"ASPECTS OF THE NEURAL ORGANIZATION OF *LITTORINA LITTOREA* (L.)"

Thesis for the Degree of Ph.D. 1975.

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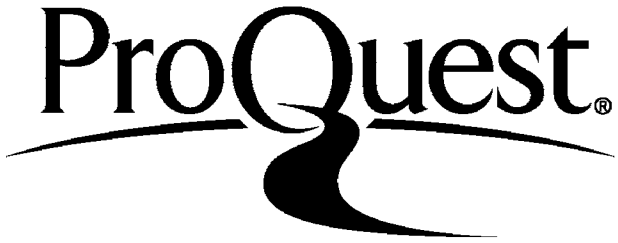

i. ACKNOWLEDGEMENTS.

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Various items of equipment were manufactured in the department, including the recording chamber and suction electrod holders, by Mr. G. Rentmore and I wish to extend my thanks to him for this and for his co-operation in many other technical aspects of the study.

During the later stages of the work Mr. J. Nicholls provided invaluable assistance and advice with respect to the histological examination of the various tissues.

Finally I wish to thank my family and friends for their unwavering support and particularly my sister and typist, Mrs. B. Pritchard.

ii. ABSTRACT.

An investigation has been made of the nervous mechanisms mediating the withdrawal response of Littorina littorea in order to establish aspects of the functional organization of its nervous system.

Observations of the withdrawal response, elicited by tactile stimulation of the tentacles and leading to partial withdrawal of the animal into its shell, revealed that the response consisted of a fast contractile phase followed by a prolonged relaxation and extension phase, without a period of prolonged contraction.

The effects of tactile stimulation on isolated tentacles were determined and the roles of the tentacle and cerebral ganglia in mediating tentacle responses were established. Four classes of response, corresponding to four types of mechanosensory neurones, were recorded from the tentacl nerves.

Two morphologically separate pathways were traced from the right tentacle between the paired cerebral, pleural and pedal ganglia to a nerve shown to innervate the columellar muscle and to originate from the left pleural ganglion. It was shown that the two pathways were excited by different classes of tentacle response.

A comparison of 'normal' contractions of the columella muscle with those induced by either of the two pathways showed that both pathways initiated apparently normal muscle contractions. These consisted of a rapid contractile phase followed by a prolonged relaxation phase of similar character to that

of the withdrawal response of the intact animal.

An explanation of the double system of pathways has been suggested after consideration of the properties of the sensory neurones found in the tentacles. One pathway is thought to function both as a high threshold slowly adapting system and also as a low threshold system, while the other functions as a high threshold rapidly adapting system.

The nerves, ganglia, tentacles and columellar muscle were examined histologically and some limited studies have been made of spontaneous central activity and conduction velocity.

iii. TABLE OF CONTENTS.

1.0. INTRODUCTION

The molluscs constitute one of the largest and most successful phyla of the Animal Kingdom with diverse representatives in many types of environment. The phylum Mollusca is divided into various classes, the largest being the Gastropoda which in turn is subdivided into three sub-classes, the Prosobranchiata the Opisthobranchiata and the Pulmonata (Thiele, 1935).

The prosobranch mollusc Littorina littorea (L.) was chosen as the subject for this investigation as it is a common inter-tidal animal that has had many facets of its biology investigated although the functional organization of its nervous system had not been studied. It was considered that a study of its neural organization would supplement the results of previous investigations and help to evaluate the possible use of Littorina in future neurophysiological studies while increasing the knowledge of the Prosobranchiata.

The wide distribution of the molluscs made them suitable subjects for many early zoological investigations: thus an extensive literature exists for certain molluscan classes. The work of Bouvier (1887) is of significance to the present work as it provides a general account of the morphology, classification and nervous anatomy of the prosobranchs.

In the late nineteenth and early twentieth centuries the gastropods, in particular, were the subject of numerous histological investigations. Such workers as Retzius (1892), Veratti (1900), and Kunze (1917a, 1917b), made extensive studies of molluscan peripheral and central nervous elements that were largely unchallenged until Schlote (1957) was able to examine

the nerves and connectives of Helix with the aid of the electron microscope.

Early experimental investigations of the functional organisation of molluscan nervous systems also date back to the beginning of the twentieth century. Drew (1908) conducted a physiological study of the nervous system of the lamellibranch Ensis directus (Conrad) while such aspects as the nervous control of foot muscle tonus were being investigated in the gastropods (Jordan, 1901.)

The development of intracellular recording techniques prompted investigations of the giant axons of cephalopods and also the large cell bodies found in the ganglia of certain pulmonates and opisthobranchs. The study of the giant axons of Sepia, Octopus and Loligo provided the basis for the understanding of the propagation of the nervous impulse, while the in situ study of the large cell bodies in Aplysia and Helix (Tauc, 1954 : Kerkut et al., 1956: Arvanitaki & Chalazonitis, 1955: reviewed by Bullock & Horridge, 1965 and Tauc, 1966). established the mechanisms of neurone interaction. Further studies established patterns of interconnections existing between various identifiable cells, particularly within the abdominal ganglion of Aplysia californica Cooper (Frazier et al., 1967; Kandel et al., 1967). The establishment of these relationships permitted the investigation of simple types of behaviour with respect to central neurone activity, for example the gill withdrawal mechanism of Aplysia californica (Castellucci et al., 1970; Kupfermann et al., 1970: Pinsker et al., 1970). More extensive studies have considered the role of fixed patterns of neural activity involved in various types of behaviour including

locomotion in Tritonia (Dorsett et al., 1973) and Aplysia fasciata Poriet (Weevers, 1971), and in feeding in A. californica (Kupfermann & Cohen, 1971). This type of study, however, has been restricted to relatively few species.

The lamellibranchs have provided various workers with the opportunity of investigating the neural mechanisms mediating simple behavioural activities, notably the siphon withdrawal reflex of Mya (Horridge, 1958; Mellon, 1965) and of the surf-clam Spisula solidissima (Dillwyn)(Prior, 1970, 1972a, 1972b).

The presence of peripheral reflexes has been long established in the molluscs (reviewed by Bullock & Horridge, 1965.) but the neural elements mediating such responses have rarely been investigated. Prior (1972a) was able to show that the withdrawal reflexes of the isolated siphons from Spisula solidissima were mediated by efferent activity from groups of neurones found at the branching points of the siphonal nerves. A complex instance of peripheral integration has been demonstrated in the cephalopods where it has been shown that compensation for loading of the arms occurred peripherally without central intervention, thus the animals were unable to distinguish between objects of different masses (Wells, 1961).

There have been comparatively few investigations of the peripheral nervous system in gastropods, and, with the exception of photic receptors, these included few detailed accounts of peripheral nervous elements. Attempts were made to classify the numerous types of nerve endings that were shown to be present in the molluscan body wall by early histologists, but such classifications were not substantiated with physiological evidence. (Schulz, 1938).

A study of tactile receptors in the mantle of Octopus was made by Gray (1960) and mechanoreceptors were reported in the mantle collar of Archachatina (Nisbet 1961). Both tactile and stretch receptors found in the body wall of the prosobranch Buccinum undatum L., were investigated by Laverack & Bailey (1963) while De Vlieger (1968) made an extensive analysis of tactile receptors found in the lips of Lymnaea stagnalis (L.). More recently a comprehensive study of mechanosensitive units in the body wall of L.stagnalis revealed a variety of primary and higher order neurones (Janse, 1974). The results of these and other investigations indicated the complexity of types of receptors to be found in the molluscan body wall. This has been confirmed by electron microscope studies of molluscan epithelia; Crisp (1971) made a comparative survey of receptors in the epithelium of Nassarius reticulatus (L.)and several other species of prosobranchs and in a further study of the epidermal sensory cells of L.stagnalis, Zylstra (1972) reported six classes of nerve endings five of which were ciliated. Other similar investigations have been restricted to the examination of specific sensory areas of epithelium including the rhinophores of nudibranchs (Storch & Welsch, 1969) and the palps of the lamellibranch Cardium edule L. (Barber & Wright, 1969).

In certain instances an attempt was made to trace functional pathways from the peripheral nervous system (P.N.S.) through the C.N.S. by stimulating sensory neurones as in Ariolimax columbianus (Gould) (Turner & Nevius, 1951) and Mya (Horridge, 1958). De Vlieger (1968) combined a similar study of L.stagnalis with a detailed investigation of the properties of sensory neurones in the lip preparation and concluded that there were specific pathways in the C.N.S. associated with particular

behavioural responses.

More recent investigations of gastropod neurophysiology used previously ignored species but the prosobranchs have remained largely ignored. The best known prosobranch is probably the common whelk Buccinum undatum as it has been the subject of neurophysiological studies concerned with the function of its osphradium and other aspects of its neural organization. (Laverack & Bailey, 1963: Bailey & Laverack, 1963: Bailey, 1965). Also some aspects of axonal conduction have been investigated in the freshwater prosobranch Viviparus contectus (Millet) (Sattelle, 1972, 1973). The family Littorinidae has been ignored from the electrophysiological viewpoint although Littorina littorea, the subject of the present study, has been the object of neurosecretory studies (Williams, 1970b: Williams & Al'Kufaishi, 1970: Al 'Kufaishi, 1970) and has also had various morphological aspects of its nervous system described (Leyon, 1947; Fretter & Graham, 1962; Newell, 1965).

The earliest studies of L.littorea included attempts to explain the distribution of the animal on the seashore in terms of its behaviour. Mitsukuri (1901) considered the animal's phototaxic responses in relation to tidal movements while later investigations were concerned with the animal's responses to light and gravity, but these gave contradictory results. The work was reviewed and furthered by Newell (1958a) who confirmed the observations of Gowanlock & Hayes (1926) and Moore (1937) that individuals tended to remain in restricted areas of a particular habitat. A more detailed investigation of natural and laboratory behaviour (Newell, 1958b) demonstrated that

the behaviour of particular specimens, during their feeding excursions, was dependent on the plane of the environment and whether the animal was light-or dark-adapted.

Further studies of phototaxic responses suggested that the eye of Littorina was capable of detecting the plane of vibration of polarized light (Charles, 1961). An examination of optical properties of the eye led Newell (1965) to conclude that it was capable of image formation in air and probably in water, while the receptor density of the retinal mosaic was calculated to be sufficiently high for the interpretation of retinal images. The visual properties of this type of eye were thought adequate to explain visual reactions of Littorina punctata (Philippi) (Evans, 1961).

The musculature of the buccal mass of L.littorea has been described (Johannson, 1939) and a histological investigation has been made of the digestive system (Jenkins, 1955). More recently some aspects of respiration have been studied including variations of oxygen consumption with temperature change, thermal acclimation and seasonal variations (Newell & Pye, 1970a, 1970b, 1971). Rumsey (1973) determined the effects on the animal's internal fluids by varying ionic concentrations in the external medium.

The growth rate of L.littorea was measured in terms of shell height by Moore (1937) and later an analysis was made of variations in the animal's biochemical composition with season (Williams, 1970a). The later study demonstrated that during active growth in the summer months, reserves of lipid and carbohydrate were accumulated to be utilized in the non-feeding winter months when gonad maturation was occurring. A

further study of Williams & Al'Kufaishi (1970) indicated that the supraintestinal and left pleural ganglia may exert a controlling influence on metabolism. A neurosecretory function was suggested for the ganglia after their reactions to neurosecretory stains had been studied.

Investigations of neurosecretory activity (Williams & Al'Kufaishi, 1970; Al'Kufaishi, 1970) revealed seasonal changes in possible neurosecretory cells in the pleural and parietal (intestinal) ganglia. All the ganglia except the pedal and buccal ganglia showed possible neurosecretory activity and evidence was presented for a flow of neurosecretory material from the right pleural ganglion and the supraoesophageal (supraintestinal) ganglion to the visceral ganglion.

In the present study the organization of the nervous system in Littorina has been studied by the investigation of the peripheral sensory elements and central pathways that, when stimulated, initiate a simple behavioural reflex, the withdrawal response. Supplementary studies of the general morphology and histological appearance of the nervous system have been included. The structures of the tentacles and columellar muscle have also been described and some limited studies of conduction velocity and spontaneous central nervous activity have been made. The results, and methods by which they were obtained, are described in the succeeding sections.

2.0 METHODS AND MATERIALS.

2.1 ANIMALS AND STORAGE.

Supplies of animals were obtained from the University Marine Biological Station, Millport, Isle of Cumbrae, Scotland. They were dispatched by passenger train and survived the journey without injury.

A stock of animals was stored in a marine aquarium containing natural sea water which was constantly aerated and filtered while its temperature was maintained at $10^{o}C$. The animals were fed occasionally with a small quantity of wrack and it was found that they survived without injury for many months, although they were usually used for experimental purposes within two or three weeks of arrival.

The animals used during the course of the experiments had a shell height in excess of 18mm. corresponding to a minimum age of about two years (Moore, 1937).

Approximately 550 animals were used in the experimental procedures.

2.2 DRUGS AND SOLUTIONS.

Natural sea water was collected from the English Channel and was used both in the aquarium and as a physiological saline for bathing the various preparations.

In some experiments a synapse blocking agent was required and this was either $10^{-5}M$. O-acetylcholine sea water solution or an artificial seawater in which the normal complement of calcium chloride had been replaced by magnesium chloride. The seawater solution was prepared according to data calculated by Barnes (1954) and contained a final magnesium

chloride concentration of 63mM/L. (see appendix 1).

In the early stages of the investigation attempts were made to find an effective anaesthetic for L.littorea. A variety of substances, both alone and in combination, were tested including Nembutal, MS222, menthol and magnesium chloride solution, as described by Runham (Runham et al., 1965). Of these only 7.4% $MgCl_2$ solution produced complete and reversible relaxation but only after immersion for several hours, even with de-shelled animals. It was found that with practice, however, prior relaxation was unnecessary for the dissection of the electrophysiological preparations, thus the possibly adverse pharmacological effects of anaesthetics were avoided.

2.3 RECORDING EQUIPMENT.

2.31 Recording Chamber.

For recording purposes all preparations were mounted in a water cooled perspex chamber (Fig.1) which was made in the Department of Zoology with the help of Mr. G. Rentmore, who also helped in the manufacture of other items of equipment. The preparations were fastened with fine entomological pins onto a layer of wax or Plasticene which was mounted on a perspex disc and which could be clamped to the bottom of the chamber.

A thermostatically controlled water circulator passed a continuous flow of water through the inner cavity of the chamber and maintained the temperature of the outer chamber at $10^{\circ}C$. The temperature in the chamber was monitored by a thermistor and calibrated bridge circuit.

2.32 Suction electrodes.

The electrical activity of nerve trunks was

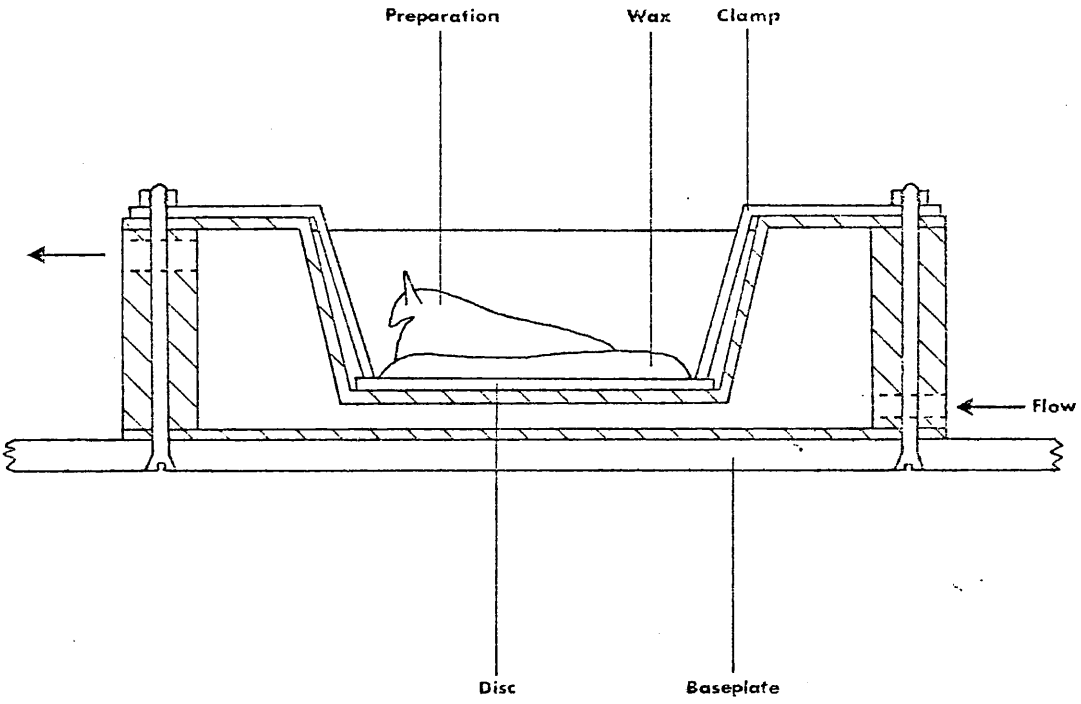


Figure 1. The recording chamber. All electrophysiological preparations were mounted in a water cooled chamber that was maintained at 10°C. Water was circulated through the inner cavity while the preparations were pinned onto a layer of wax attached to a perspex disc that could be clamped into the outer recording chamber.

monitored extracellularly usually with glass suction electrodes as these proved more convenient than wire hook electrodes because of the relative shortness of the nerves found in L.littorea. The electrodes were applied either to the cut ends of nerves, after the pipette lumen had been carefully sized to form a close fit with the nerve, or to an intact nerve, a loop of which was taken up into the lumen of the electrode, while 'en passant' recordings were made. The electrodes contained a fine silver wire which made contact with the solution bathing the nerve and potentials were recorded between this and a large diameter silver bath electrode which was also connected to earth.

2.33 Wire electrodes.

In certain experiments extracellular recordings were made with pairs of finely tapered silver wire hook electrodes mounted on perspex rod. Similar electrodes were also used to deliver electrical stimuli to the nerves.

2.34 Metal filled micropipettes.

An attempt was made to prepare metal filled micropipettes using an alloy of indium and Wood's metal, in order to facilitate extra cellular recording from short, intact connectives. A modification of the technique described by Renkin (1964) was utilized but results were handicapped by the lack of a suitable electrode puller. Attempts to fill commercially available micropipettes produced electrodes with widely variable tip diameters and impedances and their use was abandoned.

2.35 EMG. electrodes.

Extracellular muscle potentials were recorded with either electrolytically sharpened stainless steel needles insulated to within 25 microns of the tip and of high impedance, or fine pairs of silver wire electrodes, one electrode of each pair being insulated almost to its tip.

All types of electrode were mounted in micromanipulators by the use of suitable holders for both recording and stimulating purposes.

2.36 Signal amplification and display.

The electrical activity monitored in the nerves was amplified by R.C. coupled differential amplifiers (Tektronix Type 122) of gain X100 or X1,000. The high and low pass filters of the amplifiers were adjusted to the points of 3dB. attenuation at 0.8Hz. and 1,000Hz. respectively, in order to limit the recording bandwidth and to reduce the total noise level to about 12 microvolts. Occasionally a more restricted bandwidth was used to improve the signal to noise ratio. In order to eliminate mains frequency interference all recordings were made inside an electrically screened cage. Three amplifiers were available: thus simultaneous recording from three suction electrodes was possible.

The outputs of the amplifiers were passed into a switch box 'S' (fig.2) and then to a six channel oscillograph (Shandon Southern Instruments Type 650). The switch box enabled the output of a particular amplifier to be connected to one channel of a dual beam oscilloscope (Tektronix 502A) and to be monitored simultaneously by a slave oscilloscope and audio monitor.

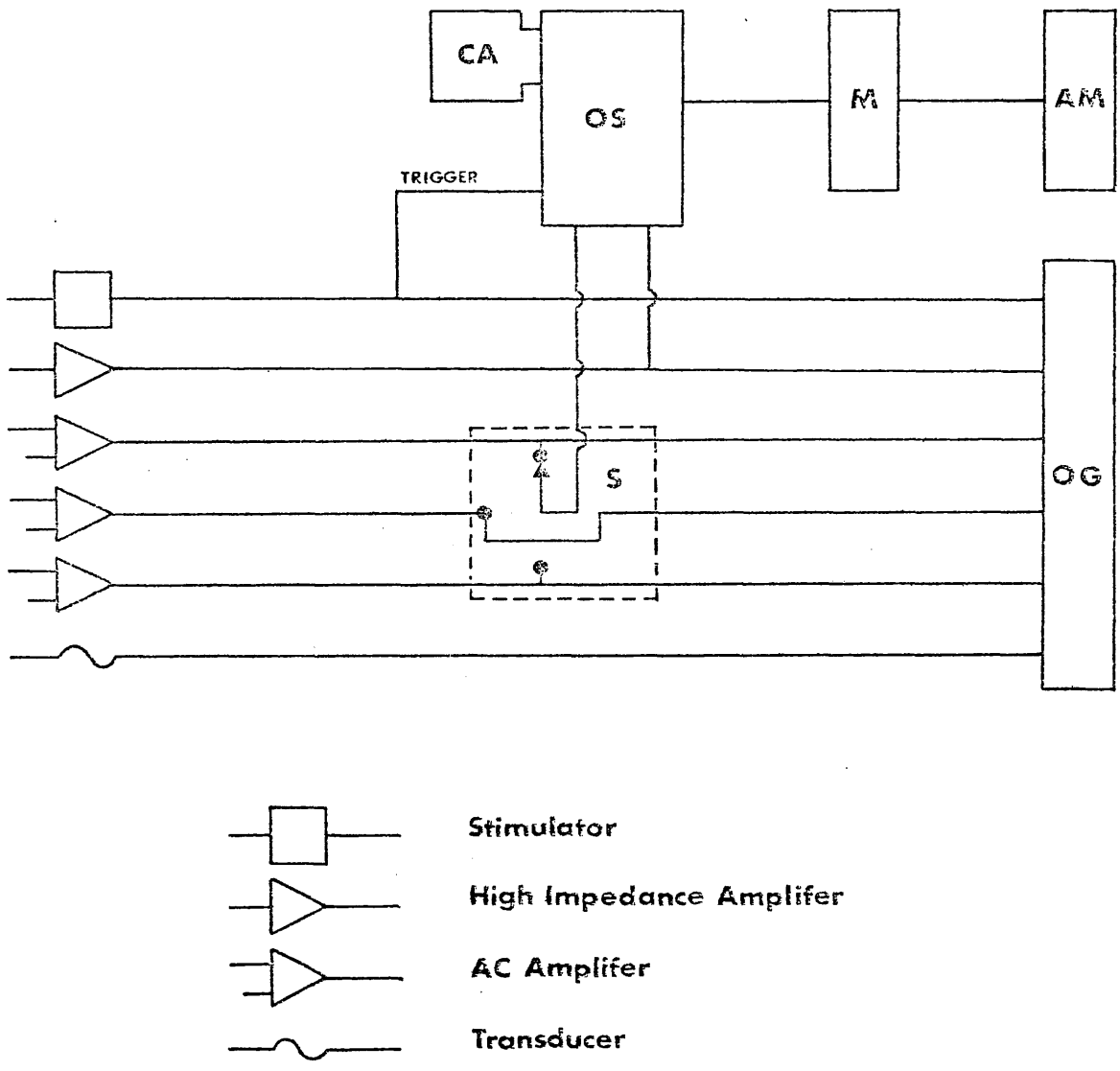


Figure 2. Diagram of the experimental system. AM, audio monitor; CA, camera: M, monitor oscilloscope; OG, oscillograph: OS. oscilloscope; S, switch box. The switch box enabled the output of either of the A.C. amplifiers to be displayed on one beam of the oscilloscope. A Grass SD9 stimulator (not shown) was occasionally incorporated.

The activity recorded by high impedance electrodes was connected via an operational amplifier, input impedance 10^{11} ohms, to a band pass amplifier of variable gain (F. Haer & Co.). This system was used for recording electromyograms (EMGs) while muscular contraction was recorded with a triode type movement transducer (R.C.A. Type 5734) and associated circuitry.

The output of the high impedance system was passed directly to the oscillograph and also, if required, to the other beam of the oscilloscope. A similar arrangement existed for displaying the output of the movement transducer.

Permanent records were made either photographically from the oscilloscope with the aid of a motorised oscilloscope camera, or by recording on U.V. light sensitive paper (Kodak Linagraph 1895) in the oscillograph.

2.4 STIMULATION.

Two types of stimulation were used in the course of the experiments; either electrical or, more frequently, mechanical.

2.41 Electrical stimulation.

Electrical stimuli of varying voltage and duration, were delivered to the nerves from a Grass SD9 stimulator by either a pair of fine wire hook electrodes, or a suction electrode and an indifferent electrode inserted into a convenient part of the preparation. In an attempt to ensure stimulus isolation the nerves, when of sufficient length, were laid across pairs of hock electrodes and then lifted into a layer of paraffin oil floated over the bathing fluid

in the recording chamber. It was found. however, that they rapidly became translucent and refractory and similar effects were observed when the preparations were bathed in isotonic sucrose solution.

The use of suction electrodes for stimulation was partially successful, but, because of the small size of the preparations, it was difficult to ensure that they were not subject to stimulation by extraneous currents. To eliminate such possibilities mechanical stimulation was used whenever possible.

2.42 Mechanical stimulation.

The various preparations were stimulated mechanically by either probing the tissues manually with a fine ball ended glass rod or by delivering stimuli from a remotely controlled electromechanical stimulator.

The stimulator (fig.3) was adapted from the central drive-unit of a 4" diameter loudspeaker. The unit was detached from the frame and cone of the speaker and a length of $\frac{1}{4}$" diameter brass rod was attached to the unit's supporting bracket. A short length of 1/16" diameter perspex rod was mounted, with an epoxy adhesive, perpendicularly at the centre of the drive unit and a natural bristle of approximately 0.23mm. diameter was mounted at the free end of the rod. The tip of the bristle was cut obliquely to form a fine point or cut squarely to form a blunt probe with an area of approximately 0.04 sq.mm.

The stimulator was advanced by applying across its coil D.C. pulses that were derived from a simple control circuit consisting of six volt battery connected to a 150 ohm potentiometer, which acted as a potential divider, and a spring-

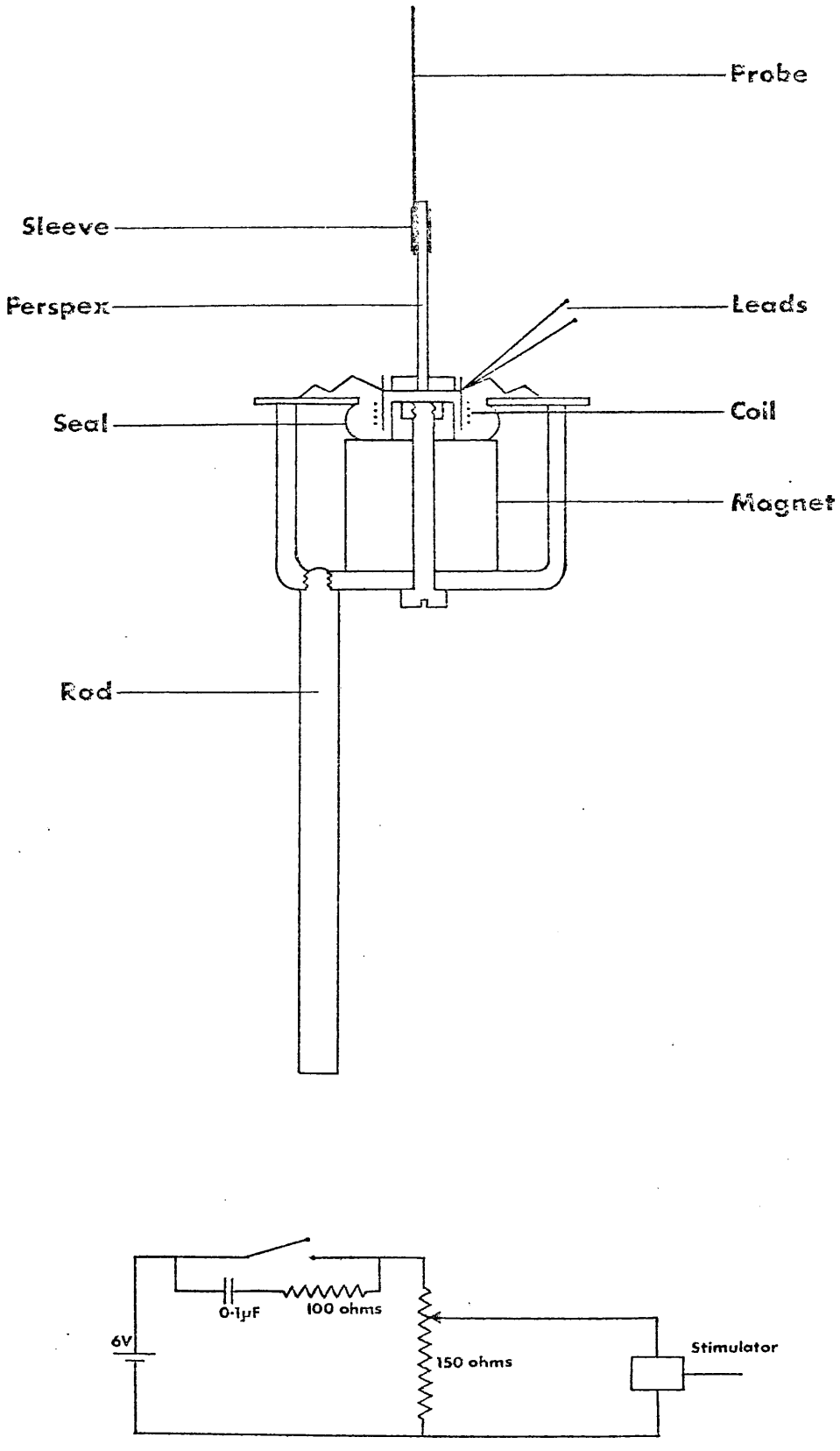


Figure 3. The mechanical stimulator and control circuit. The device was adapted from a loudspeaker and advanced by the application of D.C. pulses across its coil. See text for further details.

action toggle switch. A 100 ohm resistor and 0.1 microfarad capacitor were connected in series across the switch contacts to suppress switching transients.

