ABSTRACT

Some aspects of the anatomy, fine structure and neurophysiology of the pneumostome of the garden slug, Limax flavus (L)

The pneumostome is the aperture of the mantle edge in pulmonate gastropods through which air gains entrance to the mantle cavity. It is believed to be significant in the exchange of gases from this cavity during the process of respiration. The thesis investigates some of the anatomical and physiological aspects of the area.

The pneumostome itself is a circumscribed area invested by a sphincter muscle for its closure. Several types of gland cell provide secretions associated with the surface of the region. The muscle fibres of the sphincter are supplied by more than one neurone and it is shown that there may be up to three different types of NMJ associated with these fibres. The anal nerve that supplies the area contains over 7000 nerve fibres with four very large diameter axons (20μ) and many smaller ones.

Electrical stimulation of the distal end of the anal nerve brought about pneumostomal opening and it is suggested that this nerve, but not the right pallial nerve, is involved in pneumostomal movements. There is a relationship between the activity of certain central units of the visceral ganglion and the pneumostome, these units being rhythmic and patterned in output. During the burst the interspike interval remains constant for the first few spikes, then decreases sequentially until the end of the burst, at the same time there is a decrease in the spike amplitude. The full burst only occurs if the pneumostome opens to its fullest extent. It is not known with certainty however that these neurones are those directly responsible for pneumostome movements, but

complete isolation of the periphery from these central units led to cessation of pneumostomal opening and closing. Cutting all nerves except the anal nerve led to a reduction in time and amplitude of pneumostome closure, but the rate of opening was maintained, suggesting that sensory input through other peripheral nerves is significant in overall control of movement.

SOME ASPECTS OF THE ANATOMY, FINE STRUCTURE AND NEUROPHYSIOLOGY OF THE PNEUMOSTOME OF THE GARDEN SLUG, LIMAX FLAVUS (L)

by

Rosslyn C. Davies

Dissertation submitted to the University of St. Andrews in fulfillment of the regulations for the degree of Master of Science, May, 1975.

DECLARATION

I hereby declare that the following thesis is based on research carried out by me, that the thesis is my own composition and that it has not previously been presented for a Higher Degree.

The research was carried out under the auspices of the University of St. Andrews after my admission as a post-graduate student in October, 1969.

R.C. Davies

Candidate

Certificate

I certify that the aforesaid candicate has fulfilled the conditions of the Ordinances and Regulations prescribed for the degree of M.Sc.

/Professor M.S. Laverack

Supervisor

SOME ASPECTS OF THE ANATOMY, FINE STRUCTURE AND NEUROPHYSIOLOGY OF THE PNEUMOSTOME OF THE GARDEN SLUG, *LIMAX FLAVUS L.*

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I Introduction

Land slugs, including the garden variety, Limax flavus (L), possess a simple lung which is contained within the mantle cavity. This cavity is lined with a highly vascularized epithelium. It is this epithelium which constitutes the main part of the lung. On the right side of the mantle cavity in the body wall there is a specialized area called the pneumostome which is basically an aperture which allows air to pass in and out of the mantle cavity. It is highly contractile, both when taking part in the respiratory process and also in response to a tactile stimulus.

During the ventilation process, the pneumostomal tissue makes the following movements. When the muscles surrounding the aperture contract, the pneumostome which is in the closed position opens evenly in all directions as in Figure 1. The aperture usually remains open for several seconds. When it closes, the direction is towards the centre and is equal in all directions (arrows indicate the direction of tissue movement in Figure 1). The arrangement of the muscle fibres within the pneumostomal tissue necessary to close the pneumostome is probably that of a sphincter. If a sphincter arrangement is present, the muscle fibre within the pneumostome should be arranged in a ring around the aperture.

When the muscles surrounding the aperture contract, the dorsal and lateral walls of the mantle cavity also contract. It seems likely that the total respiratory process involves the contraction and relaxation of several systems such as the muscles of the mantle wall, the muscles surrounding the respiratory aperture and the muscles of the mantle floor.

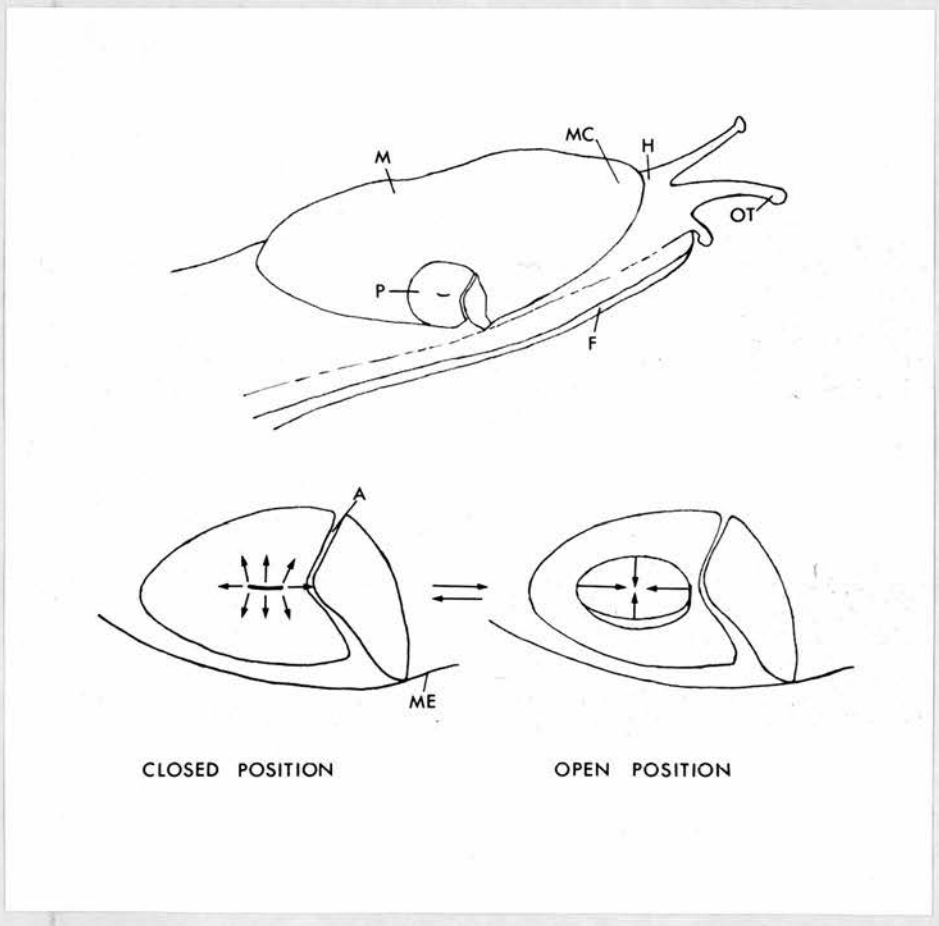


Fig 1: Diagram of the anterior part of the slug to show the position of the pneumostome in the mantle wall. This diagram illustrates the pneumostome in the opened and closed positions and the direction of the movement of the pneumostomal tissue during the ventilation process.

Legend: A: anal opening, F - foot-sole, H - head, M - mantle, MC - mantle collar, ME - mantle edge, OT - optic tentacle, P - pneumostome

Several researchers have speculated as to the mechanism of respiration in land pulmonates. Ghiretti (1966) summarizes this work and states that pulmonates possess both ventilation and diffusion lungs. He states that in the former, pressure is increased inside the lung by the following mechanism: the pneumostome is reduced to a small hole, the contraction of the muscles which make up the mantle floor causes the arched floor to flatten and air is drawn into the cavity. A valve is then drawn across the pneumostome, the muscles of the floor relax and, since this brings about a decrease in the size of the mantle cavity, pressure within the lung increases. He then states that this pressure increase enables oxygen to diffuse more easily into the blood which is located in the highly vascularized epithelial lining of the mantle cavity. When the oxygen has diffused into the blood, the pneumostome opens and carbon dioxide is released from the blood through the aperture.

Ghiretti's account of the respiratory mechanism in land pulmonates is not very satisfactory because of the lack of empirical evidence. It seems unlikely that the gas exchange between the blood and the lung would use a build up of total pressure in the lung. Diffusion that relies on small differences in partial pressure of individual gases would be adequate for gas exchange in either direction.

Some slugs appear to respire by the method of partial active ventilation. In this case, the mantle floor moves up and down while the pneumostome opens. In the case of Arion ater, the pneumostome is open for the majority of the time and the muscular floor can be seen to be moving up and down, probably drawing air in and out of the cavity (Runham and Hunter, 1970).

The rate at which slugs respire depends on certain factors such as the size and age of the animal, its metabolic rate, its locomotory activity (whether it is stationary or not) and also on certain environmental factors such as temperature, humidity and the amount of atmospheric oxygen or carbon dioxide present (Runham and Hunter, 1970).

The first description of the pneumostome and its activity was that of Simpson (1901) who included it in a larger investigation of the anatomy and physiology of Polygyra albolabris and Limax maximus. He pointed out that the respiratory orifice (pneumostome), situated at the edge of the mantle, was seen to open and close at regular intervals about 16 to 18 times per minute for normal respiration. In the open state, the orifice is circular and closure is brought about by the contraction of the muscle fibres which surround this orifice. As far as the innervation of the pneumostome is concerned, Simpson describes a nerve trunk which leaves the posterior end of the infra-oesophageal ganglion and runs posteriorly to the right side of the animal where it sends off smaller branches to the pulmonary cavity, respiratory orifice, and anus.

Laryea (1970) gives a very detailed description of the anatomy of the nervous system of the slug, Agriolimax reticulatus. He states that the anal nerve forms a plexus in the region of the anus and the pneumostome. This nerve has a common origin with the cephalic retractor and intestinal nerves from the posterior surface of the visceral ganglion. These nerves continue together in a connective tissue sheath for a short distance before separating. Laryea describes the right pallial nerve as having its origin in the posterior surface of the right parietal ganglion and innervating the right side of the mantle, the lateral musculature of the body wall along the right margin of the

pallial region and the posterior region of the right side of the anterior free mantle edge. Bullock and Horridge (1965) state that in Helix, two nerves, the pallialis dexter externus and internus both with origins in the right parietal ganglion innervate the mantle on the right including the region of the lung aperture. The anal nerve with its origin in the visceral ganglion joins a plexus in the region of the anus and the lung aperture (pneumostome).

The innervation of the pneumostomal area was investigated by Nisbet (1961A) in his work on the nervous system of the giant African land snail, Archachatina marginata (Swainson). He describes three pallial nerves, the right pallial, the anterior fine right pallial, and the middle fine right pallial, as entering the right wall of the body close to the junction of the latter with the mantle and just anterior to the position of the pneumostome. These three nerves which have their origins in the right parietal ganglion, were found to innervate the right mantle wall, the collar and the lateral body wall.

Nisbet also described another nerve, the median pallial nerve. This nerve with its origin in the abdominal ganglion, enters the wall of the body and collar close to the pneumostome. This study of the innervation of the collar and the lateral body wall was made difficult by the presence of very large gland cells in the area.

Nisbet's investigation also included observations of the movements of the pneumostome. He observed that in small snails, the pneumostomal movements are rapid but irregular. Observations in the late embryo stage revealed that the aperture opened for intervals of 5 to 10 seconds and closed for period of 17 to 45 seconds. In the adult snails,

these periods can be much longer and, in fact, the pneumostome may remain open for up to several minutes at a time. Nisbet found that if parts of the collar or adjacent pillar were touched, the pneumostome closed rapidly.

In an extended investigation of the nervous system of this snail, a limited electrophysiological study of the pneumostome and the surrounding area was done (Nisbet, 1961B). This work revealed that when tactile stimulation was applied to the body wall, reflex bursts of action potentials occurred which were obviously related to the retraction of the body and the collar. Nisbet assumed that since there were variations in the periods of opening and closure of the pneumostome that at least the initiation of this movement may be under the control of the central nervous system. He was able to observe spontaneous potential groups in nerves apparently related to the pneumostome but was unable to find any correlation between these potentials and the movement of the pneumostome.

According to Jullien et al. (1960), both the right pallial and anal nerves produce contraction of the pneumostome of the snail, Helix pomatia. The same effect is accomplished by the electrical stimulation of their peripheral ends and the tone of the sphincter (pneumostome) appears to be conditioned by impulses in these two nerves. (When a nerve is cut, the part still attached to the central nervous system is referred to as the central or proximal end; the end of the nerve on the other side of the cut is referred to as the peripheral or distal end.) Stimulation of the central (proximal) end of these two nerves had no

effect on the pneumostome. Jullien also found that the electrical stimulation of the visceral nerve induced cardiac inhibition and the opening of the pneumostome. Stimulating the peripheral (distal) end of this nerve induced the heart to stop beating; the central (proximal) end, on the other hand, induced the opening of the pneumostome and its relaxation.

The results of these experiments on the nervous control of cardiac and respiratory movements are difficult to interpret because Jullien has supplied no information on which nerves were left intact.

The most recent electrophysiological study of respiratory behaviour in pulmonates concentrates on the identification in the central nervous system of specific cells whose activity is related to the movement of parts of the respiratory apparatus (Benjamin, 1971). The animal used in this study was the freshwater pond snail, Lymnaea stagnalis. Here cells on the ventral surface of the right parietal ganglion appear to be involved in the control of movements of the pulmonary folds. Benjamin has suggested that contact with the surface of the water in which the animal is normally submerged, gives rise to sensory input which triggers the opening of the pulmonary folds via efferent activity of certain identifiable cells in the right parietal ganglion.

Identification of the neurons involved in the control of the pneumostome of the slug, Limax flavus could be achieved in the following way. If one were able to record from central identified neurons, then artificial activation of these units would show whether they had motor functions with respect to pneumostome movements. Different peripheral stimuli

which affect the output of these units could then be examined. This may be a difficult proposition because it is not known what stimulant triggers the pneumostome or in what area of the animal this stimulant acts. In fact, pneumostome movements may be triggered by a combination of different stimuli such as temperature, humidity, internal levels of carbon dioxide, etc.

Very little research has been done on the general histology of the pneumostome. The only light microscopical investigation of the pneumostomal tissue has been done on two species of freshwater pulmonates, Lymnaea stagnalis and Biomphalaria pfeifferi (Zylstra, 1972A). In this study a number of gland cell types have been classified according to certain standard histochemical techniques. Zylstra found five gland cell types present in the sub-epithelial layer of the pneumostome. These are the ubiquitous muciparous gland cells and the non-muciparous gland cells, the mantle muciparous gland cell type A and the mantle non-muciparous gland cell type A. The fifth are the pneumostome non-muciparous gland cells which are specific to this area. These cells are found only around the ventral side of the pneumostome. Muciparous means a mucin type of secretion. Zylstra noted a distinct difference in both the number and types of gland cells located in the ventral and dorsal surfaces of the pneumostome.

He found that the epidermal area of the pneumostome of Lymnaea is composed almost entirely of cilia cells and suggests that these cilia are probably involved in the production of currents. He also

suggests that the gland cells in the region probably aid in providing a mucous layer for the beating of the cilia. The mucous is probably instrumental in sealing off the pneumostome itself when the snail is under water.

In an investigation of the distribution and ultrastructure of epidermal sensory cells in Lymnaea and Biomphalaria, Zylstra (1972B) found that the rich sensory regions of the epidermis, as identified by the presence of subepithelial nerve cells, are limited to certain areas of the animal, one of these being the pneumostome.

Other light microscopical investigations of the epidermis of slugs and other pulmonates includes a description of the various mucous and skin glands found in the integument of the foot-sole, foot fringe and mantle edge of the slug, Arion ater (Barr, 1928) and a study of the structure and function of the cutaneous glands in Helix aspersa (Campion, 1961). Campion concentrates mainly on the histochemistry of the glandular secretions of the cutaneous glands. The pneumostome is described as a region appearing whiter than the rest of the mantle. She suggests that this whiteness is due to the abundance of calcium glands found in the subepithelial layer of the pneumostome.

The only description of the fine structure of pneumostomal tissue has been provided by Zylstra (1972A, 1972B) in his work on the fresh-water snails, Lymnaea stagnalis and Biomphalaria pfeifferi. One study (1972A) is limited to a description of the ciliated epithelial cells which line the pneumostome and the sub-epithelial gland cells found in the pneumostomal region. The cilia in this region tend to have well-developed roots extending up to 2μ into the cytoplasm of the cell.

The periodicity of the striations of the roots is about 640 Å and is intermediate between that of the cilia of the foot sole and those of the mouth region. The basal bodies of these cilia also have well-developed basal feet which extend to the basal bodies of the adjacent cilia. The basal feet have a filamentous structure which connects adjacent basal bodies.

Zylstra also describes the fine structure of what he calls the pneumostomal non-muciparous gland cells. These cells possess unusual membrane-like structures in their secretion granules. These structures remain intact upon release from the cell and are double layered with a distance between the layers of 150 Å. The golgi bodies are usually lamellar in form. The gramular endoplasmic reticulum is relatively sparse and the cisternae are found in a more or less parallel arrangement.

In an investigation of the ultrastructure of the sensory cells of L.stagnalis, Zylstra (1972B) describes six different types of free nerve endings found in the epithelium. He found that of these, only two were located in the pneumostome epithelium. The dendrites of these sensory cells are characterized by the presence of 10 - 40 cilia at their free surface. One type have cilia with relatively thin striated roots with a length of less than 3 u (periodicity 650 Å); the other have much thicker and longer striated roots with a length of up to 10 u (periodicity of roots: 560 - 600 Å). The cilia of both types have 3 - 5 striated roots extending from each basal body. The feet of the basal bodies and the planes joining the central filaments of the cilium have a random orientation.

General investigations of the ultrastructure of the epidermis of slugs and other pulmonates include research on the structure of glands in the epidermis of Arion rufus (L), Arion empiricorum (Fer), (Wondrak, 1967, 1969A, 1969B), on the surface epithelium of Arion rufus (L) (Wondrak, 1969) and on the microvilli found on the external surfaces of gastropod tentacles and body walls (Lane, 1963).

No study has been made of the ultrastructure of the muscle cells and neuromuscular relations in the sub-epithelial layer of the pneumostome. Some research, however, has been done on the fine structure of smooth muscle for specific regions of other pulmonate molluscs, i.e. foot of Helix aspersa (Rogers, 1969); optic tentacles of H.aspersa and Limax flavus (Rogers, 1968); optic tentacle of Vaginula soleiformis D'Orbigny (Barrantes, 1970). Grasiadei (1966) has looked at the ultrastructure of motor nerve endings in the muscles of cephalopods.

An examination of the literature which has been summarized above shows that no extensive study has yet been done on the structure of the pneumostome and its nervous control. The present investigation attempts to describe the innervation of the pneumostome of Limax flavus. Extracellular recording techniques have been used to show that some nerve cells in the visceral ganglion produce a burst of impulses which can be correlated with the movements of the pneumostome. Attempts to monitor this movement are discussed as well as the results of denervating the area from the central nervous system. In addition, the structure of the pneumostome has been studied using standard light microscopical and electron microscopical techniques and the results of these investigations are discussed.

II Materials and Methods

Animals were collected at night from garden walls and held in the laboratory for short periods of time. They were kept in small polyethylene containers on moistened filter paper and were fed small amounts of lettuce, cabbage and carrot daily until used. All the animals were kept at room temperature which was approximately twenty degrees centigrade.

A. Gross Anatomy

The slug was prepared for examination by pinning it onto a wax layer in a dissecting dish. A longitudinal incision running from the anterior edge of the mantle to the mouth was then made in the mid-dorsal line of the head. The skin was pinned back and various organs including the penis, salivary glands and oesophagus were removed to expose the central nervous system and the various nerves leading from it.

A longitudinal cut was then made along the mid-dorsal line of the mantle and the skin was pinned back on either side to expose the various organs of the mantle cavity, i.e. heart, kidney and ureters leading from it. These were removed as well as the thin muscular mantle floor to reveal the nerves running posteriorly from the central nervous system. The innervation of the pneumostome at the gross level and the muscles surrounding the aperture were examined.

Methylene blue staining techniques were used in an attempt to describe the sensory innervation of the muscles.

1. The animal was dissected as described above and the preparation was immersed in a 0.1% solution of methylene blue in Meng's saline (Meng, 1960). This solution contained the following salts:

NaCl	-	3.45 g/litre
KCl	-	0.43 g/litre
$CaCl_2$	-	1.17 g/litre
$MgCl_2$	-	1.55 g/litre
$NaHCO_3$	-	1.10 g/litre

The methylene blue solution was then washed off and replaced by Meng's saline. This operation was repeated several times. The result of methylene blue application was intense staining of the main nerve trunks within the body cavity. The branches of these nerves which are contained within the body wall failed to stain. The dissection of these branches was attempted but it was impossible to follow them to any depth in the body wall musculature.

2. Methylene blue (about a 0.1% solution in Meng's saline) was also applied by injecting it into the pneumostomal area of the live animal before dissection. The animal was then left for about anhalf hour and rapidly dissected. This method resulted in intense staining of the main nerve trunks but no staining of the nerves within the pneumostome.

3. Another method for methylene blue staining was attempted. This was the method used by Cottrell and Osborne (1969) in their work on neurosecretory systems in Helix heart. The staining solution was

prepared in the following manner. 0.4 grams of Rongalite (Gurr) and 5 drops of concentrated hydrochloric acid were mixed and added to 10 ml. of 2% methylene blue solution. The mixture was heated until yellow in colour, filtered and then diluted five times with Meng's saline. This solution was then injected into the pneumostomal area. This method was very unsuccessful in staining fine nervous elements within the pneumostome. In fact, it resulted in a fairly rapid death of the animal and very poor staining of the main nerve trunks.

B. Light Microscopy

For light microscopy, both whole animals and parts of animals were fixed in Bouin fluid or MFA (80% absolute methyl alcohol, 10% formalin, 10% glacial acetic acid; Gatty Marine Lab special fixative). Prior to fixation, the slugs were anaesthetized in the following way. The animal was placed in a small container and the latter was put into a larger container which contained cotton wool and a small amount of dry ice. The larger container was then covered and the animal was left for about four to five minutes. At the end of this time, the specimen was in an extended condition and was then fixed. The specimens were dehydrated through a graded alcohol series, cleared in xylol and embedded in paraffin wax (m.p. 56°C). Serial sections (10 μ thick) of the mantle cavity were made both longitudinally and transversely. One series of transverse sections was stained with Azan after Heidenhain: Masson's Trichrome stain was used to stain another series of transverse sections and one series of longitudinal sections.

6. Electron Microscopy

The pneumostome and surrounding mantle tissue were immersed in a 4.0% solution of glutaraldehyde in a 0.1 M PO_4 buffer, pH 7.2. After preliminary fixation, small pieces (less than 1 mm^3) were cut from the pneumostome and placed in fresh fixative at 4°C for 4 hours. After washing in 0.1 M PO_4 buffer for 24 hours at 4°C, the tissue was post-fixed in 1% OsO_4 in the same buffer for 1½ hours at 4°C. The tissue was then washed briefly in buffer and then dehydrated in a graded alcohol series through propylene oxide. The tissue was then embedded in Araldite at 60°C.

Grey-to-silver sections were cut using glass knives on a Reichert Austria OMU_2 ultramicrotome and stained with uranyl acetate and lead citrate. They were then examined using an Hitachi electron microscope at 60 kV.

In order to determine the number of axons in the anal nerve and to get an accurate picture of the axonal size distribution, it was necessary to construct a composite picture at a magnification which enabled one to make out all the individual axons. Small pieces of the anal nerve were prepared for electron microscopy by the above method and a cross section of the nerve was examined in the electron microscope. A series of photographs which covered the entire area of the nerve was then taken. The negatives were enlarged to a final magnification of 10,000 with a scale: 1 cm = 1 μ. These photographs were fitted together to give a composite picture of the cross section of the anal nerve. This picture was divided into seventy-six squares (side of each square measured 7.6 cm) to facilitate the counting and measuring of the individual axons.

As the majority of the axons in the nerve have very irregular shapes, it was impossible to measure their diameters accurately. It was decided that a more accurate representation of the size of each axon would be an area measurement. This was done in the following manner. A grid consisting of a number of squares each with an area of one square centimeter was drawn on a piece of transparency. This grid was then placed on top of the composite picture and the area of each axon underneath was measured in square centimeters, then converted to square microns. Each axon was placed in a specific category according to its area measurement.

The categories were as follows:

1. under 0.25 μ^2
2. between 0.25 and 0.50 μ^2
3. between 0.50 and 1.0 μ^2
4. between 1.0 and 2.0 μ^2
5. between 2.0 and 5.0 μ^2
6. between 5.0 and 10.0 μ^2
7. between 10.0 and 20.0 μ^2
8. over 20.0 μ^2

($1\ \mu^2 = 1\ cm^2$)

D. Electrophysiological Investigation

1. Preparation of the animal for stimulation and recording

The central nervous system of the animal was exposed by making a longitudinal incision in the mid-dorsal line of the head and then removing such organs as the salivary glands, oesophagus and penis.

The anterior aorta was pinned to one side and the tentacular retractor muscles were removed. The preparation was then flooded with Meng's saline. Care was taken to keep the mantle cavity above the surface of the saline as the pneumostome failed to open and close when submerged. A considerable amount of mucous which was secreted by the animal was removed by using a fine paint brush.

2. Recording and Stimulating Techniques

Both suction and hook electrodes were used for the recording of electrical activity. Only suction electrodes were used to stimulate the nerve trunks. The suction electrodes were made from fine polyethylene tubing and one ml. plastic syringes. Fine tips were constructed from the tubing and were fitted onto the needle of the syringe. One silver wire from the differential amplifier was connected to the saline inside the tip; the other indifferent electrode was dipped into the bath. Tips were chosen with diameters which would only just accommodate the passage of the nerve from which the recording was to be taken. Because of the tight fit of the tip around the nerve, the portion of the nerve inside the electrode was electrically isolated from that outside it. In this way, potential differences between the two silver wires could be recorded. The suction electrodes were used to record activity from either the cut end of the anal nerve or right pallial nerve or from the surface of the central nervous system. Hook electrodes made from thin silver wire were also used to record from intact anal and right pallial nerves.

