably located in the epidermis, and three types of movement receptor in the musculature. The latter comprise phasic "on" receptors, phasic "on-off" receptors and tonic position receptors.

(4) The central activity occasioned by stimulation of the mantle mechanoreceptors is concerned with local muscular contractions and also has a wider significance in relation to the retraction response, which involves the whole animal.

(5) Central nervous pathways involved in the retraction response show various levels of organisation which are postulated to relate to both short and long term co-ordination.

(6) Central nervous pathways relating to contra-lateral co-ordination show the diffuse nature of the nervous pathways. Thus paths originating from small peripheral areas take many different routes within the CNS.

(7) The convergence of afferent pathways from widely separated regions within the CNS confirms conclusion (b) and shows no indication of specific central representation of peripheral areas within the CNS.

(8) Efferent activity occasioned by preganglionic shocks shows that efferent patterns are centrally determined by the entire CNS. These results can be explained by a cascade of interneurones, by selective chemical sensitivity of the central neurones or by a system combining these features.

(9) Study of central responses to varied stimulation of the osphradium shows that this organ is involved in olfactory location of food and is insensitive to particulate matter suspended in the sea water environment, in as far as such stimulation fails to elicit activity in the central neurones.

(10) The effective stimulation of the osphradial chemoreceptors by a variety of synthetic chemicals suggests that there is a specificity favouring four to six carbon atom chain, dicarboxylic radicals.

(11) Intracellular records of central neurone activity in the

supraintestinal ganglion has demonstrated the diversity of central responses to similar afferent codes and hence the integrative powers of the CN3.

(12) By similar recording techniques it has been demonstrated that axonal branching and the relationship of soma to synaptic regions in Buccinum are similar to that described for other gastropods.

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