The device was calibrated by measuring the voltage across the speaker coil and the corresponding tip displacement for various settings of the potentiometer for which a suitable scale of settings was devised. As the device was calibrated in terms of tip displacement and coil voltage a convenient method of recording stimulus intensity was to display the coil voltage on one channel of the oscilloscope. This voltage was also used as a stimulus marker, or alternatively to synchronize the oscilloscope sweep with stimulus application.

It was found that precise tip movements from 0.040mm to 0.30mm were possible but at increasing displacements the probe tended to run off axis. In most experiments three levels of stimulus intensity were used, gentle, medium and high corresponding to tip displacements of 0.05mm, 0.15mm, and 0.30mm respectively.

The duration of the stimulus was controlled by the period of closure of the toggle switch contacts.

Other types of stimulator that have been used in similar investigations (Pringle, 1953: Bailey, 1965) have been fitted with fine nylon probes. When similar probes were used in conjunction with the present device it was found that the probe tip vibrated laterally when it was advanced so causing multiple stimulation of the preparation. This vibration was eliminated by the use of the relatively thick bristle.

The stimulator was convenient as it could be controlled remotely and did not require manual resetting. The low inertia of the moving parts permitted a short response time and the

accurate reproduction of similar stimuli. At high stimulus intensity a small amount of 'ringing' was present at the onset and offset of the coil voltage, but it was found that this was insignificant at the levels of stimulation at which it occurred.

Although the device could be described as a tactile stimulator the description would only be valid for low intensity stimulation as stimulation at higher levels resulted in relatively large distortion of the tissues.
Therefore all the responses elicited by this mode of stimulation have been termed mechanosensitive as distinction between true tactile responses and those elicited from other types of sensory neurones was not possible.

2.43 Artefacts.

The small sizes of the preparations and the low level of responses recorded from them made it necessary to ensure that stimulus artefacts were not generated by the recording and stimulating systems. Therefore a series of experiments was performed in which preparations, killed by immersion in 70% alcohol, were substituted for normal preparations. It was found that no detectable artefacts were generated at any of the stimulus intensities utilized in normal experiments.

2.5 DISSECTION.

To permit dissection the animals' shells were cracked by the application of controlled pressure in a small bench vice. The shell fragments were removed and the columellar muscle was detached from its point of insertion on the shell.

Figure 4. The dissection of Littorina littorea.

The animal is shown after the mantle and bodywall had been dissected along their midlines. In latter stages the buccal mass and associated structures were removed to expose the remaining central ganglia.

BM - buccal mass.

BG - buccal ganglion.

CBC - cerebrobuccal connective.

CG - cerebral ganglion.

CT - ctenidium.

HG - hypobranchial gland.

R - rectum.

SD - salivary duct.

SG - salivary gland.

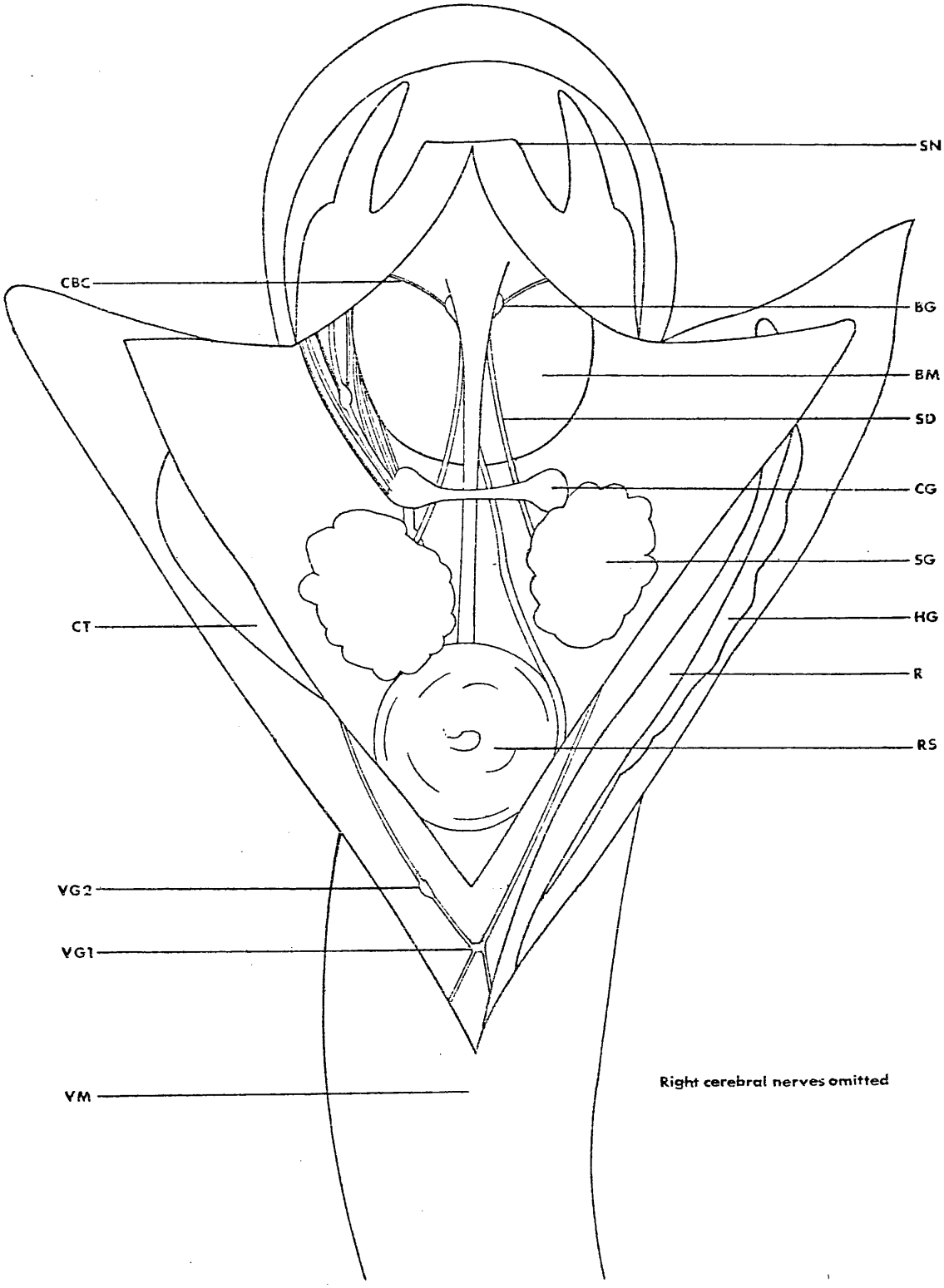
SN - snout.

RS - radula sac.

VG1 - larger visceral ganglion.

VG2 - smaller visceral ganglion.

VM - visceral mass.


SN
CBC
BG
BM
SD
CG
SG
CT
HG
R
RS
VG2
VG1
VM
Right cerebral nerves omitted

The animals were pinned out in an extended position by passing fine entomological pins through the operculum margins and the visceral mass. The mounted specimens were usually bathed in sea water and the dissection performed with the aid of a steroscopic dissecting microscope.

The first stage of the operation was to remove the mantle. Its mid-dorsal margin was lifted with fine forceps and a dorso-longitudinal incision was made and extended posteriorly as far as possible. The mantle and associated organs was either pinned out on either side of the animal, or removed completely by making incisions along its lines of insertions into the body wall on each side of the animal. The procedure exposed the visceral ganglia which could be observed lying beneath the comparatively transparent region of body wall immediately anterior to the visceral mass.

An incision was made in the head to expose the buccal mass by first lifting a fold of skin on its mid-dorsal surface between the tentacles. The fold was cut with iridectomy scissors and the opening extended towards the tip of the snout and as far backwards along the midline as possible. The sides of the incision were pulled apart to reveal the buccal mass and associated structures. (fig.4). At this stage the buccal ganglia could be observed protruding from beneath the oesophagus at either side of the buccal mass while the cerebral and pleural ganglia were positioned immediately to the posterior of the buccal mass.

The buccal mass was removed by first cutting its connections to the oesophagus, the salivary ducts and radula sac, which pass through the nerve ring formed by the cerebral and pedal commissures and connectives. The posterior margin

of the buccal mass was lifted clear of the body wall and its anterior attachments including the cerebrobuccal connectives, were cut.

The oesophagus was pulled from behind, through the cerebropedal nerve ring together with the radula and salivary glands. The intestine was cut near to the viceral mass and gently pulled from beneath the supraintestinal connective towards the anterior of the animal after any strands of connective tissue had been broken. The freed gut could now be lifted clear of the animal.

To expose the cerebral ganglia and nerves the connective tissue attached to the ganglia was removed and further cuts were made in the body wall. These were made behind the tentacles and at right angles to the longitudinal incision so forming flaps of body wall that could be pulled aside and pinned down on either side of the animal.

The intestinal ganglia were exposed by following the relevant connective from either pleural ganglion to the point at which it entered the body wall and then carefully removing the overlying tissue until the ganglia were exposed. The visceral loop arising from the intestinal ganglia was similarly exposed.

As the pedal ganglia lie beneath the buccal mass retractor muscles embedded in the upper layers of the pedal musculature, it was difficult to expose them without causing damage to the pedal nerves. The ganglia could be observed, however, after the muscles had been cut at their anterior ends and pulled backwards through the cerebropedal loop.

2.51. Preparations.

In the course of the experiments three main types

of preparation were used but occasional modifications were introduced and these have been described in the relevant sections of the results.

2.52. Tentacle preparation.

The innervation and tactile responses of the tentacles were studied in isolated tentacle preparations consisting of the tentacle and its still connected tentacle and cerebral ganglia (fig.5).

The animals were dissected to expose the cerebral ganglia and nerves; any connective tissue around the ganglia was removed and all the cerebral nerves and connectives, with the exception of the tentacle nerves (C4), were severed so isolating the ganglia from the rest of the nervous system. An incision was made around the base of the tentacle and it was lifted from the body together with its attached cerebral and tentacle ganglia.

The preparations were mounted in the recording chamber by passing pins through the posterior margin of the tentacle so immobilizing its base while leaving the tip free to move. It was unnecessary to pin out the nerves and ganglia but great care was exercised to ensure that they were not damaged during the arrangement of the preparations.

2.53. The C.N.S. preparation.

In order to investigate inter-ganglionic pathways an isolated C.N.S. preparation was devised which consisted of the tentacles, cerebral, pleural and pedal ganglia, and their interconnections (fig.6).

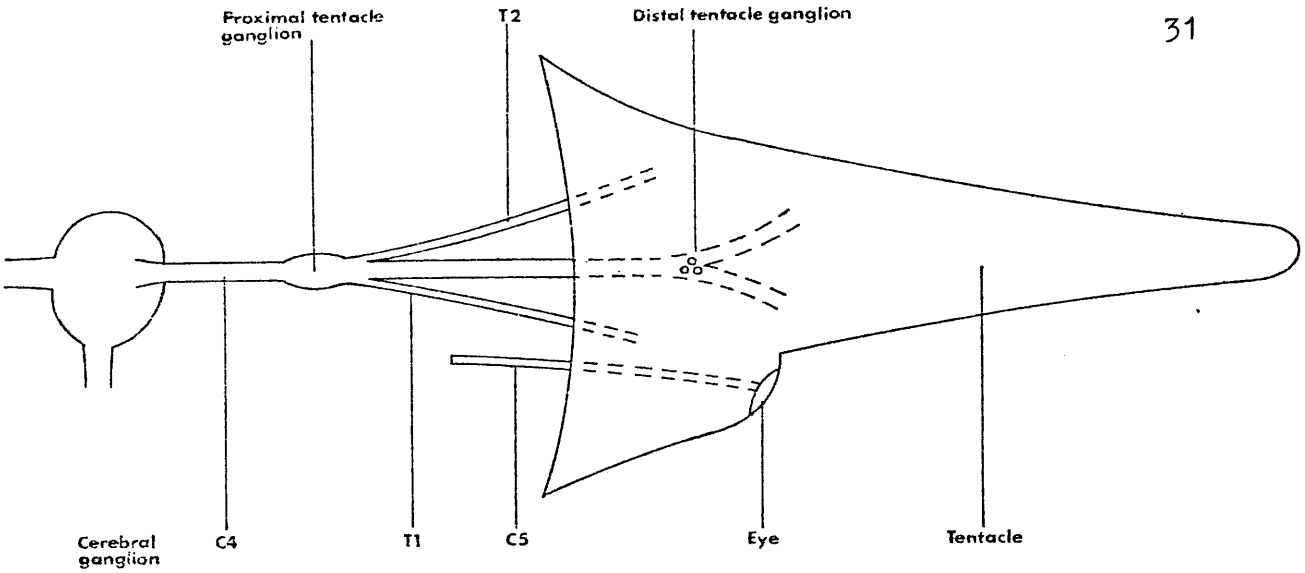


Fig.5

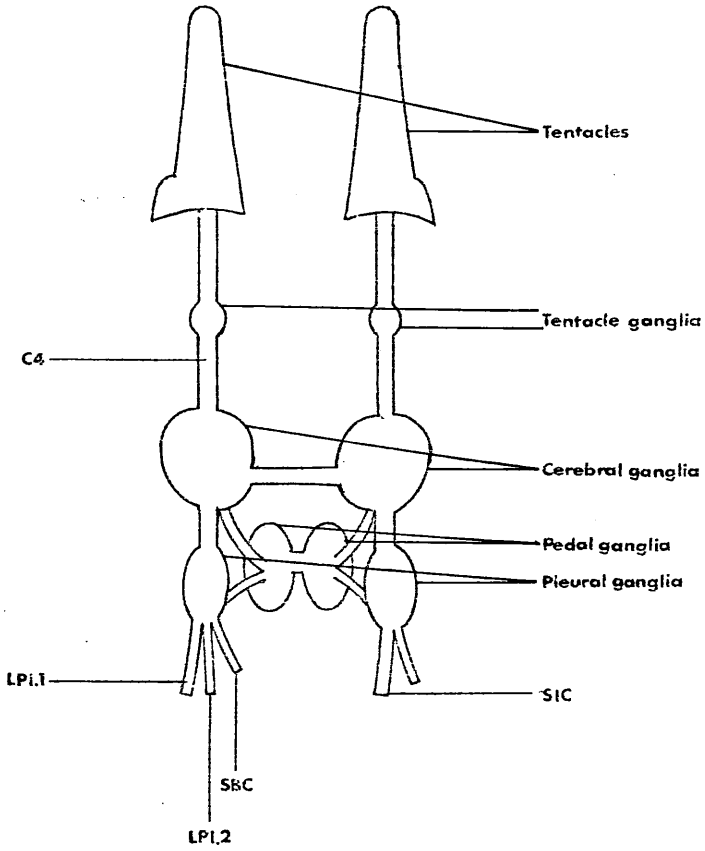


Fig.6

Figure 5. The tentacle preparation consisting of an isolated tentacle together with its tentacle and cerebral ganglia.

Figure 6. The C.N.S. preparation shown diagramatically. This was used in investigations of central sensory pathways and consisted of the tentacles, cerebral, pleural and pedal ganglia together with their respective interconnections.

The ganglia were exposed and, with the exception of the two tentacle nerves (C4), all their peripheral nerves were cut. Any connective tissue surrounding the ganglia was removed and the two tentacles were isolated from the body wall while their nerves were left intact. The tentacles and ganglia were lifted away from the animal and arranged in the recording chamber.

This type of preparation was also used during studies of spontaneous activity generated in the central ganglia.

2.54 The Columellar preparation.

In early experiments an attempt was made to investigate the innervation and activity of the columellar muscle by stimulating the columellar nerve (LPl.2) electrically. However, because of the problems of stimulus isolation, a preparation which could be stimulated mechanically was used in later experiments. The preparation (fig.7) consisted of the head and foot region of the animal separated, except for nervous connections, from the columellar muscle and visceral mass.

The animals were dissected to expose the cerebral and pleural nerves and a lesion was made to separate the foot and the columellar muscle while leaving the pleural nerves and connectives intact. The preparations were carefully pinned down so that movements of the foot and head were isolated from the columellar muscle. In the experiments designed to establish the columellar muscle innervation the visceral mass and visceral loop were left in situ, while in later experiments concerned with recording muscle contraction, the visceral mass was removed in order to expose completely

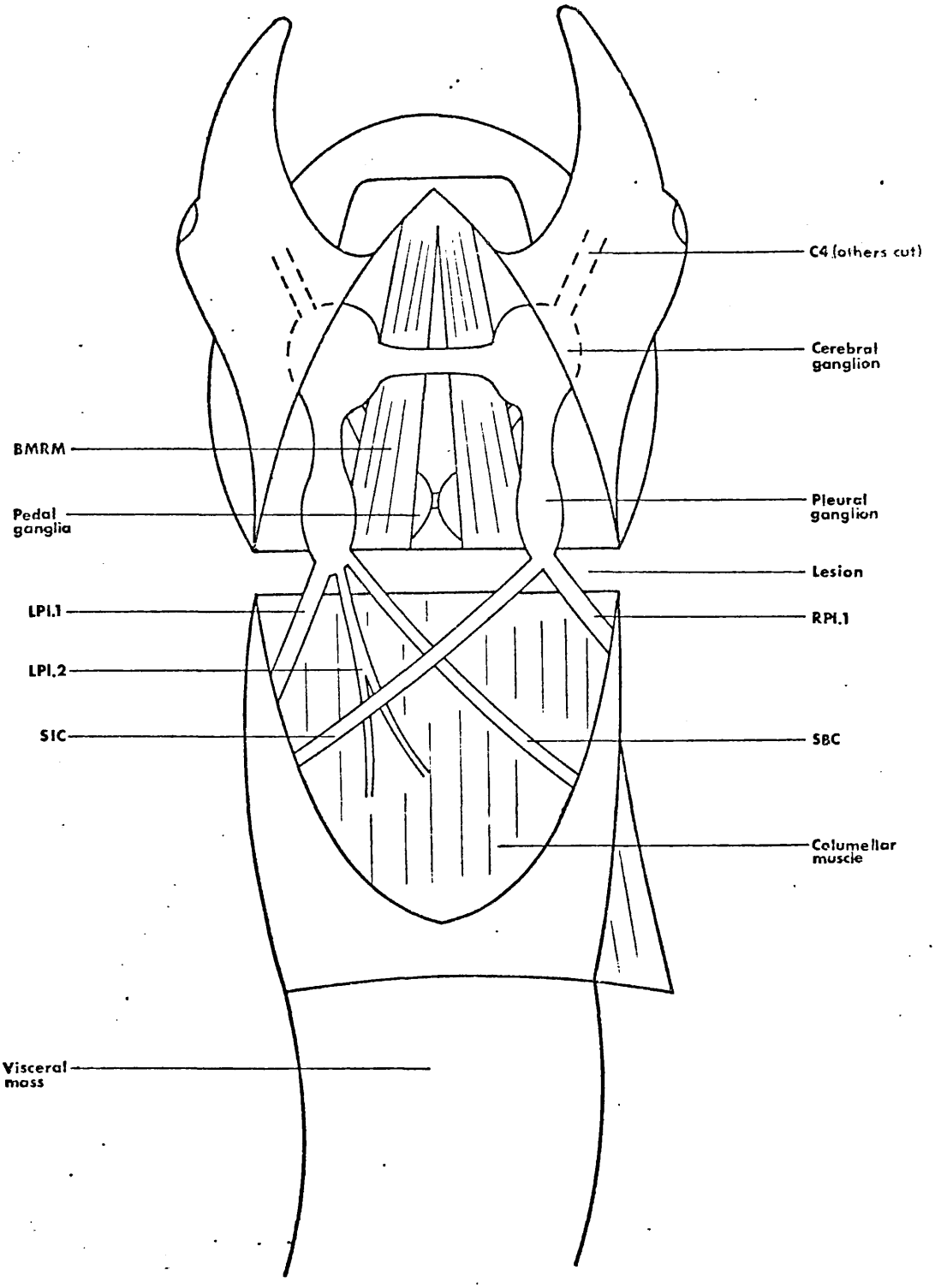


Figure 7. The columellar preparation shown diagramatically. The preparations were used for the study of columellar innervation and, in a modified form (see text), muscle activity. BMRM, buccal mass retractor muscles; SIC supra-intestinal connective: SBC, subintestinal connective.

the muscle.

It had been hoped to use the shell fragments normally left attached to the columellar muscle after deshelling, to anchor its posterior margins while the anterior of the muscle was pinned. Unfortunately the fragments were not a dependable means of attachment, therefore the muscle was pinned by its posterior lateral margins after the shell fragments had been removed and the muscle permitted to relax.

The contractions of the muscle were recorded isotonically by a transducer. A fine needle was attached to its movable anode and the device supported vertically with the needle point inserted into the centre of the posterior margin of the muscle which, despite the pinning of its lateral margins, remained relatively free to contract.

2.6 BEHAVIOURAL OBSERVATIONS.

The behavioural study was made from animals that were allowed to move freely in a large flat bottomed dish containing approximately $\frac{1}{4}$" depth of seawater. The dish was illuminated by diffuse light and the animals' reactions to manual stimulation of their tentacles were observed.

In further experiments the course of the withdrawal response was monitored by a movement transducer, as shown in figure 8. A length of 20 s.w.g. wire was tightly wound around the shell immediately behind the opercular opening. About 2 cms. of wire was left extending from the dorsal surface of the shell and adjusted so that it was perpendicular when the animal was crawling. The wire was connected by a length of fine flexible tubing to a lever mounted on the anode of the horizontally arranged movement transducer.

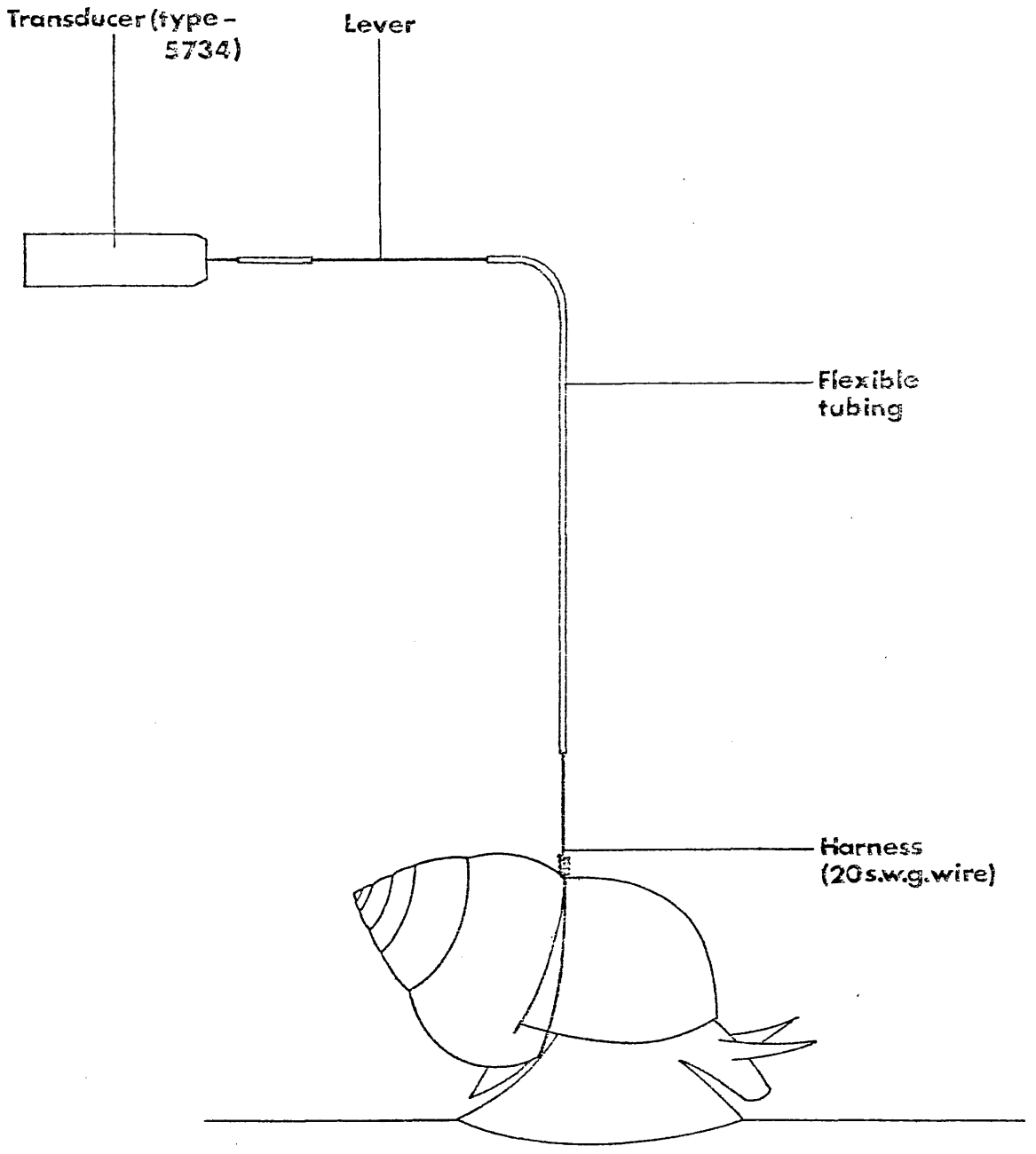


Figure 8. The behavioural recording system. In order to record behavioural responses the animals were fastened into a simple wire harness that was in turn, connected to the anode of a movement transducer by a length of flexible tubing and a lever.

The transducer was held in a micromanipulator so that it was possible to keep the link between it and the animal vertical after the animal was placed in the observation dish and had started to crawl. Again the animals were stimulated manually.

2.7 THE SENSORY INNERVATION AREAS OF THE CEREBRAL NERVES.

Those areas of the head and anterior parts of the foot that had mechanosensitive neurones linked to the various cerebral nerves were determined. The animals were dissected to expose the cerebral nerves and, in turn, each nerve was cut, and its peripheral stump taken up into a suction electrode. The foreparts of the animals were stimulated manually, and the areas from which responses were invoked in each of the nerves were noted. The procedure was repeated for the nerves of both cerebral ganglia of each specimen that was investigated.

2.8 DETERMINATION OF CONDUCTION VELOCITY.

A simple stimulating and recording arrangement was used for determining conduction velocity in the longest unbranched connective, the supraintestinal connective, and for limited studies of other nerves.

After dissection the connective was placed across two pairs of fine silver wire hook electrodes that were separated by a relatively large electrode connected to earth. Electrical stimuli were applied to the electrodes at the central end of the connective while action potentials were recorded from the distal pair of electrodes. The earth electrode served to shunt stimulus artefacts away from the recording electrodes. Both pairs of electrodes were mounted in micro-

manipulators and the distance between them was measured by utilizing the vernier calibrations on one or other of their arms.

The electrodes and connective were initially immersed in seawater but for recording purposes they were either lifted clear of the bathing fluid and recordings made in air, or occasionally the preparations were lifted into a layer of paraffin oil floated on the surface of the bathing fluid.

2.9 HISTOLOGICAL METHODS.

A histological study was made of the central ganglia, certain nerves and connectives, and the structure and innervation of the columellar muscle and tentacles.

The tissues were usually removed from animals that had been relaxed by immersion in 7.4% $MgCl_2$ solution for several hours before dissection. In some instances this was omitted, but all the tissues were fixed in Bouin's seawater fixative, before dehydration, clearing, and impregnation with paraffin wax.

The nerves and connectives of Littorina are contractile therefore those required for histological examination were slightly stretched prior to fixation. A similar problem was encountered with the tentacles as these also tended to contract on fixation even after relaxation. The relaxed tentacles were usually pinned out before fixation, but some were fixed without relaxation and pinning so that a comparison of their structure in extension and contraction could be made.

The innervation of the columellar muscle was established from specially prepared material. Animals were dissected to expose the nervous system and the visceral mass

was carefully removed. The anterior end of the muscle was separated from the foot and the nervous system anterior to the pleural ganglia was removed. A fine pin was pushed through each ganglion and they were supported in their normal positions relative to the muscle during fixation. Transverse serial sections were taken from the level of the pleural ganglia and continued along the entire length of the muscle.

2.91 General staining technique.

The prepared tissue was usually serially sectioned at 5 or 7.5 micron intervals and stained, for routine purposes in Mallory's Trichrome solution, although some sections of nerve were stained with a Malachite Green modification of Masson's Trichrome solution. (in Carleton's Histological Techniques, 1967).

2.92 Silver staining.

The silver impregnation of Palmgren (1948) was used for the treatment of thin serial sections of tentacle in an attempt to establish the presence of peripheral nervous elements.

2.93 Vital staining.

In the course of the general dissections of the nervous system difficulty was encountered in tracing some of the finer nerves: therefore a vital staining technique was employed (Alexandrowicz, 1960). The partially dissected animals were placed in a solution of 1:30,000 methylene blue in seawater and left for two to four hours before further dissection.

3.0 RESULTS

3.1 THE ANATOMY OF THE NERVOUS SYSTEM.

The morphology of the nervous system of L.littorea has been previously described in part, by Leyon (1947) who made a brief comparative study of the cerebral nerves of various prosobranchs, and in greater detail by Fretter & Graham (1962). Histological studies have been made of the receptors and neurones found in the eye and optic nerve of Littorina (Newell, 1965) and of neurosecretory activity in the nerves and ganglia (Al'Kufaishi, 1970; Williams & Al'Kufaishi, 1970).

The account of Fretter and Graham has been used as a basis for the present investigation, but it has been found to contain certain inaccuracies. The nomenclature of their account has been retained except that the previously termed oesophageal ganglia have been named intestinal ganglia in order to conform with later conventions (Bullock & Horridge, 1965).

The nervous system contains six main ganglia each of which is paired and deployed in the primitive prosobranch arrangement (fig. 9). Each ganglion is discrete and is separated from its neighbours by commissures or connectives. Each member of the paired buccal, cerebral, pedal, pleural and intestinal ganglia are approximately equal in size and share about the same relative positions within the body of the animal. The two visceral ganglia, however, show differences in relative size and position.