Standard electrophysiological equipment was used throughout the investigation. Nervous activity was amplified by a differential pre-amplifier with a gain of 3,000 and displayed on a Tektronix 502 oscilloscope. The nerve signals were recorded on film using a Cossar camera (model 1428 MK 11). For stimulation, a Tektronix waveform generator was used to trigger a pulse generator; the latter was used to deliver a chain of pulses or single pulses to the preparation. A stimulus isolation unit was used in conjunction with the pulse generator (see Fig. 2). The preparation was kept at room temperature which was approximately 20°C. The saline in the bath was changed frequently.

3. Monitoring Methods

Several methods were tried in an attempt to monitor quantitatively the movement of the pneumostome during ventilation.

a. In the first instance, a kymograph was used. This method was unsuccessful because the pneumostomal muscles failed to generate enough power to produce a noticeable deflection on the kymograph paper.

b. An RCA transducer was then tried. A needle was clamped to the transducer and this needle was inserted at right angles into the circular muscle surrounding the aperture. Movement of these muscles during the contraction as well as the movement of the general musculature of the mantle resulted in the dislodgement of the needle.

c. A third method consisted in placing a tiny fleck of mirror on the pneumostome. This mirror was prevented from slipping by the mucous

secreted by the animal during the operation. A powerful light source such as a high intensity lamp provided a narrow beam of light. This beam was reflected off the mirror and onto a photocell. Opening of the pneumostome resulted in a small movement of the mirror and, in turn, the beam of light reflected off the mirror. As this reflected beam traversed the light-sensitive area of the photocell, it caused a deflection of the oscilloscope trace.

As the above method proved unsatisfactory in monitoring quantitatively pneumostomal movements (see Results, section 7) a modification of this method was tried. Instead of the conventional photocell to measure the movement, a photodiode mounted on the end of a long rod was used. The photodiode was powered by a + 45 volt battery with a 10 mΩ resistance placed in parallel. It was thought that such an arrangement would result in easier manipulation of the photocell and perhaps give a more satisfactory recording of the movement of the pneumostome.

d. Because of the failure of the above techniques to monitor pneumostomal movements satisfactorily, the method of monitoring the movement visually was adopted. The opening of the aperture was observed and at the same time, a chain of pulses with a very high frequency was delivered from a standard pulse generator to the oscilloscope causing the lower trace to be deflected. The switch on the pulse generator was released when the pneumostome was seen to close.

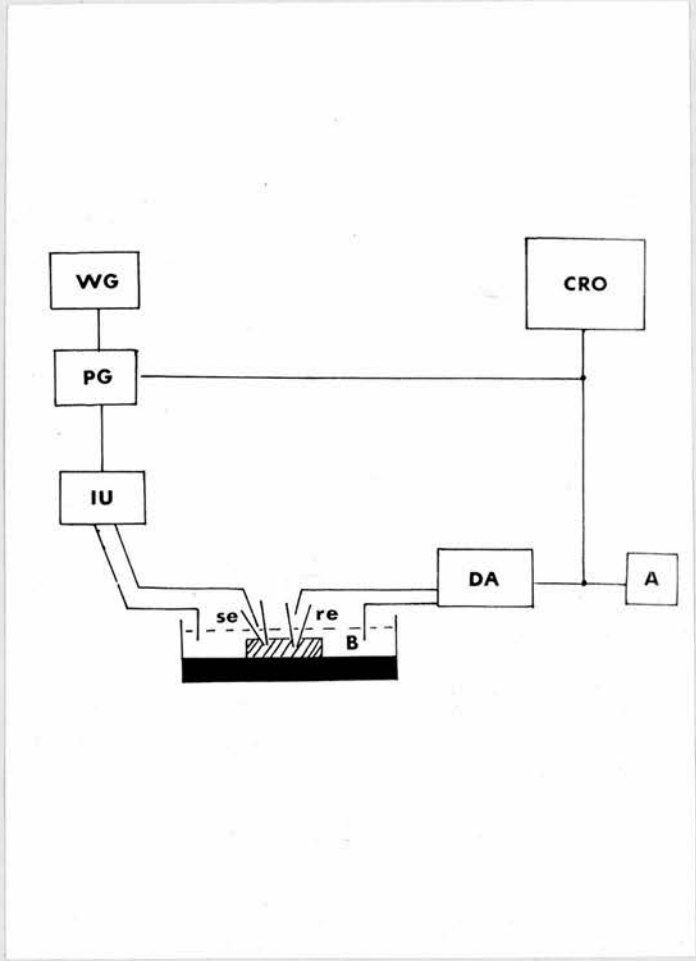


Fig. 2: Diagram of the recording and stimulating experimental set-up.
Legend: A - audio amplifier, B - bath, CRO - cathode ray oscilloscope, DA - differential pre-amplifier, IU - stimulus isolation unit, PG - pulse generator, re - recording electrode, se - stimulating electrode, WG - waveform generator

III Results

a. Gross Anatomy

The pneumostome which is circular in shape lies on the right side of the mantle (Fig. 1). It links the external environment with the mantle cavity which is bounded dorsally by the mantle roof with its highly vascularized epithelium and ventrally by the thin contractile mantle floor. The secondary ureter which is a continuation of the primary ureter of the kidney is closely applied to the rectum. It runs anteriorly and discharges into a bladder which opens to the exterior through a slit in the mantle (see Fig. 3A). The rectum runs anteriorly as well and culminates in an opening which is located in the region of the mantle slit.

The pneumostomal tissue is surrounded by a ring of pigment cells which differentiate it from the rest of the mantle. It also appears much lighter than the surrounding mantle tissue. Dissection of the area failed to reveal the arrangement of the muscles within the pneumostomal region which are responsible for ventilation movements.

The innervation of the pneumostome was studied by dissection coupled with methylene blue staining. The use of methylene blue resulted in the intense staining of the various nerve trunks which run posteriorly from the CNS within the body cavity. The anal nerve which has its origin in the ventral side of the visceral ganglion (Fig. 4), enters the right mantle wall at a point posterior to the pneumostome and where the mantle floor joins the mantle wall. This nerve then forms two branches; one runs anteriorly along the dorsal

side of the pneumostome; the other runs posteriorly along the rectum and ends in the rectal ganglion which is located on the interior side of the rectum (see Fig. 3B).

The other nerve which innervates the right side of the mantle is the right pallial. This nerve has its origin in the right parietal ganglion of the sub-oesophageal ganglionic complex (Fig. 5). This nerve runs posteriorly for a short distance within the body cavity and enters the right mantle wall at the junction of this wall and the mantle floor. It then divides into three branches: the first (1) runs outwards to the collar; the second (2) runs upwards towards the roof of the mantle and the third (3) runs posteriorly and laterally along the right mantle wall (Fig. 3B). The third branch could be traced only as far as the mantle slit due to its failure to stain by methylene blue application. The presence of a large number of gland cells in the area makes dissection of the nerve branches extremely difficult. However, from the numerous dissections carried out, it looks as though the pneumostome is innervated by a branch of the anal nerve and that the third branch of the right pallial nerve does not pass into the pneumostome but radiates upwards towards the roof of the mantle cavity.

The application of methylene blue to the pneumostomal area did not result in the staining of individual components of the nervous system within the region. Several different methods were tried in an attempt to secure information on the sensory innervation of the muscles in the pneumostome but all proved unsuccessful (see Materials and Methods).

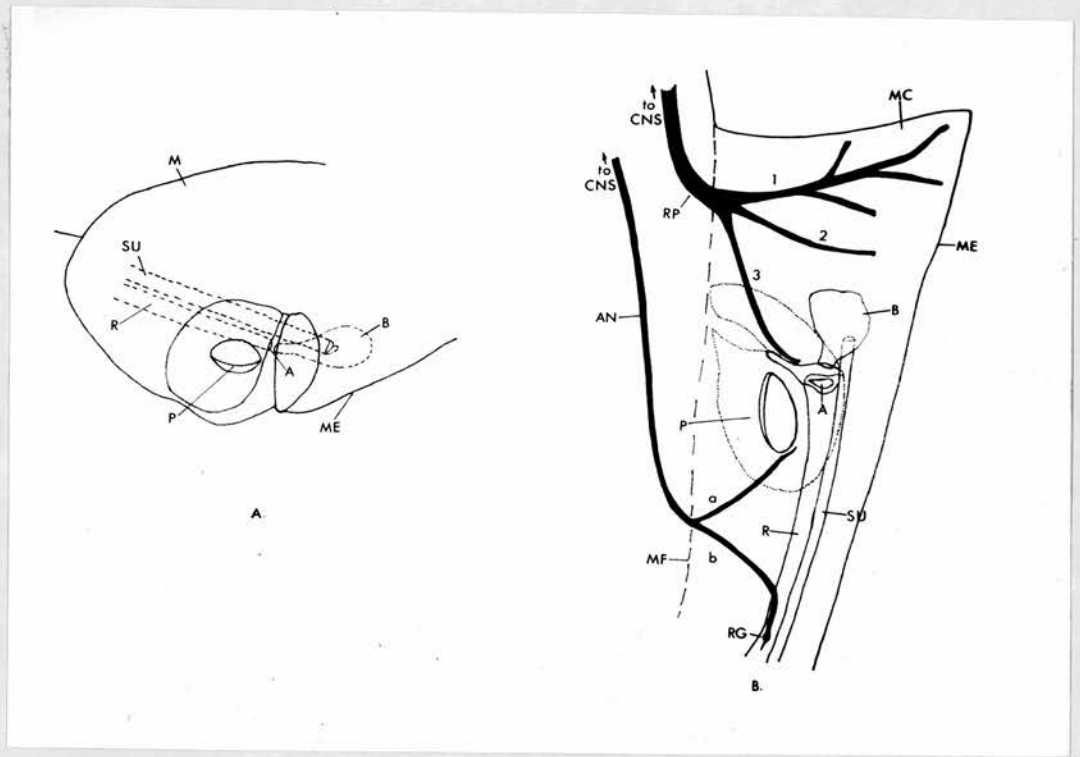


Fig. 3: Diagram of the pneumostome and related structures
A. External view of the right side of the mantle
B. Internal view of dissected right mantle wall
Legend: A - anal opening, AN - anal nerve, B - bladder, M - mantle, MC - mantle collar, ME - mantle edge, MF - mantle floor attachment, P - pneumostome, R - rectum, RG - rectal ganglion, RP - right pallial nerve, SU - secondary ureter, a - branch of anal nerve to pneumostome, b - branch of anal nerve to rectum 1, 2, 3 - branches of right pallial nerve
(for details, see text)

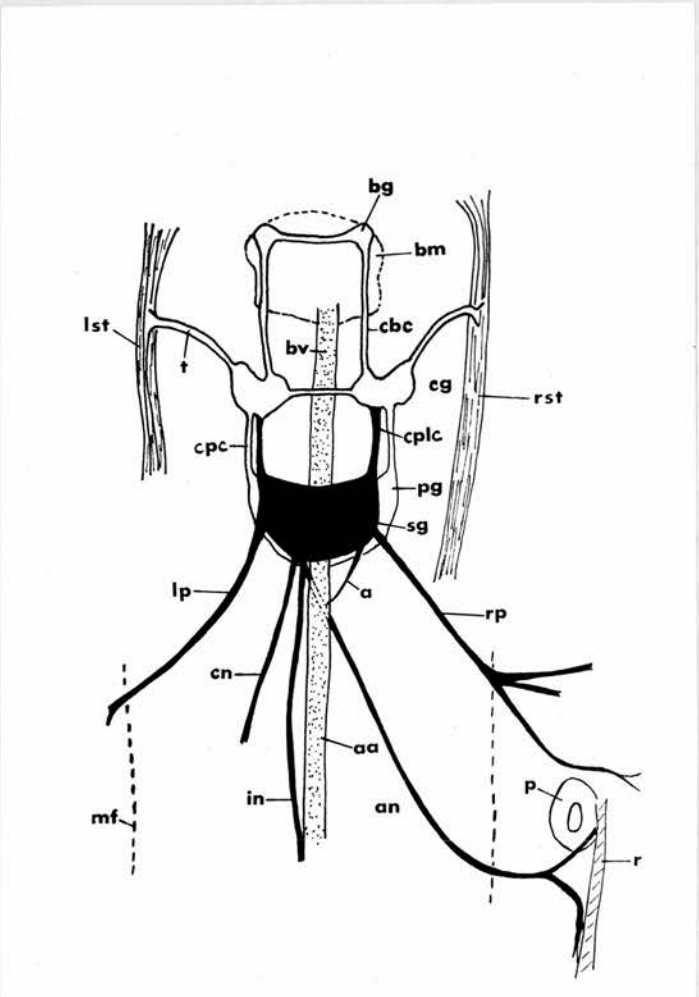


Fig. 4: Diagram of the nervous system of Limax flavus (L) to show the innervation of the pneumostome
Legend: a - aortic nerve, aa - anterior aorta, an - anal nerve, bg - buccal ganglion, bm - buccal mass, bv - buccal vessel, cbc - cerebro-buccal connective, cg - cerebral ganglion, cpc - cerebro-pedal connective, cplc - cerebro-pleural connective, cn - cephalic retractor muscle nerve, in - intestinal nerve, lp - left pallial nerve, lst - left superior tentacle muscle, mf - mantle floor attachment, p - pneumostome, pg - pedal ganglion, r - rectum, rp - right pallial nerve, rst - right superior tentacle muscle, sg - sub-oesophageal ganglion, t - tentacular nerve

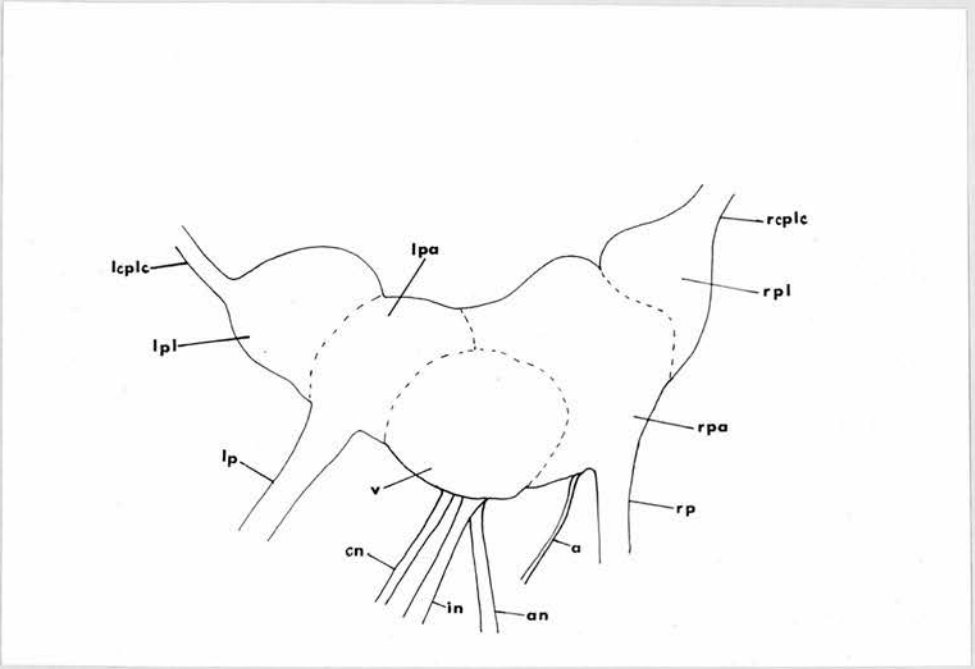


Fig. 5: Diagram of the sub-oesophageal ganglionic mass minus pedal ganglionto show the position of the visceral and right parietal ganglia.
a - aortic nerve, an - anal nerve, cn - cephalic retractor muscle nerve, lp - left pallial nerve, lcplc - left cerebro-pleural connective, lpl - left pleural ganglion, lpa - left parietal ganglion, in - intestinal nerve, rcplc - right cerebro-pleural connective, rpl - right pleural ganglion, rpa - right parietal ganglion, rp - right pallial nerve, v - visceral ganglion

The failure to stain was probably due to the presence of a large number of gland cells in the area as well as the fairly dense collagen matrix which surrounds the muscle fibres. Because of the density of the tissue, the stain was probably not able to penetrate to any depth. The large nerve trunks stained beautifully but this was most likely due to their location in the body cavity which is fluid-filled. The branches of these nerve trunks which are found within the mantle wall did not stain until they were dissected clear of surrounding tissue.

b. Light Microscopy

The epithelium which lines the pneumostomal aperture is one cell thick and contains only ciliated cells (Fig. 16). The ducts of some of the subepithelial gland cells open between the ciliated cells (Fig. 15). No pigment or goblet cells could be found in the pneumostomal epithelium. The ciliated cells are mostly columnar in shape and have a height of about 30 μ. Towards the inner part of the aperture, the cells tend to become more cuboidal in shape and have an average height of about 10 μ. The epithelium is supported by the muscle and connective tissue found in the subepithelial layer. In cross sections, these supporting muscle fibres appear to insert themselves onto the epithelium (Fig. 17).

Zylstra (1972B) has described the occurrence of a nerve plexus as identified by the presence of subepithelial nerve cells in the region of the pneumostome of Lymnaea stagnalis. Although numerous nuclei are found in the subepithelial layer of the pneumostome of Limax flavus, it cannot be concluded that they represent the ganglion cells

of a subepithelial nerve plexus. The various light microscopy stains used have indicated the presence of nerve processes running through the pneumostomal tissue (Azan staining). Cell bodies have also been seen in connection with these nerve processes. It may be that a stain such as Holmes' silver which is selective for nervous tissue might be more effective in showing up such a plexus more clearly. There are several references to the existence of such a plexus in the pneumostome of other pulmonates, Helix (Bullock and Horridge, 1965), Agriolimax reticulatus (Laryea, 1970).

Several types of gland cells are found in the subepithelial layer of the pneumostome. The most numerous type are located in a ring around the aperture in the subepithelium (Fig. 8). They occupy about 80% of the total pneumostomal tissue. We shall refer to these gland cells as the pneumostomal gland cells (gland cell type 1) because they are only found in this region of the animal (Figs. 6 and 7). These cells stain red with Masson's Trichrome and red with Azan. This indicates a non-mucin type of secretion. The cells are approximately 30 μ in diameter and have large nuclei measuring approximately 15 μ in diameter. The secretion product of these cells is granular in nature.

Another type of gland cell (gland cell type 2) found in the pneumostomal region has a large bulbous structure with a neck which extends to the outer epithelium (Fig. 15). These cells are also present in the dorsal mantle epithelium. This cell has a height of approximately 150 to 200 μ. The secretion product stains blue with Azan and green with Masson Trichrome indicating a mucin type of secretion. These cells are confined to the outer regions of the

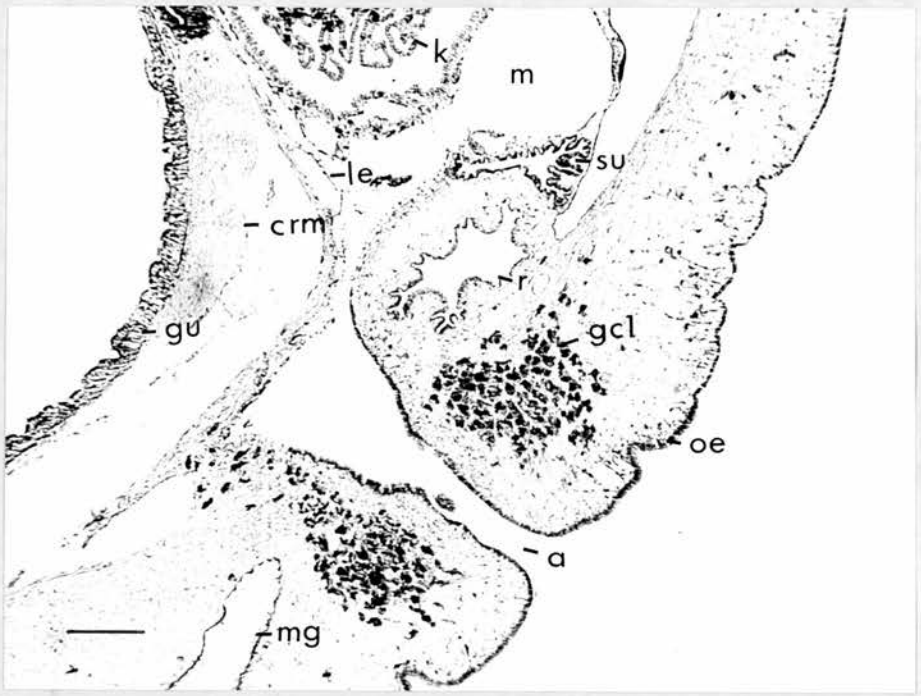


Fig. 6: Cross section through the aperture of the pneumostome. a - aperture, crm - cephalic retractor muscle, gcl - gland cell type one, gu - gut, k - kidney, le - lung epithelium, m - mantle cavity, mg - mantle groove, oe - outer epithelium, r - rectum, su - secondary ureter. (Masson Trichrome) X 46 Scale represents 200 µ.

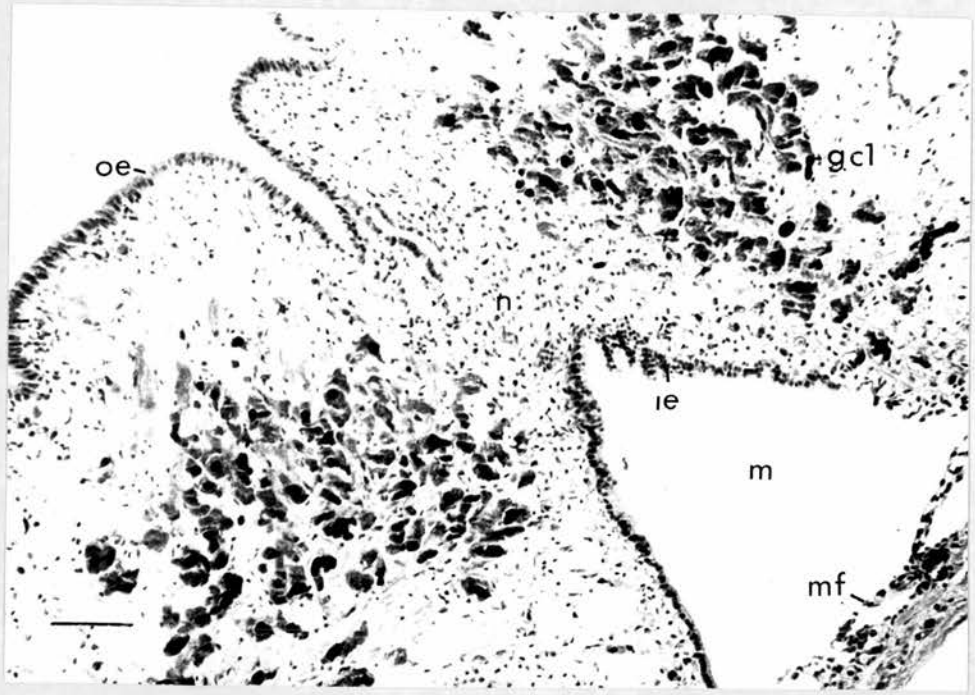


Fig. 7: Cross section of the pneumostome indicating the distribution of the pneumostomal gland cells (gland cell type 1). gcl - gland cell type one, ie - inner epithelium, m - mantle cavity, mf - mantle floor, n - nucleus, oe - outer epithelium, (Masson Trichrome) X 114 Scale represents 91 µ.

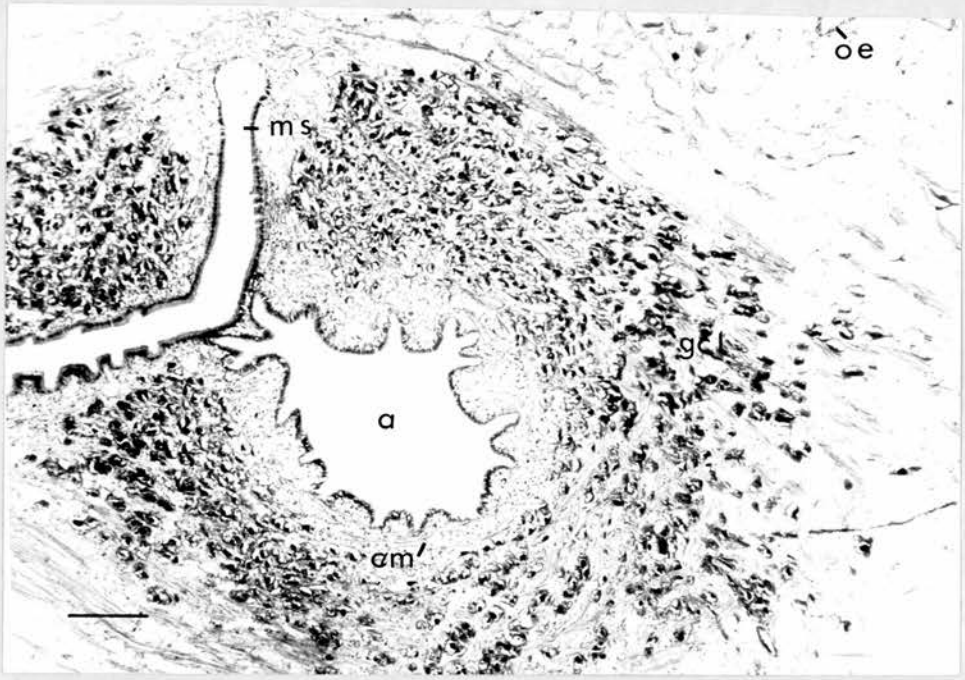


Fig. 8: Longitudinal section through the aperture of the pneumostome. This shows the arrangements of the pneumostomal gland cells (gland cell type 1) as well as the band of circular muscle surrounding the aperture. a - aperture, cm - circular muscle, gcl - gland cell type one, ms - mantle slit, oe - outer epithelium. (Masson Trichrome) X 46 Scale represents 217 µ.

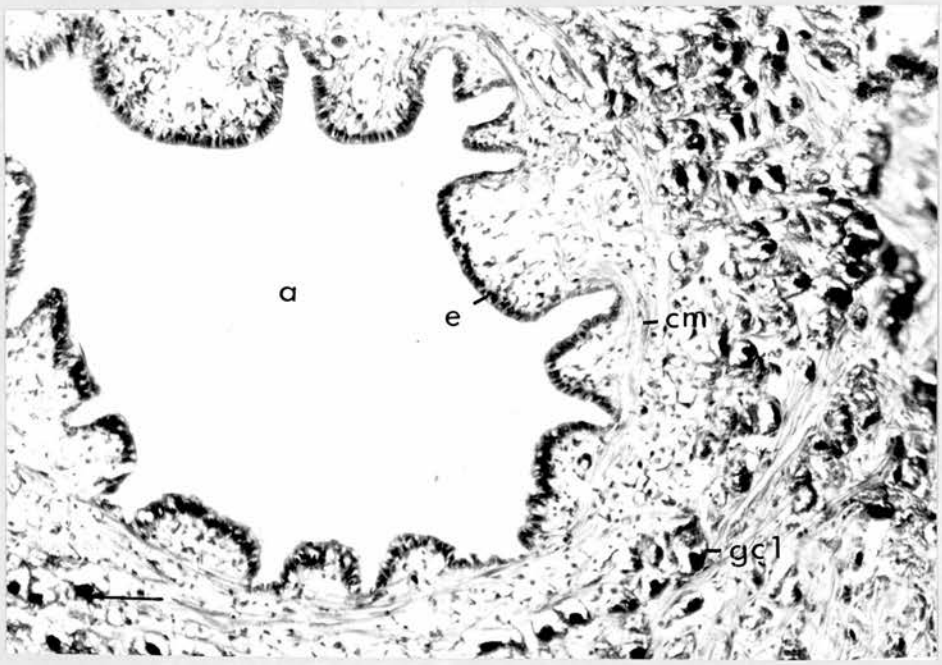


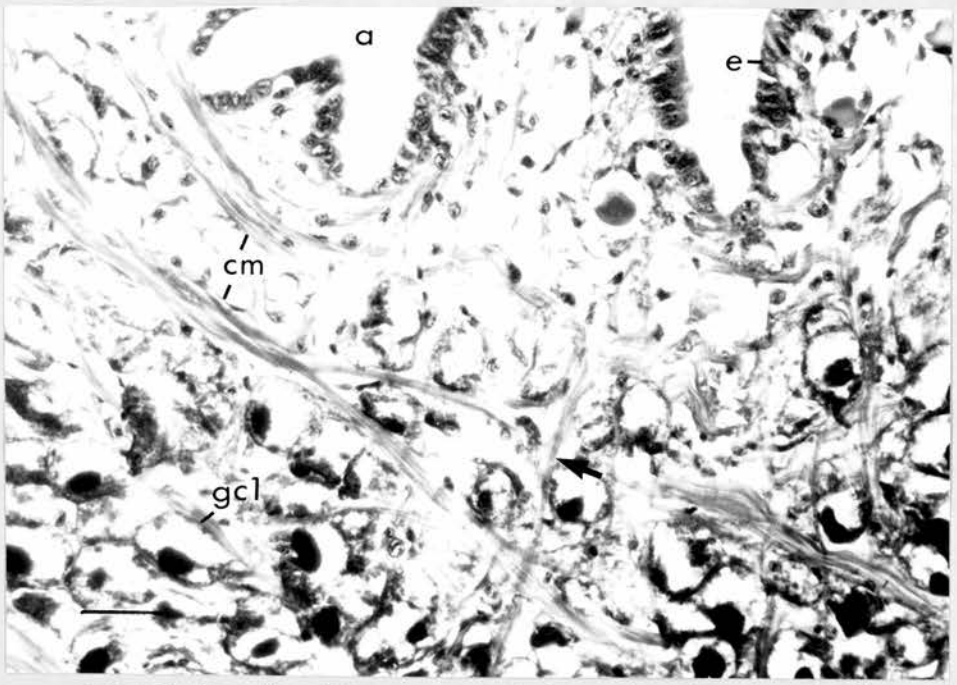
Fig. 9: Band of circular muscle surrounding pneumostome. Arrow points to muscle fibres running through the pneumostomal gland cell mass. a - aperture, cm - circular muscle fibres, e - epithelium, gcl - gland cell type one. (Masson Trichrome) X 115 Scale represents 91 µ.

pneumostomal area and are not found in the subepithelial layer which surrounds the aperture.

A third type of gland cell (gland cell type 3) is found in the pneumostomal area. These are fairly small cells located just beneath the epithelium which lines the aperture (Fig. 12). They have a diameter of about 15 to 20 μ. The nucleus is centrally placed and has a diameter of 2.5 to 5.0 μ. This gland cell type stains light blue with Azan suggesting a mucopolysaccharide type of secretion. These cells are also located in an area surrounding the rectum.

The fourth type (gland cell type 4) are similar to the calcium glands described by Campion (1961) in her work on the skin glands of Helix. These cells are confined to the outer regions of the pneumostomal area. The subepithelial layer which surrounds the pneumostomal aperture does not contain this cell type. Gland cell type 4 has a granular secretion which stains red with Azan; this indicates that the secretion product is a non-mucin type. The nucleus is located in the extreme basal portion of the cell as well as a small amount of cytoplasm (Fig. 16). The cell in Figure 16 measures 75 μ across its widest point and is about 300 μ long.

Cross sections of the pneumostomal tissue did not reveal the precise orientation of the muscle fibres in relation to the pneumostomal aperture. Fig. 17 shows the arrangement of the muscle fibres which support the epithelium lining the aperture. Longitudinal sections through the aperture were then examined and these revealed the following information. There is a band of circular muscle located immediately below the epithelium of the aperture in the subepithelial layer of the pneumostome (Figs. 8 and 9). Circular muscles also run through the region occupied by the pneumostomal gland cells



Figures 10-12: Muscle fibres comprising circular band of muscle surrounding aperture.

Fig. 10: Arrow indicates a bundle of muscle fibres at right angles to the circular muscle; these muscle fibres support the epithelium. a - aperture, cm - circular muscle bundles, e - epithelium, gcl - gland cell type one. (Masson Trichrome) X 290 Scale represents 35 µ.

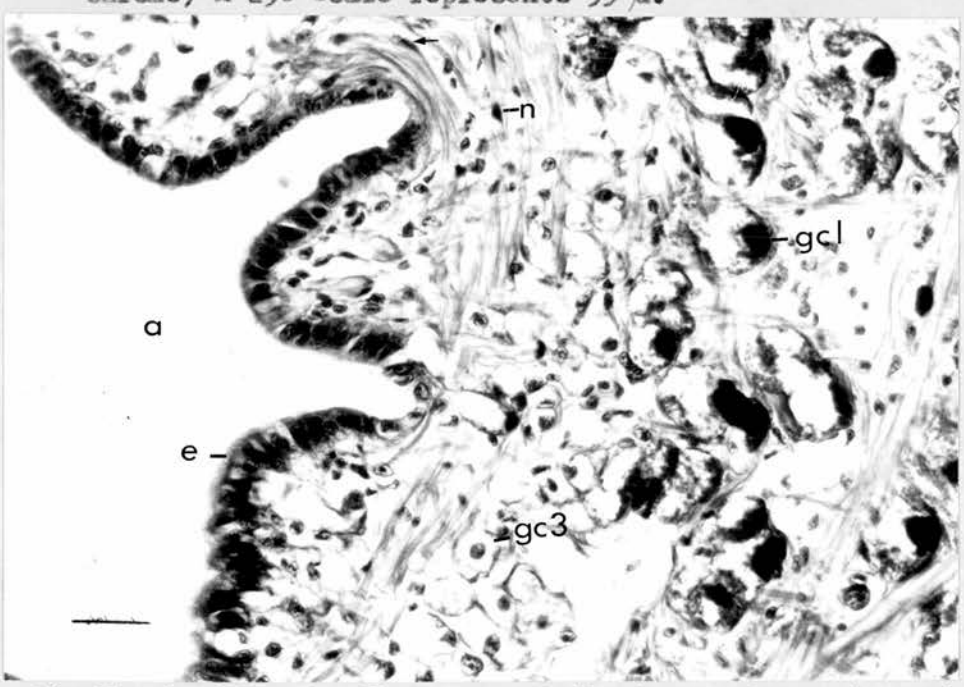


Fig. 11: Muscle fibre bundle; arrow indicates nucleus of muscle fibre. a - aperture, e - epithelium, gcl - gland cell type one, gc3 - gland cell type three, n - nucleus. (Masson Trichrome) X 290 Scale represents 35 µ.

(gland cell type 1). Figures 10, 11 and 12, show, in greater detail, the arrangement of a discrete band of muscle extrinsic to the connective tissue of the area. This band of muscle is approximately 30 μ in width. The gross morphology of several individual muscle cells can be seen clearly in Figure 12.

The muscle arrangement surrounding the pneumostomal aperture is distinct from the general body wall musculature which consists of bands of muscles running in a longitudinal direction (Fig. 13). The body wall musculature also contains bundles which interweave thus creating an extremely complex arrangement (Fig. 14). It is most likely that the band of circular muscle which surrounds the aperture fits the definition of a sphincter muscle. A sphincter is defined as a ring of smooth muscle in the wall of a tubular organ or of an opening of a hollow organ, able by contraction to narrow or close the lumen (Abercrombie et al., 1964). Equal contraction of all the muscle fibres which compose the ring would result in an even displacement of the pneumostomal tissue to produce closure of the aperture (see Fig. 1). The other muscle fibres found in the pneumostomal area are arranged at right angles to the epithelium which lines the aperture. These fibres appear very well organized to bring about an opening of the aperture when they contract. It appears then, from an examination of light microscopy sections that there are two distinct sets of muscle fibres operating the pneumostome, a circular band which closes the aperture and a number of bundles of muscle fibres set at right angles to the aperture which open the aperture. Fig. 18 is a semi-diagrammatic representation of the pneumostome in cross section and attempts to illustrate the arrangement of muscles in relation to the aperture.

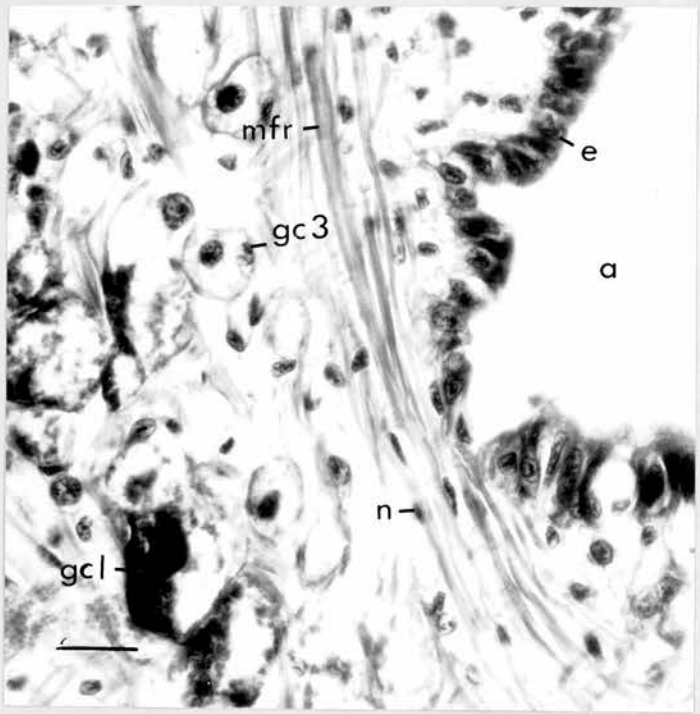


Fig. 12: The gross morphology of several individual muscle cells is distinct. The circular band of muscle runs in the space between the epithelium and the gland cell layer. a - aperture, e - epithelium lining aperture, gcl - gland cell type one, gc3 - gland cell type three, mfr - muscle fibre, n - nucleus of muscle fibre (Masson Trichrome) X 514 Scale represents 20 µ.

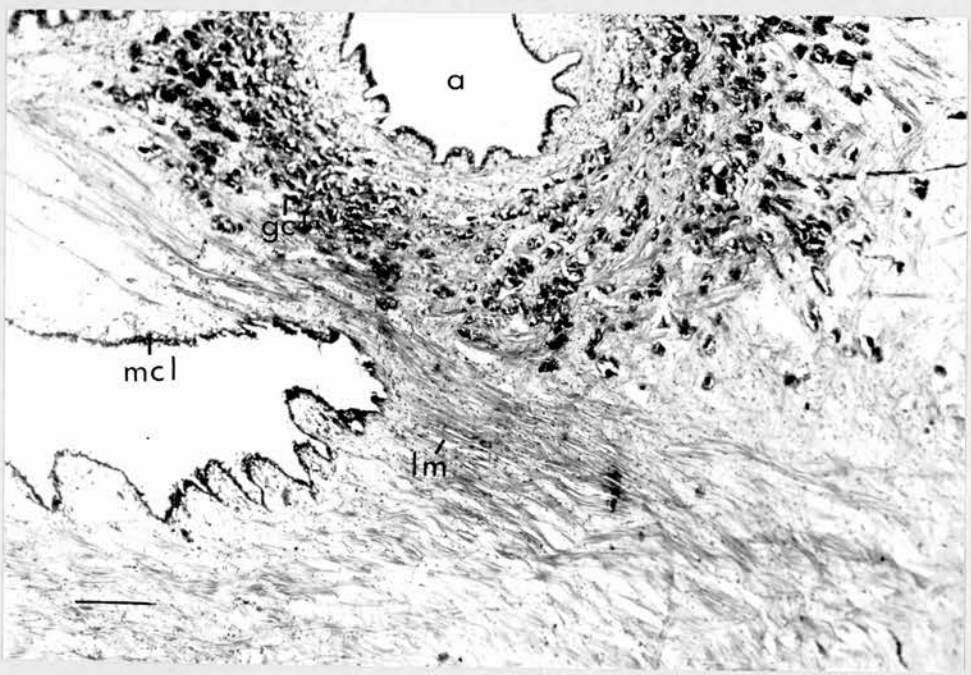


Fig. 13: Longitudinal section through the right mantle wall to show muscle bundles running in a longitudinal direction. a - aperture, gcl - gland cell type one, lm - longitudinal muscle bundles, mcl - mantle collar. (Masson Trichrome) X 46 Scale represents 217 µ.

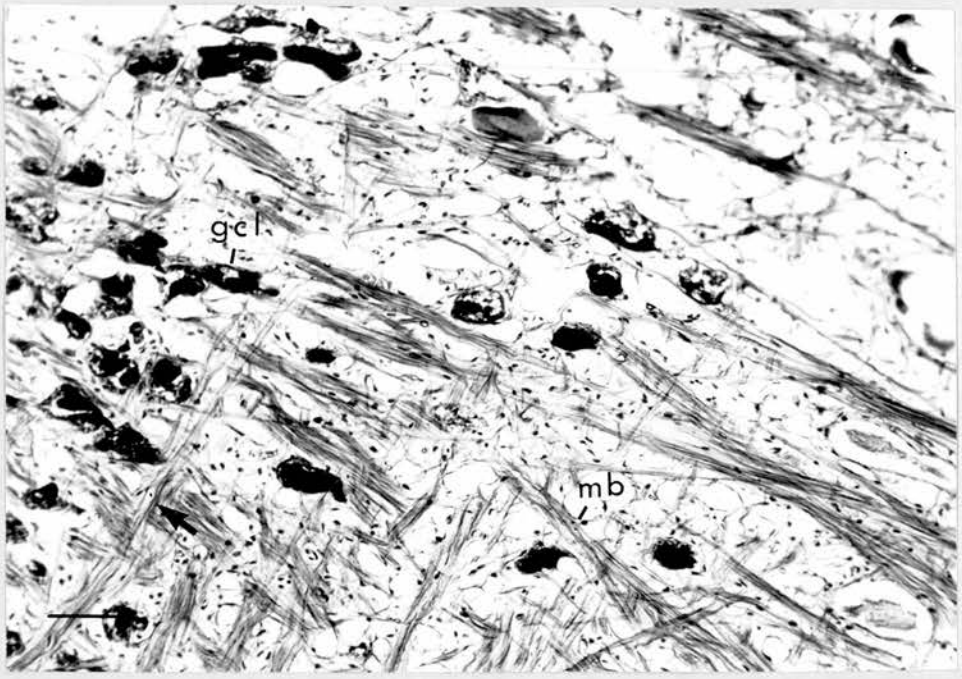


Fig. 14: Longitudinal section through the mantle wall musculature. The individual muscle bundles which are composed of several fibres run in all directions through the tissue. Arrow indicates boundary of pneumostomal tissue. gcl - gland cell type one, mb - muscle bundle (Masson Trichrome) X 115 Scale represents 91 µ.

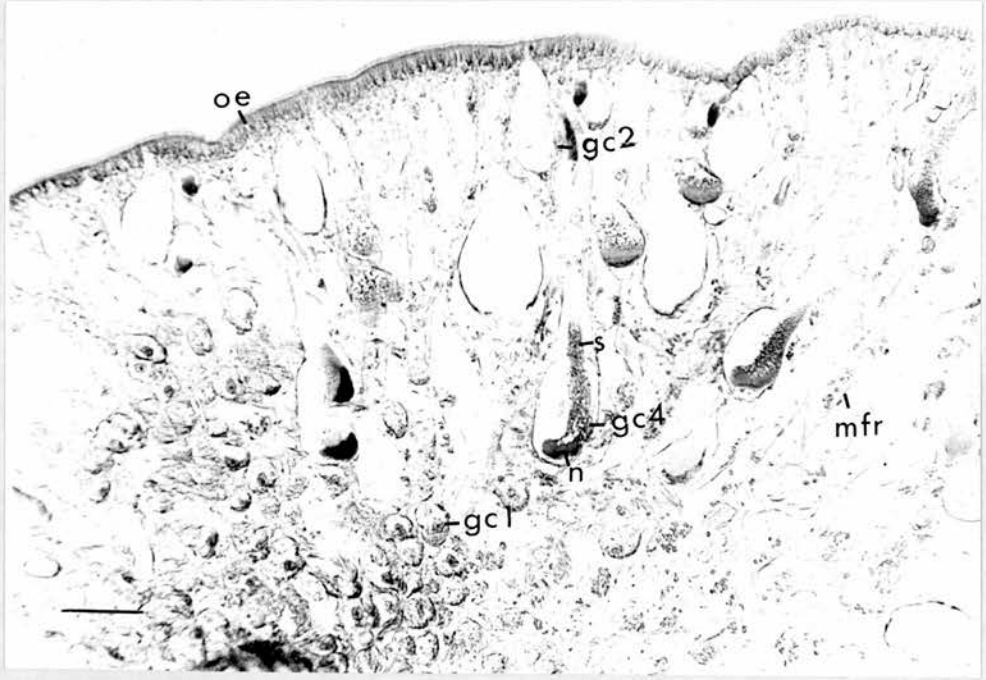


Fig. 15: Cross section of part of pneumostome to show the distribution of the various types of gland cells found in the region. gcl - gland cell type one, gc2, gland cell type two, gc4 - gland cell type four, mfr - muscle fibre, n - nucleus, oe - outer epithelium, s - secretion product. (Azan) X 115 Scale represents 91 µ.

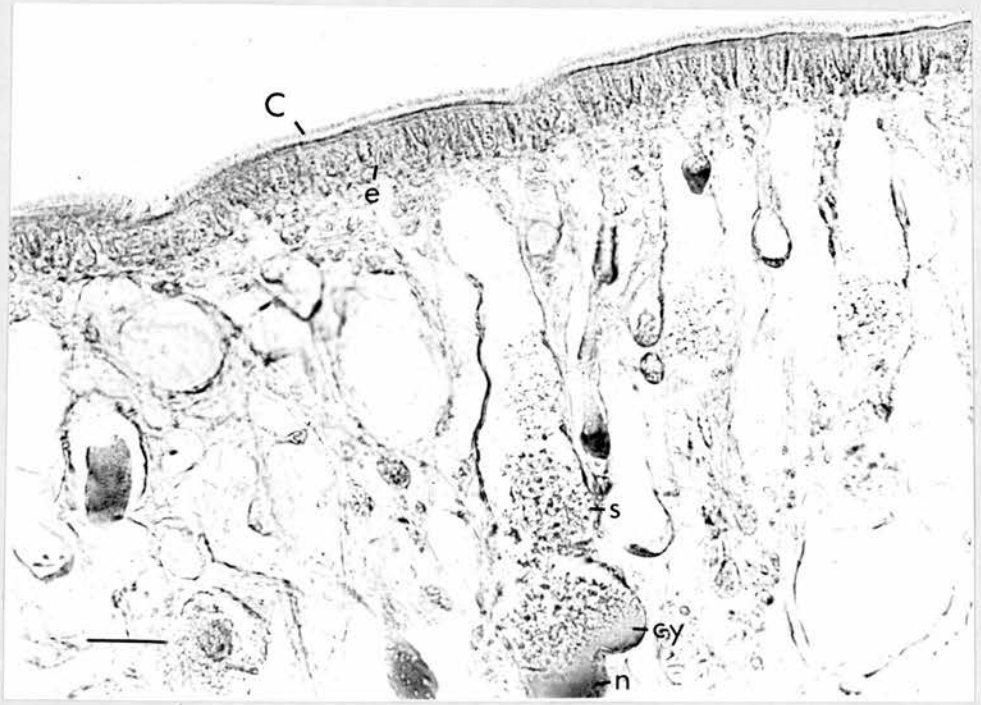


Fig. 16: Gland cell type four. C - cilia, cy - cytoplasm of cell, e - epithelium, n - nucleus, s - secretion product of gland. (Azan) Scale represents 35 μ.

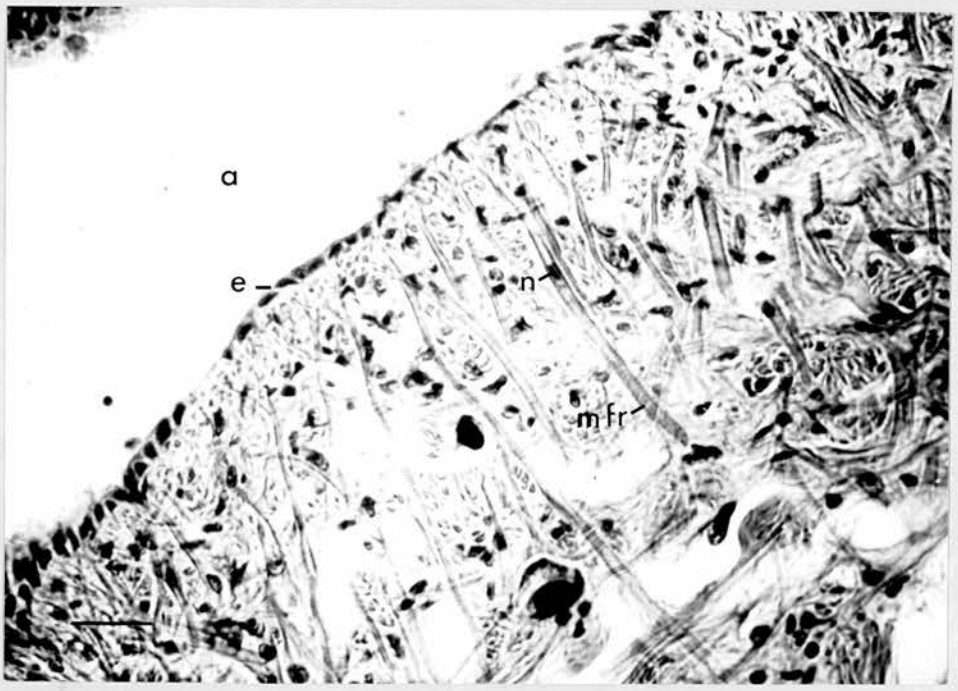


Fig. 17: Cross section through pneumostomal tissue to show the muscles supporting outer epithelium. a - aperture, e - epithelium, mfr - muscle fibre, n - nucleus of muscle fibre. (Masson Trichrome) X 312.5 Scale represents 13 μ.

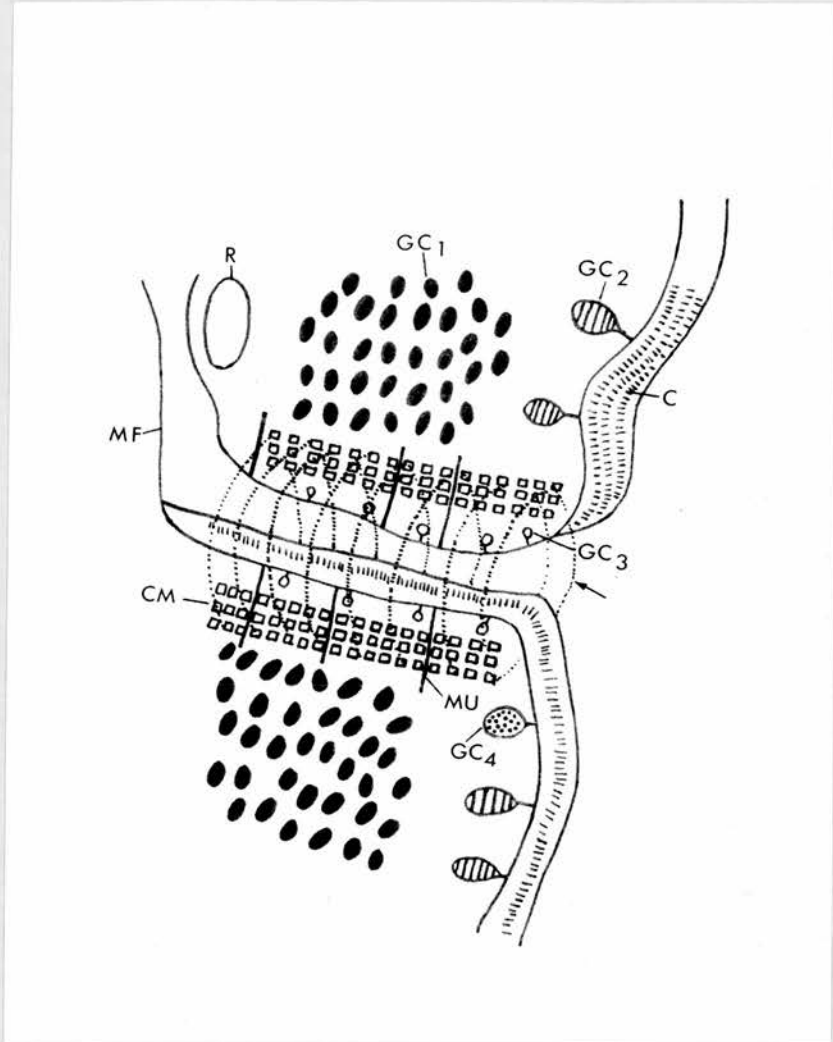


Fig. 18: Semi-diagrammatic representation of the pneumostome to show the arrangement of muscles surrounding the aperture.
Legend: C - cilia, CM - circular muscle bundles in cross section, GC1 - gland cell type one (Pneumostomal gland cells), GC2 - gland cell type two, GC3 - gland cell type three, GC4 - gland cell type four, MF - mantle floor, MU - muscle fibres attached to epithelium, R - rectum.
Arrow points to dotted line which represents the circular band of muscle surrounding aperture.

c. Ultrastructure of the Pneumostome

This section contains a description of the ultrastructure of the various cells found in the pneumostomal tissue.