The interconnections between the ganglia and the main nerves arising from them are shown in figure 10, while their

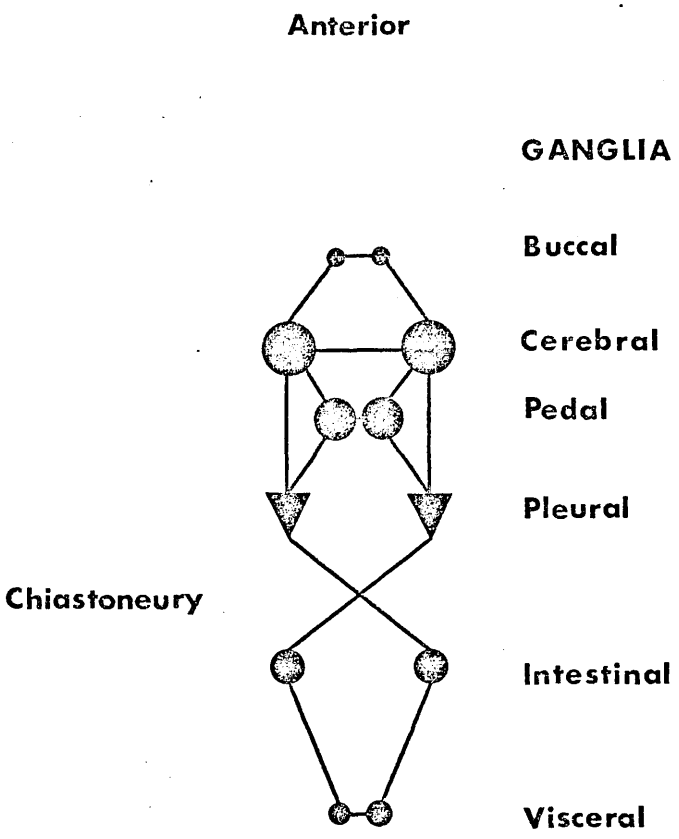
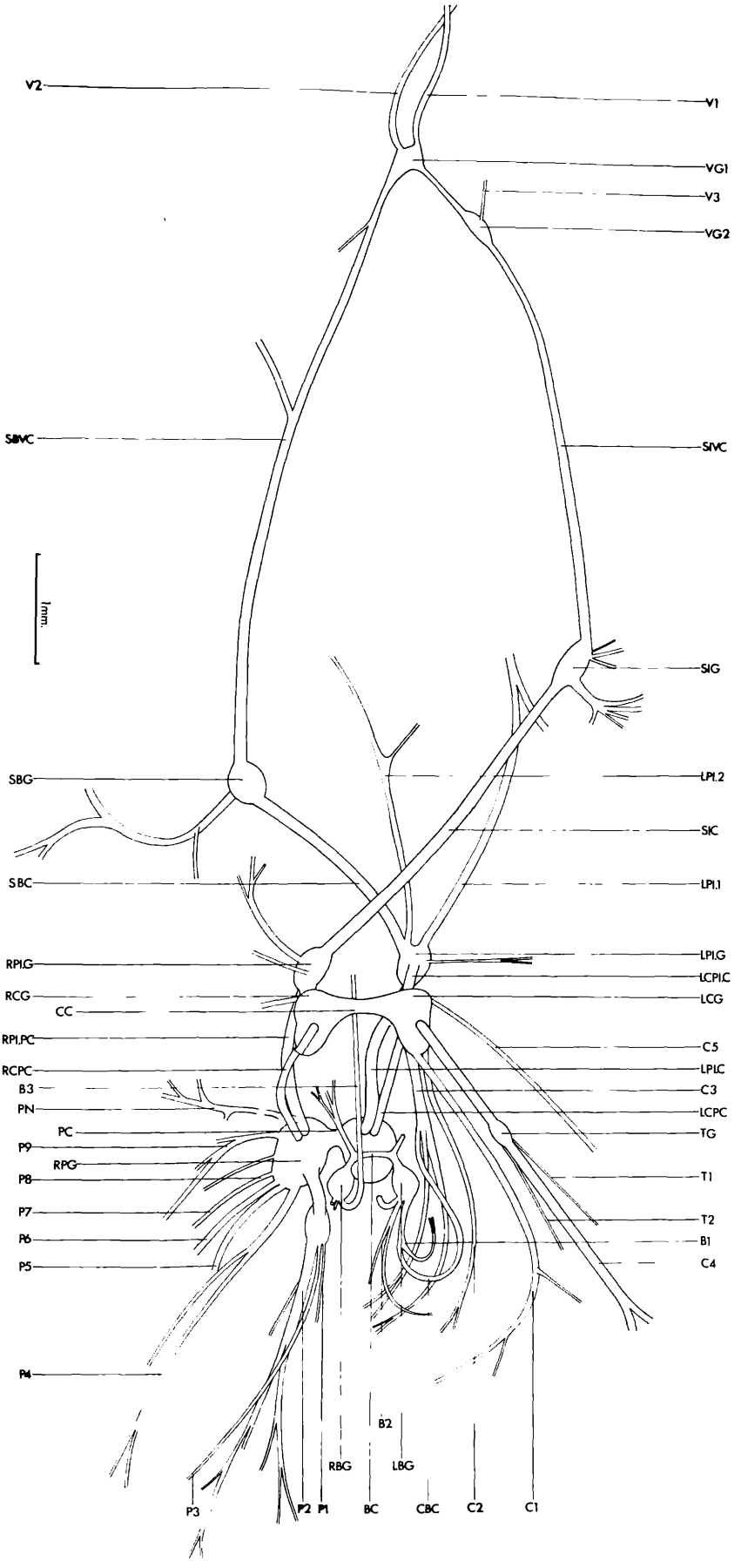


Figure 9. The basic arrangement of ganglia and their interconnections in the nervous system of Littorina littorea.

Figure 10. The nervous system of Littorina littorea; diagram was prepared from several dissections and it is shown in inverted orientation to Fig. 9. See following pages for Key.


V2
V1
VG1
V3
VG2
SIVC
SIVC
1mm.
SIG
SBG
LPI.2
SIC
SBC
LPI.1
RPI.G
LPI.G
LCPI.C
RCG
LCG
CC
RPI.PC
C5
RCPC
LPI.C
B3
C3
PN
LCPC
PC
TG
P9
RPG
T1
P8
P7
T2
P6
B1
P5
C4
P4
B2
RBG
LBG
P3
P2
P1
BC
CBC
C2
C1

Key to figure 10.

Ganglia.

RBG, LBG	right and left buccal ganglia.
RCG, LCG	right and left cerebral ganglia
RPG, LPG	right and left pedal ganglia
RPl.G, LPl.G	right and left pleural ganglia.
SBG	subintestinal ganglion
SIG	supraintestinal ganglion.
TG	tentacle ganglion (proximal).
VG1	visceral ganglion (larger).
VG2	visceral ganglion (smaller)

Commissures and connectives.

BC	buccal commissure.
CBC	cerebrobuccal connective.
CC	cerebral commissure.
PC	pedal commissure.
RCPC, LCPC	right and left cerebropedal connectives.
LCPl.C	left cerebropleural connective (right not shown)
RPl.PC, LPl.PC	right and left pleuropedal connectives.
SBC	subintestinal connective.
SIC	supraintestinal connective.
SBVC	subintestinal-visceral connective.
SIVC	supraintestinal-visceral connective (SBVC and SIVC form the visceral loop)

Nerves.

B1 - B3	buccal nerves
C1 - C5	cerebral nerves
C4	tentacle nerve.
P1 - P9	pedal nerve.

PN.	penis nerve.
LPl.1, LPl.2	left pleural nerves (LPl.2, columellar nerve).
T1, T2	tentacle nerves.
Vl - V3	Visceral nerves.

relative positions within the body of Littorina have been shown previously in figure 4.

The effects of torsion are apparent between the pleural and intestinal ganglia where crossing of the intestinal connectives, or chiastoneury, occurs. Fretter & Graham stated that a connection or dialyneury, existed between a nerve (LPl.1) arising from the left pleural ganglion and the supra-intestinal ganglion, but such a connection has not been confirmed in the present investigation.

The buccal ganglia (BG) are situated dorsally resting on the buccal mass with their commissure (BC) passing beneath the oesophagus. The cerebral ganglia (CG) lie posteriorly to the buccal mass with the cerebral commissure (CC) passing over the oesophagus. They are connected to the respective buccal ganglia by the left and right cerebrobuccal connectives (LCBC, RCBC) which lie on either side of the buccal mass. Slightly ventral to, and posterior to, the cerebral ganglia are the pleural ganglia (Pl.G); these are connected to the cerebral ganglia by the short left and right cerebropleural connectives (LCPl.C, RCPl.C) respectively. A pleural commissure is not present, while both the cerebral and pleural ganglia are connected to the ventrally placed pedal ganglia (P.G) by the left and right cerebropedal connectives (LCPC & RCPC) and the left and right pleuropedal connectives (LPl.PC & RPl.PC). The pedal ganglia are separated by a short commissure and lie embedded in the upper layers of the foot musculature which is beneath the oesophagus and the buccal mass retractor muscles. Fretter and Graham reported a second para-pedal commissure linking the two ganglia, but this has not been found in every animal examined.

The pleural ganglia give rise to the intestinal connectives: the subintestinal connective (SBC) passes from the left pleural ganglion beneath the intestine to the right or subintestinal ganglion (SBG), while the supraintestinal connective (SPC) passes over the intestine to the left or supraintestinal ganglion (SIG). The two intestinal ganglia are embedded in their own side of the body wall, a little behind the point of insertion of the mantle collar.

The visceral loop extends posteriorly from the intestinal ganglia to the two visceral ganglia (VG) situated just anterior to the visceral mass. The ganglia usually appear as two unequally sized masses separated by short commissure although they were previously described by Fretter & Graham as being represented by a single mass. The larger ganglion (VG1) is situated near the margin of the visceral mass while the smaller ganglion (VG2) is displaced anteriorly and to the left of VG1.

The nerves arising from the ganglia were traced as far as possible by dissection and the results largely confirmed the previous descriptions with the exception of the columellar muscle innervation, therefore the overall distribution of nerves has been described only where necessary.

The cerebral ganglia each give rise to five major nerves two of which innervate the single tentacle found on either side of the head. Each main tentacle nerve (C4) bears a small tentacle ganglion from which two or occassionally three thin nerves (T_1 & T2) arise, and which is situated at about the midpoint between the tentacle base and the cerebral ganglion. The tentacle nerve C4 was found to branch repeatedly after entering the tentacle and a further ganglionic region was

found at the site of a major nerve junction near the tentacle base. The two ganglionic regions have been termed the proximal and distal tentacle ganglia respectively. The other tentacle nerve arising from each cerebral ganglion is the optic nerve (C5) which passes, apparently without branching, to the single eye situated near the base of each tentacle.

Fretter & Graham described the columellar muscle as being innervated by a nerve V3 arising from the smaller visceral ganglion (VG2), but it has been demonstrated in the present investigation that the muscle is innervated by a previously undescribed nerve LP1.2 which arises from the left pleural ganglion.

3.2 HISTOLOGY.

3.21 Central ganglia.

The histological examination of the C.N.S. was restricted to the cerebral, pleural and pedal ganglia. The structure of the ganglia conformed to a common pattern with an outer fibrous perineurium overlying a cortex of unipolar ganglion cells each with its process directed towards a fibrous medulla, or neuropile. Little extracellular space was found in the ganglia.

The perineurium covering the ganglia was continuous with that of the interganglionic connectives and nerves (fig. 11). It varied in thickness but was approximately 4 microns over the greater part of the ganglia and attained a maximum thickness of 8 microns in the covering of the cerebral commissure and adjacent areas of the cerebral ganglia.

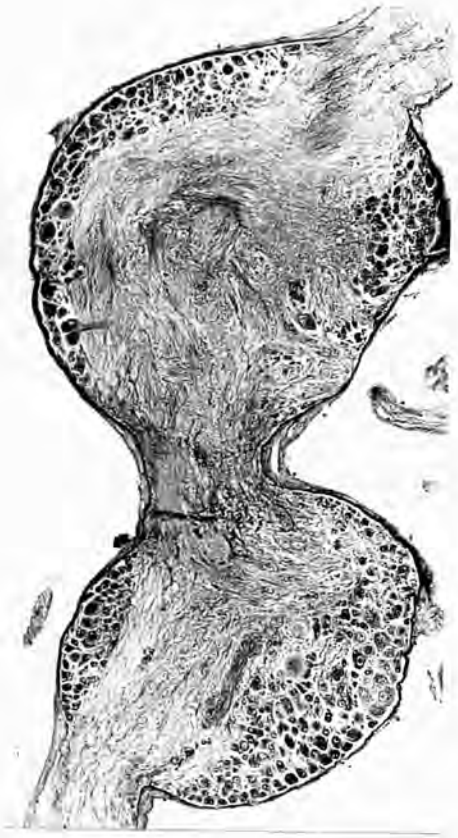


Figure 11. A horizontal section of the right cerebral ganglion (upper) and the right pleural ganglion showing the origins of the supraintestinal connective and the right cerebral nerve C2. (Mallory's stain). Typical ganglion structure is illustrated with an outer perineurium, continuous with that of the nerves and connectives, overlying a cortex of ganglion cells and an inner neuropile of complex tracts of nerve fibres.

The observered ganglion cells were, without exception, unipolar and generally possessed oval cell bodies, the longest axis of which coincided with that of the stem process. After staining with Mallory's Trichrome Solution the cell bodies were observed to have a blue staining granular cytoplasm and a relatively large light staining nucleus containing a densely red staining nucleolus. A second type of ganglion cell was present that was larger and more rounded in outline. When treated with Mallory's solution the staining reaction of its cytoplasm was light, whereas its nucleus was more deeply stained. These cells were found scattered throughout the ganglia but were particularly conspicuous in the mid-ventral regions of the cerebral ganglia and the posterior section of the right pleural ganglion near to the origin of the supraintestinal connective (fig.12).

The majority of ganglion cells gave rise to fine stem processes less than 1 micron in diameter (fig.13), but other cells were found in the pedal ganglia that possessed relatively thick stem processes of up to 4 microns in diameter (fig.14).

The size of the ganglion cells, measured along the greatest axis, varied from 1 - 2 microns to a maximum of 40 microns. An estimate of the distribution of cells of particular sizes was made by measuring the cells found on ten randomly selected sections of each ganglion or, in the case of the pedal ganglia, pairs of ganglia. The numbers of cells found in particular size categories were summed and expressed as a percentage of the total cell count for each ganglion (Table 1).

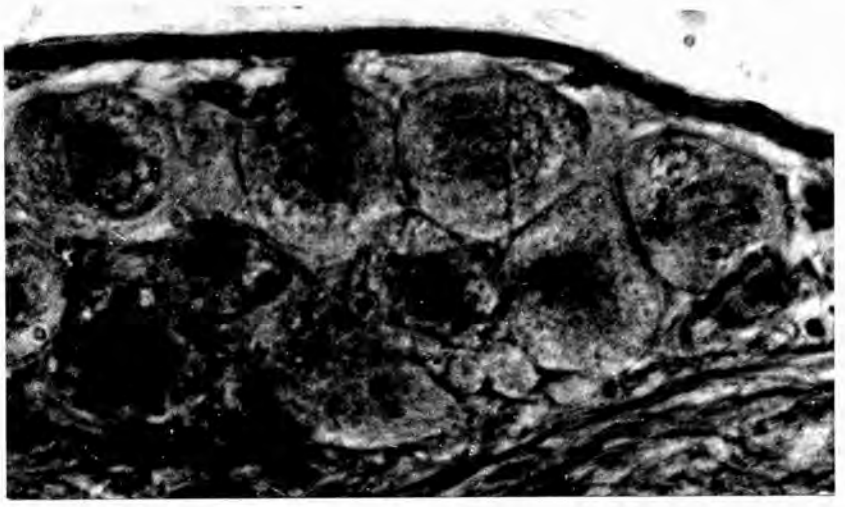


Figure 12. A transverse section of the right pleural ganglion showing the large cells found near to the origin of the supraintestinal ganglion (Mallory's stain). Typically the cells were rounded and faintly staining with slightly darker staining nuclei.

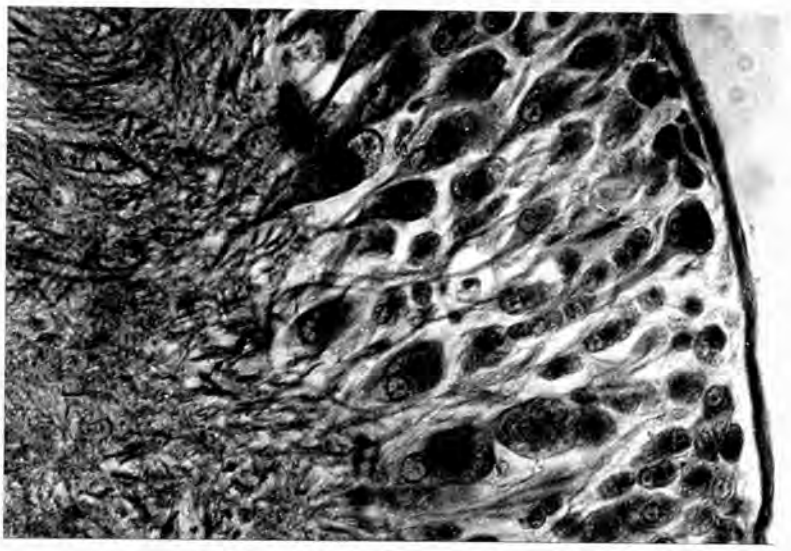


Figure 13. A section of the right pedal ganglion showing typical ganglion cells (Mallory's stain). The cells were usually elongated with a darkly staining cytoplasm and a lighter staining nucleus with a distinct nucleolus. All the observed cells were unipolar with their processes directed into the neuropile.

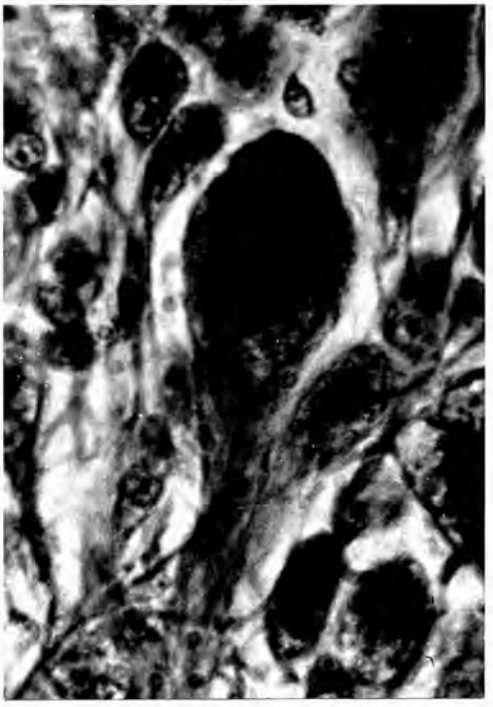


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Figure 14. Large ganglion cells in the left pedal ganglion (Mallory's stain). All the central ganglia contained occasional large ganglion cells that gave rise to thick stem processes of up to 4 microns in diameter.

GANGLION	% CELLS IN SIZE CATEGORIES OF 5 MICRONS						
	TO 5	5.-10	10-15	15-20	20-25	25-30	30-35
Left cerebral	68.7	18.4	7.4	4.7	0.7	-	0.1
Right cerebral	58.4	25.6	10.0	5.3	0.5	0.1	0.1
Left pleural	35.8	32.3	18.6	11.3	1.8	0.2	-
Right pleural	36.5	34.5	15.6	8.8	3.9	0.6	0.1
Left pedal	42.1	31.7	15.3	8.7	1.6	0.6	-
Right pedal	57.0	28.0	9.4	4.8	0.7	0.1	-

Table 1. The size distribution of ganglion cells in the central ganglia of Littorina littorea. A few scattered cells of up to 40 microns were occasionally observed in the cerebral ganglia.

Cells of less than 10 microns accounted for the highest percentage of cells in all the ganglia. In the right pedal ganglion these accounted for 85% of the total cell population; of this 57.0% of the total consisted of cells of less than 5 microns diameter. In other ganglia, for example the left pleural ganglion, the number of cells in these two size categories was about equal. The percentage of cells in the remaining size categories decreased rapidly with increasing cell size, thus the highest incidence of cells in 20 - 25 microns category was 1.7% in the left pedal ganglion whilst the largest cells of between 30 - 35 microns accounted for less then 0.1% of the total cell count of the right and left cerebral ganglia. Cells in excess of 30 microns were not found in the left pleural or pedal ganglia.

The cortex of the ganglia consisted of several layers of ganglion cells varying from one or two cells to ten or more cells in depth. It was most extensive in the pedal ganglia where, in the anterior ventral regions, a very densely packed area of cortex occurred that was composed of numerous layers of closely associated cells less than 8 microns in size (fig.15). This type of cortex was more extensive in the right ganglion while the remainder of the cortex in both ganglia was composed of larger, less densely packed cells.

The cortex of the cerebral ganglia was less densely packed than that of the pedal ganglia but a greater depth of tissue was found in the anterior region of the cerebral ganglia adjacent to their commissure. In the posterior section of either cerebral ganglion a column of ganglion cells extended from the dorsal margin of the cortex into the neuropile and presented the appearance of an island of ganglion cells

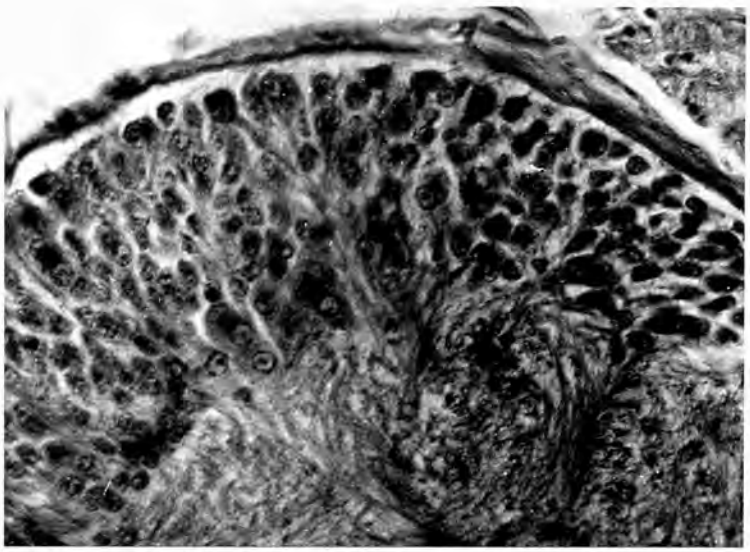

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Figure 15. Transverse section of the right pedal ganglion showing 'dense' cortex (Mallory's stain). The cortex of parts of the pedal ganglia, particularly the anterior ventral region of the right ganglion, consisted of layers of small densly packed ganglion cells.

surrounded by neuropile when viewed in transverse sections.

As far as could be determined with the light microscope no synapses were formed in the cortex of the ganglia. Similarly the fine detail of the masses of axons that formed the ganglionic neuropile was beyond resolution.

The component axons of the neuropile formed complex patterns of fibre tracts that, in some instances, formed the fibrous core of the ganglionic nerves and connectives, however the merging and interweaving of the tracts made it difficult to trace their origins.

3.22 Connectives and Commissures.

The ganglia of Littorina are linked by commissures or connectives that were found to be histologically similar to the larger nerves. In longitudinal section the commissures contined occasional small ganglion cells and an extensive network of endoneurium extending from, and at right angles to, the perineurium. There were also numerous glial nuclei present (fig.16).

3.23 Peripheral Ganglia.

The presence of a small proximal tentacle ganglion on each of the tentacle nerves (C4) of Littorina was described by Leyon (1947). This ganglion has been examined histologically in the present investigation together with the distal ganglionic region found at the site of the first major nerve junction within the tentacle body.

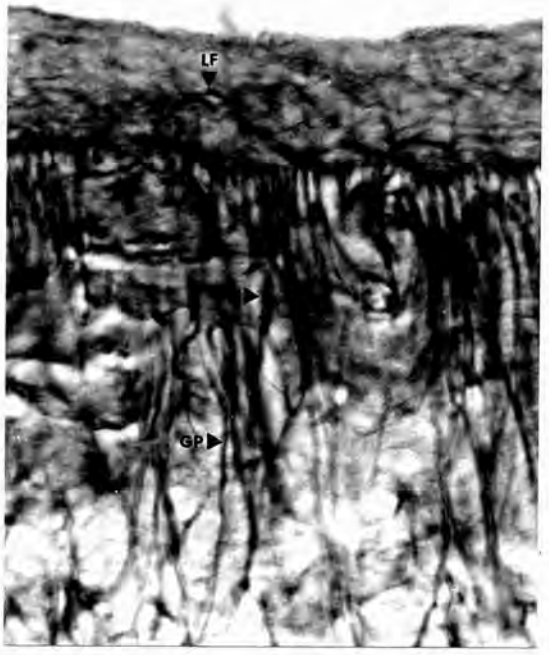

Figure 16. Longitudinal section of the cerebral commissure (Mallory's stain). Under high power the fibrous perineurium was seen to contain longitudinal muscle fibres (L.F). which assumed a characteristic zig-zag pattern when relaxed. The endoneurium extended at right angles to the perineurium and contained numerous glial processes (GP) and glial nuclei (GN).

3.24 Proximal tentacle ganglion.

The structure of the proximal tentacle ganglion was similar to that of the central ganglia with a fibrous perineurium overlying a cortex of small unipolar ganglion cells and an inner neuropile, but the ganglia were small with an overall diameter of about 150 microns (fig.17).

The cortex consisted of a layer of ganglion cells no more than two cells deep that were largely similar to those found in the central ganglia. The cells did not exceed 15 microns in size and their processes were directed towards the centre of the ganglion. A second type of small pink staining ganglion cells was also present that were less than 5 microns in size and gave rise to very fine cell processes. Some of these cells appeared to be bipolar, but certain identification was not possible because of the fineness of their processes. It is possible that these cells were non-nervous in function but their processes lacked the characteristic staining reaction of glial cells found in the nerves after similar staining.

The characteristics shown in the structure of the tentacle nerve on either side of the ganglion were lost in its body and the fibrous medulla assumed the characteristics of ganglionic neuropile with transverse tracts of nerve fibres in evidence.

3.25 Distal tentacle ganglion.

An examination of serial sections of the tentacles revealed the presence of a ganglionic region in the tentacle nerve at the site of the first major nerve junction within the basal region of the tentacle (fig.18). A smaller cluster of ganglion cells was found slightly posterior to the major

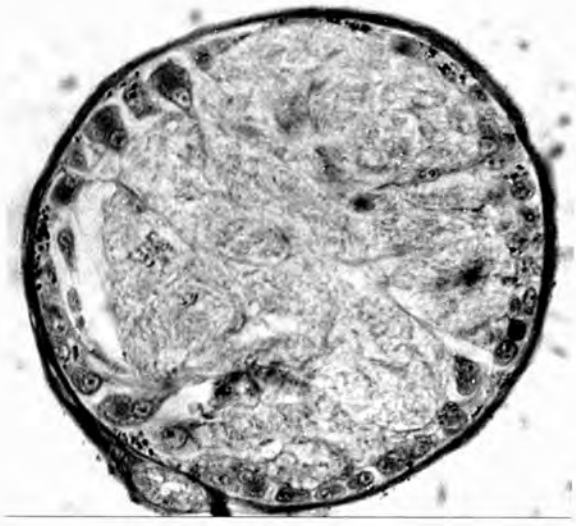

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Figure 17. Transverse section of the proximal tentacle ganglion showing the peripherally arranged ganglion cells overlying the inner neuropile (Mallory's stain).

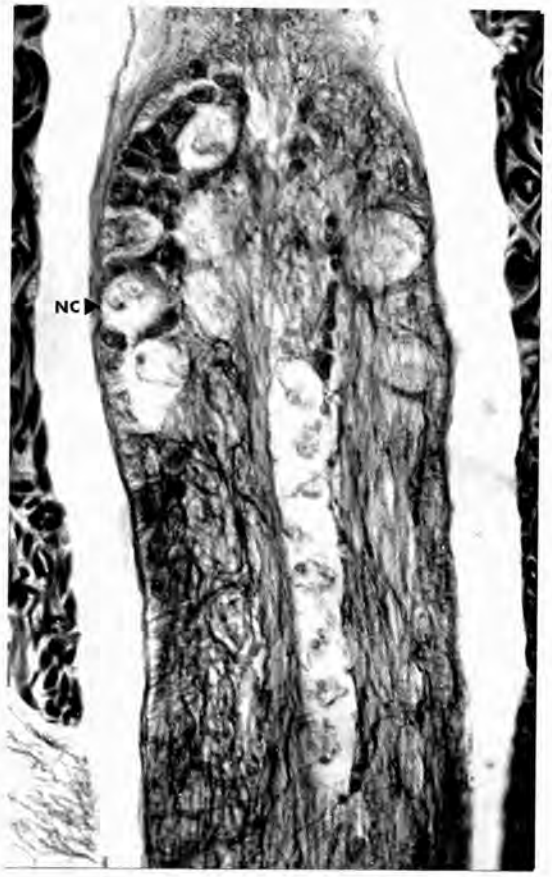


Figure 18. A longitudinal section through the distal tentacle ganglion. (Mallory's stain) The ganglion contained large faintly staining 'neurone-like cells' (NC) and small ganglion cells. The cells were arranged mainly at the periphery of the ganglion but a few other cells occurred at another minor nerve junction posterior to the ganglionic region while others were scattered in a longitudinal group in the centre of the nerve.

group of neurones at a minor nerve junction.

Two types of cell were observed: the smaller and more numerous type consisted of about 60 - 70 ganglion cells measuring approximately 3 to 4 microns along their greatest axis and having a similar structure to neurones of same size found in the central ganglia. These cells were closely packed together, but in some instances stem processes were observed and the cells appeared to be unipolar. This type of ganglion cell was arranged mainly at the periphery of the nerve junction but a small group of similar cells appeared in the central region of the nerve.

There were also about 30 of the second type of cell which stained only faintly and varied in size from 18 - 20 microns. These cells were rounded in outline and contained a nucleus which was also faintly staining, but they were unlike the types of cell observed in the C.N.S. As far as could be determined the cells lacked stem processes although observation was difficult because of the dense packing of the surrounding material. Therefore the cells have been termed 'neurone-like cells' because their position implied a nervous function but evidence of typical neurone structure was not observed.