A. Epithelial Cells:

The epithelium of the pneumostome contains only ciliated cells. These cells bear microvilli in addition to cilia (Fig. 19). It is very difficult to distinguish sensory endings, which normally bear cilia, and these indifferent epithelial cells. The characteristic 9 + 2 fibre arrangement is found in the cilia of these cells (Fig. 20). The two central fibres appear to extend to the basal plate but do not pass through into the basal body, as do the peripheral fibres (Fig. 19). A well-developed basal foot extends laterally from the basal body. These basal feet are arranged parallel to each other. Well-developed striated rootlets extend from the basal bodies into the cytoplasm of the cell. The diameter of the rootlet varies from about 350 Å at the tip to 700 Å at the point where it joins the basal body. The periodicity of these rootlets is approximately 600 Å. Mitochondria are very numerous in the region of these striated rootlets.

All of the epithelial cells examined using the electron microscope contained cilia having a parallel orientation and only one striated rootlet. Zylstra (1972B) used these characteristics as a means of distinguishing indifferent ciliated epidermal cells from free nerve endings. He found that the cilia of the free nerve endings have a random orientation whereas those of the indifferent ciliated cells have a parallel orientation. Furthermore, the cilia of the free

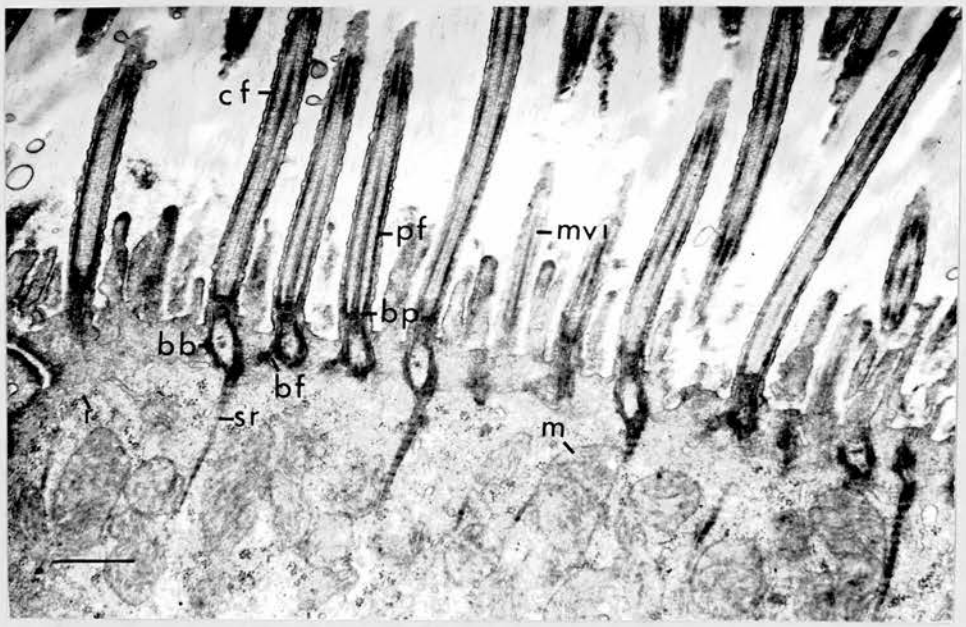


Fig. 19: Electron micrograph of epidermal cilia cell. bb - basal body, bf - basal foot, bp - basal plate, cf - central subfibres of cilium, m - mitochondrion, mvi - microvillus, pf - peripheral subfibres of cilium, r - free ribosomes, sr - striated rootlet.
Fixation: glut./OsO_4 X 20,400 Scale represents 0.5 µ.

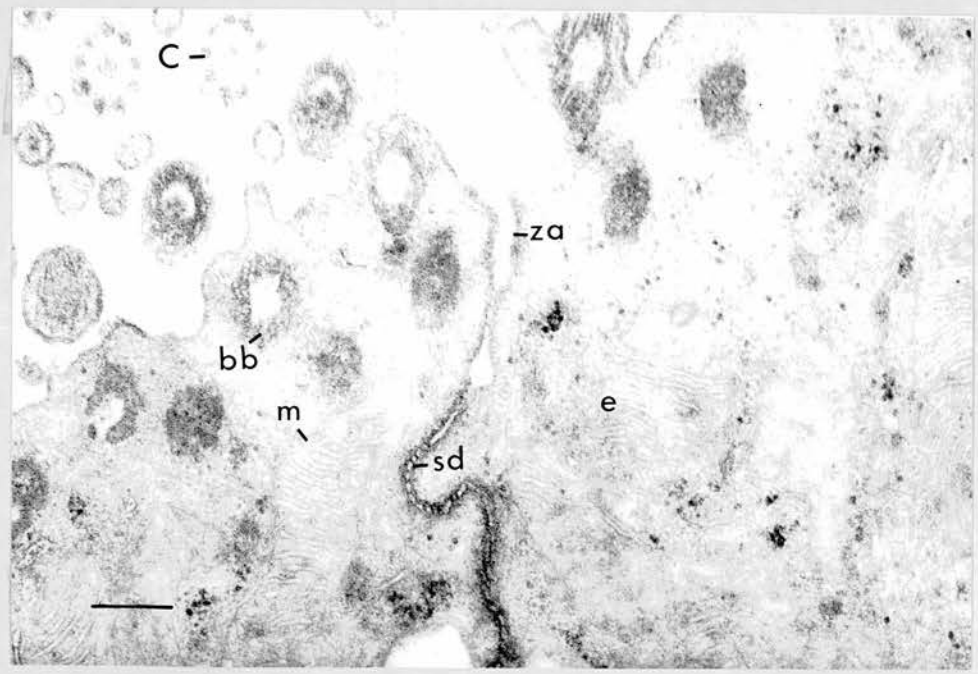


Fig. 20: Septate desmosome of epithelial cell. bb - basal body, c - cilium, e - epithelial cell, m - mitochondrion, sd - septate desmosome, za - zonula adhaerens.
Fixation: glut/OsO_4 X 51,000 Scale represents 0.2 µ.

nerve endings have usually 3 - 5 roots whereas the cilia of the epidermal ciliated cells have only one striated root. Using these criteria, it appears that all of the ciliated epithelial cells examined in this investigation are indifferent epithelial cells and not the free nerve endings of subepithelial sensory cells.

There are considerable interdigitations between the adjacent epithelial cells which probably allow for the stretching of the epithelium. Several junctions are also found between the epithelial cells. The most apical, the zonula adhaerens (Farquhar and Palade, 1963) extends for a length of 0.3 μ and begins about 0.3 μ in from the apical surface (Fig. 20). Below the zonula adhaerens, there is a septate junction which is found approximately 1 μ from the apical surface of the epithelial cell. The adjacent plasma membranes of this junction are about 150 Å apart and are joined by septa which are approximately 200 Å apart. These septate desmosomes seem identical to those described by Wood (1959) and Locke (1965). The occurrence of a macula adhaerens described by Wondrak (1968) in his work on the epithelial structure of Arion rufus has not been established.

B. Gland Cells:

Three types of subepithelial gland cells have been distinguished using the electron microscope. The first type is found in a region just beneath the epithelium and is probably gland cell type 2 of Figure 15. The most distinguishing characteristic of this cell is the prominent golgi apparatus. This structure is composed of a stack of up to 20 lamellae. The length of the longest stack in one of

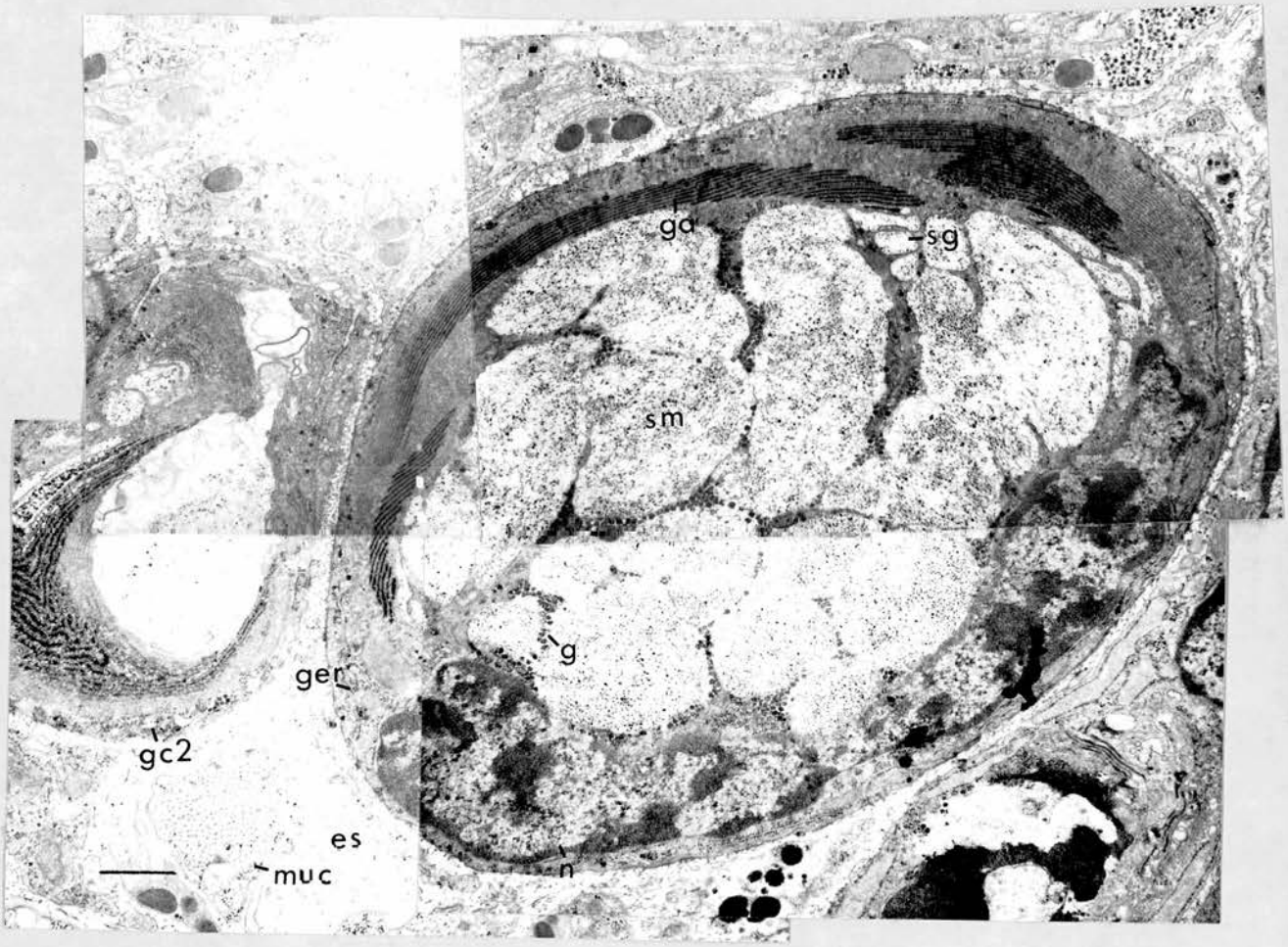


Fig. 21: Composite electron micrograph of gland cell type two. es - extracellular space, g - granules, gc2 - gland cell type two, ga - golgi apparatus, ger - granular endoplasmic reticulum, muc - muscle cell, n - nucleus, sg - secretion granule, sm - secretion mass. Fixation: glut/OsO_4; X 15,300 Scale represents 0.7 u.

these cells (Fig. 21) is approximately 6.5 μ. The lumen of the lamellae has a greater electron density than that of the surrounding hyaloplasm. The central portion of the cell contains a very large heterogeneous secretion mass. This mass is probably formed by the fusion of smaller secretion granules (Fig. 21). These secretion granules are closely associated with the golgi apparatus. It may be that the formation of these secretion granules takes place within the golgi complex of the cell (see Fig. 23). A large number of microvesicles are associated with the golgi apparatus. The granular endoplasmic reticulum appears to be confined to the peripheral area of the cytoplasm.

Several complete membrane-bound secretory vacuoles have been seen within the epithelial layer of the pneumostome. In some cases, the membrane surrounding these vacuoles has been ruptured and the contents are released at the epithelial surface (Fig. 22). These secretory vacuoles appear to be the same as those contained within gland cell type 2. The secretory vacuole must pass between adjacent epithelial cells on its way to the exterior.

These gland cells are very similar to the "Manteldrüsen zelle" (mantle glands) described by Wondrak (1969B) for the slug Arion rufus (L). The "Manteldrusen zelle" have the same elaborate golgi apparatus and large secretion mass.

Another type of gland cell is found in the deep subepithelial area of the pneumostome and is probably gland cell type 1 of Figure 15. These gland cells are specific to the pneumostomal region of the slug in contrast to gland cell type 2 which is also found in large numbers

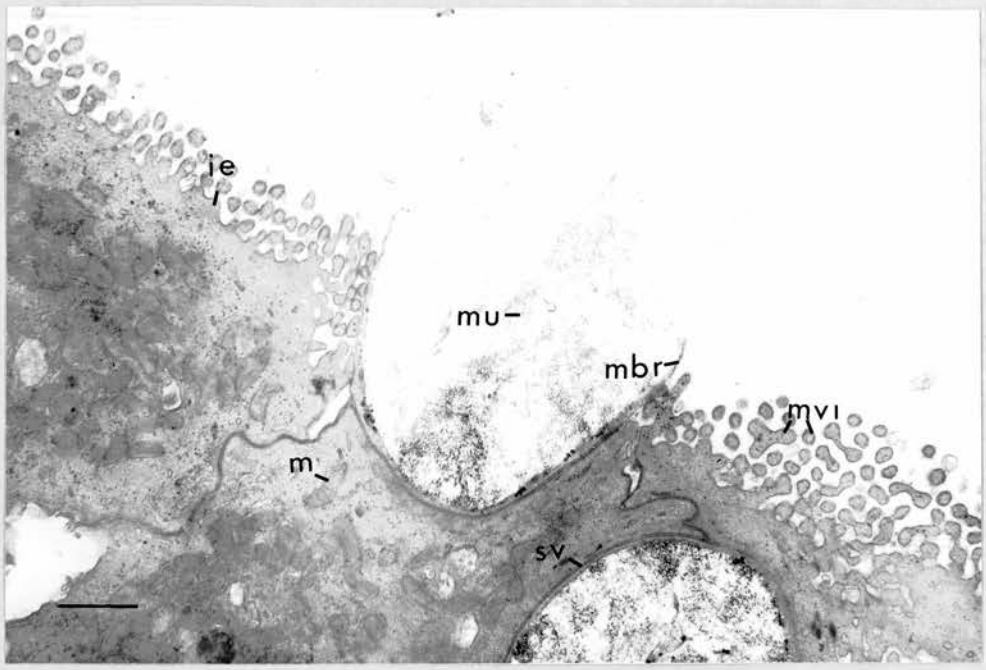


Fig. 22: Secretory vacuole releasing contents at the epithelial surface of the pneumostome.
ie - indifferent epithelial cell, m - mitochondrion, mbr - membrane of secretory vacuole, mu - mucous, mvi - microvilli, sv - secretory vacuole
Fixation: glut/OsO_4 X 12,600 Scale represents 0.8 µ.

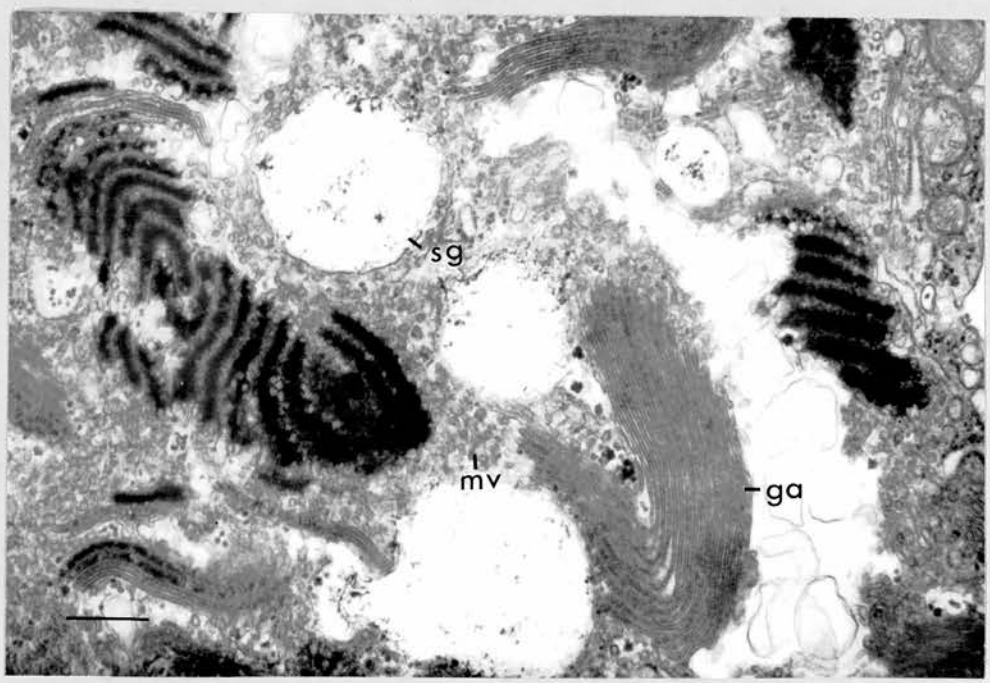


Fig. 23: Golgi complex of gland cell type two.
ga - golgi apparatus, mv - microvesicles, sg - secretion granule
Fixation: glut/OsO_4 X 28,900 Scale represents 0.3 µ.

throughout the entire mantle wall. Gland cell type 1 is characterized by a large number of very homogeneous secretion granules which contain material of considerable electron density (Fig. 24). These granules do not fuse to form a larger secretion mass which is typical of gland cell type 2. These granules appear to be membrane-bound (Figs. 25 and 26). The cytoplasm which surrounds these secretion granules is composed of a well-developed granular endoplasmic reticulum (Fig. 25). The cisternae of the GER are well-developed and their contents have a greater electron density than the surrounding hyaloplasm. Ribosomes are not only associated with the membranes of the GER but are also scattered throughout the cytoplasm. The cisternae of the GER have a width of up to 0.20 µ.

The golgi apparatus consists of a curved stack of six to seven lamellae (Fig 26). The lumen of these lamellae has a greater electron density than the surrounding hyaloplasm. Microvesicles are associated with the golgi complex. The ends of the inner lamellae of the stack are swollen. These swollen ends probably break off to form the secretion granules characteristic of this type of gland cell. Mitochondria are scattered throughout the cytoplasm.

The ultrastructure of these pneumostomal gland cells is completely different from that of the pneumostomal non-muciparous gland cells described by Zylstra (1972A) for the freshwater snail, Lymnaea stagnalis. The secretion granules of the latter contain double-layered membrane-like structures whereas the secretion granules of the pneumostomal gland cells of Limax flavus are solid entities.

The ultrastructure of another type of gland cell found in the pneumostomal region is shown in Figure 27. These cells occur very

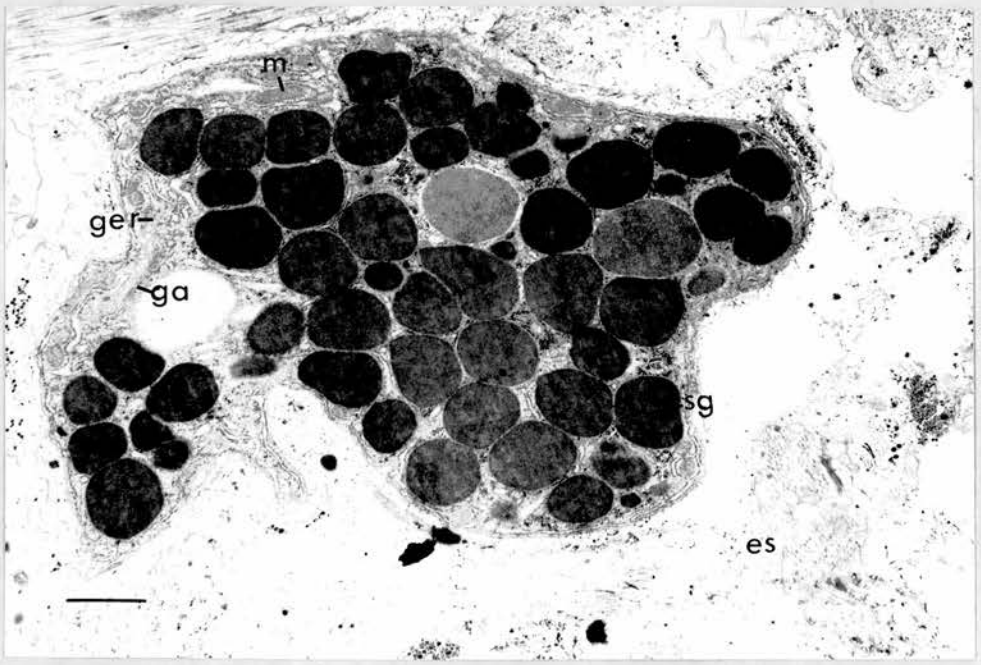


Fig. 24: Profile of gland cell type one (Pneumostome gland cells) ga - golgi apparatus, es - extracellular space, ger - granular endoplasmic reticulum, m - mitochondrion, sg - secretion granule
Fixation: glut/OsO_4 X 8,800 Scale represents 1.1 µ.

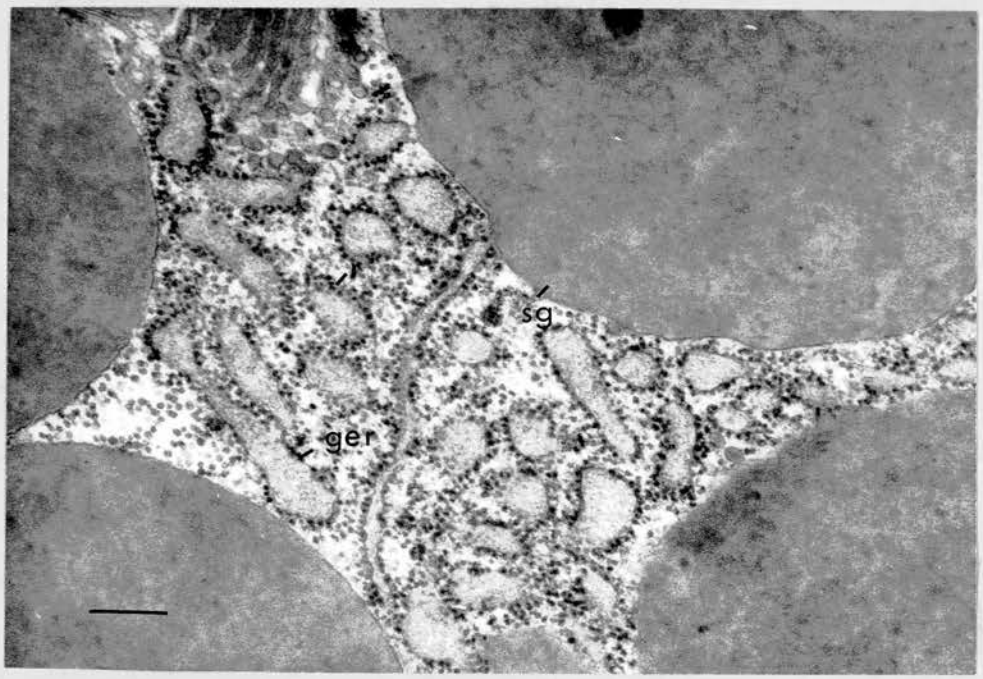


Fig. 25: Granular endoplasmic reticulum of gland cell type one. ger - granular endoplasmic reticulum, r - ribosomes attached to GER membrane, sg - secretion granule
Fixation: glut/OsO_4 X 44,000 Scale represents 0.2 µ.

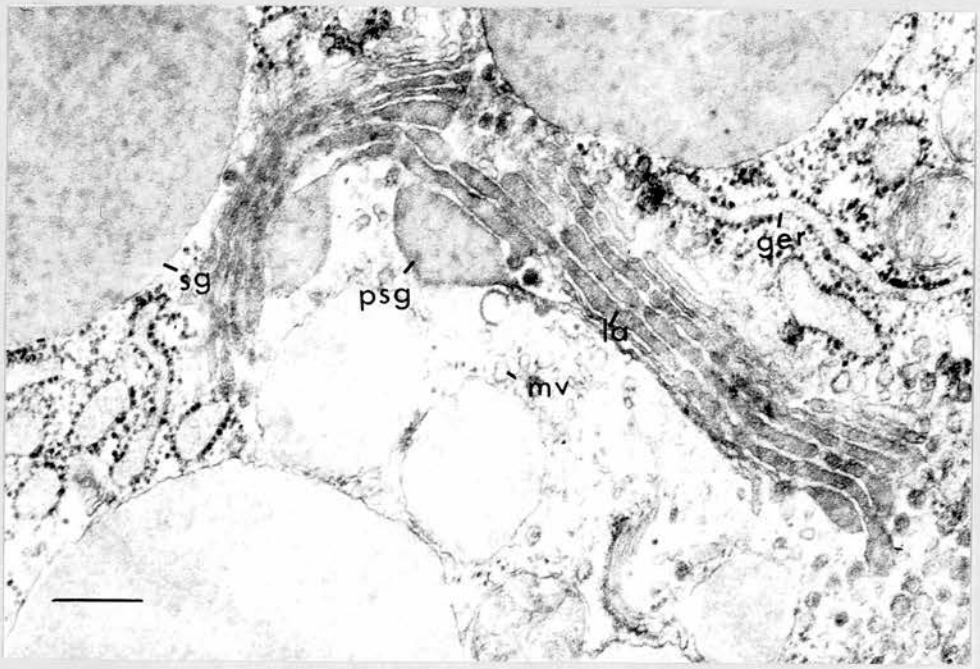


Fig. 26: Golgi apparatus of gland cell type one. ger - granular endoplasmic reticulum, la - lamellae of golgi apparatus, mv - microvesicles, psg - pro-secretion granule, sg - secretion granule. Fixation: glut/OsO_4 X 44,000 Scale represents 0.2 µ.

infrequently in the sections examined and it has not been possible to relate them to any gland cell found in the light microscopy sections. This gland cell contains a large number of irregularly-shaped heterogeneous secretion granules. Some of these granules contain filamentous material (sg_1, Fig. 27); others contain small, moderately dense bodies (sg_2, Fig. 27). These secretion granules can be seen in greater detail in Figure 28. The granular endoplasmic reticulum has a tubular structure and is widely distributed throughout the cytoplasm of the cell. Ribosomes are scattered throughout the cytoplasm as well as being associated with the membranes of the endoplasmic reticulum. The cytoplasm also contains numerous mitchondria and well-defined Golgi bodies. The Golgi apparatus is lamellar in nature and is associated with microvesicles.

The most distinguishing feature of these gland cells is the presence of rather large multivesicular bodies. These membrane-bound bodies are composed of numerous small clear vesicles and a dense substance which fills in the space between the vesicles. In some cases, there is a gap in the membrane where these vesicles open directly into the cytoplasm of the cell (Fig. 29, see arrow).

C. Muscle Cells

The circular band of muscle which surrounds the pneumostome is composed of smooth muscle cells. It is assumed that this circular muscle is involved in the ventilation movements of the pneumostome.

These smooth muscle cells contain two types of filaments: (a) thin filaments which are approximately 50 Å in diameter and (b) thick filaments which are approximately 200 - 300 Å in diameter. Each thick

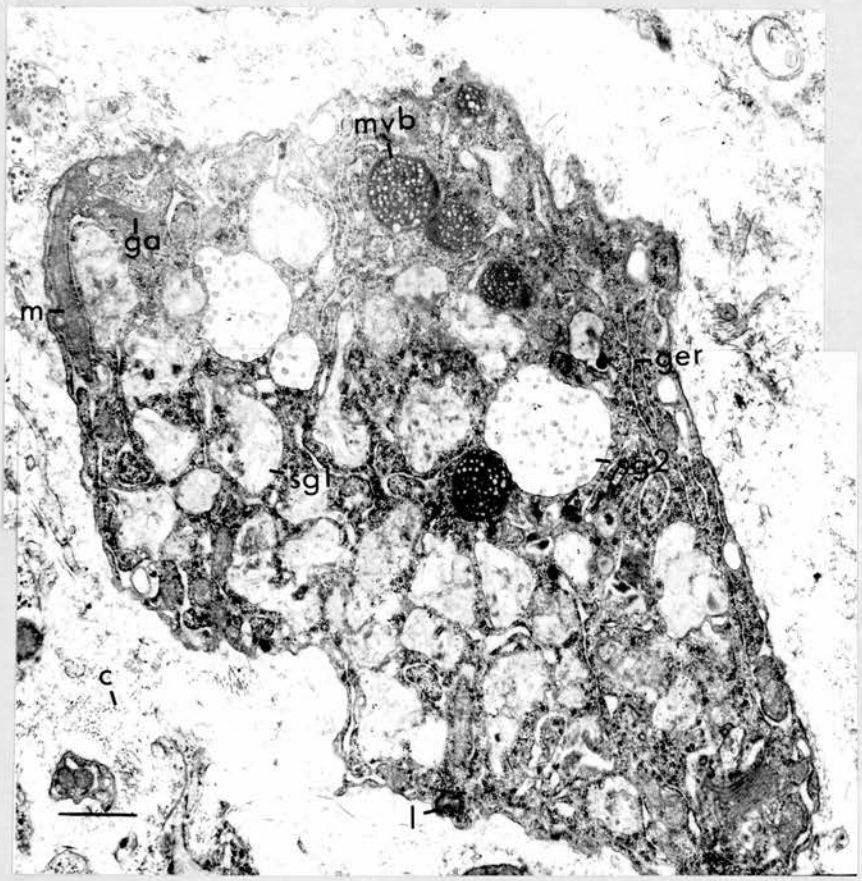


Fig. 27: Subepithelial gland cell in pneumostomal region. c - collagen fibres, ga - golgi appratus, ger - gramular endoplasmic reticulum, l - lamellated body, m - mitochondrion, mvb - multivesicular body, sgl - secretion granule type one, sg2 - secretion granule type two.
Fixation: glut/OsO_4 X 15,300 Scale represents 0.7 μ.

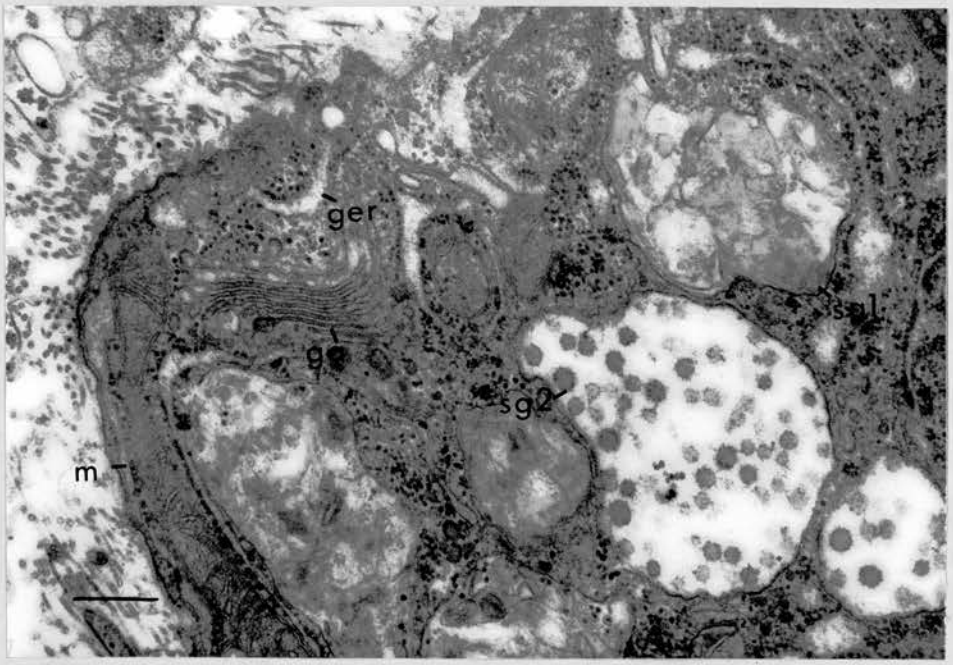


Fig. 28: Golgi appratus and secretion granules of subepithelial gland cell (see Fig. 27).
c - collagen fibres, ga - golgi apparatus, ger - granular endoplasmic reticulum, m - mitochondrion, r - free ribosomes, sgl - secretion granule type one, sg2 - secretion granule type two.
Fixation: glut/OsO_4 X 39,000 Scale represents 0.25 µ.

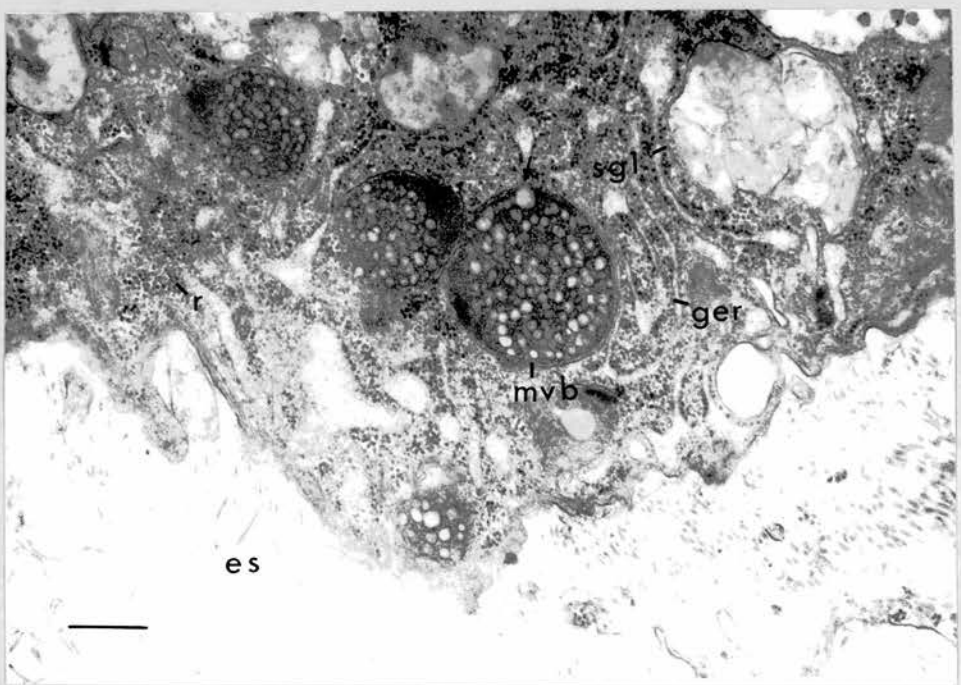


Fig. 29: Multivesicular bodies of subepithelial gland cell. One of the vesicles opens into the cytoplasm through a break in the membrane (arrow).
es - extracellular space, ger - granular endoplasmic reticulum, mvb - multivesicular body, r - free ribosomes, sgl - secretion granular type one.
Fixation: glut/OsO_4 X 29,000 Scale represents 0.3 µ.

filament appears to be surrounded by a number of thin filaments (Fig. 31). There are a number of dense bodies distributed throughout the mass of myofilaments. These bodies have a diameter of approximately 700 - 800 Å and appear to be filamentous in nature. It has been suggested that these dense bodies are attachment sites for the thin myofilaments (Heyer et al., 1973).

Dense patches are also found at the muscle cell membrane. These dense areas appear to be composed of a filamentous material which extends across the membrane and into the extracellular space. There is a thickening of the muscle cell membrane at this point. Figure 33 shows apposing dense patches in adjacent muscle cells.

The non-contractile areas of the muscle cell contain a number of different inclusions. These include numerous mitochondria which are orientated both longitudinally and transversely within the cell (Fig. 32). Mitochondria are also found in the central portion of the cell (Fig. 30). The granular endoplasmic reticulum is extensive and tubular in form. Ribosomes are scattered throughout the cytoplasm of the cell.

Numerous large vesicular profiles are found at the periphery within the non-contractile cytoplasm of the cell (Fig. 30). The limiting membrane of the vesicular profiles which open into the intercellular space is continuous with the plasma membrane and has the same dimensions (Fig. 34). It may be that some of these submembrane vesicles are formed by sarcolemmal invaginations. Tubular elements which are both obliquely and longitudinally aligned (Fig. 31) are found among the myofilaments. The locations of these tubular elements make them morphologically comparable to the sarcoplasmic reticulum of vertebrate striated muscle (Heyer et al., 1973).

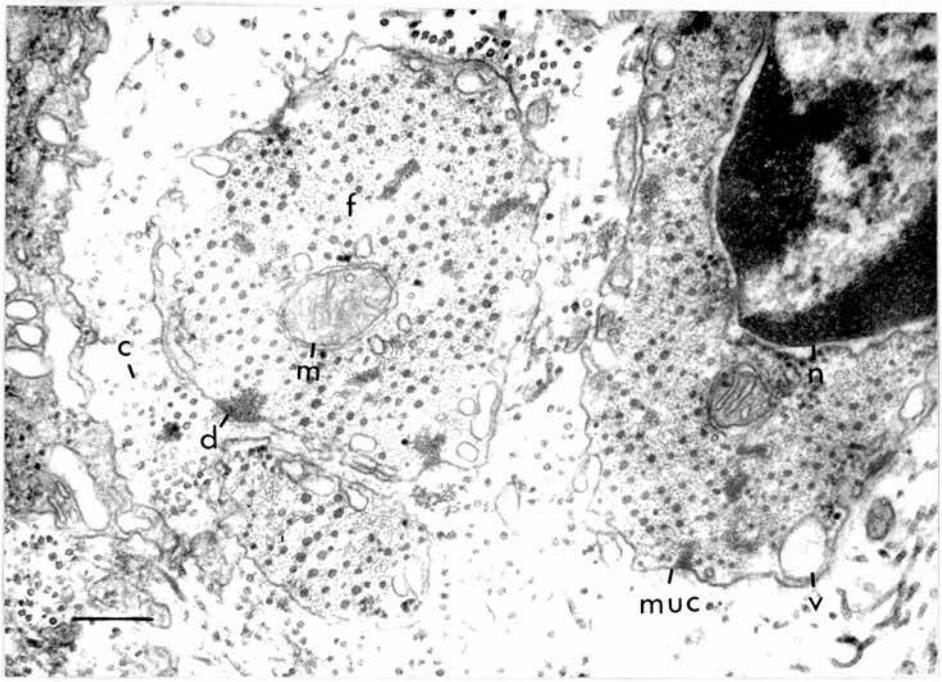


Fig. 30: Cross section of a muscle cell in pneumostomal tissue.
c - collagen fibres, d - dense body, f - thick and thin myofilaments, m - mitochondrion, muc - muscle cell, n - nucleus, v - vesicular profile.
Fixation: glut/OsO_4 X 44,000 Scale represents 0.25 µ.

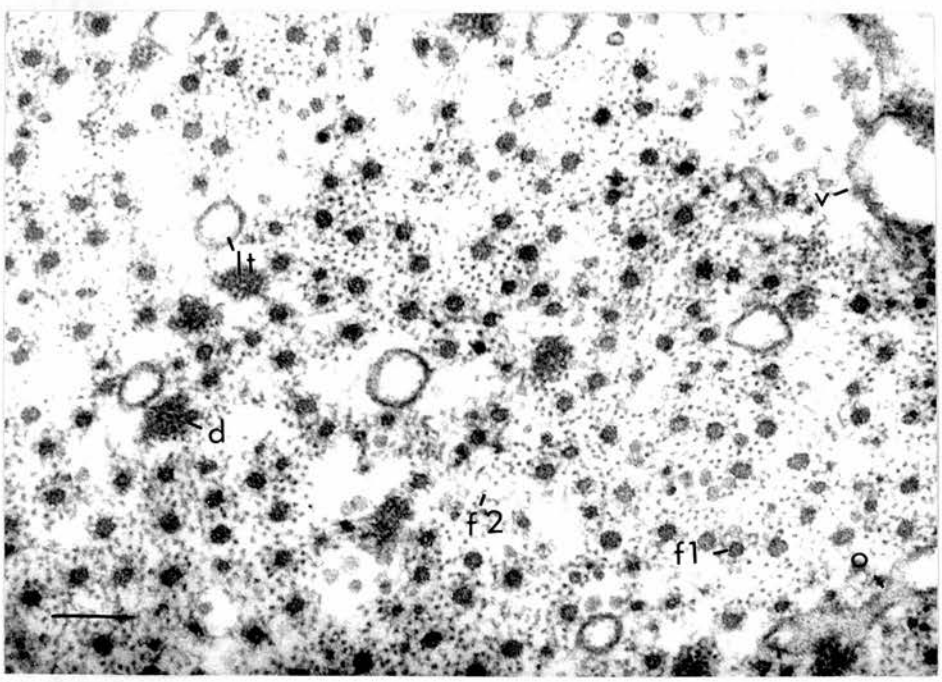


Fig. 31: High power micrograph of muscle cell to show the arrangement of thick and thin myofilaments.
d - dense body, f1 - thick myofilament, f2 - thin myofilament, lt - longitudinally aligned tubule, o - obliquely aligned tubule, v - vesicular profile
Fixation: glut/OsO_4 X 87,000 Scale represents 0.1 µ.

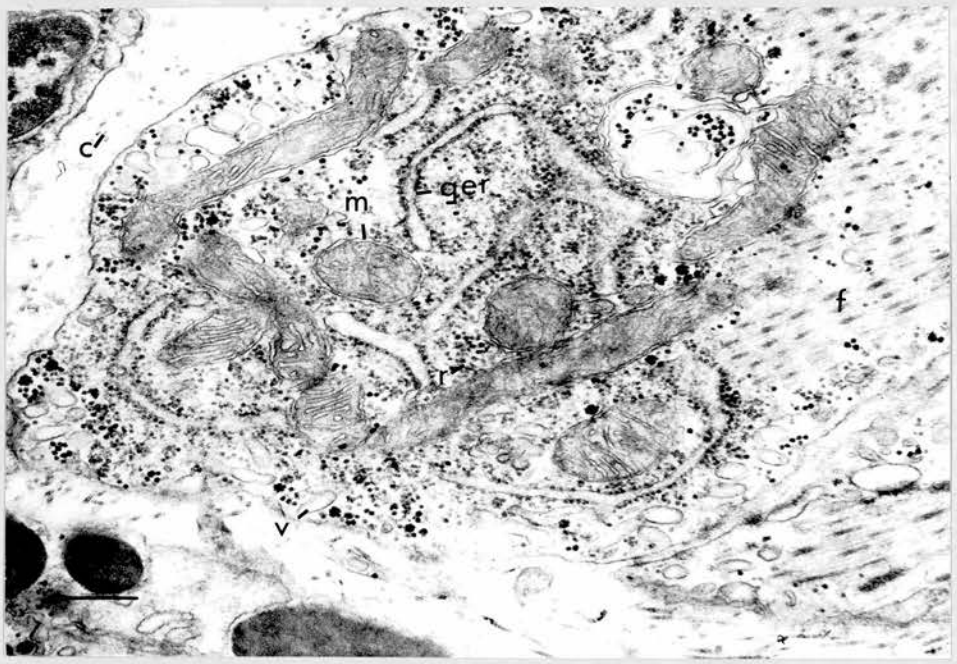


Fig. 32: Non-contractile portion of a muscle cell.
c - collagen fibres, f - thick myofilaments, ger - gramular endoplasmic reticulum, m - mitochondrion, r - free ribosomes, v - vesicular profile.
Fixation: glut/OsO_4 X 39,000 Scale represents 0.25 µ.

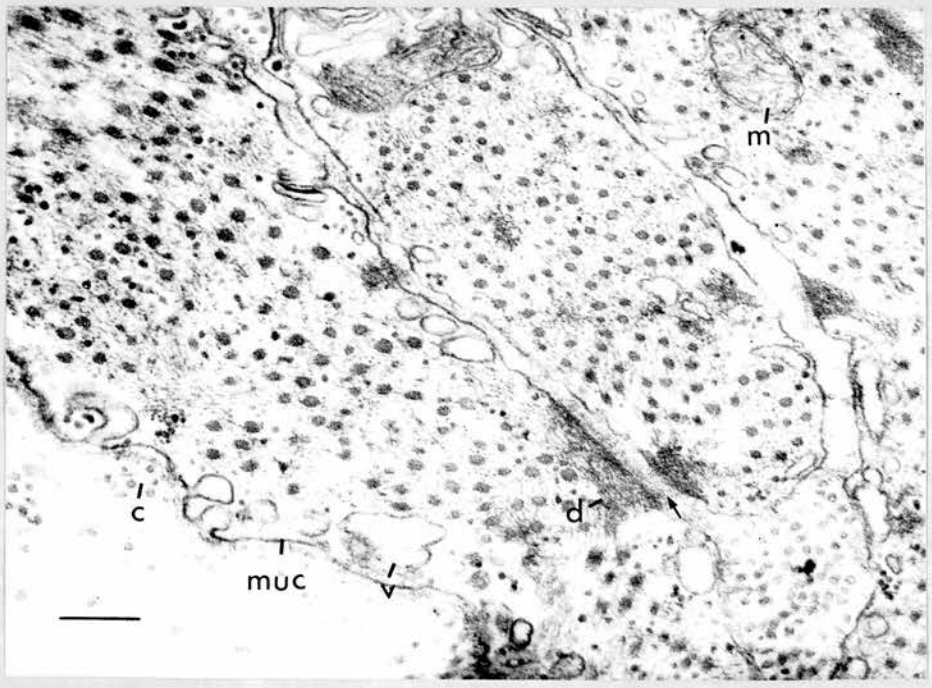


Fig. 33: Adjacent muscle cells; note apposing dense patches (arrow).
c - collagen fibres, d - dense body, m - mitochondrion, muc - muscle cell, v - vesicular profile.
Fixation: glut/OsO_4 X 51,000 Scale represents 0.2 µ.

D. Axons:

The muscles surrounding the pneumostomal aperture appear to be innervated by axons which run in the anal nerve from the visceral ganglion (Fig. 3B). Bundles of nerves are found among the muscles cells which compose the circular muscle surrounding the aperture. These bundles are composed of a number of small axons ranging in size from 0.2 - 1.5 μ in diameter (Fig. 35). Teloglia (Nicaise, 1973) may be associated with these nerve bundles. The teloglial cell processes contain large homogeneous dense granules with diameters ranging from 2000 to 5000 Å (Figs. 35, 36, 37). The teloglia may also contain mulberry granules (Simpson, 1969) which are most probably composed of glycogen (Figs, 35, 38).

The axons which make up these small nerve bundles contain different types of inclusions. For example, in Figure 35, axon one contains a number of vesicles of moderate electron density; axon two contains small agranular vesicles similar to those found in neuromuscular junctions in the area; axon three contains a couple of small electron dense granular vesicles which are also found in neuromuscular junctions, and axon four contains no vesicles but numerous neurofilaments. It is highly likely that these axons which possess synaptic vesicles form synaptic junctions with the muscle cells found in the pneumostomal region.

A large nerve trunk was found in the deep subepithelial area of the pneumostome dorsal to the aperture. This is probably a large branch of the anal nerve. This nerve trunk consists of a large number of naked axons surrounded by the processes of glial cells (Fig. 37). The axons in this nerve trunk possess different types of vesicles:

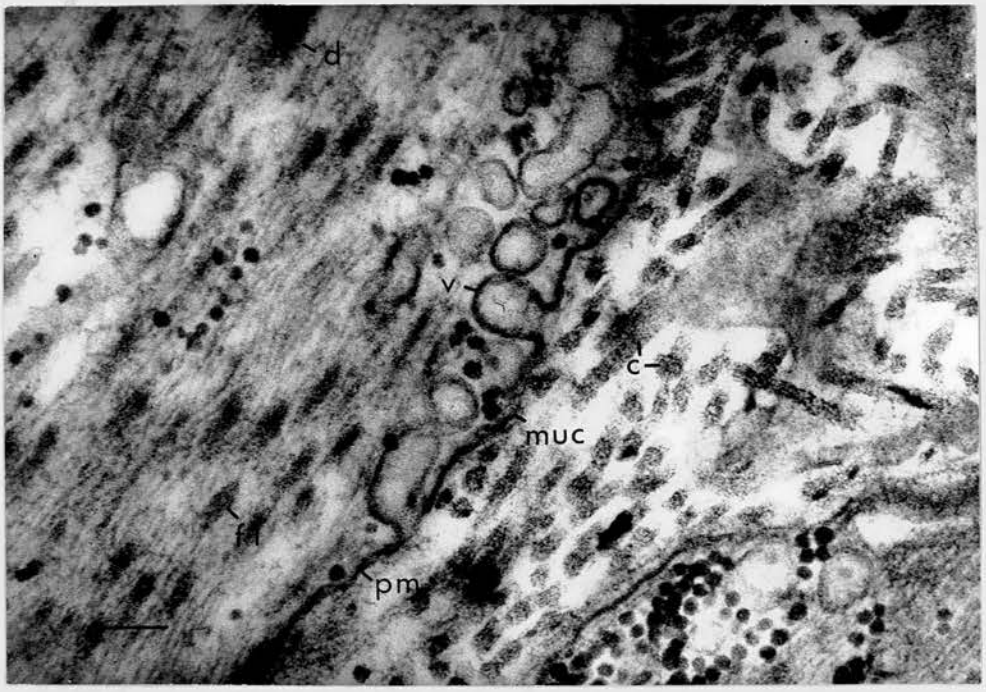


Fig. 34: High power micrograph to illustrate the arrangement of vesicular profiles at the plasma membrane.
c - collagen fibres, d - dense body, fl - thick myofilament, muc - muscle cell, pm - plasma membrane, v - vesicular profile.
Fixation: glut/OsO_4 X 87,000 Scale represents 0.1 μ.