The structure of the fibrous core of the nerve was unmodified in the region of the ganglion cells. The tracts of fibres associated with the neuropile of larger ganglionic regions was absent while an extensive endoneurium was retained.

3.26 Nerves and Connectives.

All the nerves and connectives encountered in the course of the histological investigation, with the exception

of the columellar nerve, LPl.2., shared a common structure. In transverse section the nerves were approximately circular and bounded by an outer fibrous perineurium. An extensive endoneurium extended inwards from the perineurium towards the centre of the nerves. The axons lay between the branches of the endoneurium, but, except for the columellar nerve, it was impossible to distinguish any detail of axon structure.

The perineurial covering of the nerves and connectives was approximately 2 to 3 microns in depth, but the perineurium of the cerebral commissure attained a thickness of 8 microns. The appearance of the perineurium, after staining in Mallory's Trichrome Solution, was of a light blue staining ground material containing large and small longitudinally arranged fibres. The larger of the two types of fibre was identified as smooth muscle fibres while the small fibres were thought to be strands of connective tissue or collagen. In longitudinal sections of the cerebral commissure the arrangement of the longitudinal fibres and connective tissue cells was observed. In the relaxed state the longitudinal fibres assumed a zig-zag arrangement as shown in figure 16.

The glial processes of the endoneurium formed an extensive network arising from the inner margin of the perineurium. Clusters of processes were grouped together and formed branched radiating septa that penetrated towards the centre of the nerves and between which the axons were situated (fig.,19).

3.27 The columellar nerve.

The columellar nerve was exceptional because a number

Figure 19. A transverse section of the supraintestinal connective (Masson's stain). The larger nerves and connectives of Littorina conformed to a common pattern with an extensive branching network of endoneurium extending inwards from the perineurium. No details of axon structure could be observed but numerous glial nuclei (GN) were present in the endoneurium.

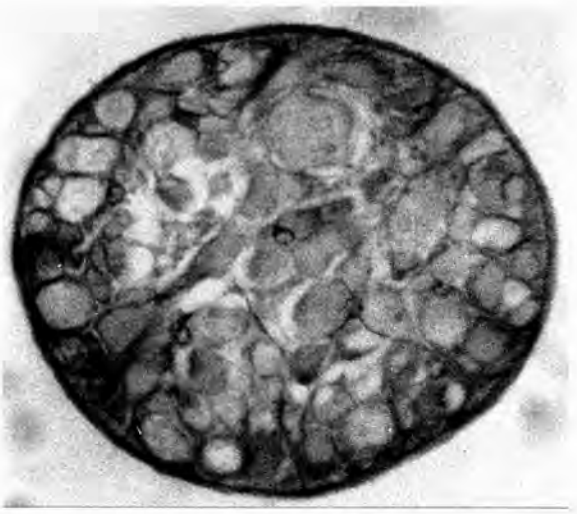
of distinct axons were visible in transverse section. The nerve measured about 100 microns in diameter and contained approximately 60 readily distinguishable axons (fig.20). These varied in diameter with a maximum size of about 23 microns and were generally regular in outline without visible infoldings. Towards the centre of the nerve, however, the axons were more irregular and flattened, and some extracellular space was apparent. The axons were invested in the processes of an endoneurium but this lacked the characteristic arrangement of fibres observed in the other nerves.

3.28 Tentacle Structure.

The tentacles of Littorina are complex structures that have a segmented appearance because of a number of regularly spaced transverse constrictions along their lengths. A single longitudinal groove is also present extending medially along the outer lateral margin. The constrictions and groove are bordered with black and iridescent green pigments while the general appearance of the tentacles is black or grey mottle similar to the remainder of the exposed sections of body wall.

A large degree of flexibility was exhibited during the searching movements made by the tentacles. They were also seen to be contractile in response to noxious stimulation, rapidly shortening to a third of their extended length, 5 to 6 mm. in a large specimen, with corresponding changes in diameter.

In transverse section the tentacles were approximately elliptical in outline and largely composed of longitudinal and transverse muscles with numerous nerve branches in evidence. The outer epithelial covering consisted of a deep layer of



15μ

Figure 20. Transverse section of the columellar nerve (Mallory's stain). The columellar nerve was exceptional as about sixty axons could be observed between the much reduced endoneurium. Towards the centre of the nerve the axons became flattened in outline and a small amount of extracellular space was apparent.

columnar, pseudostratified cells and occasional large globular cells that penetrated to the outer epithelial margin. These cells were probably secretory in function and responsible for the large quantities of mucus that was produced in response to stimulation. Immediately beneath the epithelial basement membrane an irregular layer of longitudinally arranged peripheral muscle fibres was present together with a variety of interstitial cells. When viewed in longitudinal section the muscle fibres were particularly obvious between small transverse epidermal folds formed during contraction (fig.21). In sections of stretched tentacles the folds were no longer apparent and the peripheral muscle fibres formed a thin layer of tissue immediately beneath the epithelium.

The main complement of longitudinal muscle was arranged in blocks towards the centre of the tentacle and was interspersed by branching bundles of radial muscle fibres (fig.22). The peripheral ends of these passed between the peripheral muscle fibres and merged into the tissue beneath the epithelial basement membrane. The remaining spaces in between the longitudinal muscle blocks and between these and the peripheral tissues were filled with large parenchyma cells and occasional interstitial spaces. Towards the base of the tentacles the spaces between the muscle blocks increased with consequent increases in the quantity of the parenchymous tissue. The eye stalks, found fused to the tentacles, lacked similar muscular organization and were largely composed of parenchyma tissue.

The main bodies of the tentacles were each innervated by a single cerebral nerve (C4) and by two nerves, T1, and T2,

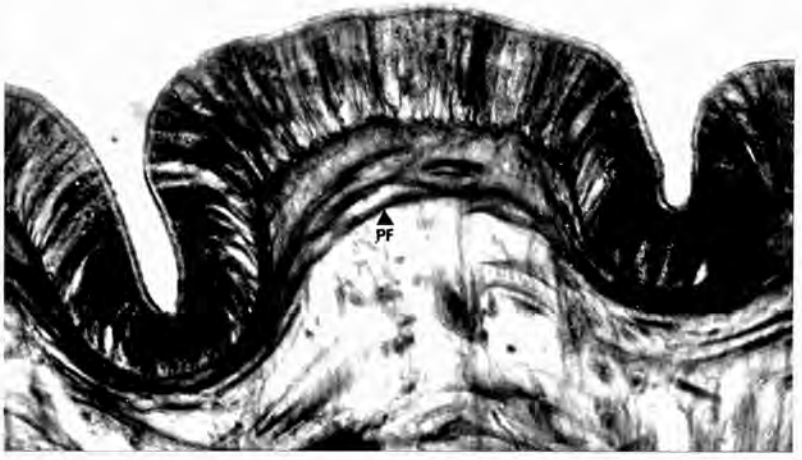
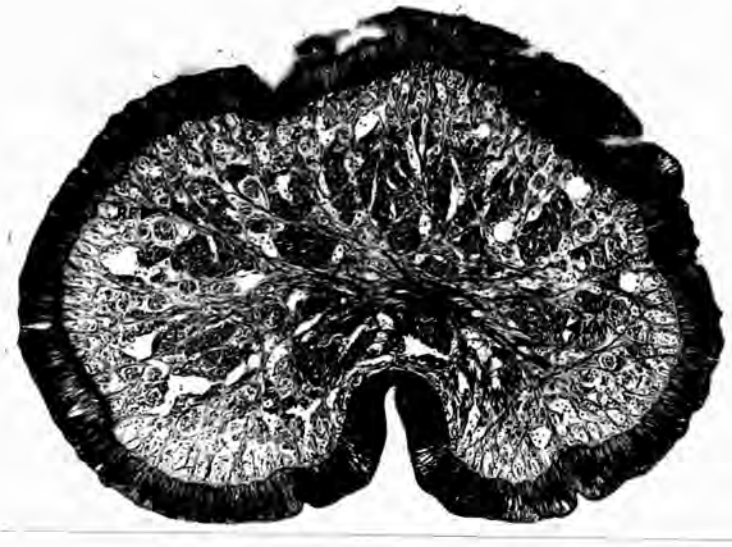


Figure 21. Peripheral muscle fibres in the tentacles of Littorina. (Mallory's stain). In longitudinal sections of contracted tentacles the peripheral muscle fibres (PF) where particularly apparent between the temporary folds formed in the tentacle walls. The muscle fibres were thought to be responsible for mediating local reflexes in response to mechanical stimulation.



150μ

Figure 22. A transverse section of a tentacle (Mallory's stain). The main muscle complement was longitudinal muscle blocks (LM) interspersed by radial muscle fibres (RF). The peripheral muscle fibres were found immediately beneath the epidermal layer. Spaces between the muscle fibres were occupied by extracellular space and large parenchymous cells.

arising from the tentacle ganglion, while the optic stalks each received a single cerebral nerve, the optic nerve C5. The nerves, after entering the main body of the tentacle, branched repeatedly and numerous scattered nerves were observed which decreased in diameter as the tentacle tip was approached. The ganglionic region, found at the first major nerve junction of C4 in the basal region of the tentacle, has been described previously in section 3.25.

An attempt to stain peripheral nervous elements in the tentacle wall by treatment of thin sections with a silver impregnation technique was unsuccessful. (section 2.92)

3.29 Columellar muscle structure.

The columellar muscle of Littorina is a large block of tissue that forms the only connection between the animal's soft parts and its shell. Its function is to control the posture of the shell when the animal is extended while its contraction pulls the shell over the animal during the withdrawal response.

The muscle originates from the ventral surface of the body immediately posterior to the foot and extends posteriorly to the columellar of the shell, on to which its distal end is inserted. After relaxation and fixation the muscle of a medium sized specimen measured approximately 7 mm. square with a maximum thickness of about 2 mm. at its proximal end. Following induced contraction its length could be reduced by up to 50% but this may not represent the normal limits of physiological contraction.

In transverse section it was observed that the muscle consisted of mainly longitudinally arranged muscle fibres with a tract of transverse muscle fibres extending across the

muscle near to its ventral surface. At the lateral margins the transverse tract divided and individual muscle fibres curved upwards and merged into the main mass of tissue. The arrangement of these fibres was more complex on the left side of the muscle where the margin of the muscle merged with the body wall tissues. The main body of longitudinal muscle was penetrated by vertically arranged fibres, extending from the transverse muscle layer to the dorsal surface of the muscle, and also by numerous scattered interstitial blood sinuses (fig. 23).

The normally exposed surfaces of the muscle were covered in an outer fibrous epithelium containing a single layer of large rounded cells and scattered secretory cells that were probably mucus secreting. A basement membrane separated the epithelial layer from a thin layer of circularly arranged muscle fibres and fibrous connective tissue lying on the outer surface of the longituinal fibres and which extended over most of the muscle surface (fig.24).

The examination of horizontal longitudinal sections showed that the longitudinal muscle fibres were more or less parallel but some interweaving occured. The vertical fibres were arranged in narrow extended groups of twenty to thirty fibres interspersed between the longituinal fibres (fig. 25). Some further interstial spaces were observed other than blood sinuses, that contained large faintly staining cells of unknown function.

The longitudinal muscle fibres varied in width from about 7 to 10 microns while the vertical muscle fibres were thinner measuring approximately 3 microns. Some shrinkage

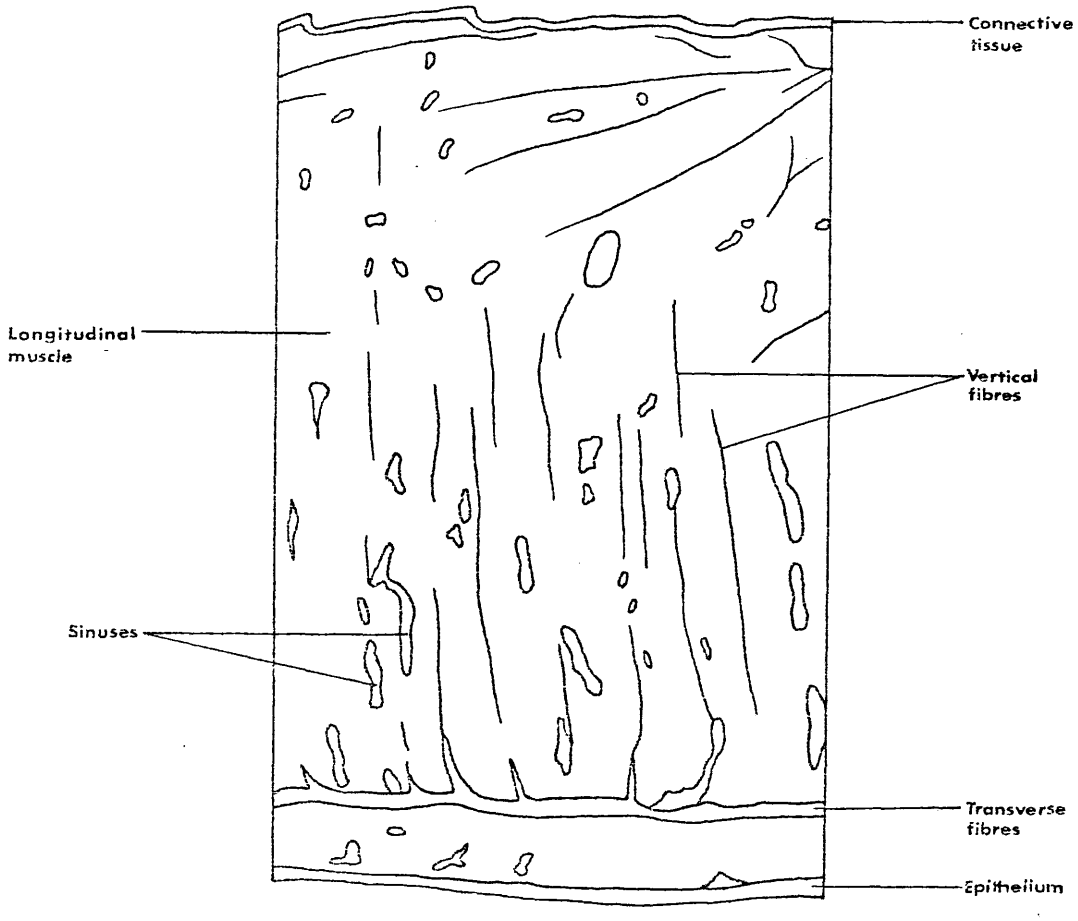
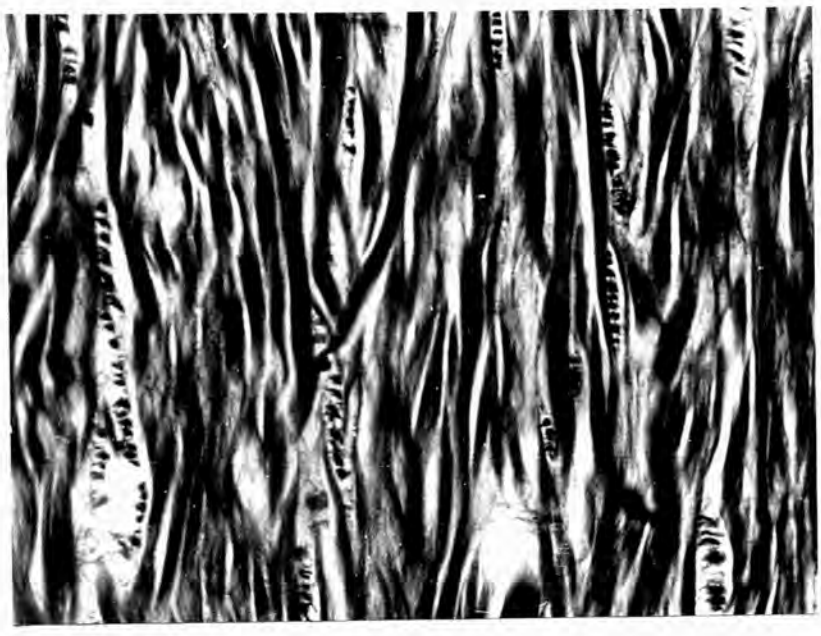


Figure 23. A transverse section of the columellar muscle showing the arrangement of vertical, longitudinal and transverse muscle fibres. Numerous blood sinuses may be observed between the tissues.

Figure 24. Transverse section of the outer epithelial covering of the columellar muscle. (Mallory's stain). The epithelium consisted of a single layer of large rounded cells interspersed with scattered mucus secreting cells containing secretory grannules. Beneath the epithelial basement membrane a layer of circular muscle may be observed overlying the blocks of longitudinal muscle and small sinuses.



10μ

Figure 25. A horizontal longitudinal section of the columellar muscle. (Mallory's stain). The longitudinal muscle fibres measured approximately 7 to 10 microns in width and were more or less parallel. The vertical muscle fibres occurred in scattered longitudinal groups of up to 30 fibres between the horizontal fibres and measured about 3 microns in diameter.

was apparent after fixation and staining but this helped to reveal the thin layers of connective tissue between groups of muscle fibres.

The innervation of the muscle was followed by serially sectioning specially prepared tissues. The only nerve that was seen to enter and remain in the main body of the muscle was the left pleural nerve LPl.2. It was observed to give rise to fine branches that entered the dorsal surface of the muscle while its main branch eventually entered the muscle capsule to give rise to further branches that could be followed between the longitudinal muscle fibres (fig.26).

Figure 26. A transverse section of the columellar muscle and nerve (Mallory's stain). The left pleural nerve LP1.2 was observed to give rise to nerve branches that entered the body of the muscle. Eventually the nerve, shown immediately before and after branching, entered the muscle tissue. No other nerves were observed to do so.

3.3 THE SENSORY INNERVATION AREAS OF THE CEREBRAL NERVES.

As a preliminary experiment those areas of the snout and anterior margins of the foot that are innervated by particular cerebral nerves were determined experimentally. The peripheral stump of each of the cerebral nerves was in turn, taken up into a suction electrode and the foreparts of the preparation were stimulated manually. The responses were observed directly from the oscilloscope, and the areas from which responses were elicited were noted for the cerebral nerves C1, C2, both major branches of C3, and C4.

Responses were obtained from all the nerves but these varied according to the area stimulated and also between the individual preparations. The nerves that gave the most consistent responses were C1 and the tentacle nerve, C4.

The areas from which responses could be obtained for particular nerves were not sharply defined, there was a wide overlap between adjacent areas and also across the midline of the animal. In figure 27 the results of six determinations have been compared and represented as a sensory map.

Nerve C1. The nerve was responsive to stimulation of the dorsal section of the snout and also to stimulation of the dorsal lip margins.

Nerve C2. The responses recorded from C2 were usually elicited by stimulation of the ventral lip margins and in some specimens, by stimulation of the ventral region of the snout.

Nerve C3I. The nerve was comparatively insensitive but some activity was elicited by stimulation of the posterior ventral section of the snout and adjacent regions of the foot.

Nerve C3II. This branch of C3 was also comparatively insensitive but some responses were invoked by stimulation of the body regions immediately beneath the tentacles.

Nerve C4. The tentacles were very sensitive to touch and responses were recorded from the nerves to gentle stimulation of any part of their surface. The area of innervation of C4 was more sharply defined than for other nerves and did not extend from the tentacle base into the head regions.

As the nerve that showed greatest sensitivity and also innervated an easily isolated section of the body wall was the tentacle nerve C4., the tentacles were chosen as suitable areas for detailed analysis of mechanosensitive responses. The tentacles were also stimulated to elicit withdrawal responses in intact animals, although similar behavioural responses were obtained by stimulating other areas of the animals' foreparts.

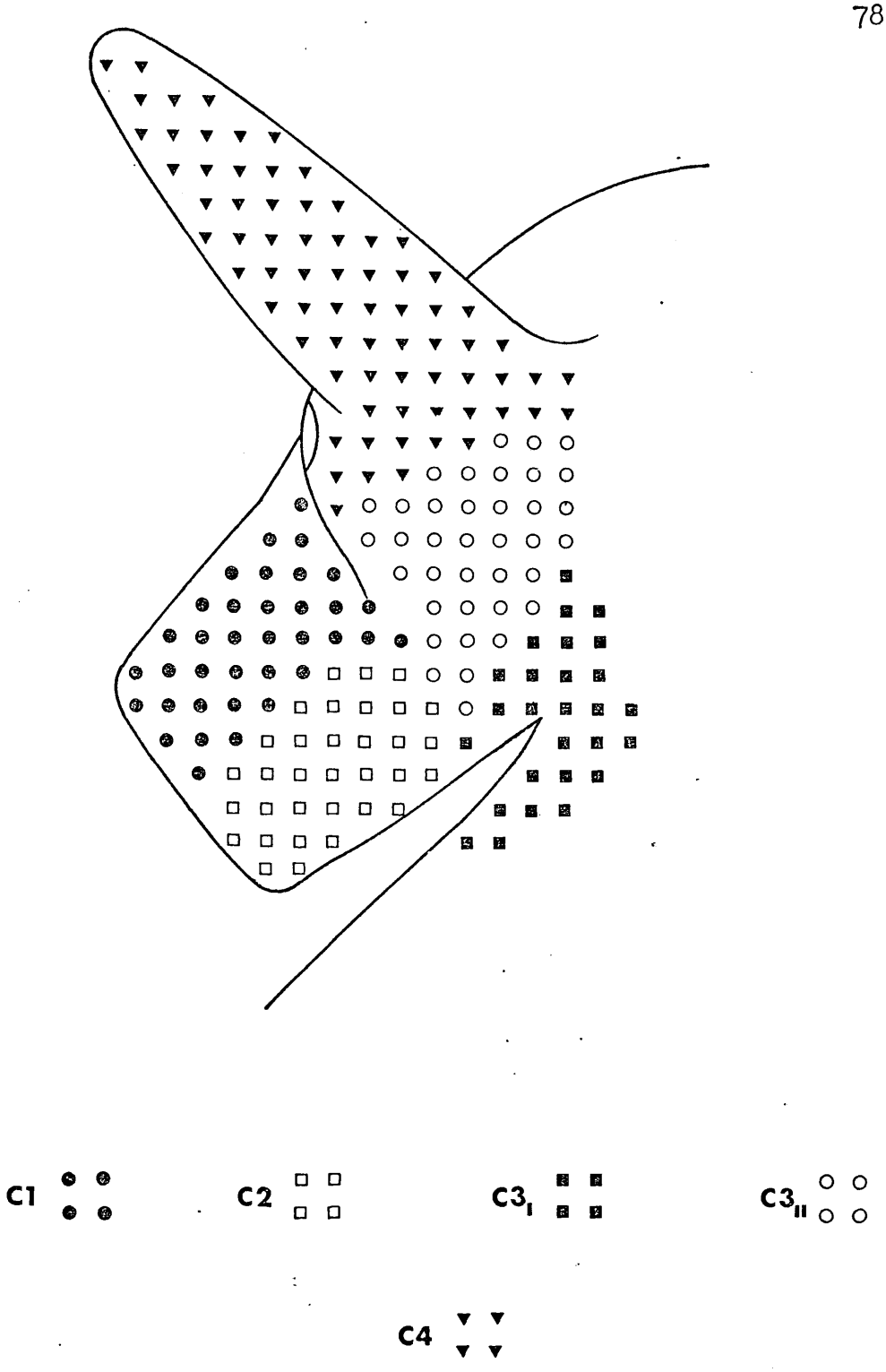


Figure 27. A map showing the distribution of mechanosensory areas of the cerebral nerves in the anterior region of L.littorea (lateral view).

3.4 BEHAVIOURAL OBSERVATIONS.

Behavioural observations were limited to the animal's withdrawal response: it was shown that this could be divided into several phases that were dependent on the stimulus intensity and the animal's position at the time of stimulation.

Initially the observations were made from animals allowed to move freely in a circular dish containing a small quantity of sea water. Soon after being placed in the dish an animal emerged from its shell, righted itself and started to crawl. During active crawling the fully extended position was attained with the foot held in firm contact with the substrate (fig.28a). The animal's snout protruded and curved downwards so that the mouth was held near to the substrate while the tentacles were fully extended and made active searching movements.

The gentle application of a fine glass probe to either tentacle caused the immediate contraction of both tentacles followed by a downward movement of the shell that resulted in the partial covering of the head and foot (fig.28b). The retractile phase of the response was relatively short and it was immediately followed by a slow relaxation phase during which the animal slowly emerged from its shell until it was again fully extended. During the response the forward movement of the animal did not appear to be inhibited but objective measurements were difficult to make because of the short time-course of the response and the animal's slow forward progress.

The degree of covering by the shell increased with increasing stimulus intensity until the maximum response was

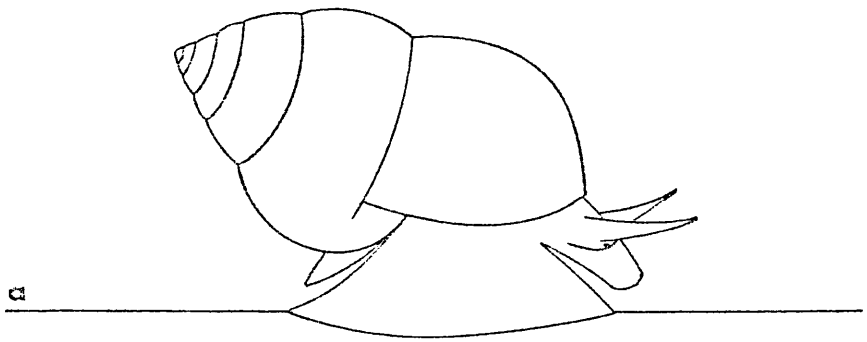
observed. At this point the shell was pulled down over the animal's exposed head and foot until its anterior margins and the ventral section of the largest whorl touched the floor of the dish (fig.28c). This resulted in the complete covering of the snout while the foot, although partially retracted, retained its hold and was protected by the margins of the shell aperture and the whorl.

Usually an animal could not be induced to break its foothold and withdraw completely into the shell provided the foot was in contact with a solid object. If the foothold was broken the animal withdrew completely and sealed the shell aperture with its operculum. Similarly, if animals were placed on a dry surface, they retracted completely after crawling for a few centimetres.

Phasic withdrawal responses could also be elicited by other modes of stimulation including rapid changes in light intensity and sudden vibration, as caused by sharply knocking the bench.

The rapid application of several stimuli to the same tentacle, each applied as soon as the tentacle re-emerged from the shell, led to a progressive reduction in the extent and duration of the withdrawal response until, after the third or fourth stimulus, the animal remained extended while it moved away from the direction of stimulation. The stimulated tentacle was held in a partially contracted state against the snout, while the contralateral tentacle was extended and made searching movements. The application of a higher intensity stimulus to the originally stimulated tentacle elicited a phasic withdrawal response.

Figure 28. Successive stages in the withdrawal response. In (a) the fully extended position is shown while (b) shows the partial retraction of the animal into its shell in response to a single moderate stimulus. At higher stimulus intensities (c) the animals retracted until the shell margins made contact with the substrate, but the foot, although partially contracted, retained its hold on the substrate.



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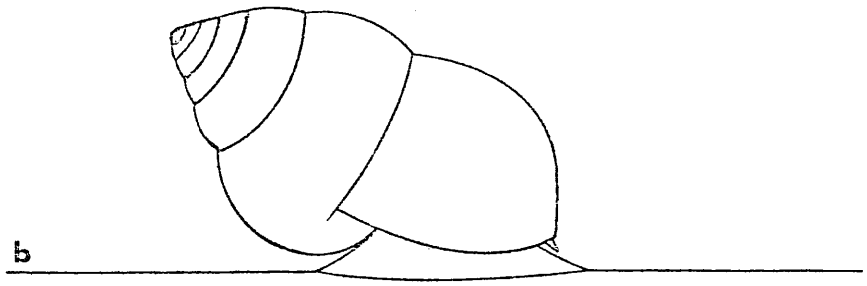

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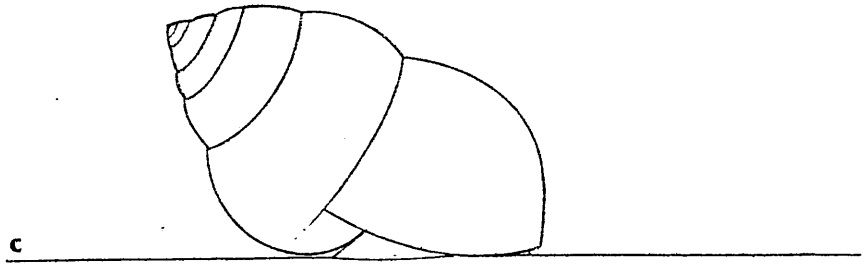


c

3.41 Transducer recordings.

The preliminary observations of the withdrawal response indicated that the retractile phase was followed immediately by a slow relaxation phase. In order to observe the response in greater detail its time course was monitored electronically by using the transducer arrangement described previously (section 2.6).

The typical response that could be elicited by stimulation of either tentacle was superimposed on a background activity of small shell movements (fig. 29). Its duration was typically about 5 secs. and it could be divided into two phases: an initial retractile phase, lasting for about 0.5 secs. from the onset of the response and followed immediately by a prolonged relaxation phase lasting for about 4.5 secs. An intervening phase of tonic, or maintained contraction was not observed thus the response could be described as phasic. In a later section a comparison of the withdrawal response characteristics has been made with those of the columellar muscle contraction.

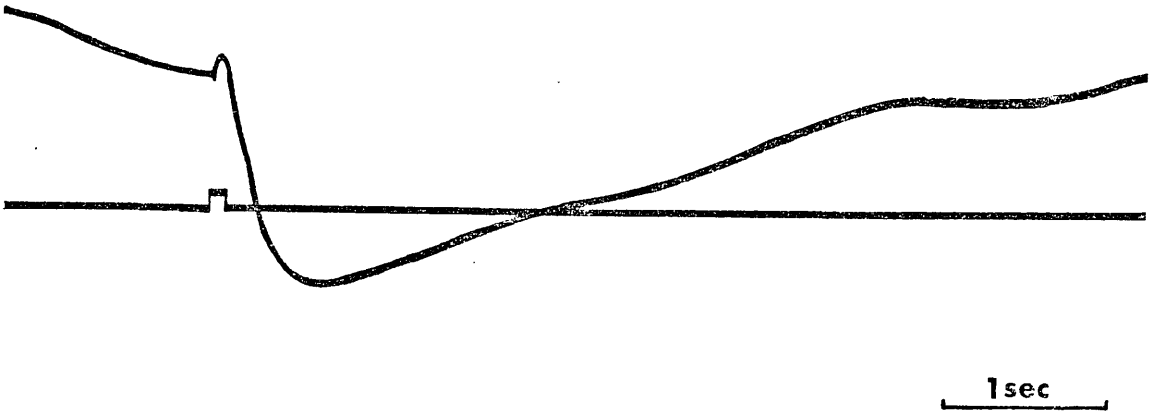


Figure 29. Transducer recording of the withdrawal response. The response consisted of a rapid contractile phase followed immediately by a phase of prolonged relaxation. The animal re-emerged completely after about 5 seconds.

3.5 THE TACTILE RESPONSES OF ISOLATED TENTACLES.

Before it was attempted to record nervous responses from the tentacles a study was made of their overall reactions to tactile stimulation. Also the roles of the cerebral and tentacle ganglia in mediating the observed responses were investigated.

Shortly after a tentacle preparation was placed in a bath of sea water it resumed a pattern of spontaneous movements similar to that observed in the intact animal.

A gentle stimulus, applied manually to the tip of the tentacle with a fine glass probe, resulted in a rapid twitch of the tip away from the point of stimulation. The response was limited to the tip and the overall pattern of tentacle movement was unchanged, consequently the response has been referred to as the 'tip twitch'. A similar stimulus applied to other areas of the tentacles elicited a localized contraction of the tentacle wall, at the point of stimulation.

At higher stimulus intensity the tentacle responded by a rapid overall contraction, the extent of which varied proportionally with the stimulus intensity. The contraction elicited by a single stimulus was not prolonged and the normal pattern of tentacle movement was soon resumed. The duration of contraction was increased by repeated stimulation.

These observations indicated that the tentacle exhibited at least two classes of response to tactile stimulation that depended on the stimulus intensity. The first was a localized response restricted to the immediate point of stimulation and without apparent effects on overall tentacle activity. The tip twitch response was of this type, but it was effected by a localized contraction of the

tip tissue directly away from the point of stimulation, therefore it may have represented a more complex type of response than the localized contractions observed at all other points on the tentacle body.

The second class of response was a rapid but not prolonged contraction of the tentacle invoked by a high intensity stimulus. The nature of this response suggested that it may have been centrally mediated in contrast to the localized response that indicated possible peripheral mediation, therefore the experiments were repeated after the tentacle nerve (C_4) had been severed between the cerebral and proximal tentacle ganglia, in order to isolate the cerebral ganglion from the preparation, and also after the isolation of the tentacle ganglion.

3.51 Cerebral ganglion removal.

The removal of the cerebral ganglion resulted in the abolition of spontaneous tentacle movements. The response of the tentacle to low intensity stimulation was unchanged but the rapid overall contraction, elicited by high levels of stimulus intensity, was replaced by a slow overall contraction of greatly increased duration which lasted for several minutes.

3.52 Proximal tentacle ganglion removal.

After the proximal tentacle ganglion was removed the movements, and the reactions of the tentacles to mechanical stimulation, were unchanged and remained similar to those observed after the removal of the cerebral ganglion.

It was concluded that the spontaneous movements and

rapid withdrawal and extension of the tentacles were controlled by the cerebral ganglion as its removal leads to the abolition of these functions. In contrast, the local responses initiated by low intensity stimulation and the slow contraction elicited by high intensity stimulation in the absence of the cerebral ganglion, must have been the result of peripheral interactions as both types of response occurred in the absence of the cerebral and proximal tentacle ganglia.

Further conclusions could be drawn concerning the observed responses, namely that their central control was derived solely from the cerebral ganglia without any apparent participation of the tentacle ganglia. While the consideration of tentacle structure (section 3.28) suggested that the centrally placed longitudinal muscle blocks were responsible for the rapid contraction response and were therefore under central control derived from the cerebral ganglia. The slow contraction and local contractions of the tentacle were probably induced by the peripheral muscle elements in response to stimulation from peripheral neurones.

In the absence of central ganglia tentacle extension was also greatly inhibited, thus the antagonistic muscles responsible for extension must also have been largely controlled from the cerebral ganglia. As no circular muscle was found in the tentacle walls, the radial muscle fibres must have been responsible for extension.

3.53. Responses of tentacle mechanosensitive neurones.

The properties of various mechanosensitive neurones of the tentacles were investigated by recording with a suction electrode from the peripheral end of the tentacle

nerve C4 after it had been severed close to the distal side of the tentacle ganglion. The responses of the neurones to stimuli of various intensity and frequency, and their reactions to synapse blocking agents were investigated.

3.53 (i) Response characteristics.

A stimulus of medium intensity applied to the distal section of the tentacle elicited a complex response consisting of several units (fig. 30). The response coincided with the stimulus onset and did not exhibit tonic discharge during prolonged stimulus contact, or further activity following the offset of the stimulus. Thus the units were rapidly adapted.

When stimulated repeatedly the response showed prolonged adaptation but it was not possible to accurately determine the rate of adaptation because each stimulus initiated a local contraction of the tentacle wall, thus preventing accurate orientation of repeated stimuli. Usually responses were absent after the rapid application of two or three similar stimuli.

These observations revealed that the responses to medium intensity stimulation consisted of various rapidly adapting mechano-sensitive units, further studies revealed that four classes of sensory neurone were present.

3.53 (ii) Response Type 1.

A threshold intensity stimulus applied to the tentacle tip elicited two types of response; that having the shortest latency from the onset of the stimulus was termed

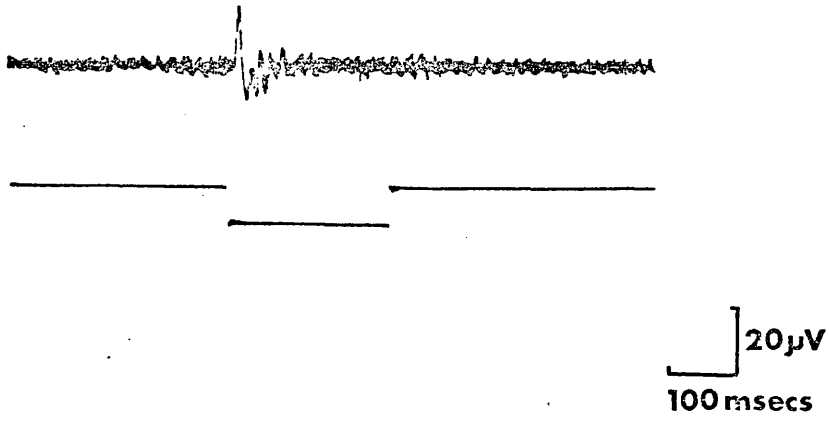


Figure 30. The responses of mechanosensory neurones in the tentacles. The response to a medium intensity stimulus, consisted of several discrete rapidly adapting units coinciding with the stimulus onset (bottom trace). Tonic discharge and responses to the stimulus offset were absent.

Type 1. It was characterised by a simple action potential of approximately 30 microvolts amplitude and 12 msecs. duration (fig. 31a). The latency of the response was approximately 20 msecs. from the onset of stimulation at the tentacle tip. The stimulation of other points on the tentacle surface elicited similar responses the latency of which varied as the length of the conduction pathway between the point of stimulation and the recording electrode changed.

The response adapted rapidly and remained so for relatively long periods of up to ten seconds.

3.53 (iii) Response Type 2.

The Type 2 response had a similar stimulus threshold to the Type 1 response but it was readily distinguished as it occurred as a low amplitude compound potential of long latency, approximately 65 msecs.(fig. 31b). Both types of response could be recorded singly or together, however the Type 2 response was less rapidly adapted than the Type 1 therefore it was more commonly recorded alone.

3.53 (iv) Response Type 3.

The application of a high intensity stimulus to the tentacle tip elicited a complex response that included Type 3 units. The overall response consisted of a compound potential comprising the summed activity of Type 1 and Type 2 responses and a third type of fast high amplitude potential of up to 100 microvolts and about 6 msecs. duration, superimposed on the compound potential (fig. 32a).

A single stimulus evoked numerous type 3 units, but further stimulation reduced the number of these units

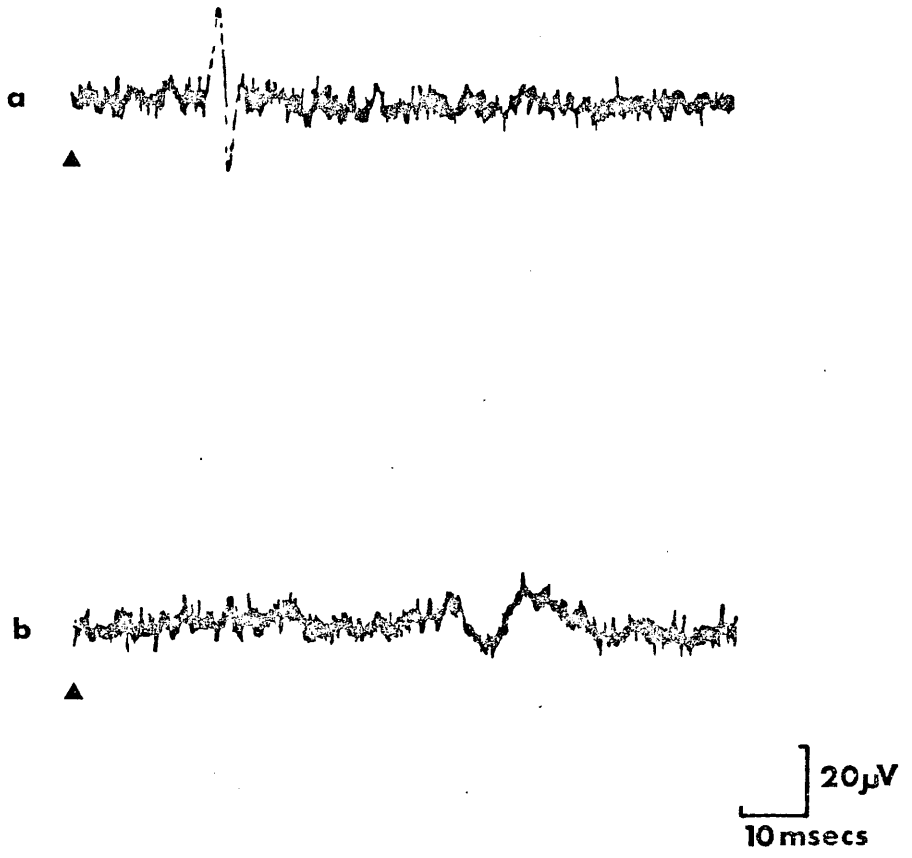


Figure 31. The low threshold tentacle mechanosensory responses. The onset of stimulation corresponds to the beginning of the trace (as shown) and stimulus duration was in excess of the sweep time.

(a) The Type 1 response was characterised by a simple action potential of approximately 30 microvolts amplitude and 12 msecs. duration.

(b) The Type 2 response was a low amplitude slow compound potential of long latency, about 65 msecs from the stimulus onset.

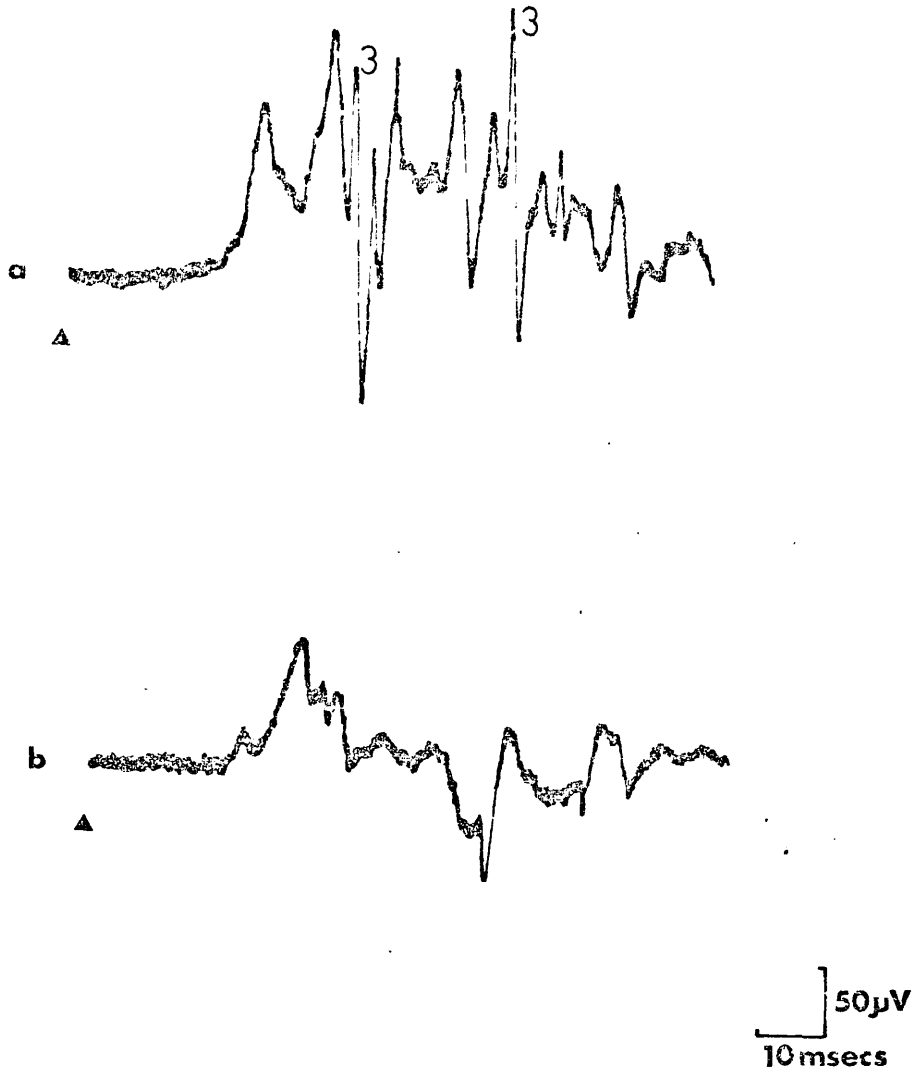


Figure 32. The Type 3 mechanosensory tentacle response elicited from the tentacle tip.

(a) High stimulus intensity elicited fast high amplitude potentials of over 100 microvolts, that were superimposed on summed Type 1 and Type 2 activity.

(b) The response showed adaptation and subsequent stimulation elicited only the summed Type 1 and Type 2 activity.

until only a compound potential composed of the summed Type 1 and Type 2 activities remained (fig. 32b). This, in turn, was greatly reduced in amplitude and duration after further repeated stimulation but the response was usually not eliminated completely, probably because each stimulus caused a local contraction of the tentacle wall, thus succeeding stimuli were delivered to slightly different areas of the preparation. The response returned to normal after a resting period of five to ten minutes.

3.53 (v) Response Type 4.

Repeated stimulation of the tentacle tip failed to elicit further classes of response even at high stimulus intensity, but when other regions of the tentacle were stimulated at high intensity a fourth response was recorded (fig. 33). This was a compound potential of short latency, about 5 msecs. for stimulation of the mid-point of tentacle, and variable amplitude of 30-50 microvolts. Unsuccessful attempts were made to record single units of the response by utilizing a pointed stimulator probe in order to reduce the area of stimulus to a minimum. Unfortunately the high stimulus threshold of the response required high stimulus intensity that caused a relatively large deformation of the tentacle surface with consequent sensory activity.

3.54 Primary and H.O.S. neurones.

In order to determine the type of neurones responsible for the propogation of the responses the experiments were repeated with preparations that had been previously bathed in high magnesium sea water (Mg.SW.) or 10^{-5}M. Acetylcholine

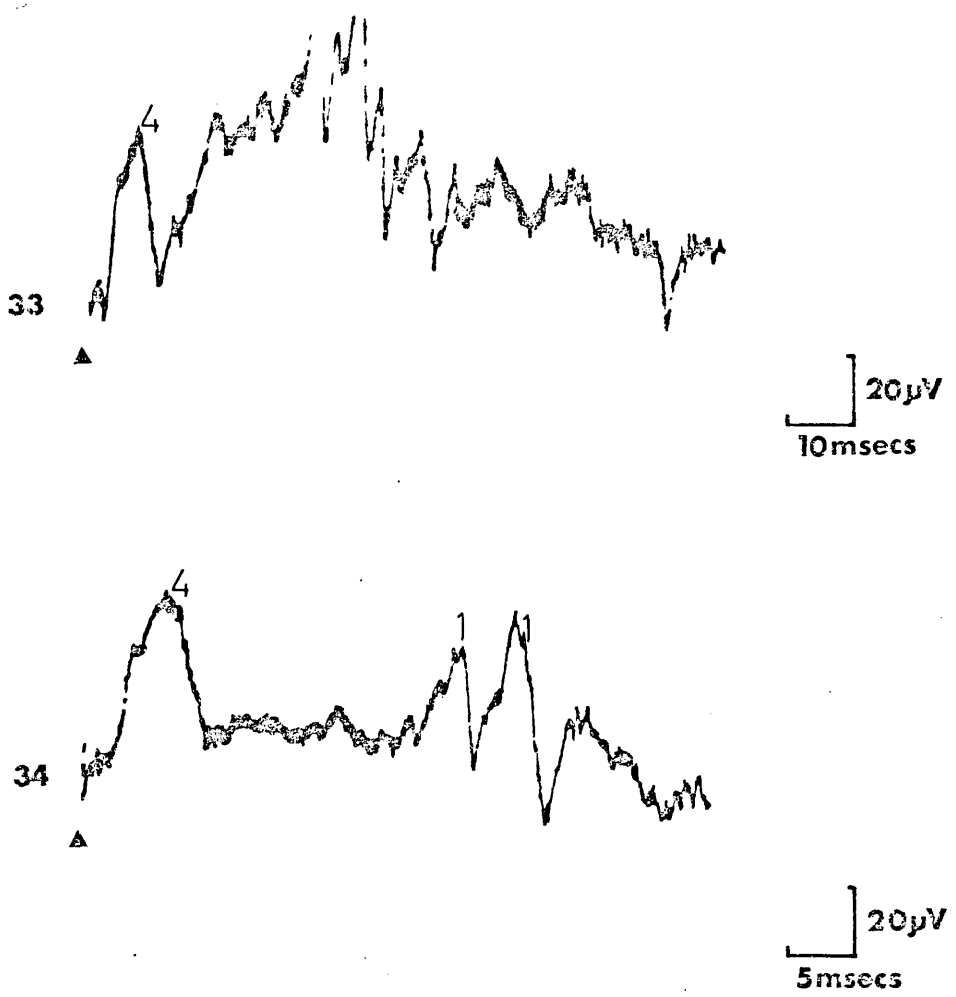


Figure 33. The Type 4 mechanosensory tentacle response. At high stimulus intensity the Type 4 response, characterised by a compound potential of short latency of about 4 to 5 msecs, could be elicited from regions immediately behind the tentacle tip.

Figure 34. Type 1 and Type 4 responses recorded simultaneously from a synapse blocked preparation. (Mg.SW). Only Type 1 and Type 4 responses could be recorded from this type of preparation and it was concluded that they were propagated by primary sensory neurones.

seawater solution for periods of two to three hours. Both solutions caused similar effects which could be reversed by returning the preparations to normal sea water.

The stimulation at both high and low intensities of the tentacle tip elicited only Type 1 responses, while stimulation of other tentacle areas at low intensity invoked Type 1 responses, and, at high intensity, typical Type 4 responses. Figure 34 shows Type 1 and Type 4 responses recorded from a synapse blocked preparation.

Thus the Type 1 and Type 4 responses, as recorded in the tentacle nerve, corresponded to primary sensory neurone activity, while the Types 2 and Type 3 responses were shown to be synaptically mediated, therefore their responses were propagated by higher order sensory neurones (H.O.S. neurones) in the tentacle nerve.

3.55 Responses in the fine tentacle nerves (T1 & T2).

The tentacle nerves T1 and T2 were severed in turn, near to their insertion into the tentacle ganglion and their proximal ends taken up into a fine suction electrode. The tentacle was stimulated as before and the responses recorded from T1 and T2 were compared to those previously obtained from C4.

The responses of both nerves were similar when low intensity stimuli were applied to the tentacle. In both instances two responses (fig. 35) were recorded which were similar to the Type 1 and Type 2 responses recorded from C4. Further similarity was suggested when the experiment was

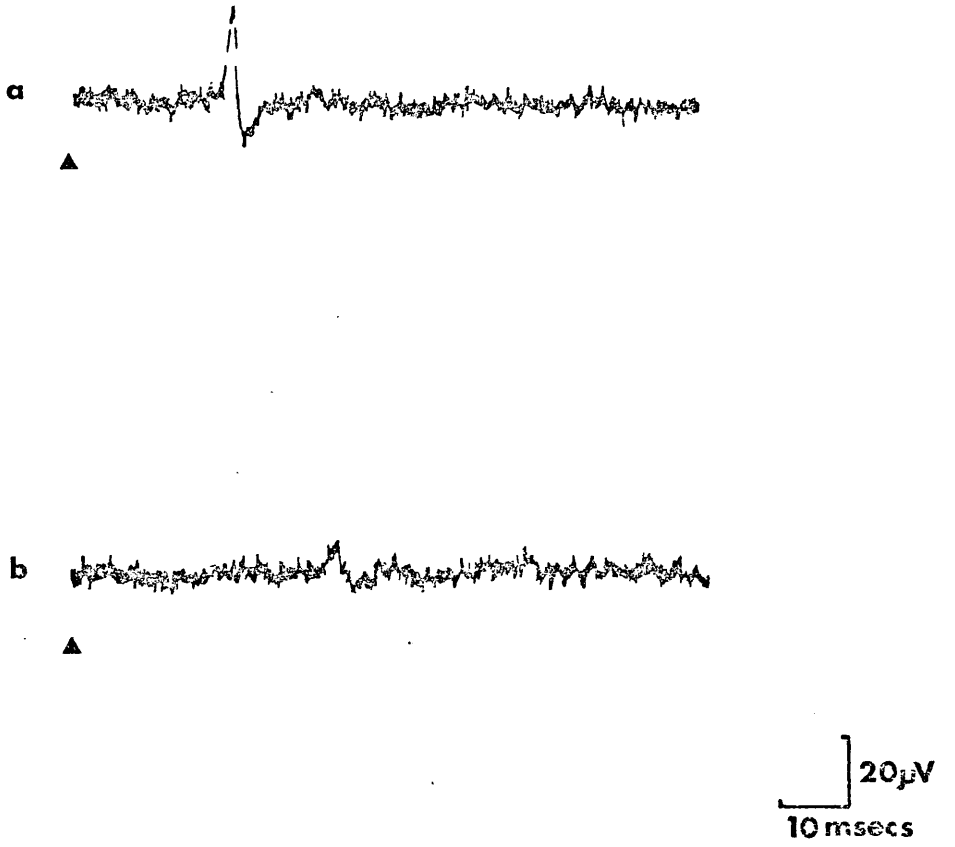


Figure 35. The mechanosensory responses of the fine tentacle nerves T1 and T2. Both nerves showed similar low threshold responses corresponding to the Type 1 (a) and Type 2 responses (b) recorded from the main tentacle nerve C4.

repeated with synapse blocked preparations (immersion in Mg.SW.). The response corresponding to the Type 2 response was abolished while that corresponding to the Type 1 response was unaffected.

The distribution of the sensory units responsible for these responses was different from that previously observed. Stimulation of most areas of the tentacle surface evoked responses in T1 and T2 but the most sensitive area, indicating a higher density of relevant sensory neurones, was the outer lateral margin of the tentacle near to its base.

3.56 The proximal tentacle ganglion.

The early investigation of the tentacle ganglia (section 3.52) suggested that they were of limited importance in controlling tentacle movements, but this did not preclude a possible relay or integrative function in relation to nervous activity in the nerves T1, T2 and C4. The possibility that the primary sensory neurones of C4 formed a relay with higher order sensory neurones in the ganglion was first investigated.

As a preliminary step the original experiment was repeated with recordings made from C4 at the central side of the tentacle ganglion after both T1 and T2 had been severed in order to eliminate extraneous sensory activity. The results of this experiment were compared to those obtained from similar experiments performed on synapse blocked preparations (Mg.SW.).

In the normal preparations it was possible to record two low threshold responses and two high threshold responses

which shared similar properties to the four classes of response recorded peripherally from C4. In the synapse blocked preparations only the responses corresponding to the original Type 1 and Type 4 were recorded. Thus the Type 1 and Type 4 primary sensory neurones were shown not to synapse with H.O.S. neurones in the tentacle ganglion. A similar conclusion could not be drawn concerning the Types 2 & 3 neurones as these have been shown to be susceptible to synapse-blocking agents at the periphery, but it is considered that a further synaptic junction is unlikely as the characteristic responses of these neurones were observed in the postganglionic recordings.

3.57 Reflex ganglion activity.

The fine tentacle nerves T1 and T2 were shown to propagate activity to the tentacle ganglion, therefore recordings were made from the severed ends of C4, on either side of the ganglion, in order to observe the effects of afferent input on the sensory and motor activity of the ganglion.

Both the distal and proximal stumps of C4 propagated 'spontaneous' activity and, when stimuli were applied to base region of the tentacle, complex responses were recorded (fig. 36).

A higher level of spontaneous activity was recorded from the central branch than from the peripheral branch of C4. In both instances the activity contained numerous asynchronous units of which some appeared to occur on a 1:1 basis in the recording sites (marked in fig. 36). The

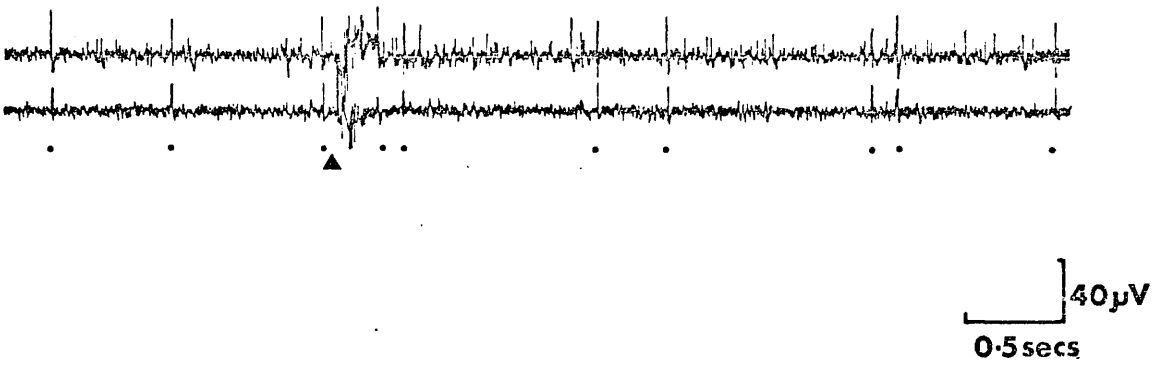


Figure 36. Spontaneous activity and mechanosensory responses recorded from C_4 at the peripheral side (lower trace) and the central side (upper trace) of the proximal tentacle ganglion. Certain spontaneous units (dotted) appeared to correspond on a 1:1 basis while the application of a single stimulus (marked) elicited ccmplex responses at both recording sites. All activity was abolished by Mg.SW.

responses observed on stimulation contained numerous units and activity was maintained after the stimulus offset. The level of activity returned to normal after approximately 0.5 secs.

The frequency of the synchronous units appeared to increase on stimulation but individual units could not be identified in the complex response. After stimulation the rhythm of these units was more irregular than before.

By replacing the bathing fluid with Mg.SW. all activity in C4 was abolished. This returned to normal after the substitution of normal seawater. Consequently it was concluded that all the activity observed on both sides of the ganglion was synaptically mediated.

Cutting either T1 or T2 while leaving the second nerve intact produced little change in the base level of activity, or in the response to stimulation observed in the stumps of C4. Therefore it seemed probable that axons in both T1 and T2 formed synapses with motor and sensory neurones in the ganglion because, when either of the two nerves was intact, both centrally and peripherally directed activity could be elicited from the stumps of C4.

3.58 Motor activity in T1 and T2.

It was possible to record efferent activity from the central stumps of T1 and T2 in response to stimulation of the tentacle. The responses were abolished by synapse blocking agents and therefore synaptically mediated, but further investigations were not made.

3.59. Summary of tentacle sensory innervation.

The results of the experiments have been summarized

and represented by figure 37.

The Type 1 and Type 4 responses were shown to be propagated by primary sensory neurones with different spatial distributions. The two neurones have been shown as simple sensory neurones, the Type 1 being present at the tentacle tip and the Type 4 occurring in regions below the tip. In contrast the Type 2 and Type 3 responses were synaptically mediated and therefore propagated by H.O.S. neurones.

The Type 2 response occurred at a similar threshold to the Type 1 but as the responses could be recorded separately, it was unlikely that the slow Type 2 response was induced by the faster Type 1 response. The neurones responsible for the Type 2 response have been represented as one or more primary sensory neurones forming peripheral synapses on an H.O.S. neurone with its axon extending into C4. The compound nature of the recorded response was probably due to several such units firing more or less synchronously.

The characteristics of the Type 3 response suggested that it was propagated by relatively large and consequently fast neurones in C4. The response was synaptically mediated and never recorded without preliminary Type 1 and Type 2 activity, but it could be eliminated by repeated stimulation to leave a base level of Type 1 and Type 2 activity. The Type 3 neurone has been shown as an H.O.S. neurone in C4, which received synaptic excitation from Type 1 and, or, Type 2 neurones in the periphery. The short latency between the onset of the general response and that of the Type 3 response suggested that the Type 1 neurones were responsible for excitation of the Type 3 H.O.S. neurone. Possible involvement,

however, of the Type 2 neurones could not be dismissed, therefore both connections have been included in the diagram.