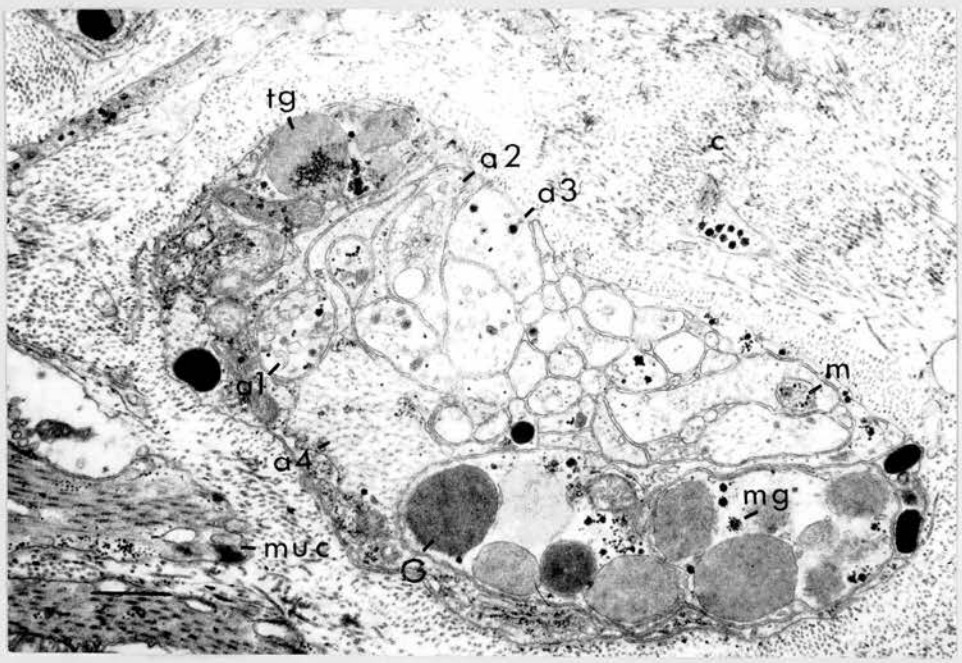


Fig. 35: Small nerve bundle from the subepithelial layer of the pneumostome.
a_1 - axon one, a_2 - axon two, a_3 - axon three, a_4 - axon four (see Text for explanation), c - collagen fibres, m - mitochondrion, muc - muscle cell, mg - mulberry granule, G - large dense granule, tg - teloglial cell process.
Fixation: glut/OsO_4 X 15,000 Scale represents 0.7 μ.

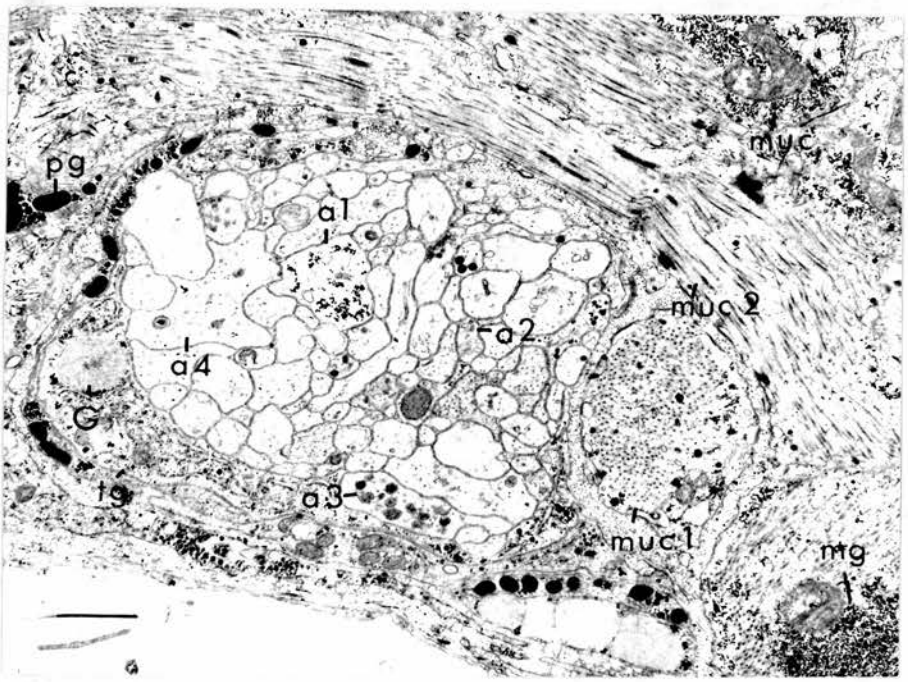


Fig. 36: Nerve bundle in deep subepithelial layer of the pneumostome to show variation in the contents of the axon profiles.
a_1 - axon one (neurofilaments and mulberry granules)
a_2 - axon two (agranular vesicles)
a_3 - axon three (dense granular vesicles)
a_4 - axon four (neurofilaments)
c - collagen fibres, muc_1 - muscle cell in transverse section, muc_2 - muscle cell in longitudinal section, muc - muscle cell containing mulberry granules, mg - mulberry granules, pg - pigment granule, G - large dense granule, tg - teloglial cell process.
Fixation: glut/OsO_4 X 10,700 Scale represents 0.9 µ.

(1) small agranular vesicles, 400 - 600 Å in diameter (2) moderately dense granular vesicles, 900 Å in dameter (3) large agranular vesicles, 800 Å in diameter. Mitochondria and neurofilaments are often found within the axon profiles (Fig. 38).

E. Neuromuscular Junctions:

The size and shape of the nerve endings vary greatly. The nerve membrane may fit tightly against the muscle cell membrane (Figs. 39, 41) or lie in a groove in the muscle cell (Fig. 42). In some cases, there does not appear to be any specialization of either the axonal membrane or the muscle cell membrane where the two come together. In other cases (Fig 41), there seems to be an increase in density of the apposed nerve-muscle membranes. The synaptic cleft is approximately 200 Å wide and is filled with a moderately dense amorphous material (Fig. 40).

Occasionally, an axon or number of axons lie in close apposition with a protrusion of the muscle cell (Fig. 40). More than one axon may make contact with a single muscle cell (Fig. 40). It is, however, very difficult to determine the amount of axonal branching and folding and thus, to estimate the number of neurons which innervate a single muscle cell.

The synapses may occur anywhere along the muscle cell membrane. The cytoplasm of the muscle cell near the synapse may or may not contain contractile elements. Large vesicular profiles are often associated with the post-synaptic region (Figs. 40, 42). Teloglial processes may be associated with muscle cells in the vicinity of neuromuscular synapses (Fig. 40).

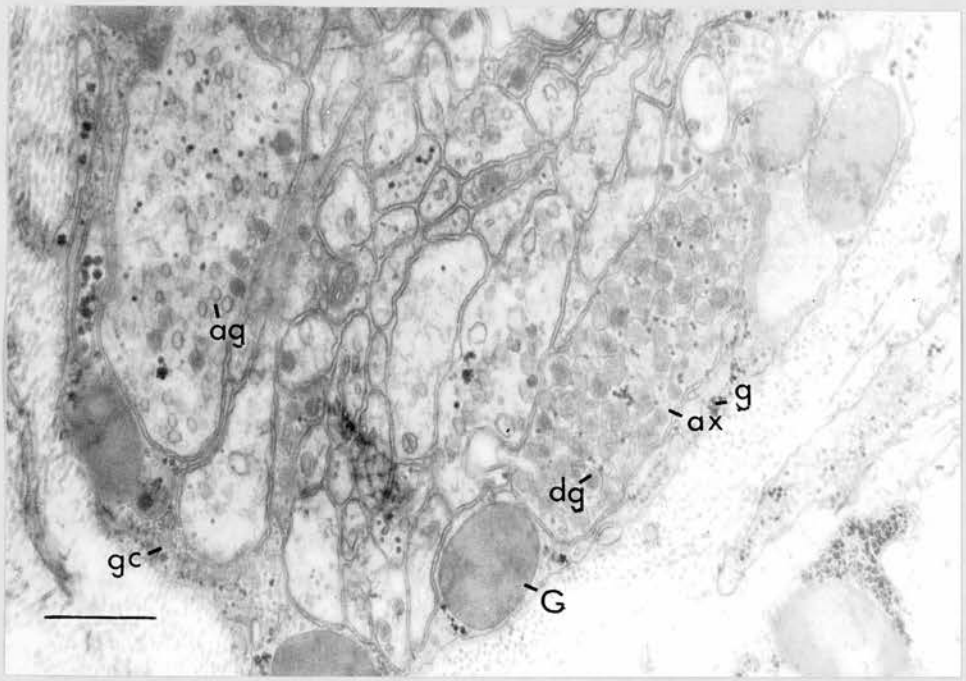


Fig. 37: Part of a large nerve trunk in the area of the pneumostome dorsal to the aperture. ax - axon profile, ag - agranular vesicle, dg - dense granular vesicle, gc - glial cell process, g - small dense granules, G - large dense granule. Fixation: glut/OsO_4; X 27,000 Scale represents 0.5 µ.

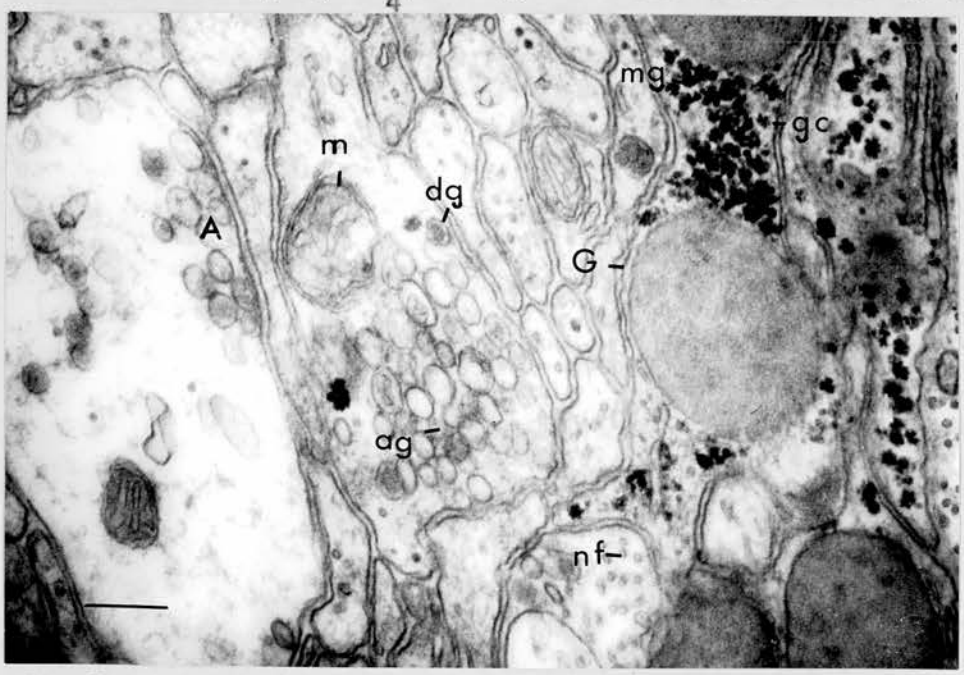


Fig. 38: High power micrograph of axons in large nerve trunk. A may represent a synaptic region. ag - agranular vesicles, dg - dense granular vesicle, G - large dense granule, gc - glial cell process, mg - mulberry granules, m - mitochondrion, nf - neurofilaments. Fixation: glut/OsO_4; X 51,000 Scale represents 0.2 µ.

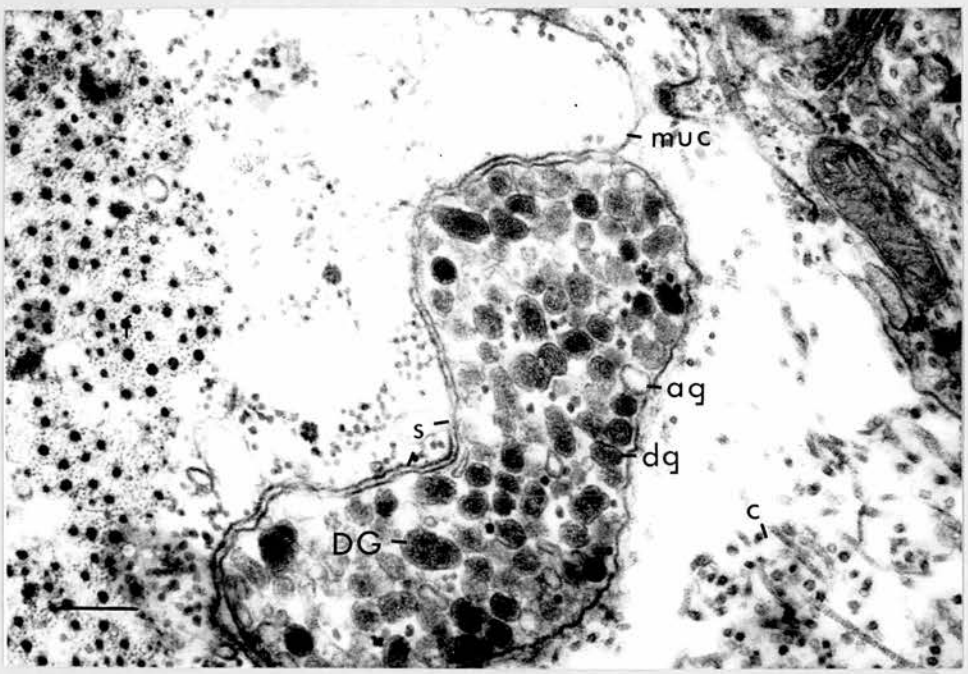


Fig. 39: Type 3 neuromuscular junction. This nerve ending contains three types of vesicles - large dense granular, small dense granular, and agranular. ag - agranular vesicle, c - collagen fibres, dg - small dense granular vesicles, DG - large dense granular vesicle, f - contractile portion of muscle cell, muc - muscle cell, s - synaptic region. Fixation glut/OsO_4; X 51,000 Scale represents 0.2 μ.

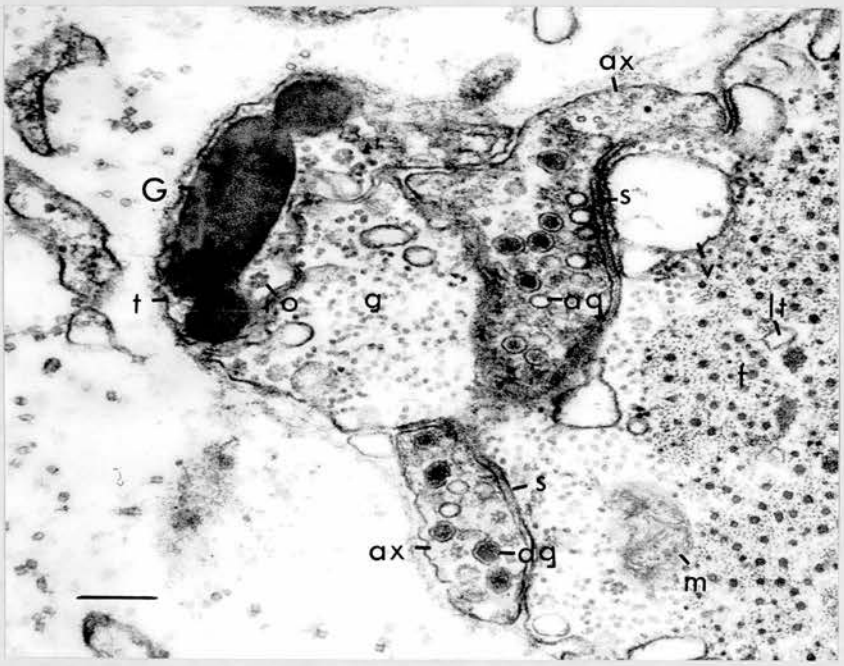


Fig. 40: Protrusion of a muscle cell in close contact with two axons. These two axons form Type 2 neuromuscular junctions with the muscle cell. The protrusion of the muscle cell is also in close contact with a teloglial cell process.
ax - axon profile, ag - agranular vesicle, dg - dense granular vesicle, f - contractile part of muscle cell, G - large dense granule, lt - longitudinally aligned tubule, g - small dense granules, m - mitochondrion, ro - small dense granules arranged in a rosette pattern, s - synaptic region, tg - teloglial cell process, v - vesicular profile.
Fixation: glut/OsO_4; X 44,000 Scale represents 0.2 µ.

Several types of synaptic vesicles have been found in the nerve endings of neuromuscular junctions in the pneumostomal region. They are as follows: (1) clear synaptic vesicles (agranular), 400 to 500 Å in diameter, (2) granular vesicles possessing a moderate electron dense core, 600 to 800 Å in diameter, (3) large granular vesicles possessing a high electron dense granular substance, 1400 to 1800 Å in diameter. The neuromuscular junctions in the pneumostomal region can be classified according to their vesicle populations into three different types:

Type 1: This ending contains only clear synaptic vesicles (Fig. 42)

Type 2: This ending contains a mixed population of agranular and granular vesicles. The granular vesicles are 600 - 800 Å in diameter (Figs. 40, 41).

Type 3: This ending contains a mixed population of agranular, small granular (the same as those in Type 2 endings) and large granular vesicles. The latter have a diameter of 1400 to 1800 Å (Fig. 39).

F. Anal Nerve Examination

It has been established through gross dissection and the examination of serial light microscopy that the anal nerve innervates the pneumostomal region. An examination of cross sections of this nerve using the electron microscope might provide information on the number of sensory and motor components within the nerve. This information would be useful in a broader analysis of the neurophysiological control of pneumostomal muscles involved in the ventilation process.

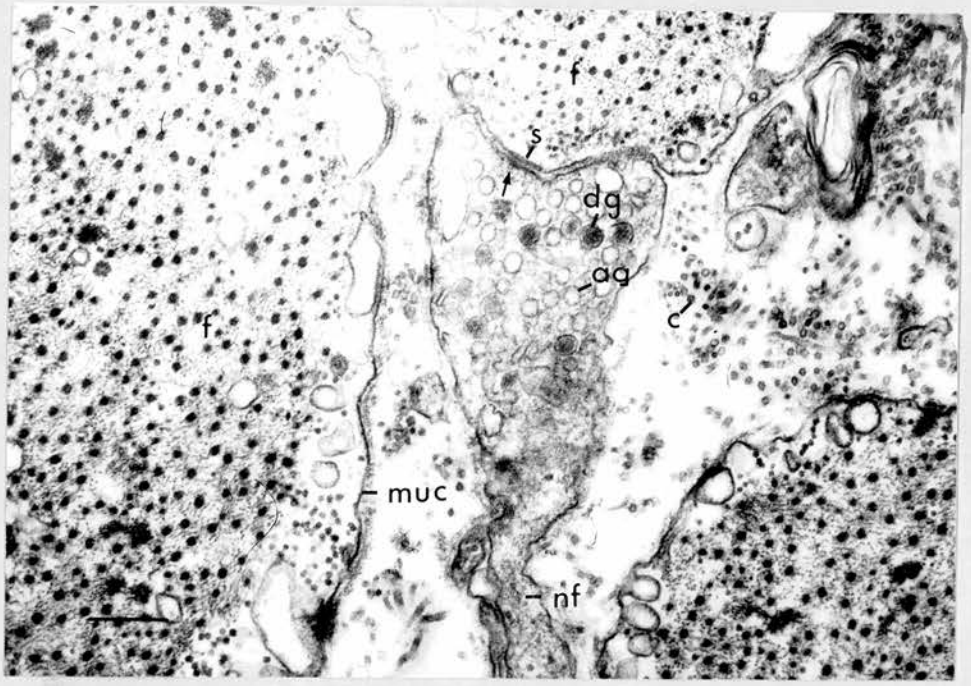


Fig. 41: Neuromuscular junction type 2. The apposing membranes of the axon and muscle cell seem to be slightly thickened (arrow). ag - agranular vesicle, c - collagen fibres, dg - small dense granular vesicle, f - contractile part of muscle cell, muc - muscle cell, nf - neurofilaments, s - synaptic region. Fixation: glut/OsO_4 X 44,000 Scale represents 0.2 µ.

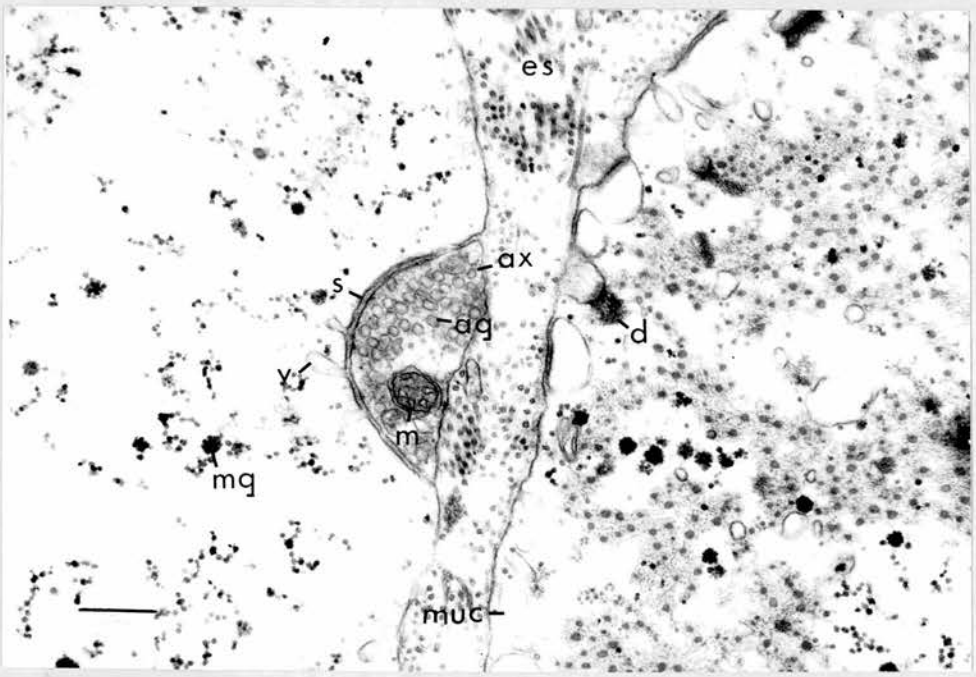


Fig. 42: Neuromuscular junction type 1. This nerve ending contains only agranular vesicles. ax - axon, ag - agranular vesicles, d - dense body, es - extracellular space, m - mitochondrion, mg - mulberry granule, muc - muscle cell, s - synaptic region, v - vesicular profile. Fixation glut/OsO_4 X 32,000 Scale represents 0.3 µ.

Such an examination could be achieved by cutting thin sections further and further out towards the periphery (i.e. epithelium which lines the pneumostome) to see which axons contained within the nerve disappear first. Sections cut at various intervals should be examined to determine the number of axons still present within the nerve.

As the muscle fibres disappear in the sections, the large (or small) nerve fibres may also disappear. These nerve fibres could probably be labelled as motor (i.e. they innervate the muscle fibres of the area). As the extreme periphery is reached, the sections will probably contain no muscle cells but only a small number of axons (which have been traced through all the previous sections) and which could be labelled as sensory axons.

In the present investigation, only the first step in this long and laborious exercise was achieved. The entire anal nerve before it entered the right mantle wall, was sectioned and counts of axons and measurements of axon size were made on a composite electron micrograph of this cross section (see Materials and Methods section). The number of axons in the anal nerve in each of eight categories is as follows:

1. under 0.25 μ^2 - 6171
2. between 0.25 and 0.50 μ^2 - 300
3. between 0.50 and 1.0 μ^2 - 257
4. between 1.0 and 2.0 μ^2 - 253
5. between 2.0 and 5.0 μ^2 - 161
6. between 5.0 and 10.0 μ^2 - 16
7. between 10.0 and 20.0 μ^2 - 4
8. over 20.0 μ^2 - 4

The total number of axons contained within the anal nerve was estimated at 7166. The distribution of axons for the first five

categories throughout the cross section of the nerve is very random. On the other hand, the axons in categories six, seven and eight tend to be concentrated towards the middle of the nerve.

The anal nerve consists of a large core of axons surrounded by a sheath or perineurium. This perineurium (Fig. 43) is composed of scattered muscle cells which run longitudinally along the length of the nerve, and collagen fibres. The nerve is divided by a number of septae which are arranged radially and which project inward from the perineurium. These septae may contain the nuclei of glial cells.

This micrograph (Fig. 43) illustrates very clearly the diversity of axon size in this nerve. The majority of small axons are grouped together in small bundles and these bundles are surrounded by glial cell processes. The larger axons are individually enveloped by several layers of glial cell processes.

d. Electrophysiology

Nine different experiments were carried out in order to provide information regarding various aspects of the overall innervation and operation of the pneumostome. These experiments with their results are outlined in the following sections.