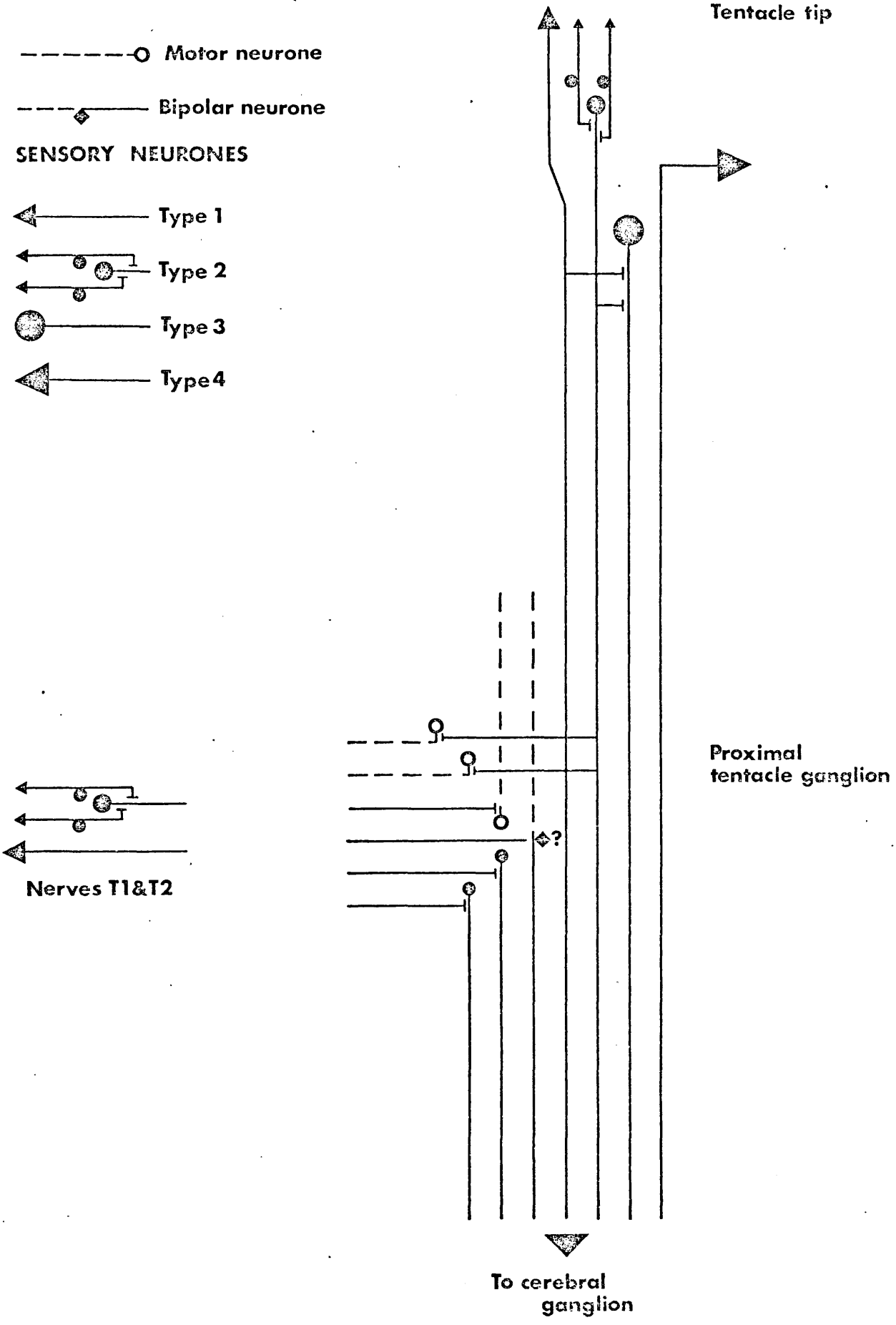
Neither the Type 1 nor Type 4 neurones were shown to relay with H.O.S. neurones in the tentacle ganglion and it was concluded that the possibility of the Type 2 and Type 3 neurones forming similar junctions was unlikely. Consequently no relay synapses have been included at the level of the tentacle ganglion and the axons of the respective neurones have been shown to extend directly to the cerebral ganglion, although it was demonstrated that synaptically invoked activity could be recorded in the nerves arising from the tentacle ganglion.

The sensory responses from T1 and T2 were similar to the Type 1 and Type 3 responses recorded from C4 and similar neurones have been shown in the diagram. Afferent input to the tentacle ganglion evoked both motor and sensory activity, the former having a greater intensity than the latter, thus the afferent neurones of T1 and T2 have been shown to synapse with peripherally directed and a greater number of centrally directed neurones in the tentacle ganglion.

The synchronous units recorded at the peripheral and central sides of the ganglion have been represented as a possible bipolar neurone with its processes arranged in either direction. This type of activity increased after stimulation so a synapse with an afferent unit originating from T1 or T2 has been included. It is possible that the synchrony of the units was fortuitous or that a more complex system may have been involved in propagating the responses, a possible arrangement being two or more unipolar neurones receiving excitation from a single afferent neurone.

Synaptically mediated motor activity was recorded from both T1 and T2 in response to low intensity stimulation of the tentacle, thus motor neurones have been included which received excitation from both of the low threshold sensory neurones, Type 1 and Type 2. No evidence was found to suggest that either type of neurone was solely responsible for the motor activity and no conclusion could be drawn concerning Type 3 or Type 4 involvment.

Figure 37. The sensory innervation in the tentacles of Littorina littorea. See text for discussion.


Tentacle tip
Motor neurone
Bipolar neurone
SENSORY NEURONES
Type 1
Type 2
Type 3
Type 4
Proximal tentacle ganglion
Nerves T1&T2
To cerebral ganglion

3.6 THE ORGANIZATION OF INTERGANGLIONIC PATHWAYS.

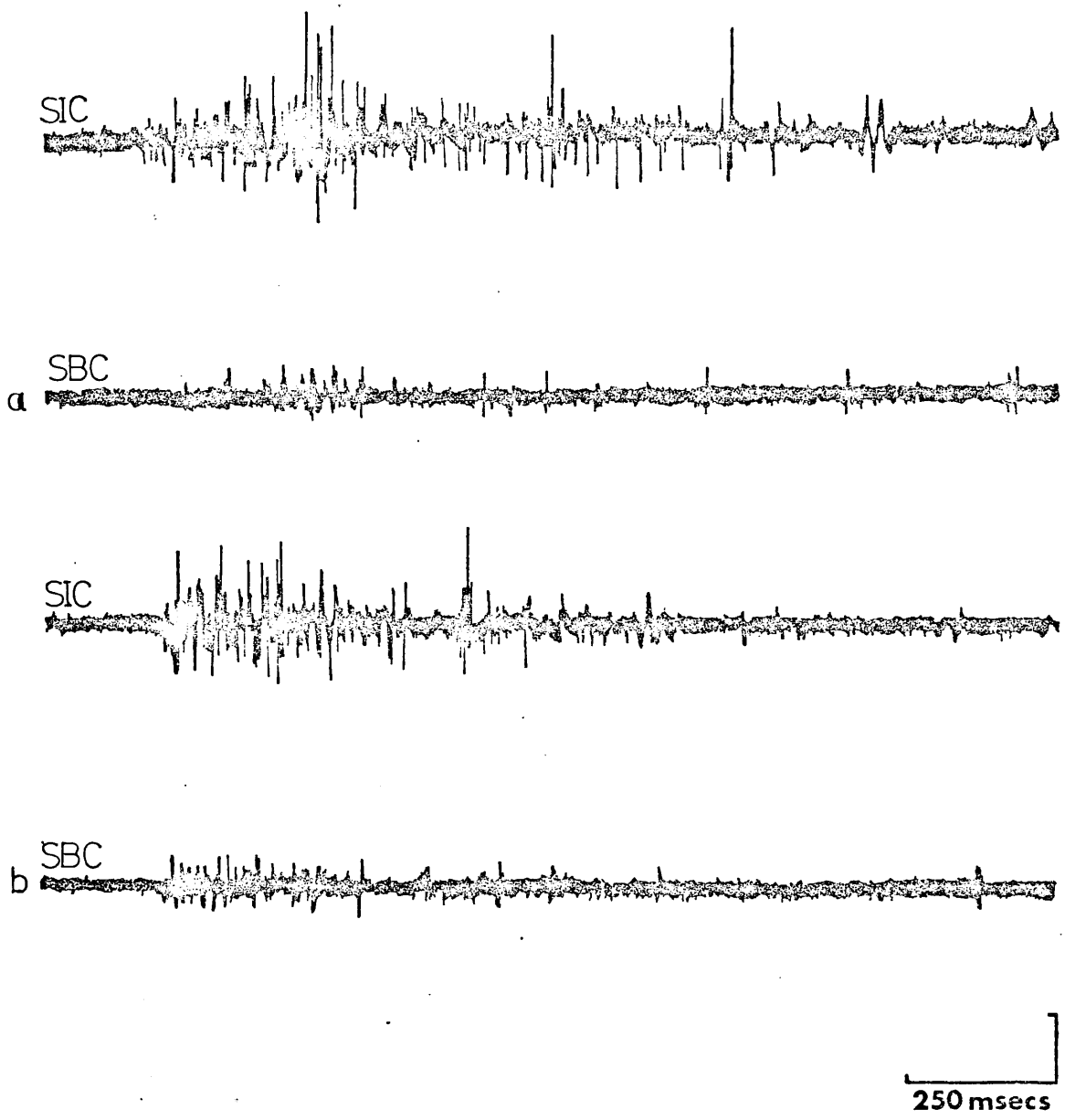
The interconnections between the six central ganglia suggested a number of possible pathways for the propagation of sensory information from the tentacles to the pleural ganglia and ultimately to the columellar muscle. To establish whether there were preferential pathways between the ganglia recordings were made from the pleural nerves of responses elicited by stimulation of the tentacles in C.N.S. preparations (section 2.53). A series of lesions was made in the interganglionic connections and their effects on the activity in the pleural nerves were noted. At this stage it was not attempted to make a qualitative analysis of the responses as they could be analysed on a simple present or absent basis.

Initially responses were recorded from the subintestinal and the supraintestinal connectives and later from the two pleural ganglion nerves LPl.1 and LPl.2 and the supraintestinal connective.

From each of the nerves complex characteristic responses could be elicited that were similar for both ipsilateral and contralateral stimulation of the tentacles. Figure 38 shows the responses obtained from the supraintestinal and subintestinal connectives by stimulation of the left and right tentacles.

The results of the lesion experiments have been summarised in figure 39. The first lesion was made in the cerebral commissure (fig. 39b) and, when either tentacle was stimulated, responses were recorded from both sides of the nervous system. As the pedal commissure was the only intact

Figure 38. Activity recorded from the supra-and subintestinal connectives in response to tentacle stimulation. Characteristic responses were observed in both connectives for (a) stimulation of the right tentacle (upper trace, supraintestinal connective; lower trace, subintestinal connective) and (b) stimulation of the left tentacle (traces as above). The supraintestinal response was characterised by numerous large amplitude spikes while that of the subintestinal connective consisted of low amplitude units.


SIC
a
SBC
SIC
b
SBC
20 µV
250 msecs

cross connection it must have propagated activity across the nervous system and had connections with two possible pathways: either via the ipsilateral cerebropedal connective to the pedal ganglia and then to the contralateral pleural ganglion by way of the pleuropedal connective, or, via the ipsilateral cerebropleural connective and the pleuropedal connectives to the contralateral pleural ganglion.

A second lesion (fig. 39c) was made in the left cerebropedal connective and the left tentacle stimulated: both contralateral and ipsilateral responses were recorded. Similarly when the right cerebropedal connective was cut (fig. 39d) and the right tentacle stimulated, responses were observed at both sides of the nervous system. From this it was concluded that the cerebral commissure and the cerebropedal connectives were not essential connections for the propagation of information across the nervous system. In order to determine if they were redundant within the system a further series of lesions was made in fresh preparations.

Firstly, the left cerebropleural connective was severed (fig. 39e) and the left tentacle was stimulated. In this instance only the contralateral response was recorded from the supraintestinal connective, but, when the cerebral commissure was severed (fig. 39f), the response was abolished. Stimulation of the right tentacle elicited responses from both sides of the system (fig.39g) which were abolished when a lesion was made in the right cerebropleural connective (fig. 39h). The only remaining connections and possible pathways were the cerebropedal connectives, consequently these must have been non-functional with respect to the

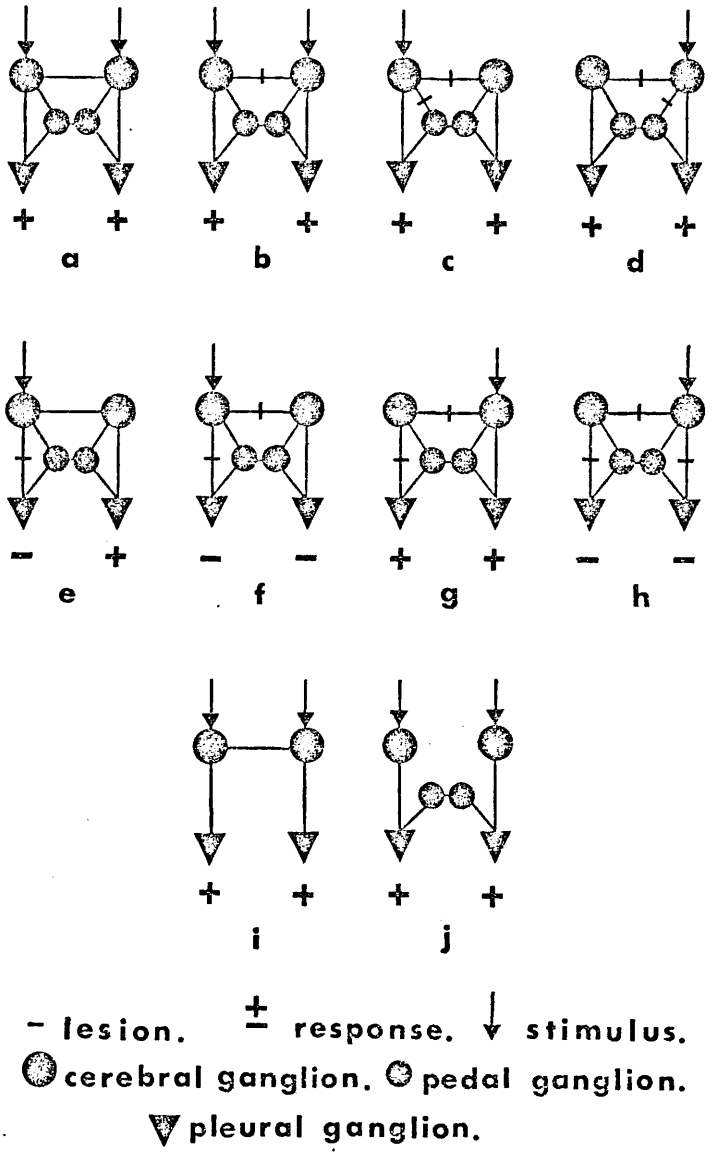


Figure 39. A summary of successive stages in the determination of mechanosensory central pathways. A systematic series of lesions was made in the interganglionic connectives and two pathways were revealed, the pedal pathway (j) and the cerebral pathway (i).

propagation of tactile information from the tentacles to the posterior sections of the nervous system.

The general conclusion was that two pathways existed for the propagation of tactile information across the nervous system. The first series of lesions (fig. 39b - d) indicated that information could travel from either cerebral ganglion to the ipsilateral pleural ganglion and then by way of the pleuropedal connective, the pedal ganglia and ultimately via the contralateral pleuropedal connective to the contralateral pleural ganglion, a pathway referred to as the pedal pathway (fig. 39j). The second series (fig. 39 e-h) showed that the cerebral commissure and the contralateral cerebropleural connective provided a second functional pathway referred to as the cerebral pathway (fig. 39 i).

The cerebropedal connectives were shown to be non-functional as far as the transmission of sensory information from the tentacles to the pleural ganglia was concerned, because when they were the only intact connections between the cerebral and pedal ganglia (fig 39 h) no post ganglionic responses could be recorded when the tentacles were stimulated.

3.61 Pathways and Receptors.

The significance of the two functional pathways for the propagation of tactile information across the nervous system was investigated in a series of experiments designed to reveal whether the pathways were associated with particular mechanosensitive neurones in the tentacles.

As before C.N.S. preparations were used and recordings were made from sites chosen to facilitate a comparison of

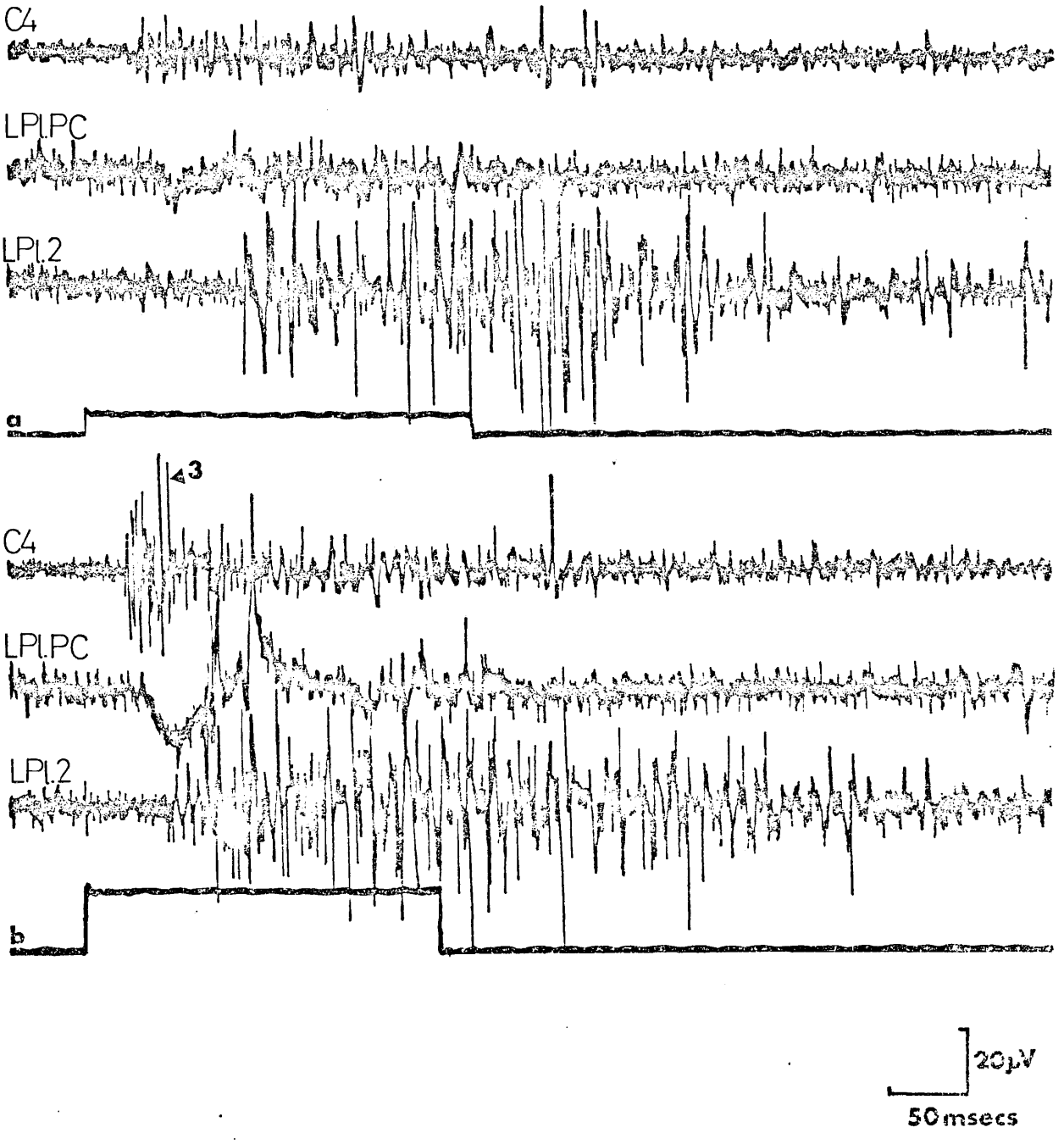
the activity propagated by the two pathways with sensory responses in the right tentacle nerve. The activity of the cerebral pathway was recorded from the central stump of the columellar nerve LPl.2, while the activity of the pedal pathway was recorded from left pleuropedal connective (LPl.C), after it had been cut close to the left pleural ganglion. Simultaneously en passant recordings were also made from the intact right tentacle nerve.

Throughout the experiments precautions were taken to ensure that the preparations were maintained under constant conditions between recordings and at least thirty minutes was allowed between repeated recordings taken from the same preparation. At first the effects of low and high intensity stimulation were compared (fig. 40) in the same preparation and without disturbing the position of the recording electrodes.

The low intensity stimulus (fig. 40a) elicited a compound response from the tentacle nerve (top trace) that lacked the large spike activity associated with Type 3 neurones while the response from the LPl.C (middle trace) corresponding to the pedal pathway, was a small compound potential barely discernible over the noise level. In contrast the pleural nerve (LPl.2) (bottom trace), representing the cerebral pathway, responded with a complex burst that contained numerous large amplitude units and showed prolonged after discharge following the stimulus offset.

High intensity stimulation (fig.40b) of the tentacle elicited a burst response from the tentacle nerve (top trace) that contained several large amplitude Type 3 potentials.

Figure 40. The effect of (a) low and (b) high intensity stimulation on activity in C4 (upper trace), the pedal pathway (middle trace) and the cerebral pathway (lower trace). (a) At low stimulus intensity the tentacle response lacked Type 3 sensory responses and there was little activity in the pedal pathway while the cerebral pathway showed a complex response (b) At high stimulus intensity Type 3 units were present in the tentacle response and there was an increase in the duration and complexity of the pedal pathway. The cerebral pathway showed an increase in response duration and a reduction in latency from approximately 70 msecs. to 35 msecs.


C4
LPl.PC
LPl.2
a
3
C4
LPl.PC
LPl.2
b
20μV
50msecs

The response of the pedal pathway (middle trace) showed a large increase in the amplitude of the slow component and several large spikes also appeared. The activity of the cerebral pathway (bottom trace) was increased both in intensity and duration compared to that observed at low stimulus levels.

A comparison of the latency between the onset of the tentacle response and the onset of other responses showed that the latency of the pedal response was more or less constant at about 20 msecs. for both levels of stimulation. The latency of the cerebral response, however, showed a marked decrease in latency from approximately 70 msecs. at low intensity stimulation to approximately 35 msecs. at high levels of stimulus intensity. These results were interpreted after consideration of the properties of the mechanosensitive neurones found in the tentacles.

At low levels of stimulus intensity the response of the tentacle nerve could be ascribed to Type 1 and Type 2 sensory neurones as these have been previously shown to be low threshold types, while the after discharge of the response may have been caused by induced reflex activity from the cerebral ganglion. Thus the activity observed in the two central pathways must have originated from one or both types of sensory neurone. As the responses showed relatively short latencies, the faster Type 1 neurones were probably responsible.

At higher stimulus intensity the tentacle response was more complex and contained large amplitude action potentials that were characteristic of Type 3 sensory neurones.

Coincident with the appearance of the Type 3 activity there was a large increase in the complexity and intensity of the pedal pathway response and a reduction in the latency of the cerebral pathway response. It was possible that the Type 3 activity was responsible for both of these effects but the Type 4 sensory neurone has also been shown to be a high threshold type. To determine the functions of these two sensory neurones further experiments were performed which exploited the rapid adaptation of the Type 3 responses.

Preparations were taken as before and the right tentacle was stimulated by the rapid application of two identical high intensity stimuli to the same point on its surface. The first stimulus was required to stimulate Type 3 neurones while the second stimulus was applied during their refractory period in order to elicit the Type 4 responses alone.

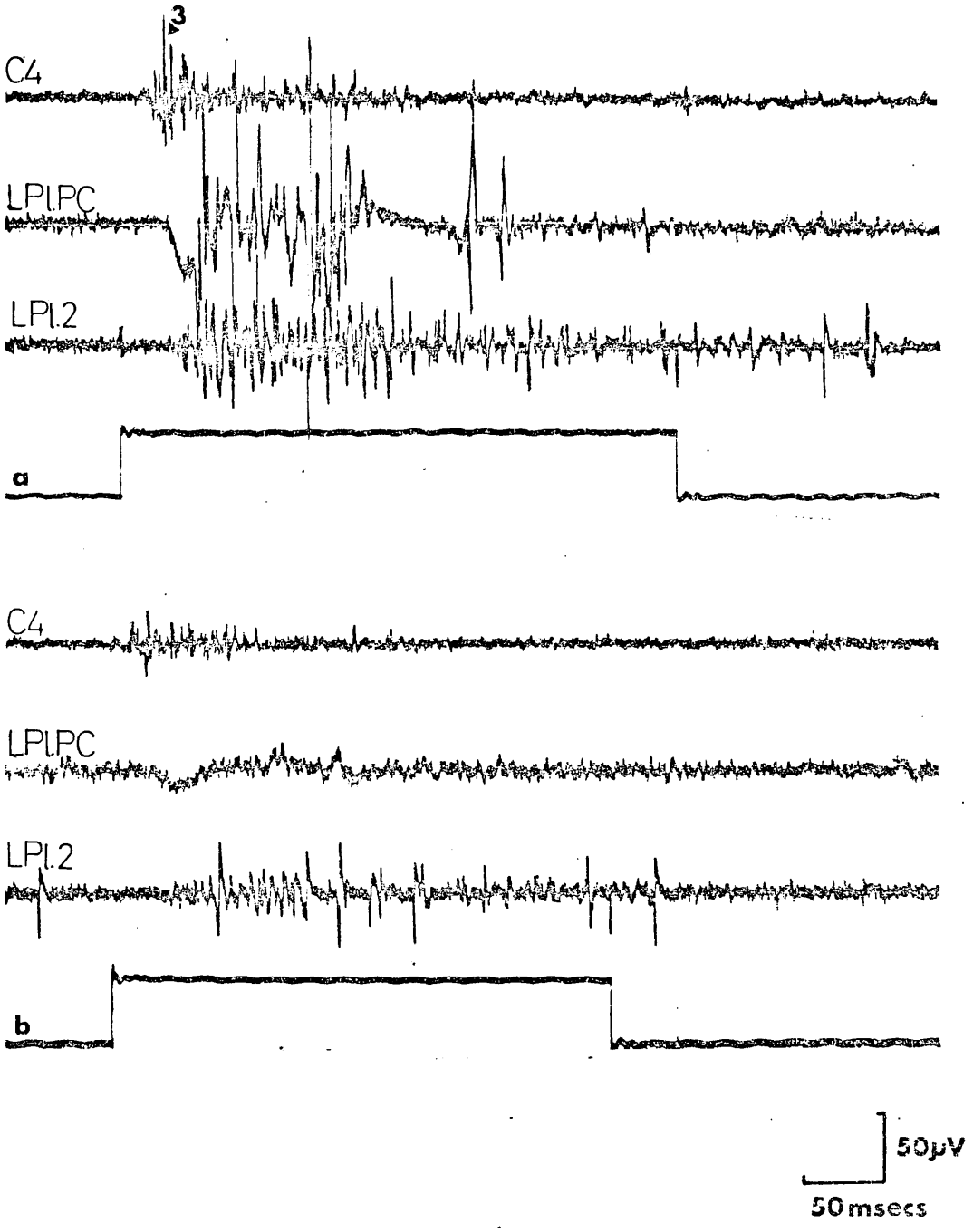
The results of one such experiment are shown in fig. 41. The first stimulus (fig.41a) elicited a normal response comparable to that observed in earlier experiments. In contrast, the second stimulus elicited a response from the tentacle nerve (fig. 41b, upper trace) that lacked Type 3 activity. The responses recorded from the pedal pathway (fig. 41b, middle trace) showed complete lack of the previously observed spike activity leaving only several low amplitude, slow potentials. Similarly the cerebral pathway reflected a reduction in spike activity but most significantly the latency of the response was not reduced.

The results discussed below, have been summarised diagramatically in figure 42. The cerebral pathway has been shown as receiving sensory input from Type 1 and Type 4 sensory neurones while the pedal pathway has been shown to

Figure 41. A comparison of the cerebral pathway responses to repeated stimulation (traces as in Fig. 40).

a) A single high intensity stimulus elicited the usual responses from the pathways and tentacle nerve.

b) A second stimulus, applied during the refractory period of the Type 3 tentacle responses, elicited reduced responses from both pathways but the latency of the cerebral pathway response remained constant although its onset was reduced in amplitude.


3
C4
LPl.PC
LPl.2
a
C4
LPl.PC
LPl.2
b
50μV
50msecs

be innervated mainly by Type 3 neurones with a small contribution from the Type 1 neurones.

At low stimulus intensities only Type 1 and Type 2 responses were elicited from the tentacles, but, because of the greater velocity of the Type 1 response, the cerebral pathway has been represented as receiving part of its excitation from Type 1 neurones. There was recorded from the pedal pathway at similar stimulus intensities a low level of activity that was characterized by a comparatively short latency from the onset of the tentacle response. Thus the pathway has been shown to receive some activity from Type 1 units, but the level of response recorded from the pedal pathway at low stimulus intensity suggested that its role was insignificant under these conditions.

As far as can be seen from the evidence the Type 2 neurones do not play an active role in this system and it is possible that they were only concerned with simple reflexes with motor neurones situated in the cerebral ganglia and supplying the muscle of the tentacles.

At higher stimulus intensities both pathways showed responses of increased intensity while the latency of the responses from the cerebral pathway were greatly reduced. When the Type 3 tentacle response was eliminated by repeated stimulation the overall activity of the pedal pathway was greatly reduced on subsequent stimulation, but, despite a similar reduction in the intensity of the cerebral pathway activity, its latency remained constant. If this activity had depended on Type 3 neurone responses then at least a change in latency should have been observed. Therefore other factors must have been involved and the cerebral pathway has

been shown as receiving input from the low intensity Type 1 units and the high intensity Type 4 mechanosensitive neurones from the tentacles.

The reduction in the intensity of the cerebral pathway response after the adaptation of Type 3 tentacle responses, was interpreted as either reflecting the adaptation of various Type 1 units within the stimulated area of the tentacle, or due to similar effects manifesting themselves in interneurones between the sensory units and those responsible for the observed responses.

Further investigations of the functional significance of the two pathways have been described in section 3.72, but the results of an investigation concerning the innervation and contraction of the columellar muscle have been included first.

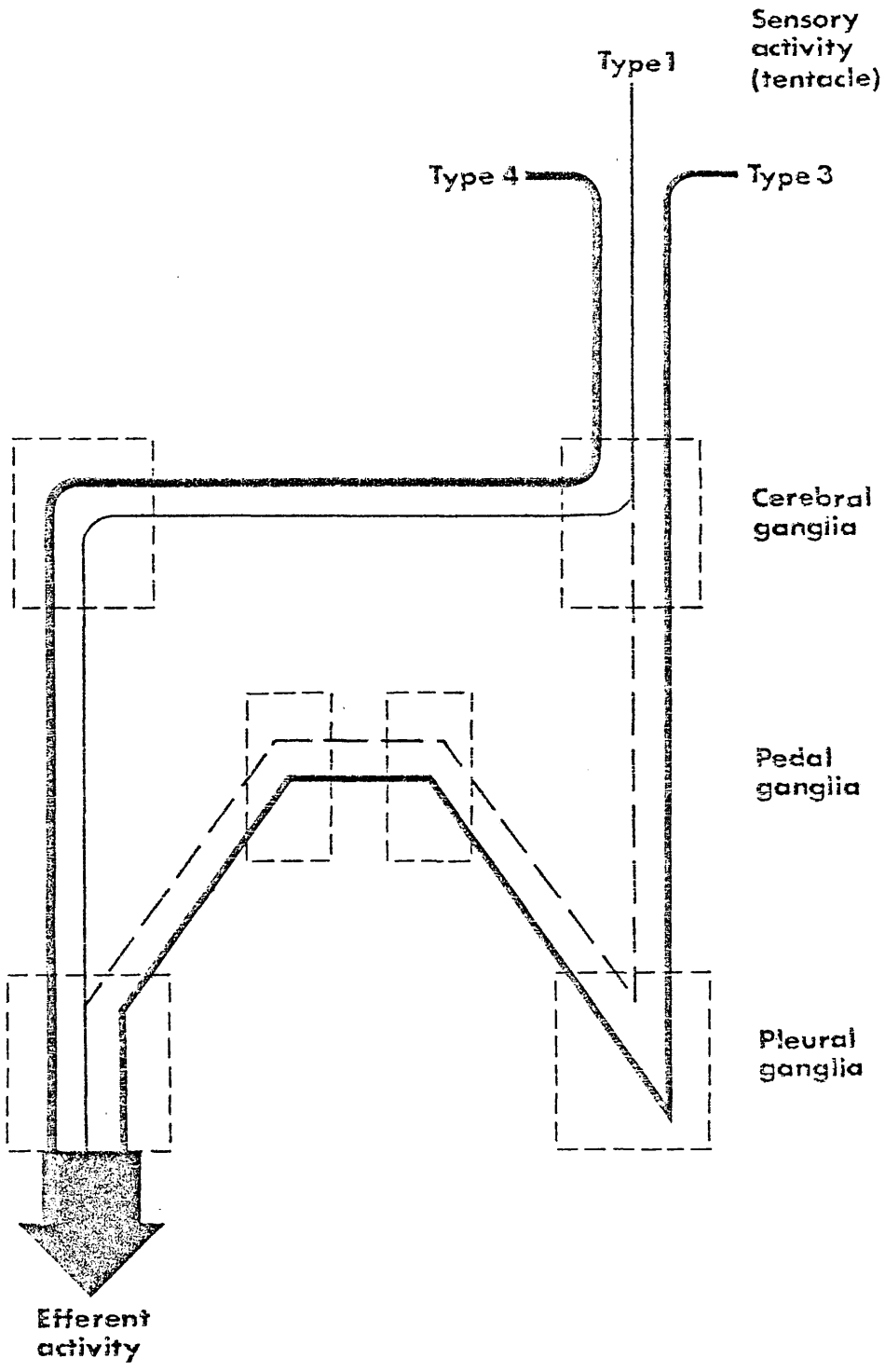


Figure 42. The system of central pathways and their sensory innervation. It was concluded that the pedal pathway received excitation from Type 3 and a few Type 1 neurones while the cerebral pathway was excited by Type 1 and Type 4 tentacle mechanosensory neurones.

3.7 INNERVATION OF THE COLUMELLAR MUSCLE.

In the course of detailed dissection it was noted that a nerve LPl.2, originating from the left pleural ganglion, appeared to enter the columellar muscle. This nerve had not been mentioned in earlier descriptions where it was stated that the columellar muscle was innervated by a nerve (V3) arising from the smaller visceral ganglion (Fretter & Graham, 1962). Further dissection showed that V3 did not enter the muscle but remained in the body wall immediately above it, whereas the pleural nerve gave rise to fine branches that entered the muscle's dorsal surface. Further details could not be observed by dissection therefore the motor innervation of the muscle was determined experimentally and the results compared with the evidence from the histological examination (section 3.29).

The experimental evidence was obtained by comparing the electrical activity of the muscle, and later its contractions induced by the stimulation of the pleural nerves. Initially it was attempted to stimulate the left pleural nerve LPl.2 electrically by the use of a suction electrode applied to its cut central end.

Electrical activity (EMGs) could be recorded from the muscle in response to stimulation of the nerve but stimulus isolation was poor. This could be improved by careful positioning of the indifferent stimulating electrode but an unacceptably large stimulus artefact remained. In order to eliminate the possibility of direct stimulation of the muscle by extraneous currents further preparations were

bathed in a non-conducting isotonic solution of sucrose. In these instances the nerve was laid across a pair of fine silver hook electrodes and lifted clear of the bathing solution prior to stimulation. Again transitory responses could be recorded from the muscle but the nerve rapidly became refractory after immersion, possibly because of excessive stretch incurred during the lifting procedure. Further attempts to stimulate the nerve while it was immersed were unsuccessful as contact between it and the electrodes could not be maintained.

Eventually the problems were eliminated by using a preparation which enabled the muscle to be stimulated indirectly by mechanical stimulation of the tentacles (section 2.54) and its nervous connections were established by observing the effects of a series of lesions made in the pleural nerves. The EMG's recorded from the muscle consisted of a complex potential of several hundred microvolts amplitude and long duration (fig. 43).

At first the muscle responses were recorded with all the pleural nerves intact. Then one side of the visceral loop was broken by cutting the supraintestinal connective and the effects on muscle responses were noted. A similar procedure was followed when the remaining part of the visceral loop was interrupted by cutting the subintestinal connective. In both cases the muscle's response induced by stimulation of the tentacles were unaffected. Thus it appeared unlikely that the muscle received motor innervation from either of the intestinal or visceral ganglia.

The experiment was continued to determine which of the pleural nerves was involved in mediating the muscle's

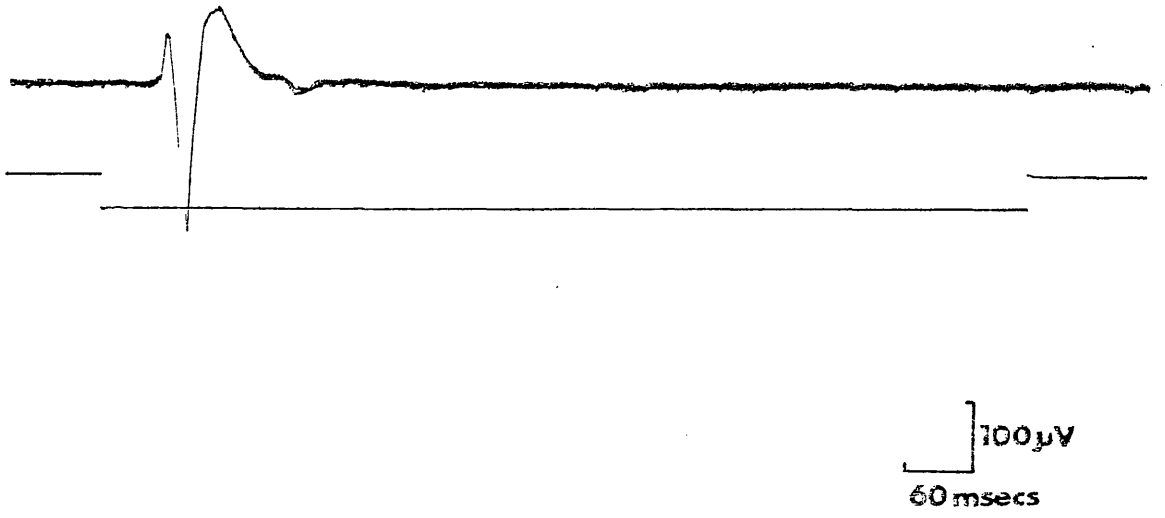


Figure 43. The columellar muscle EMG induced by stimulation of the right tentacle. The EMG consisted of a compound potential of large amplitude and long duration. Stimulus indicated by lower trace.

responses. Each nerve was severed in turn and the effects on the muscles responses were noted as before. The muscle responded normally when the right pleural nerve RPl.1 or the left pleural nerve LPl.1 were severed, but the responses were abolished when the left pleural nerve LPl.2 was broken.

It was concluded that the left pleural nerve LPl.2 was probably the only nerve carrying motor fibres to the muscle, but to ensure that no other nerves were mediating muscle responses a further experiment was performed.

Further preparations were made and, except for the left pleural nerve LPl.2, all the pleural nerves were left intact. It proved impossible to record muscle activity from this type of preparation when either of the tentacles was stimulated. Consequently the remaining pleural nerves and connectives were not implicated in the innervation of the muscle

Thus, within the limitations of the stimulating technique, it was established that the pleural nerve LPl.2 carries the motor fibres to the columellar muscle, and this conclusion was supported by the histological evidence.

3.71 Contraction of the columellar muscle.

After the innervation of the columellar muscle had been established the course of its contractions was recorded isotonically using the methods and preparations described previously (section 2.54). The contractions were induced by stimulation of the right tentacle in order to maintain a comparable point of stimulation for each stage of the experiment.

The responses were recorded under 'normal' conditions

from preparations in which all the pleural nerves, except for the columellar nerve LPl.2, were severed while the inter-ganglionic connections were left intact. Later a comparison was made with contractions recorded from preparations in which only one or other of the central nervous pathways was present. In all cases the columellar muscle was isolated, except for its nervous connections, from the remainder of the preparation in order to ensure that the observed contractions were not influenced by movements of other parts of the animal.

3.72. Normal contraction.

The typical contraction elicited by applying a single stimulus to the right tentacle is shown in figure 44. The response was phasic and could be divided into two stages: the initial stage was contractile and lasted for approximately 3.25 secs. The second stage followed immediately and it was seen as a period of slow relaxation proceeding at an almost constant rate until the muscle returned to its original length after a period of about 25-30 seconds following the point of maximum contraction. An intervening stage of tonic or maintained contraction was not observed.

The effects of repeated stimuli were additive and resulted in incremental increases in the degree of contraction (fig. 45). Each increment consisted of the normal contractile phase followed by the start of relaxation. The overall pattern of contraction was not changed except in degree, as after the final stimulus the muscle slowly relaxed until it

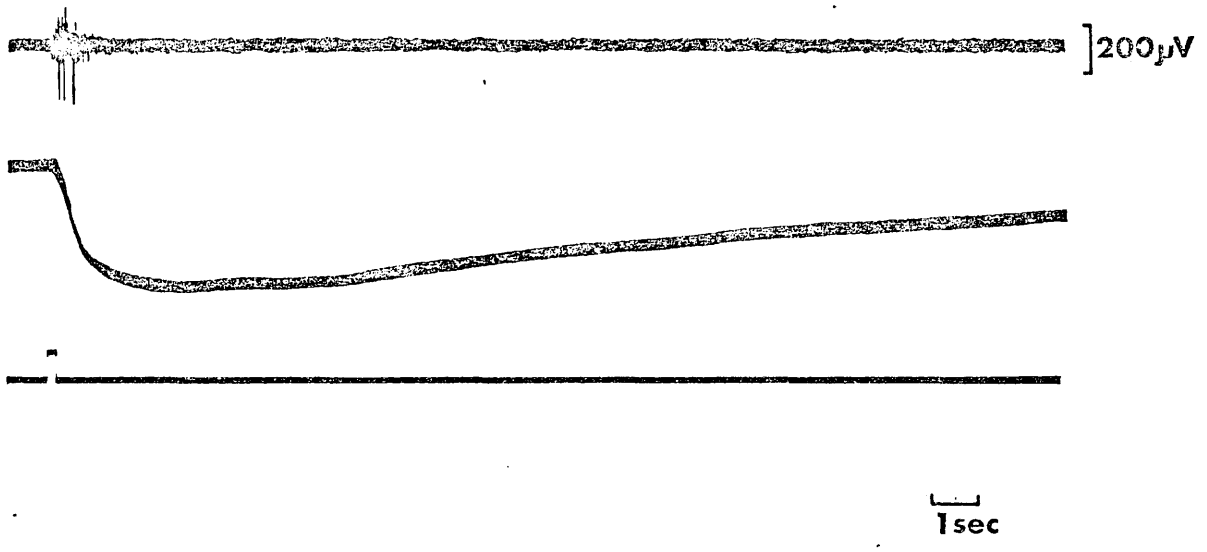


Figure 44. Columellar muscle contraction induced by stimulation of the right tentacle with both central pathways intact. Upper trace, EMG: middle trace, contraction indicated by downward movement: bottom trace, stimulus. The contraction was phasic with a typical duration of about 30 secs. During the period of prolonged relaxation the muscle lacked obvious electrical activity.

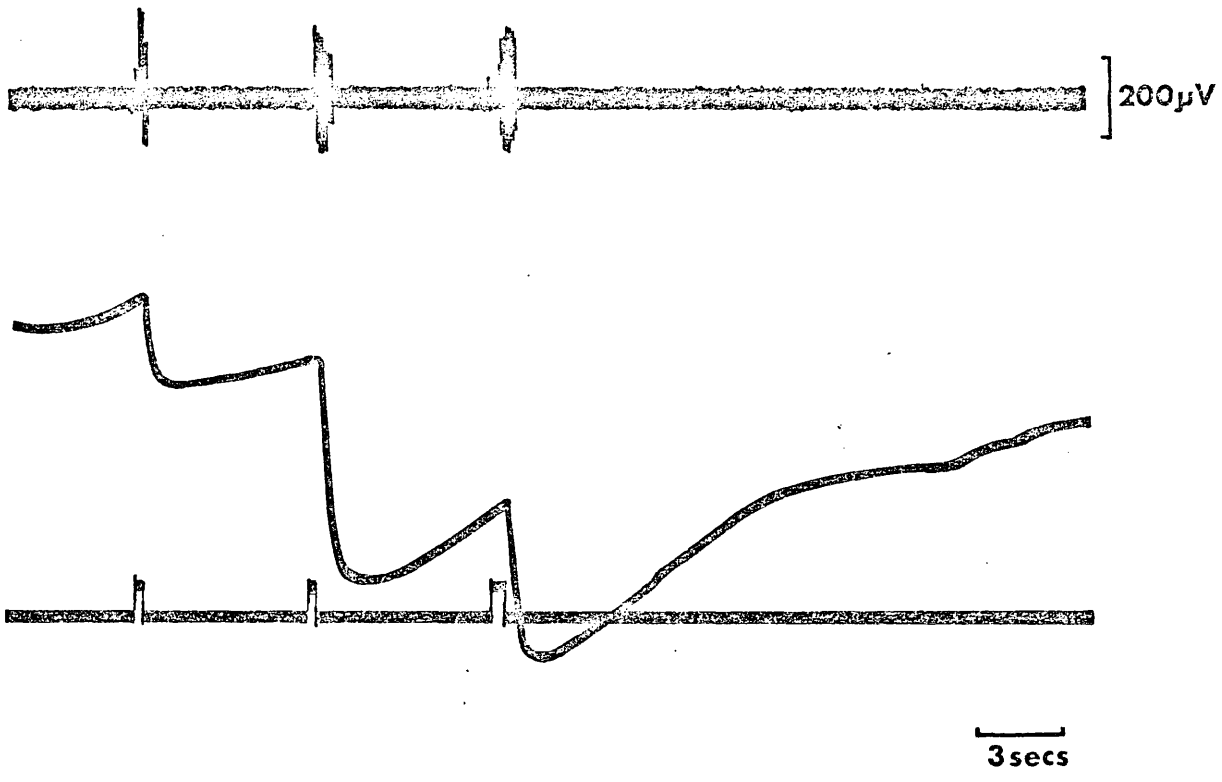


Figure 45. The muscle response of an intact preparation to repeated stimulation. Each successive stimulus induced a normal contraction and an increase in the overall degree of muscle contraction, while relaxation after the final stimulus continued as before.

returned to its normal resting length.

3.73 Central pathways and columellar muscle contractions.

In order to observe the muscle responses that could be elicited by stimulation via the two central pathways, fresh preparations were taken and a comparison was made of the muscle contractions that could be induced via either the cerebral or the pedal pathway, in response to a single stimulus of the right tentacle. Subsequently the effects of repeated stimulation were similarly compared. Initially contractions were recorded when both pathways were intact, then a lesion was made in the cerebral commissure to leave only the pedal pathway intact, and the observations were repeated.

Both pathways were capable of transmitting information that initiated apparently normal contractions of the columellar muscle (fig. 46) in response to a single stimulus. When the effects of repeated stimulation were investigated it was found that after the cerebral commissure was cut the muscle usually responded to only the first stimulus of a series (fig. 47) while prior to cutting the commissure the responses were similar to those observed previously (fig. 45). In some cases two or three successive contractile phases could be recorded in response to successive stimuli after the commissure had been cut, although responses were usually absent after several stimuli had been applied to the tentacle. Even in those preparations which failed to give obvious responses after the initial stimulus, the relaxation phase of the response was not the usual steady extension associated

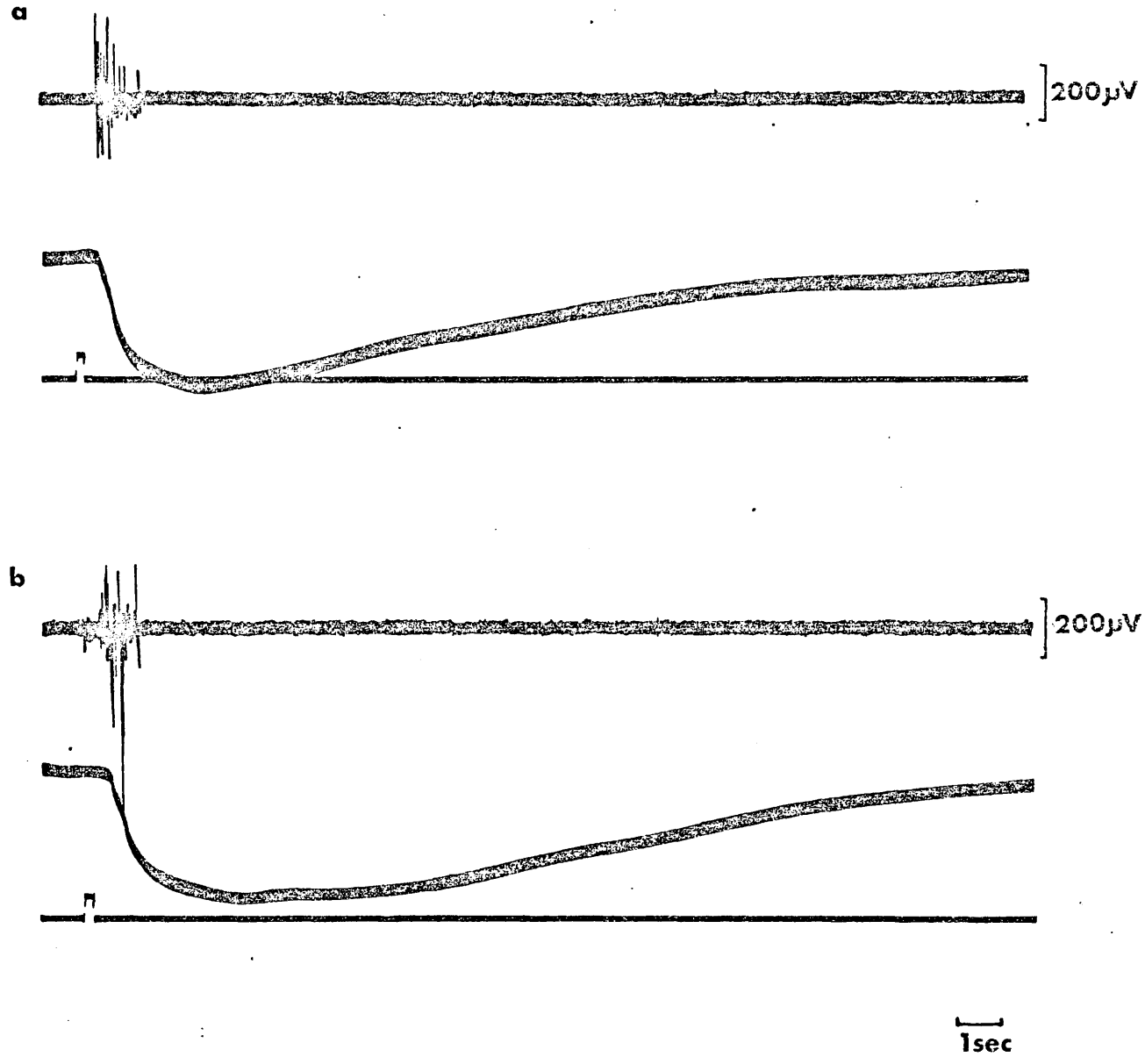


Figure 46. The columellar muscle contractions elicited by stimulation of the right tentacle of preparations in which a) the cerebral pathway and b) the pedal pathway was intact. In both instances apparently 'normal' muscle responses were observed. Compare with figure 44.

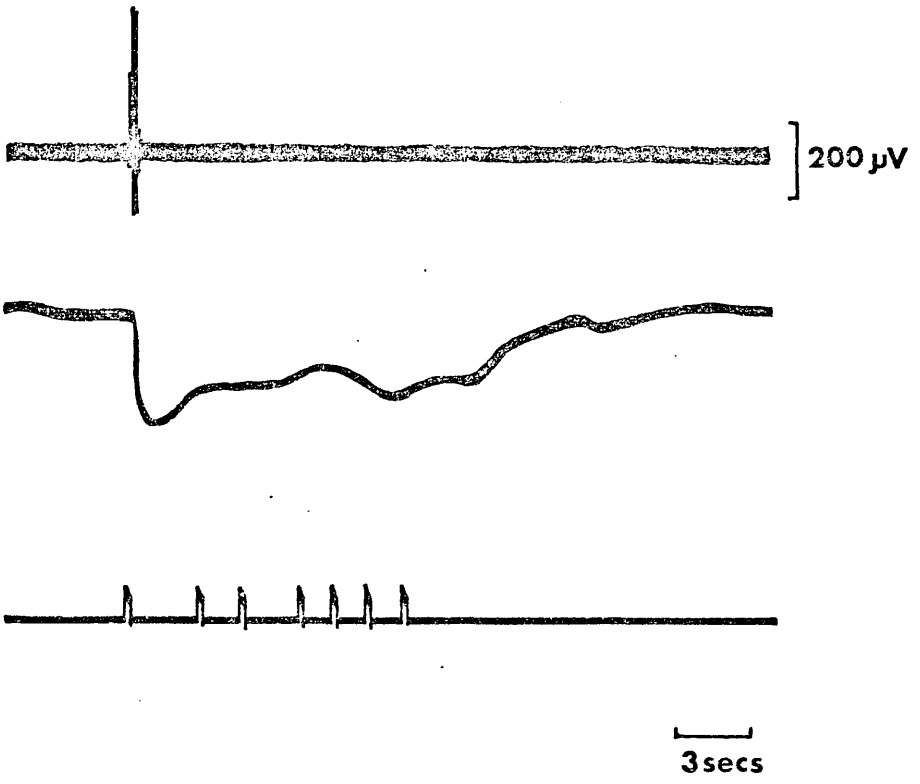


Figure 47. The effects of repeated stimulation on muscle contraction via the pedal pathway. The muscle showed a definite contraction to only the first of a series of stimuli although small muscle movements were apparent during the relaxation phase. Compare with figure 45.

with normal preparations, but showed small irregular changes in muscle length.

A comparison of the above data with that concerning the mechanosensitive neurones of the tentacles and their links with the central pathways shows them to be complementary. It was shown that the cerebral pathway was innervated by low threshold Type 1 neurones, and slowly adapting, high threshold Type 4 neurones (section 3.61). In contrast the pedal pathway was shown to receive its excitation from a limited number of Type 1 neurones and the rapidly adapting, high threshold Type 3 neurones. Thus the adaptation of muscle contraction after cutting the cerebral commissure to leave only the pedal pathways intact, can be attributed to the characteristic adaptation of the Type 3 mechanosensitive neurones in the tentacles. Whereas the repeated muscle responses in those preparations in which both pathways were intact can be said to reflect the properties of the Type 4 tentacle neurones. Those preparations with severed cerebral commissures that showed less rapid adaptation of muscle responses may have had a greater contribution from Type 1 neurones in the pedal pathway than in other animals. Similarly the activity of Type 1 neurones may have been responsible for the irregularity of the relaxation phase observed in those preparations which showed rapid adaptation.

It is suggested that the cerebral pathway is functional at low and high stimulus intensity and, at high intensity, functions as a high threshold non-adapting escape system. In contrast the pedal pathway functions as a rapidly adapting system that is operational at slightly

lower threshold than that of the cerebral pathway.

Further experiments were attempted in which the differences of threshold between tentacle neurones were further exploited. Unfortunately the tentacles of the preparations were particularly active and accurate discrimination between small differences in applied stimulus intensity was not possible. In some cases attempts were made to immobilize the tentacles, but, without the use of anaesthetics, they became extensively damaged and further attempts were abandoned.

A comparison of the system with similar systems in other molluscs, notably Lymnaea stagnalis, has been included in the general discussion.

3.74 Motor activity of the left pleural ganglion.

As it had been shown that the columellar muscle is innervated by the left pleural nerve LP1.2 the possible occurrence of groups of motor neurones within the ganglion was investigated as these may have proved suitable for an intracellular study.

The preparations were similar to those used in the studies of the columellar muscle and responses were induced by stimulation of the right tentacle. Recordings were taken from restricted areas of the ganglion's surface by the application of fine tipped suction electrodes and the recorded responses were compared with responses recorded simultaneously from the central stump of the nerve LP1.2.

A variety of activity could be recorded from almost all points on the ganglion's surface but the most active area was found to be between the two nerves LP1.1 and LP1.2

Despite careful recording from all areas of the ganglion it was impossible to correlate surface activity with response recorded from the nerve. Therefore it was concluded that the ganglion lacked extensive motor areas concerned with the columellar muscle, but the possibility remained that scattered ganglion cells were involved in such activity. As specific sensory areas were not found further studies were not made.

3.8 SPONTANEOUS ACTIVITY IN THE C.N.S.

In Littorina complex activity was recorded from the central stumps of all the nerves present in isolated C.N.S. preparations, but activity was usually recorded from the sub - and supraintestinal connectives. The activity consisted of numerous units some of which were completely arhythmic, while others maintained a more regular level of firing. No completely rhythmic units were observed. The overall level of activity showed considerable variations when considered over long periods and consisted of prolonged bursts of activity followed by periods of lesser activity. Overall the activity could be recorded for many hours from individual preparations and, although the life-span of preparations was never determined, it was in excess of twenty four hours.

The supra - and subintestinal connectives showed characteristic activities (fig. 48) : that of supraintestinal connective contained large amplitude and many smaller units, while that of the subintestinal connective was characterised by a lower level of activity and lacked the large amplitude units present in the supraintestinal connective.

By cutting the connections between the pleural ganglia and the other central ganglia the effects of isolation on the levels of activity were observed. The effect on activity of both ganglia was the same when their respective pleuropedal and cerebropedal connectives were severed. In each case the frequency of activity was greatly increased immediately after the connective was cut but it gradually returned to about the level observed before the cuts were made. Activity could be recorded for many hours from the isolated ganglia thus they must have contained auto-active

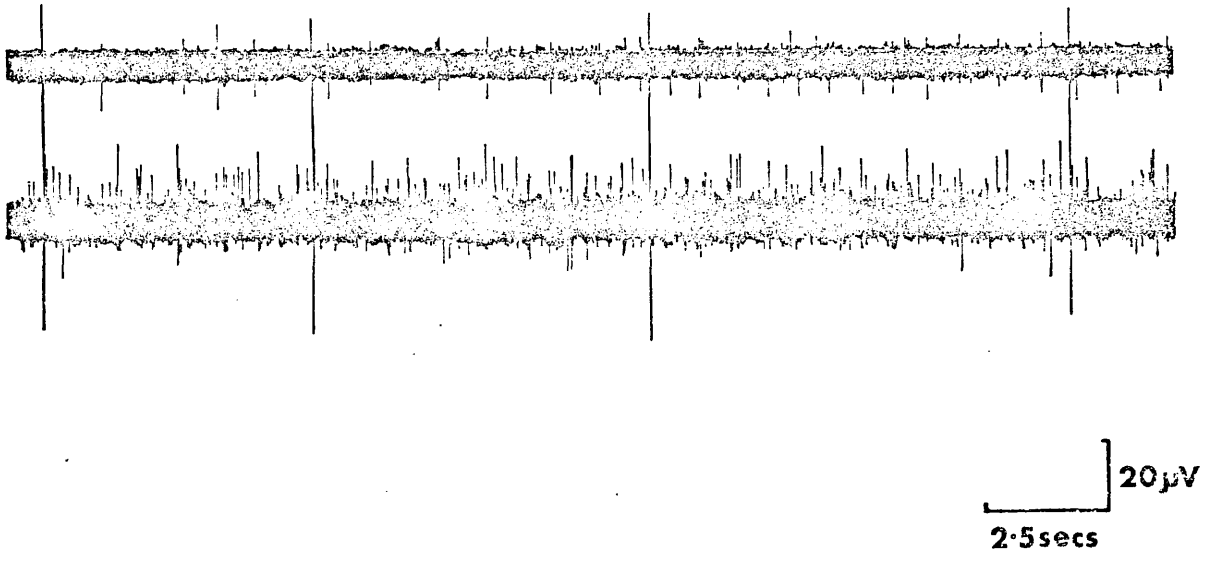


Figure 48. Spontaneous activity recorded from the supra and subintestinal connective in isolated C.N.S. preparations. The activity of the supraintestinal connective (lower trace) contained large amplitude fairly regular, units and numerous smaller asynchrous units while the subintestinal connective (upper trace) showed a lower level of activity without large amplitude spikes. The activity of both connectives showed alternating periods of high and low intensity activity.

cells which may have been responsible for much of the activity observed in intact C.N.S. preparations.