Section 1: Electrical Stimulation of the Anal and Right Pallial Nerves

A. The anal nerve was cut and its distal end sucked into the end of a fine tipped suction electrode. All other nerves were left intact. A chain of square-wave pulses was delivered via the electrode and a stimulus isolation unit to the nerve. The frequency of this pulse was 10/ second, the pulse amplitude was 30 Volts, its pulse width was 10 msec and the duration of the

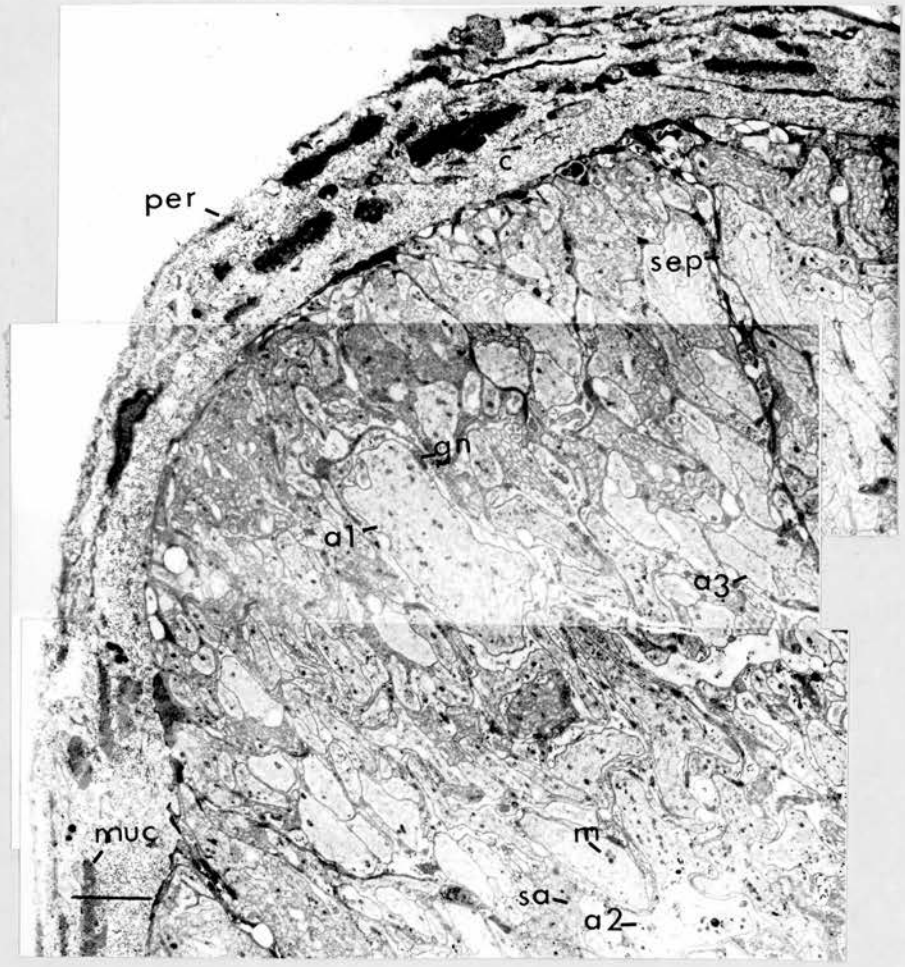


Fig. 43: Composite micrograph of a cross section of the anal nerve.
a_1 - very large axon profile; area measurement - 19.0 u^2
a_2 - axon profile with an area between 5.0 and 10.0 u^2
a_3 - medium-sized axon profile with an area of 3.0 u^2
c - collagen fibres, gn - glial cell nucleus, m - mitochondrion, muc- muscle cell, per - perineurium, sa - very small naked axons with area measurements of less than 0.25 u^2, sep - septa.
Fixation: glut/OsO_4; X 5,300
Scale represents 1.0 μ.

stimulus was three or four seconds. Stimulating the nerve resulted in the opening of the pneumostome. When the preparation no longer received the stimulus, the aperture was seen to close.

B. The distal end of the right pallial nerve was also stimulated using a fine-tipped suction electrode. All other nerves were left intact. The frequency of the pulse used was 10 per second, the pulse amplitude was 35 Volts, the pulse width was 10 msec and the pulse duration was about four seconds. The effect of such stimulation was a strong contraction of the entire right side of the mantle wall. There appeared to be no effect on the pneumostome alone.

Section 2: Electrical Stimulation of the Intestinal Nerve

The intestinal nerve was cut and the proximal end was placed in a suction electrode. All other nerves were left intact. The stimulus was a rectangular pulse with a duration of 1 msec and amplitude of 3 Volts and a frequency of 5.0 per second. It was applied for three or four seconds through the electrode. The pneumostome was observed throughout the operation. Stimulation of the intestinal nerve resulted in the pneumostome opening. The time from the onset of the stimulus to the opening of the pneumostome was estimated to be approximately one second. After it closed, it took a minute or so before a second stimulus was followed by opening of the aperture.

Stimulation of the intestinal nerve had the same effect on the pneumostome as direct stimulation of the distal end of the anal nerve. Both resulted in the pneumostome opening.

Section 3: Recording Motor Activity from the Anal Nerve

The proximal end of the anal nerve was sucked into a fine tipped suction electrode. The anterior aorta and all nerves to the sub-oesophageal ganglion other than the anal nerve were left intact. Activity was recorded from this nerve over a fairly long period in order to determine whether rhythmicity was present.

Rhythmical activity was recorded from the proximal end of the anal nerve. This activity was in the form of a burst of action potentials which repeated itself very irregularly. Because other activity not related to the burst was also recorded by the electrode, it was impossible to determine the exact number of spikes of which the burst is composed, the inter-spike interval, or the exact duration of the burst (see Fig. 44).

Section 4: Recording Nervous Activity from the Sub-Oesophageal Ganglion

Because of the difficulty encountered in analysing the nature of the burst from the anal nerve recordings, an investigation of the dorsal and ventral surfaces of the sub-oesophageal ganglion was done in order to locate this activity without too much interference from other unrelated nerve signals. A very fine tipped suction electrode was used to scan the surface of the ganglion. All nerves leading to the CNS were left intact. The burst was recorded from an area immediately anterior to where the intestinal, anal and cephalic retractor muscle nerves leave the visceral ganglion (Fig. 45).

Figure 46 shows the burst activity over a nine minute period. This recording illustrates that this activity is somewhat irregular.

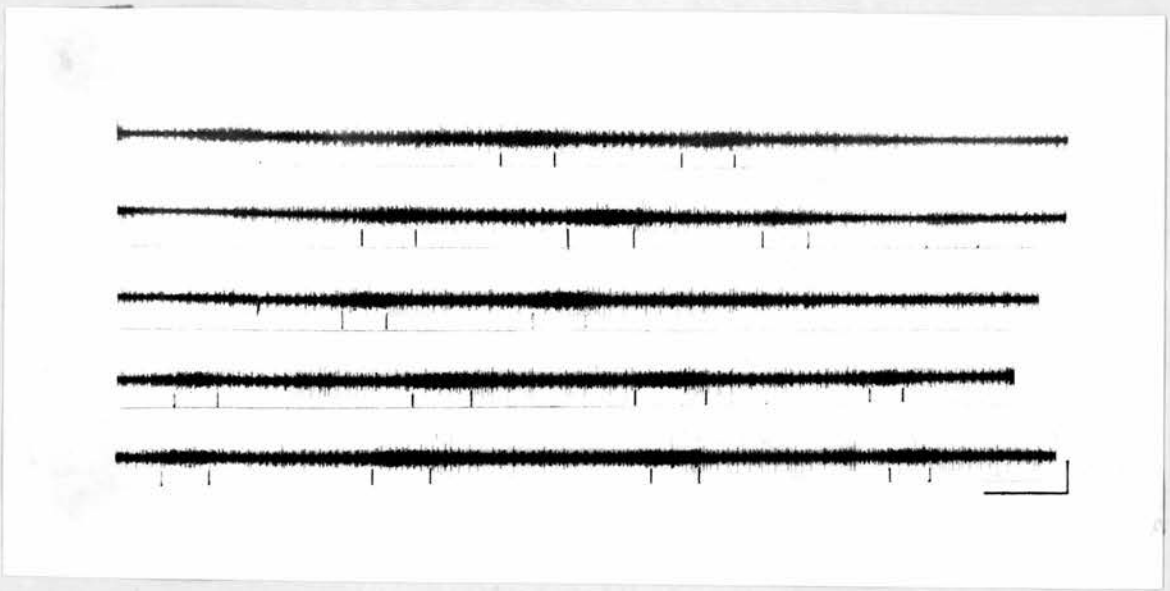


Fig. 44: Continuous extracellular recording of efferent activity in the anal nerve. Suction electrode used. Bars indicate the burst periods. Calibration equals 33 μvolts and 10 seconds.

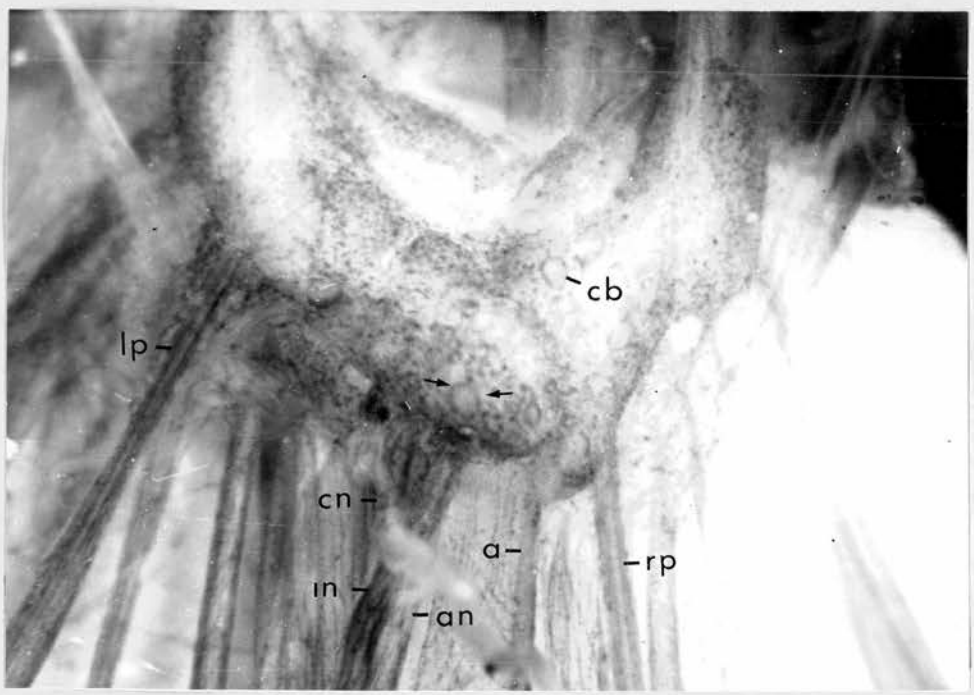


Fig. 45: Sub-oesophageal ganglion. Arrows indicate the area in the visceral ganglion from which the burst is recorded. The outline of several cell bodies can be seen. a - aortic nerve, an - anal nerve, cn - cephalic retractor muscle nerve, cb - cell body, in - intestinal nerve, lp - left pallial nerve, rp - right pallial nerve.

The burst tends to repeat itself every half minute. This result compares very favourably with the anal nerve recording which also shows a burst rate of 2 per minute. Figure 47 shows the burst in greater detail. The unit of which the burst is comprised continues to fire between successive bursts. However, during the inter-burst interval, this unit tends to fire at a much lower frequency (about 1.5 - 2.0 impulses per second) than it does during the burst.

The interval changes of this activity are plotted in Figure 48. A ten-spike moving average was used to construct the slope of the graph. The Y-axis represents the inter-spike interval measured in seconds and the X-axis represents time also measured in seconds. The graph illustrates clearly that the burst initially fires at a low frequency and ends with its highest frequency. There is an increasing inter-spike interval between the bursts which indicates a decreasing frequency.

Section 5: Simultaneous Recordings from the Visceral Ganglion and the Anal Nerve

Having located activity in the visceral ganglion which can be roughly correlated with pneumostomal movements, the following experiment was done in an attempt to relate the burst activity recorded in the anal nerve (see Section 3) with this ganglionic activity. A very fine tipped suction electrode was used to record the activity from the dorsal surface of the visceral ganglion. Activity from the intact anal nerve was recorded en passant using a suction electrode. The anterior aorta and all nerves to the sub-oesophageal ganglion were left intact. A recording was made and the results are shown in Figure 49. The upper trace shows a

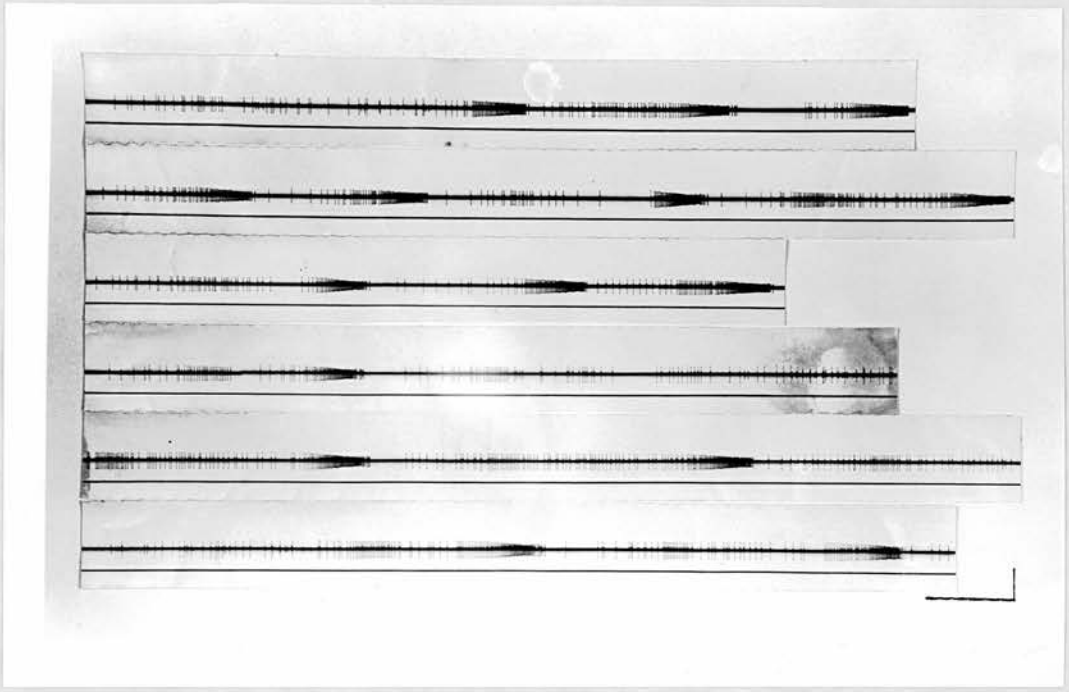


Fig. 46: Continuous extracellular recording of burst activity from the dorsal surface of the visceral ganglion. Suction electrode used. Calibration equals 83 μvolts and 10 seconds.

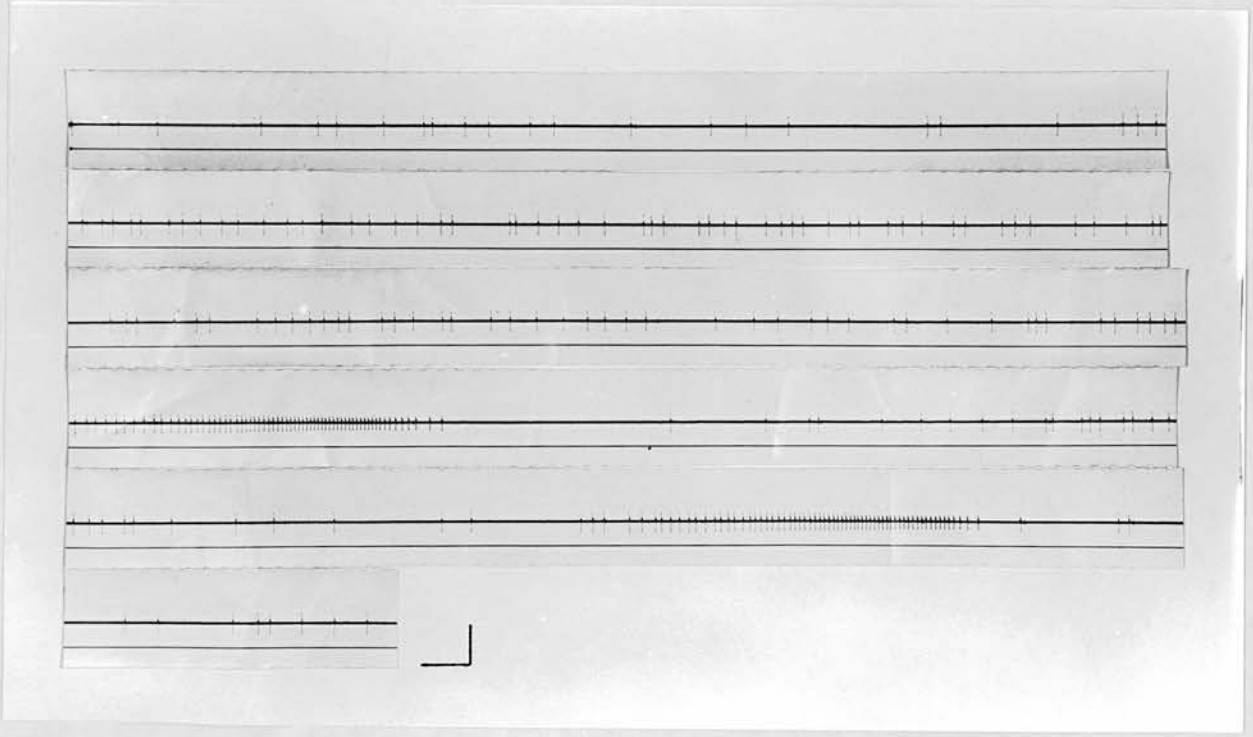


Fig. 47: A more detailed recording of the burst activity from the dorsal surface of the visceral ganglion. Suction electrode used. Calibration equals 83 μvolts and 100 seconds.

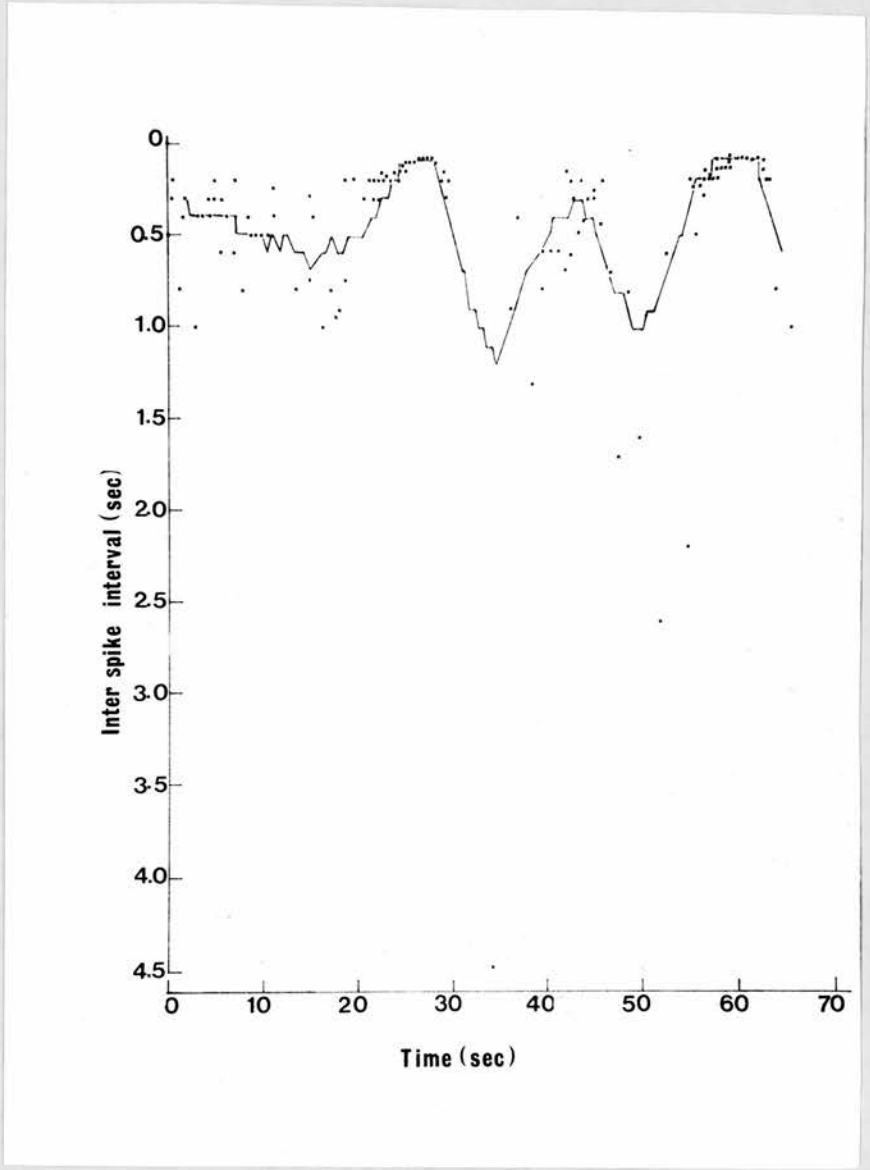


Fig. 48: Plot of inter-spike interval changes for two bursts recorded from the dorsal surface of the visceral ganglion shown in Figure 47.

burst which occurs simultaneously with a burst of activity recorded in the anal nerve (lower trace). This recording suggests that the central neurons which produce this characteristic burst pattern send their axons into the anal nerve.

Section 6: The Recording of Synchronous Units in Burst

The recordings of efferent activity in the anal nerve do not show whether the burst consists of synchronous units. However, several different recordings from the dorsal surface of the visceral ganglion illustrate that the burst activity is composed of more than one unit (Fig. 50). At least two cells in the ganglion are firing in a similar burst pattern.

Other recordings, however, show only one unit firing in a burst pattern (see Fig. 46). In this case, a suction electrode with a much finer tip than those used in the preceding experiments was used to record from the dorsal surface of the ganglion. This finer tipped electrode most probably covered only one of the two nerve cells.

Section 7: Monitoring the Opening of the Pneumostome

In order to determine whether or not the cell or cells which fire in a burst pattern are part of the motor control centre of the pneumostome, it is essential to illustrate some correlation between these burst potentials and the movements of the pneumostome, i.e. opening and closing.

The pneumostome has been observed to open to irregular sizes. During an experiment, it was observed that the characteristic burst only occurred if the pneumostome opened to its full extent. If there

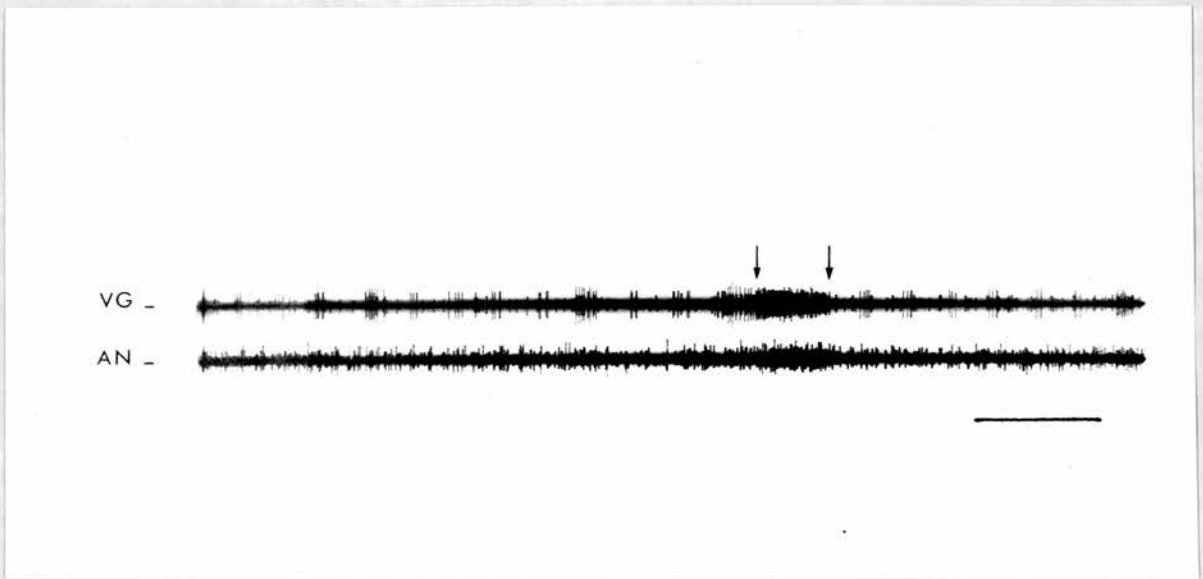


Fig. 49: Simultaneous recording of burst activity from both the dorsal surface of the visceral ganglion and the anal nerve. Arrows indicate period of burst. VG - visceral ganglion (upper trace), AN - anal nerve (lower trace). Suction electrodes used. Calibration equals 10 seconds.

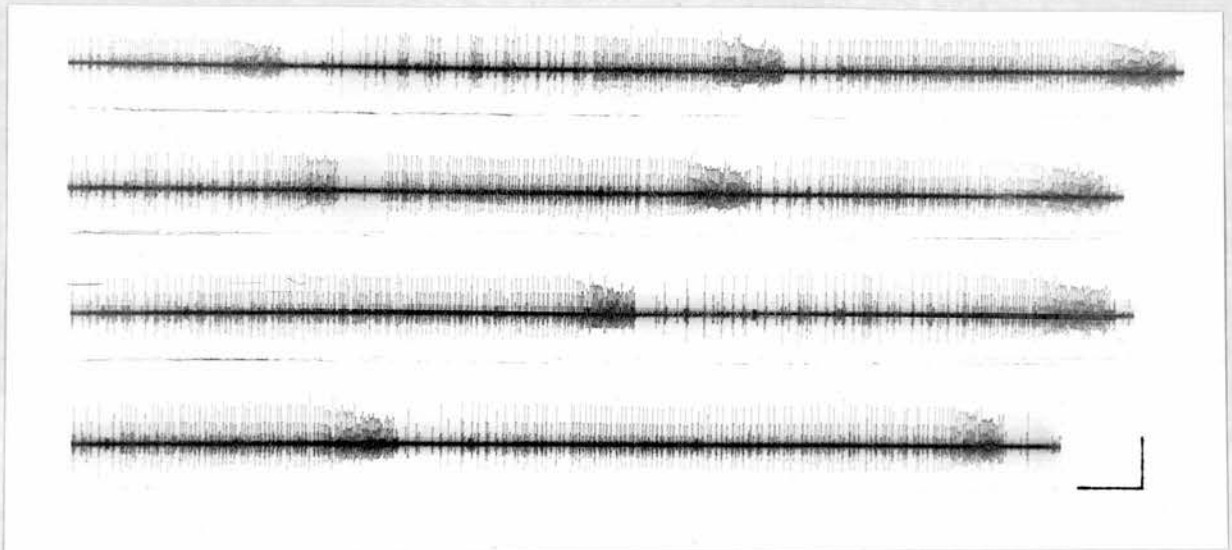


Fig. 50: Continuous extracellular recording of burst activity showing two synchronous units from the dorsal surface of the visceral ganglion. Suction electrode used. Calibration equals 33 μ volts and 5 seconds.

was only a partial opening of the pneumostome, the burst unit did not reach the frequency characteristic of a full opening. There may well be a correlation between the variation in the firing pattern of the units which comprise the burst and the extent to which the pneumostome opens. Clearly if such a correlation could be established, it would contribute significantly to the understanding of how the pneumostome works. It was felt, therefore, that a method of monitoring quantitatively the movement of the pneumostome was desirable.