It was planned to make further investigations of these phenomena by building and using an instantaneous frequency analyser and level selector as described by Matthews and Searle (1972). Unfortunately the experiments had to be postponed and eventually abandoned because of difficulties in obtaining some of the required components during the industrial disruption of 1974.

3.9 CONDUCTION VELOCITY.

The problem of determining the rates of nervous conduction in molluscan nerves is complicated by their inherent elasticity. Various workers have measured conduction velocity in the nerves of a range of species (reviewed by Bullock & Horridge), but only some of these studies have included the effects of stretch on their results (Goldman, 1961).

Turner & Nevius (1951) found that the fastest rate of conduction in the pedal nerves of Ariolimax columbianus was 0.64 M/sec at 19.8°C, while Turner (1951) showed that the rate of conduction, also in the pedal nerves of A.columbianus, was unchanged for a twofold increase in length. The problem of measuring the functional length of nerves in the giant African land snail Archachatina marginata (Swainson) was overcome by Nisbet (1961a) who devised a formula that defined functional nerve length in terms of shell dimensions. The formula was used in investigations of conduction velocity and other properties of the pallial and visceral nerves of Archachatina (Nisbet 1961b)

The supraintestinal connective was chosen for conduction velocity studies as it is the longest nerve or connective without major nerve branches that Littorina possesses, measuring 4 - 5 mm. in length in a large specimen. The experimental technique has been described previously (section 2.8), but the length of the connective created some problems as it was difficult to arrange it over the electrodes without the risk of damage and difficulty was encountered when it was attempted to measure its functional length.

The preliminary results were variable: the range of conduction velocities calculated for the fastest component of the invoked response extending from 0.24 M/sec. to 0.69 M/sec.

The range of values was thought to reflect inaccuracies in measuring the connective's length between the stimulating and recording electrodes compounded by the effects of varying degrees of stretch. The shortness of the connective meant that any small error inherent in the measuring technique was large when considered as a percentage of the inter-electrode distance, usually 1.5 to 2.0 mm. Therefore it was not attempted to use a similar method to that used by Nisbet (1961a) in order to overcome the problem of functional nerve length determination. By exercising great care to ensure that a similar degree of stretch was present in each preparation when it was arranged across the electrodes, more consistent results were obtained. Unfortunately the connective proved too short and delicate to permit more objective studies of the effects of stretch to be made and similar problems were encountered when attempts were made to utilize the cerebral nerves C2 and C4, the tentacle nerve.

The stimulus intensity was varied by increasing the duration of constant voltage pulses rather than by increasing the pulse voltage as it was found that this method provided better stimulus isolation although similar results were obtained when the latter method was used. A maximal response was elicited by a stimulus of 2.5 volts and 0.1 msecs. duration. It consisted of an initial fast component followed by a large compound potential comprising several units and

several slower potentials of smaller amplitude (fig. 49) The slower components of the response usually had a higher stimulus threshold than the faster components.

The conduction velocity of the fast component of a series of ten preparations was determined and the mean of the results was calculated to be 0.52 M/Secs. (Table 2). Several series of determinations were made from both the supraintestinal connective and the cerebral nerve C2 but only one series of results from ten supraintestinal connectives has been included as the remainder were not satisfactory.

Conduction Distance (mm.)	Conduction Time (M/secs.)	Conduction Velocity (M/sec.)
2.90	6.25	0.46
1.50	5.60	0.50
2.75	6.87	0.40
2.90	6.25	0.46
2.30	5.31	0.43
1.50	3.37	0.44
2.75	4.06	0.67
2.50	4.68	0.53
2.50	3.75	0.66
2.60	3.75	0.69
	MEAN	0.52 M/Sec.

Table 2. Conduction velocity of the supraintestinal connective: mean calculated from ten determinations in separate preparations.

The results suggested that the supraintestinal connective contained several fast, low threshold, and therefore

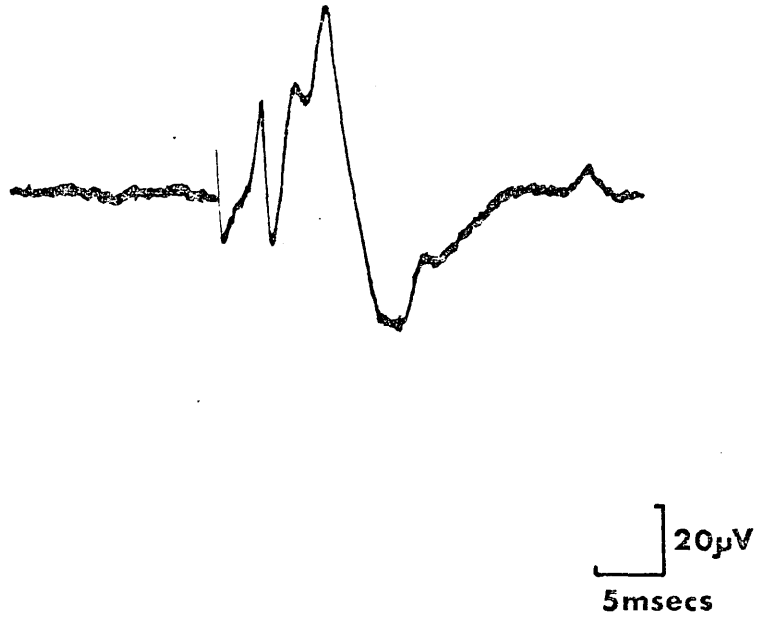


Figure 49. Electrically invoked responses from the supra-intestinal connective. The response consisted of a fast component followed by a higher threshold compound potential and other smaller potentials. The conduction velocity of the fastest component, the mean of ten determinations, was 0.52 M/sec.

relatively large diameter axons, with a conduction velocity of about 0.52 M/secs. While the higher threshold component of the maximal response was the activity of various smaller diameter axons with correspondingly lower conduction velocities.

4.0 DISCUSSION.

The winkle *Littorina littorea* has had many aspects of its biology investigated, but, in common with many other prosobranch molluscs it had been previously ignored from the neurophysiological viewpoint. Therefore it was chosen as the subject for the present investigation in which various aspects of its nervous organization have been studied. Particular attention has been paid to the sensory elements and central pathways mediating the animal's withdrawal response which is brought about by contraction of the columellar muscle. The response may be induced by various modes of stimulation but the experimental analysis was restricted to mechanical stimulation of the tentacles.

Both the morphological and the histological studies of the nervous system gave results that, with the important exception of the columellar muscle innervation and some other minor differences, were largely in agreement with those of other workers. The muscle was originally described by Fretter and Graham (1962) as receiving a nerve from the smaller visceral ganglion, but histological and physiological evidence has shown that it is innervated from the left pleural ganglion by the nerve LPl.2.

The physiological experiments were limited because of the difficulty in ensuring stimulus isolation when possible motor nerves supplying the muscle were stimulated electrically. This difficulty was overcome by using indirect mechanical stimulation of the tentacles and observing the effects on muscle activity as the nerves and connectives from the pleural

ganglia were systematically severed. An experiment of this type leaves the possibility that motor nerves, other than that indicated by the results, were present and were not excited by the mode of stimulation. In the present study this seemed unlikely as the induced muscle response formed the basis of the animal's escape reaction in which rapid and synchronized contraction of the muscle is necessary. Also the histological results supported the physiological evidence for a single motor nerve supplying the muscle. Kater et.al., (1971) mentioned that the columellar muscle of Helisoma trivolvis (Say) is innervated by a single nerve trunk, but its origin was not stated. The literature concerning the columellar muscle innervation in other species is vague, but the visceral ganglia are usually described as innervating the organs of the visceral mass only (Barnes, 1968). The further points of disagreement with other accounts of the nervous system may, in part, be due to variability between individuals thus the significance of the separation of the two visceral ganglia, described by Fretter and Graham as being formed from a single mass, cannot be regarded as significant without further investigation. The failure, however, to confirm a connection between the left pleural ganglion and the supraintestinal ganglion was probably due to the difficulty of tracing the finer nerves through the tissues. The functional significance of such a link is probably limited because of the small diameter of the nerves in that region of the animal.

If the vital staining technique (Alexandrowicz, 1960) had proved successful further details of the nervous system would have been revealed. A similar failure was reported by Bailey (1965) after attempting to stain the nerves of Buccinum undatum. Thus it appears that the marine prosobranchs may be unsuitable subjects for this technique.

The histological appearance of the central ganglia and nerves is typical of the gastropods and agrees with the earlier observations of Littorina by Al'Kufaishi (1970). The size range of the ganglion cells was similar to that reported for Buccinum (Bailey, 1965), varying from less than one micron to 40 microns in diameter, although the distribution of cell sizes was differert. In Littorina the majority of cells in the central ganglia were less than 10 microns in diameter whereas in Buccinum the most common cells were between 6 - 20 microns diameter. Al'Kufaishi described a seasonal variation in the size of fuchsinophilic cells in the ganglia and recorded a maximum diameter of 60 microns for a small group of cells in the left cerebral ganglion of the female which were assumed to be neurosecretory. In this study the largest cells that were observed were also scattered in the cerebral ganglia and measured about 40 microns along their greatest axis. A possible explanation for the differences in maximum cell size is that in the present study the animals were examined between December and March corresponding to the period of minimum size in the cycle of variation of cell size described by Al'Kufaishi. Generally Littorina may be said to share the characteristics of Buccinum in lacking giant ganglion cells.

The cortex of ganglion cells was extensive in all the central ganglia but it was particularly complex in the ventral margins of the pedal ganglia. It is usual to associate such complexity with high integrative properties, thus the pedal ganglia have been assumed to be of great importance. Similar observations have been made for other species (reviewed by Bullock & Horridge) and Janse (1974), after considering the

tactile areas innervated from the central ganglia in Lymnaea stagnalis, came to the same conclusion.

The proximal tentacle ganglion share the same structure as the central ganglia and usually gave rise to two or occasionally three fine tentacle nerves although they were described by Fretter and Graham as giving rise to numerous fine nerves. The organization of the distal ganglionic region lacked a comparable neuropile while the ganglion cells were either similar to normal cells or were large 'neurone like' cells. The presence of peripheral neurones in molluscs has been inferred from a number of studies of isolated molluscan tissues such as the gill of Aplysia (Peretz, 1970), Mytilus adductor muscle (Bowden &Lowy, 1955) and they have been observed in the nerve trunks of other species as in the nervus intestinalis of Helix pomatia (Schlote, 1957) and the visceral nerve of Archachatina (Nisbet, 1960). Prior (1972a) has shown that neurone-like cells at the junction of the siphonal nerves of Spisula solidissima mediated local contractions of the siphons and it is possible that the local responses and tonic contraction of the tentacles in Littorina are mediated by the cells of the distal ganglion. Unfortunately its position, embedded in the tissues of the tentacles, precluded further investigation as it could not be exposed without causing major damage. A further possibility was that local reflexes were mediated by peripheral nerves situated in the tentacle walls as it has been shown that the body walls of many species are richly supplied with such elements (reviewed by Bullock & Horridge). Although it was unsuccessfully attempted to reveal peripheral elements in the tentacles of Littorina by the use of a silver

staining technique, their presence cannot be discounted, particularly as silver staining techniques are known to be capricious.

The lack of detail that was observed in transverse sections of the nerves, except for the left pleural nerve LPl.2. is a feature in common with many gastropods including Buccinum (Bailey, 1965) because the constituent axons are very fine, often less than 1 micron in diameter (Scholte, 1957). The columellar nerve LPl.2 was exceptional because in transverse section about 60 axons could be observed, measuring up to 23 microns in diameter. In the nerves of other species where large axons have been observed, such as Aplysia (Bantham 1961), Archachatina (Nisbet, 1957) and Helix (Schlote, 1957), these have shown longitudinal glial inpushings while the fine axons were more regular in outline and the distribution of axon diameters was irregular. As far as could be observed in Littorina the large axons were without glial inpushings and the distribution of axon diameters appeared fairly regular, but the fine structural details of the axons were below the resolving power of the light microscope. The unique structure of the nerve is a reflection of its function in that it is required to cause the rapid contraction of a large muscle in order to initiate the animal's escape response. Bullock (in Bullock & Horridge 1965) has suggested that the columellar nerves of snails may be useful for structural studies of molluscan nerves and it is hoped that the columellar nerve of Littorina will be examined by electron microscopy at a later date.

The induced contractions of the columellar muscle were essentially phasic and were similar in outline to the animal's

withdrawal response. The time course of the muscle contraction was about 25 secs while that of the withdrawal response was typically 5 secs. This difference may be accounted for if the loading factor of the shell and the antagonistic effects of other regions of body musculature are considered in the withdrawal response. Without these factors the contraction of the muscle must be greatly increased with a corresponding increase in the time for relaxation. The greatest degree of contraction that was observed was about 50% of the normal free length of the muscle. It is unknown if this is within the muscle's normal range of contraction although Bozler (1930) has described the columellar muscle of Helix pomatia as contracting to 10% of its free length. The columellar muscle of Helisoma trivolvis has been shown to have a similar structure to that of Littorina and also to contract in a similar manner (Kater et al., 1971).

Relaxation of the muscle proceeded at a slow, almost constant rate until it returned to its normal length. In other molluscan muscles which showed gradual relaxation, such as those of the mantle of Spisula (Wilson and Nystrom, 1968), the relaxation phase was accompanied by electrical activity, but this was absent in Littorina. It seems unlikely that the columellar muscle was innervated by the double system suggested for Spisula (Wilson, 1969) and for other molluscan muscles, and it is possible that slow contractions of the vertically arranged fibres of the muscle were involved in its extension.

The behavioural response that has been utilized as a basis for much of the investigation is an escape response involving a rapid withdrawal of the exposed head and foot

into the safety of the shell. In the Littorinacea and other operculate prosobranchs, the shell aperture may be sealed by the operculum in order to give greater protection against predation and changing environmental conditions. The various stimuli which invoked the response in Littorina were similar to those that invoked withdrawal in Lymnaea (De Vleiger, 1965) and included vibration, shadow and tactile stimulation. A similar variation in the extent of the response with stimulus intensity was also observed, starting with a local contraction of the tissue at the point of stimulation for low intensity stimulation. Higher intensity stimulation elicited the phasic withdrawal response in Littorina, but the animal could not be induced to withdraw completely even by repeated stimulation. In contrast Lymnaea could be made to withdraw completely and to remain so for several minutes. De Vlieger described how the response varied according to the animal's orientation; if an animal was creeping over a submerged substrate tactile stimulation led to the loosening of the foot from the substrate and, because of the air in the lung cavity, the animal tended to float to the surface. The reverse was observed when a snail was creeping upsidedown at the water surface because air was expelled from the lung cavity and the animal tended to sink.

The different responses of Lymnaea and Littorina reflect the environments which they inhabit. Lymnaea is a fresh water pulmonate that is usually found amongst the vegetation of ponds and streams where water currents are gentle and where there is little danger in temporary loss of attachment. Littorina inhabits the rugged intertidal zone where

it is exposed to the elements, predators and to violent wave action. In these conditions the animal must retain a firm hold on to a substrate to resist being dislodged, consequently its foot is not usually withdrawn in the normal escape response. It is interesting to note that Littorina may often be found crawling over sand and soft mud in rockpools although the effects of these substrates on the response have yet to be investigated.

Accommodation of the response in Littorina to repeated stimulation enables the animal to adapt to an environment where frequent spurious stimulation is present, for instance bombardment with fine debris and disturbance by wave action. Despite this the animal retains its sensitivity to stimuli of greater intensity and is therefore able to discriminate between spurious stimuli and a threatening stimulus caused by a predator, or a significant change in the environment, partly because of the arrangement of the mechanosensitive neurones in its tentacles.

The first step in the electrophysiological experiments was to map the tactile sensory areas of the various cerebral nerves. The degree of overlap between the areas and the wide variations that have been recorded from different individuals has been observed in other molluscan species including the bivalve Ensis directus (Clivo, 1970), Aplysia fasciata (Hughes, 1971) and Lymnaea stagnalis (Janse, 1974), and has been observed in individuals belonging to species of different phyla including the annelids (Mill & Knapp, 1967: Nichols & Baylor, 1968). Yet wide variability of sensory areas is not a universal feature of the invertebrates as discrete sensory

areas have been reported in the crustaceans (Hughes & Wiersma, 1960).

The complexity of the tentacle innervation in Littorina, is comparable with that of the peripheral nervous organization of other species and in contrast to the simple system of tentacle innervation suggested by Hanström (1926). This consisted of a simple reflex between tentacle sensory and motor nerves in the cerebral ganglia and was without mention of the tentacle ganglia or peripheral neurones and reflexes.

The local reflexes that were observed in the absence of central connections were similar to those observed by De Vlieger in the isolated lip preparations of Lymnaea. De-Vlieger noted that the time required for relaxation of tissues after stimulation was greater when the tissues' central connections had been severed. A similar phenomenon was not observed in Littorina with respect to restricted contractions, but the overall contractions of the tentacles were greatly prolonged in the absence of the cerebral ganglia. De Vlieger concluded that in Lymnaea the relaxation of locally contracted tissues was at least partially controlled from the C.N.S. In Littorina the relaxation of locally contracted tentacle tissue appeared to be peripherally controlled, whereas the overall motor control of the tentacles must have been centrally derived and, as shown by the experiments, derived from the cerebral ganglia without the apparent involvement of the tentacle ganglia.

In further experiments the tentacle ganglia were shown to be largely sensory, but reflexes involving efferent activity in both the fine tentacle nerves T1, and T2, and the main tentacle nerve Ch, were found to occur in the proximal

tentacle ganglia. Their significance appears to be limited as the tentacle responses were similar in both the presence and absence of the proximal tentacle ganglion, although they may play some role, together with the other elements, in the mediation of peripheral reflexes. A possible involvement of the cells of the distal tentacle ganglia in mediating similar peripheral responses has been discussed previously.

Studies of molluscan peripheral nervous systems have shown them to be complex and to contain a variety of sensory neurones. Laverack & Bailey (1963) described a single class of tactile receptor and three classes of movement receptor in the mantle wall of Buccinum undatum, while De Vlieger (1968) demonstrated that different tactile signals were propagated by nerve fibres which had different characteristics and originated from different areas in the skin of lip preparations. In a later study Janse (1974) described a system of complex primary and H.C.S. neurones that supported the electron miscroscope study of epidermal sensory nerves by Zylstra (1972). While Crisp (1971) in a study largely concerned with the epithelial sensory cells of Nassarius reticulatus, briefly mentioned a non-ciliated type of sensory cell in the epithelium of L.littorea.

The tactile responses recorded by Laverack and Bailey occurred at both the onset and offset of the stimulus whereas in Littorina and in Lymnaea (De Vleiger and Janse) the responses occurred only at the onset of the stimulus. It was observed that with certain types of stimulator probe both onset and offset responses could be recorded from the tentacles of Littorina, while the offset response was abolished when the

probe was replaced by a more rigid material. When a flexible probe was used lateral vibrations were induced as it was advanced and retracted, so that the preparation received multiple stimulation, but this does not necessarily reflect the situation in Buccinum.

A comparison of the low threshold responses recorded from Littorina with those described by Janse from Lymnaea showed that the Type 1 response from Littorina was similar to that of Lymnaea as it was not blocked by synapse blocking agents (Mg.SW. and 10^{-5}M acetylcholine solution) and was therefore propagated by primary sensory neurones. Both types of response also showed rapid adaptation. The Type 2 response in Littorina was a compound potential of long latency that was abolished by Mg.SW. while similar activity in Lymnaea was propagated by primary sensory neurones and was not abolished by synapse blocking agents. It was concluded that the Type 1 and Type 2 responses in Littorina, despite their similar thresholds, were propagated by different neurones. In the diagrammatic summary of tentacle innervation (page 105) the units responsible for Type 1 responses were shown as primary sensory neurones while the Type 2 units were represented as several primary peripheral sensory neurones synapsing with an H.O.S. neurone. The Type 1 responses were relatively fast and discrete, thus they probably originated from large and therefore fast axons, while the slow compound Type 2 potentials were propagated by thinner, slower axons that may have been active more or less synchronously. Slow potentials have been recorded from the nerves of Aplysia californica (Jacklet, 1969) and from the central connectives of Viviparus contectus (Sattelle, 1972).

As the Type 1 and Type 2 responses were elicited by low threshold simulation they could be regarded as originating from touch sensitive neurones whereas the higher threshold responses may have been elicited from neurones responding to the deformation of the tentacle surface caused by higher stimulus levels. Unfortunately a sharp distinction between different types of sensory neurone was not possible. Unlike the tissues that have been used in similar investigations, for instance the lip preparation of Lymnaea, the tentacles of Littorina are active structures capable of powerful contractions and were found to be unsuitable for investigations of stretch responses. The Type 4 response was distinguished from the Type 3 by its short latency, high threshold and spatial distribution. At first the response was viewed with suspicion but the control experiments showed the experimental apparatus to be free of artefacts and later consideration of the conduction velocity determinations showed that a velocity of the required magnitude, about 60 cm/sec for a conduction pathway of approximately 2.5 to 3 mm. in the tentacle, could be maintained in other nerves.

Throughout the study difficulties were encountered when it was attempted to determine the receptive areas and the rates of adaptation of the sensory nerves, as each stimulus elicited a local contraction that changed the relationship between the point of stimulation and the stimulator. All the responses were susceptible to some degree of adaptation. In particular, the Type 1 response was usually completely adapted after the stimulus onset although occasionally two or three successive responses could be recorded. The receptive areas

of the low threshold responses were indeterminate but appeared to be considerably smaller than the $1mm^2$ areas reported for touch sensitive units in the mantle wall of Buccinum (Laverack & Bailey, 1963). In other instances smaller receptive fields have been reported particularly in the anterior regions of the animals concerned, as in Lymnaea (Janse, 1974) and Ensis directus (Olivo, 1970).

The ultrastructure of epidermal sensory nerves was studied by Crisp (1971) and Zylstra (1972) while Janse (1974) considered the properties of sensory nerves in order to establish their structure. He described the primary touch sensitive nerves of Lymnaea as having a complex dendritic tree connected by an axon to a centrally situated cell body. Similar suggestions have been made for other molluscs including Aplysia californica (Carew, et al., 1971) Spisula solidissima (Mellon, 1972), and a similar arrangement seems likely for the primary sensory neurones in Littorina. The overall significance of the sensory neurones has to be discussed in the light of the central pathways and their effects on the contractions of the columellar muscle.

In order to follow the pathways between the central ganglia of Littorina, similar methods to those of De Vlieger (1968) were used. In both instances pre-ganglionic stimulation was affected by stimulation of peripheral sensory neurones and the effects of lesions in the interganglionic connections on the post ganglionic responses were observed. This method is preferable to the use of electrical stimulation as the chances of antidromic stimulation are avoided.

There have been a variety of investigations of central

pathways in other species of mollusc (Herter, 1931: Turner & Nevius, 1951: Horridge, 1958: Hughes & Tauc, 1961, 1962) and the work of De Vlieger (1968, 1970) revealed a similar system of pathways in Lymnaea to those in Littorina. In the earlier work De Vlieger demonstrated that the cerebral commissure was activated by high intensity tactile stimulation of the lips and its activity was associated with the animal's escape response. The lip responses were propagated by a few large axons which, unlike the comparable units in Littorina, were rapidly adapting, while a low threshold response was propagated by slow, small diameter axons. In the later work De Vlieger showed that these units elicited two classes of postganglionic response one of which could initiate an escape reaction, a condition which is analogous with the excitation of the cerebral pathway in Littorina from the Type 4 sensory neurones and the slower Type 1 sensory neurones.

The involvement of the pedal ganglia, via the pedal pathway, in a system concerned with the withdrawal response is not surprising as the ganglia have the characteristics of major centres of integration and probably co-ordinate sympathetic movements of the foot and shell.

During the pathway experiments it was noted that the cerebropedal connectives were redundant with respect to the system of tactile pathways. Although no evidence has been collected it seems likely that these propagate information from the pedal ganglia to the cerebral ganglia.

The remaining sections of the investigation were concerned with conduction velocities, which have been discussed previously, and spontaneous activity in the C.N.S. In both

cases the small size of the nervous system in Littorina proved to be a handicap but the results that were obtained were comparable to those reported in other studies. The overall pattern of spontaneous activity was similar to that recorded from the isolated C.N.S. of Lymnaea (De Vlieger, 1968) and it appeared that similar long term changes in activity were present. If these experiments could have been continued an intracellular study of spontaneously active cells may have been possible, but such experiments may be pursued at a later date.

In general the investigation has accomplished its primary aims in that some aspects of gross organization of the nervous system in Littorina have been established and the knowledge of the prosobranchs has been increased. The study of mechanosensory neurones of the tentacles, together with the complementary investigation of central pathways and columellar muscle innervation, has permitted an explanation in terms of nervous activity, for a simple behavioural reflex, the withdrawal response, despite the fact that the 'simple' reflex involved the main central and peripheral ganglia of the nervous system.

Some limitations were imposed on certain experiments by the size of Littorina and its use in future electrophysiological experiments may be limited. Some interesting questions, such as the role of the neurone like cells of the distal tentacle ganglion and the ultrastructure of the columellar nerve, may form the basis of further studies.

5.0 SUMMARY.

In the present investigation the neural elements involved in a simple behavioural response have been investigated in order to establish some aspects of the functional organization of the nervous system. It was found that Littorina, in common with other gastropods, withdraws into its shell when subjected to adverse stimulation, particularly mechanical stimulation of the tentacles. This response was investigated and experiments were devised to establish the functional organization of the neural elements that mediated withdrawal.

Observations of the responses of isolated tentacles suggested both peripheral and central control of tentacle reflexes while an analysis of activity, recorded from the tentacle nerves, revealed four classes of response, elicited from different mechanosensitive neurones by stimulation of the tentacles. In further experiments a system of pathways was traced through the C.N.S. to a nerve that had been established as the motor nerve supplying the columellar muscle, and the effects of stimulating the muscle by way of the separate pathways were compared. A suggestion as to the functional significance of the pathways has been put forward after consideration of the available data.

The ganglia of the C.N.S. and other tissues have been examined histologically and some limited studies of conduction velocity and spontaneous activity within the nervous system have been included.

5.1 CONCLUSIONS.

1). The morphology of the nervous system of *Littorina littorea* has been examined and found to be in agreement with earlier descriptions, apart from the innervation of the columellar muscle. This has been shown to be innervated by a previously undescribed nerve LP1.2 arising from the left pleural ganglion.

2). The nerves and connectives, except for the columellar nerve, have a typical gastropod structure with an extensive endoneurium and lack visible axon detail when viewed under the light microscope. The columellar nerve contained approximately sixty easily discernible axons which had a maximum diameter of 23 microns.

3). The ganglion cells were unipolar and the majority were less than 10 microns when measured along their greatest axis.

4). The tentacle nerve C4, bears two ganglionic regions, the proximal tentacle ganglion and the previously undescribed distal ganglion which occurs at the site of a major nerve junction in the tentacle.

5). The animal's escape response is usually a rapid withdrawal into its shell and consists of a brief contractile phase followed immediately by a phase of prolonged, gradual extension. The response is modified according to the stimulus intensity and the position of the animal.

6. There is a wide overlap between adjacent areas of sensory innervation by the cerebral nerves.

7). Motor innervation of the tentacles is derived from the cerebral ganglia but the tentacles show two types of peripheral reflex that may possibly be controlled from the distal tentacle ganglia.

8). Four classes of mechanosensory neurones are present in the tentacles of which two are H.O.S. neurones and two are primary sensory neurones.

9). Two pathways exist across the nervous system for the transmission of sensory information from the right tentacle to the columellar muscle.

10). The activity of the pathways was correlated with that of the mechanosensitive neurones in the tentacle. The cerebral pathway received excitation from Type 1 and Type 4 neurones while the pedal pathway received excitation from Type 3 and some Type 1 neurones.

11). The induced contractions of the columellar muscle were phasic and showed a similar pattern to the withdrawal response although the muscle responses were of longer duration.

12). It was possible to induce similar contractions of the columellar muscle via either the cerebral or the pedal pathway but the pathways showed different characteristics when stimulated repeatedly.

13). It is suggested that the pedal pathway acts as a high threshold rapidly adapting system while the cerebral pathway functions as a low threshold and a high threshold slowly adapting escape system.

14). Conduction velocity studies of the supraintestinal connective showed that it contained groups of axons with various conduction velocities. The fastest component had a velocity of approximately 0.52 M/secs.

6.0 APPENDIX 1.

The composition of the artificial sea water was taken from data calculated by Barnes (1954) and reproduced below. The values shown are in grams/litre.

NaCl	23.991		
KCl	0.742		
$CaCl_2$	1.135	($CaCl_2.6H_2O$	2.240)
$MgCl_2$	5.102	($MgCl_2.6H_2O$	10.893)
Na_2SO_4	4.012	($Na_2SO_4.10H_2O$	9.100)
$NaHCO_3$	0.197		
NaBr	0.085	($NaBr.2H_2O$	0.115)
$SrCl_2$	0.011	($SrCl_2.6H_2O$	0.018)
H_3BO_3	0.027		

The constituents were dissolved in 1 litre of distilled water.

Salinity = 34.33‰

Chlorinity = 19‰

High magnesium sea water was made by substituting the usual complement of calcium chloride by magnesium chloride. The final solution contained a Mg^{++} concentration of 63mM/L (equivalent to 1.512 gms Mg^{++}/L of solution).

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