Several monitoring methods were tried but all proved unsuccessful (see Materials and Methods section for a discussion of the technical problems encountered). Figure 51 is an extracellular recording of nervous activity in the anal nerve on the top trace and monitoring of the movement of the pneumostome by the mirror-photocell method on the bottom trace. This recording clearly indicates that the unit appears to begin to fire before the pneumostome opens. However, it is not at all clear at what stage of the burst the pneumostome opens or to what extent the muscles of the aperture contract.

Figure 52 is an extracellular recording of the burst activity from the dorsal surface of the visceral ganglion on the top trace and monitoring the movement of the pneumostome by the mirror-photodiode method on the bottom trace. Once again, it is difficult to see when the pneumostome opens in relation to the burst. It appears from the recording that the unit fires before the pneumostome opens. This appearance, however, may be due to the inadequacy of the monitoring method. The ideal monitoring system would seem to require a small deflection of the oscilloscope trace downwards when the pneumostome opens and another deflection of the trace in the opposite direction

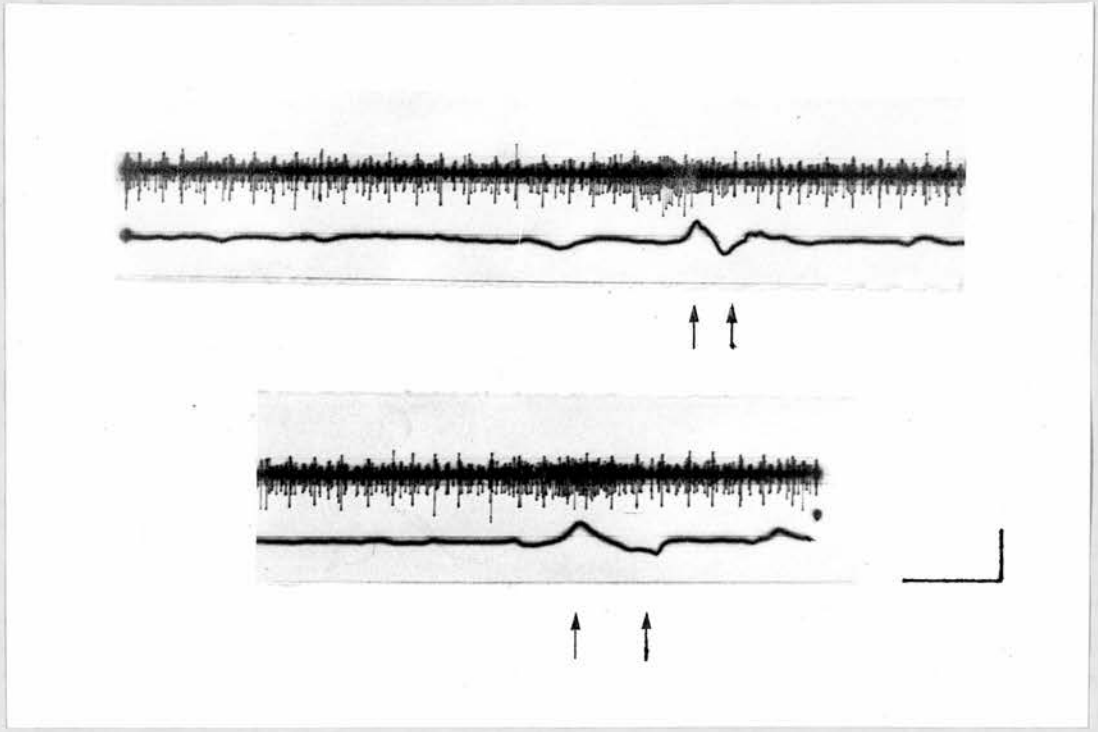


Fig. 51: Extracellular recording of burst in anal nerve on top trace; monitor of pneumostome using photocell on lower trace. Arrows indicate when pneumostome opens and shuts. Hook electrodes used to record nervous activity. Calibration equals 33 μ volts and 5 seconds, and can be applied to the top trace only.

when the pneumostome shuts. Using conventional monitoring techniques, it was impossible to get a neat deflection of the trace during the opening and closing of the pneumostome because of the overall movement of the animal.

The final method tried, that of monitoring the movement visually, gave a fairly good indication of when the pneumostome opens and shuts and how long it remains open. Figure 53 is an extracellular recording of the burst as recorded from the dorsal surface of the visceral ganglion and the monitoring of the pneumostome by the above method. As a method of measuring the pneumostomal movements quantitatively it is unsatisfactory because it does not show to what extent the pneumostome opens or how much the muscles of the aperture contract.

Section 8: Recording from Pneumostomal Muscles

In order to determine that the cells producing the characteristic burst are the motor neurons which directly innervate the muscles of the pneumostome, it is necessary to show that when these cells fire, excitatory junction potentials of a similar frequency can be recorded from the muscle fibres.

An extracellular suction electrode was used to record the burst potentials in the ganglion and a similar electrode was used to record potentials from the circular band of muscle which surrounds the pneumostomal aperture. The tip of the electrode was inserted into a slit made in the pneumostomal epithelium. The insertion of the electrode stimulated the gland cells of the area which resulted in a large secretion of mucous by these cells. This mucous was very effective

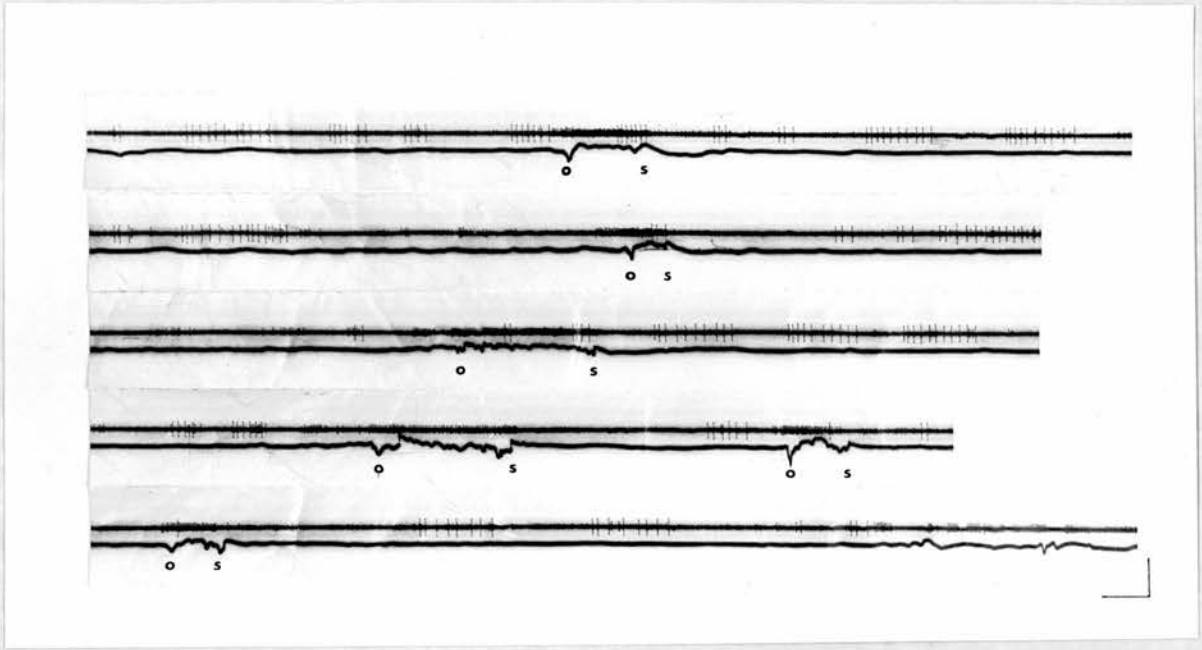


Fig. 52: Upper trace: extracellular recording of burst activity from the dorsal surface of the visceral ganglion. Suction electrode used. Calibration equals 33 μ volts and 5 seconds.
Lower trace: pneumostome monitored using photodiode.
c - open, s - shut.

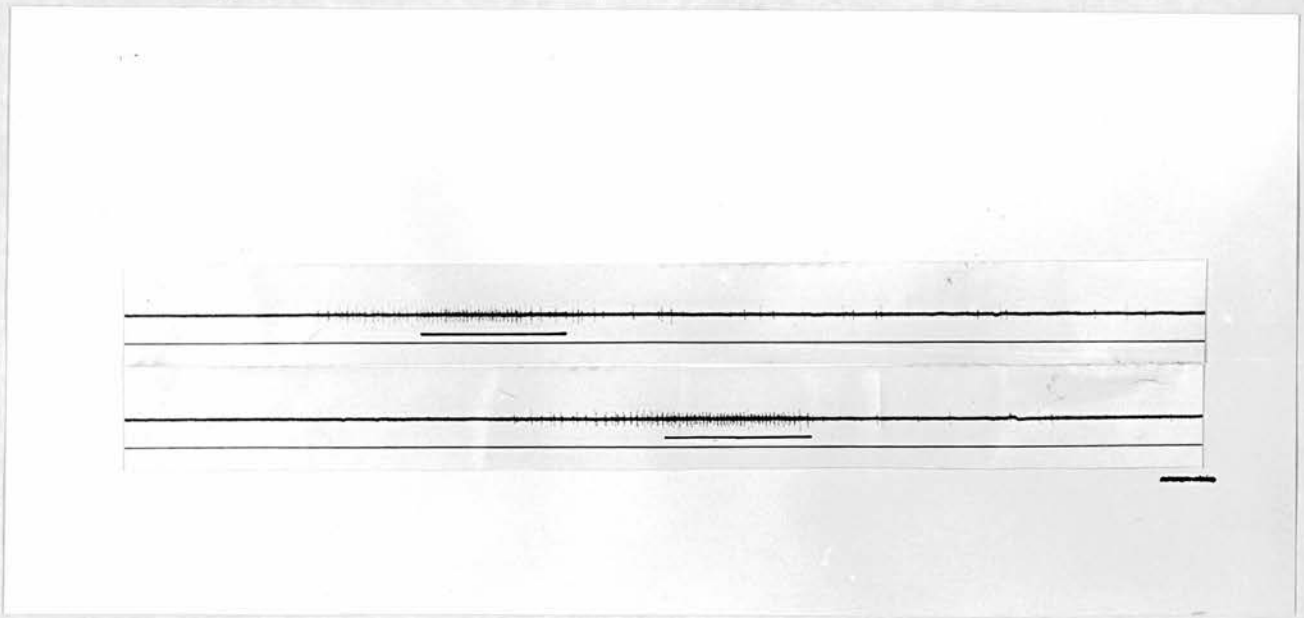


Fig. 53: Upper trace: burst recorded from the dorsal surface of the visceral ganglion. Suction electrode used. Calibration equals 1.0 second.
Lower trace: pneumostome monitored visually. Bar above bottom trace indicates length of time pneumostome remains open.

in blocking the fine tip of the suction electrode. Several attempts were made to record myograms from the muscle but in each case, the tip of the electrode was blocked.

Both light and electron microscopical studies of the area show that the individual muscle fibres are contained within a very compact collagen matrix. They are also surrounded by a large number of different types of secretory cells. The failure of the attempt to record muscle potentials is almost certainly due to a structural problem. Other researchers have been able to record potentials from molluscan muscle (Mellon and Prior, 1970) using extracellular recording techniques but these muscles were not contained within such a compact collagen matrix.

Section 9: Denervation of the Pneumostome

Three denervation experiments were carried out and are outlined below.

1. Complete central denervation:

The animal was dissected to expose the central nervous system and all the nerves running posteriorly from it. The dissected part was flooded with saline. Care was taken to preserve the mantle cavity and to keep it above the level of the saline as the pneumostome failed to open and close when submerged. The opening and closing of the pneumostome was then recorded over a ten minute period. All the nerves leading from the CNS were cut and the pneumostome movements were recorded for a further ten minutes.

When the nerves were left intact, the pneumostome opened 2.0 times per minute. The average length of each opening was approximately three seconds. The amplitude of opening was slightly less than for

animals not dissected. It seems that dissection results in partial deflation of the mantle cavity which alters the shape of the pneumostome slightly. When the open pneumostome was prodded gently, it closed rapidly. For a period of four minutes after complete central denervation, the pneumostome remained closed. It then opened slightly for a second but the amplitude of opening was considerably reduced. Movement continued around the pneumostome but the aperture did not open normally. After a period of ten minutes, the pneumostome remained completely closed. The area contracted when prodded in a manner similar to the mantle wall which has been severed from the CNS. This movement is probably due to a local reflex action. No normal ventilation movements of the pneumostome were observed after complete central denervation.

2. The central nervous system was exposed as in Part 1 of this section and the preparation was flooded with saline. The opening and closing of the pneumostome was then recorded over a ten minute period. The anal nerve was cut and the pneumostome movements were recorded for a further ten minutes.

When all nerves were left intact, the rate of opening was about 4.0 times per minute. After cutting the anal nerve, the pneumostome was observed to open 3.7 times per minute. It seems that the rate of opening is not significantly altered from the normal rate by severing this connection with the CNS. However, the operation does seem to affect the length of time the pneumostome remains open. When the anal nerve is left intact, the pneumostome is open for an average of 18.6 seconds per minute; when the nerve is cut, it is open for an average of 14.2 seconds per minute. The amplitude of opening is also altered

when the anal nerve is cut. Cutting this nerve resulted in a reduction in amplitude by about one-third. This experiment appears to indicate that the pneumostome can continue to operate even when the anal nerve is severed.

3. The central nervous system was exposed as in Part 1 of this section. The opening and closing of the pneumostome was then recorded over a ten minute period. All nerves except the anal were then cut and the pneumostomal movements were recorded for a further ten minutes.

When all nerves were left intact, the rate of opening was about 3.3 times per minute. After cutting all nerves to the CNS except the anal, the pneumostome was observed to open 2.9 times per minute. When all nerves were left intact, the pneumostome remained open 18.6 seconds per minute. Cutting all nerves except the anal reduced this to an average of 6.4 seconds per minute. The amplitude of opening is reduced by about one-third after this operation. The results of this experiment indicate that pneumostomal movements are modulated by sensory information arriving at the CNS through these other nerves.

IV Discussion

This investigation is the first to describe some aspects of the electrophysiology of the pneumostome in land slugs. A description of the gross anatomy, light microscopy and fine structure of the pneumostome is also included. Other workers, such as Nisbet (1961A, 1969B), Simpson (1901) and Jullien et al. (1960) have looked at the innervation of the pneumostome in other land pulmonates and in the case of Jullien and Nisbet, electrophysiological techniques were used.

The pneumostomal area is a highly specialized region of the mantle wall in that it contains an unusually high density of gland cells. Several gland cell types have been distinguished in light microscopy sections. Gland cell type 1, the pneumostomal gland cells, is specific to this region of the animal. Gland cell type 2 is similar to mucous gland cell type A described by Campion (1961) and the large mucous cells described by Barr (1928). Gland cell type 4 is similar to the calcium gland described by Campion (1961). As far as gland cell classification is concerned, it is necessary to use histochemical stains to identify the different types of secretion products. Standard light microscopy stains such as those used in this investigation are not adequate for this.

The pneumostome needs to be well lubricated in order for the cilia to beat and thus produce currents which aid in the flow of gases in and out of the lung (Zylstra, 1972A). Gland cells type 2 are very large and produce a considerable amount of mucous. The mechanism of mucous secretion

may depend on the formation of individual secretion granules (or vacuoles) which separate from the cell, work their way towards the epithelium and eventually release their contents at the surface. However, some of the cells that have been examined seem to reach the exterior via short necks which run between the epithelial cells.

Only gland cell types 1 and 2 have been identified in electron microscopy sections. Zylstra (1972A) found that the pneumostomal gland cells of Lymnaea stagnalis contain a membraneous type of secretion product. He suggests that this particular type of secretion is probably instrumental in sealing off the pneumostome when the snail is underwater. An examination of the ultrastructure of the pneumostomal gland cells of Limax flavus revealed that these cells do not possess this membraneous type of secretion. As the pneumostome of Limax is never submerged, there is no reason why this type of structure should be present within gland cells found in this region.

The arrangement of muscle fibres within the pneumostomal region has been investigated. Some of these muscle fibres appear to be arranged in the form of a sphincter which is distinct from the general body wall musculature and is composed of distinct bundles. The contraction of all the fibres produces an even displacement of the pneumostomal tissue to form a closed aperture. The muscle fibres that are arranged at right angles to the pneumostomal epithelium contract to bring about an opening of the pneumostome.

These muscle fibres which are involved in the movement of the pneumostome have all the characteristics of smooth muscle cells as described by Heyer et al. (1973). There is no clear organization of thick and thin myofilaments which form the contractile part of the cell.

The dense bodies are not arranged in any particular pattern and there is no high degree of vesicular specialization. In some muscles such as the fast adductor of Pecten, the dense bodies are arranged in rather precise rows perpendicular to the long axis of the fibre (Heyer et al., 1973). These muscles also have a highly ordered myofilament structure and a fairly extensive vesicular system. An examination of the ultrastructure of muscle cells found in the pneumostome indicates that a slow muscle system is probably involved in the opening and closing of the aperture.

Neuromuscular junctions found in the pneumostomal area have the following characteristics: narrow synaptic cleft, 200 Å wide, presynaptic structure containing different types of vesicles and a relatively unspecialized post-synaptic membrane. In one case, there appeared to be a slight thickening of the apposing membranes.

More than one axon has been seen to make contact with a single muscle cell. Rogers (1968) found a number of axons simultaneously forming close contacts with a large bulbous protrusion of a muscle cell in the optic tentacles of Limax flavus.

Different types of neuromuscular junctions may be tentatively classified according to the vesicle population of these endings. Three types of neuromuscular junctions have been found in the pneumostomal tissue of Limax flavus. Barrantes (1970) has postulated the existence of two different types of neuromuscular junctions in the slug, Vaginula soleiformis, based on two distinct populations of synaptic vesicles. It may be that different synaptic populations may represent the presence of different types of transmitters.

However, vesicles have not been used to classify neuromuscular junctions because of a lack of data relating the morphology of the endings to transmitter identity or synaptic function (Heyer et al., 1973).

An examination of the ultrastructure of the anal nerve has shown that this nerve is composed of over 7000 individual axons varying in area from less than 0.25 μ^2 to over 20.0 μ^2. The central portion of the nerve contains four very large axons with areas exceeding 20.0 μ^2. These are most probably the axons of motor neurons. From this initial study, it would appear that an attempt to trace the paths of axons in the anal nerve to the periphery would be very difficult because of the large number of axons involved.

Gross dissection has revealed that the anal nerve definitely innervates the pneumostomal tissue. However, it was not possible to distinguish the details of branching of this nerve within the pneumostome because of the failure of methylene blue staining. The only other nerve from the CNS that could possibly innervate the area as well is the right pallial nerve. Because of the failure of methylene blue staining, it was impossible to follow branch 3 (see Fig. 3B) of this nerve to see if it enters the pneumostomal tissue.

Electrical stimulation of the distal cut end of the anal nerve caused the pneumostome to open. On the other hand, electrical stimulation of the distal cut end of the right pallial nerve did not have this effect. Only the muscles of the right mantle wall contracted in response to the stimulus. This finding does not agree with the results of Jullien et al. (1960) who examined the innervation of the pneumostome in the land snail, Helix pomatia. They found that both the right pallial and anal nerves produce contraction of the pneumostome and that

stimulation of the distal cut end of the right pallial nerve has an effect on the pneumostome. Jullien's account does not indicate, however, if the anal nerve was left intact while the right pallial nerve was stimulated or vice versa. Hence, these results must be regarded with suspicion.

It would appear from these initial experiments that axons in the anal nerve innervate the muscles surrounding the pneumostomal aperture either directly or indirectly and that the right pallial nerve is not involved in the initiation of pneumostomal movements.

The experiments involving the recording of activity from the central nervous system of Limax flavus show that there is a relationship between the firing of certain units in the visceral ganglion and the movements of the pneumostome. This patterned activity is not regular. There is considerable variability in the number of action potentials per burst and in the duration of the interburst interval. Kater and Kaneko (1972) found that for the cell PAL-B in the left parietal ganglion of Helisoma trivolvis, the duration of the interburst interval (first spike of burst to first spike of following burst) is a function of the preceding burst's size, i.e. number of spikes in burst. This relationship has not been established for the burst activity recorded from the visceral ganglion of Limax flavus.

The pattern of action potentials which comprise the burst is unusual. The inter-spike interval remains constant for the first few spikes; then there is a sequential decrease in the inter-spike interval until the end of the burst. Accompanying this decrease in the inter-spike interval, there is a corresponding decrease in spike amplitude. The largest amplitude is recorded at the onset of the

burst and the smallest at the end of the burst. This decrease in spike amplitude within the duration of the burst is characteristic of the bursting pattern of cell R 15 which is found on the dorsal surface of the abdominal ganglion of Aplysia (Frazier et al., 1967). It would be interesting to know the characteristic action potential durations of each of these spikes within the burst but this could only be determined by using intracellular recording techniques.

The unit or units which comprise the burst continue to fire between successive bursts. Pneumostomal movements appear to be sensitive to the discharge rate of these units. It has been observed that the full burst only occurs if the pneumostome opens to its full extent. The firing rate did not reach its maximum (which is about 13 impulses/sec) when there was only a partial opening of the pneumostome. It appears that there is some correlation between the variation in the firing rate of the units which comprise the burst and the extent to which the pneumostome opens. Unfortunately, it was impossible to find an adequate method of monitoring pneumostomal movements, i.e. one which showed if the pneumostome opened to its full extent and when the pneumostome opened in relation to the initiation of the burst. Hence it was impossible to establish the existence of such a correlation.

It may be that this burst activity is endogenous in nature since its firing pattern is similar in some respects to that of PAL-B (Kater and Kaneko, 1972) and R15 (Frazier et al., 1967). Kater and Kaneko demonstrated the endogenous nature of the patterned activity in PAL-B in the following manner. Artificially imposed depolarization of the

soma gave rise to increased action potentials in one burst, thus delaying the succeeding burst. On the other hand, hyperpolarization deleted spikes from the burst and the interval between that burst and the succeeding burst was decreased. Since this artificially imposed polarization had a significant effect on the subsequent firing pattern of the neuron, Kater and Kaneko concluded that this patterned activity was endogenous and not dependent on synaptic input. However, they did find that the variability in the pattern can be attributed to synaptic modulation of sensory origin. It would be necessary in a wider investigation of the nervous control of pneumostomal movements to establish whether the burst activity recorded from the visceral ganglion is endogenous. This could be determined by using the above method.

It would appear from recordings where monitoring methods were used, that the cell or cells producing this unusual burst pattern are involved in the control of pneumostomal movements. It has also been established that burst activity in the anal nerve is related to burst activity recorded from the visceral ganglion. However, it is not known whether the neurons innervate the muscles of the pneumostome directly or via a peripheral plexus. Such a nerve plexus has not been positively identified in light microscopy sections, although numerous nerve processes, some with accompanying cell bodies, were found in some of the sections. It is highly likely that such a plexus exists. Laryea (1970) states that he found such a plexus in the region of the pneumostome and anus of the field slug, Agriolimax reticulatus.

It may be that the central pathways converge on peripheral ones in the pneumostomal tissue. In order to determine whether these central neurons innervate the muscles directly, it is necessary to record from the appropriate cells using intracellular recording techniques

and at the same time, from the muscles they innervate. One would have to show that action potentials recorded from the cell body produced excitatory junctional potentials in the muscle fibres. This was attempted in the present investigation but technical problems prevented the recording of myograms from the pneumostomal muscles.

The analysis of the neuronal control of pneumostomal movements in land slugs requires an understanding of the relative contributions of the central nervous system and the peripheral nerve plexus. Recent research on the neural control of certain behavioural responses in Aplysia has shown that the peripheral plexus plays an important part in the mediation of such responses. Peretz (1970) found that habituation and dishabituation of gill movements continues in a preparation from which the entire central nervous system has been removed. Kupfermann et al. (1971) showed that peripheral pathways are necessary and sufficient for the pinnule response in Aplysia.

Extracellular recordings from the visceral ganglion and anal nerve suggest that central neurons are involved in the control of pneumostomal movements. Denervation experiments were undertaken in an attempt to establish the contribution of the peripheral system in the control of these movements.

Complete central denervation of the pneumostomal area resulted in the cessation of normal activity of the pneumostome, i.e. the pneumostome did not open and close after this operation. The conclusion that can be drawn from this is that central pathways are necessary for the pneumostome to operate normally.

Another denervation experiment involved the cutting of all nerves

to the CNS except the anal nerve. This operation resulted in a reduction in the amplitude and length of time the pneumostome remained open but a normal rate of opening was maintained. The conclusion to be drawn here is that movement is modulated by sensory input arriving at the CNS through these other nerves.

The final denervation experiment involved the cutting of the anal nerve only. The pneumostome continued to open at a normal rate but various parameters such as amplitude and length of opening were reduced. This result was completely unexpected because it had been assumed from extracellular recordings from the visceral ganglion and anal nerve, that the latter contains processes of central neurons which innervate the muscles of the pneumostome.

The results of extracellular recordings show that central neurons in the visceral ganglion are in some way involved in the control of movements of the pneumostome. It may be that these neurons which fire in the characteristic burst pattern represent the overall control centre for the entire respiratory process, i.e. coordinating the movements of the mantle wall, mantle floor and pneumostome. A simultaneous recording of the burst activity from the visceral ganglion and anal nerve would indicate that these bursting neurons have an effect on the pneumostomal muscles through the anal nerve. However, the results of cutting the anal nerve indicate that this nerve does not contain the final motor pathway for the pneumostomal muscles. This experiment also shows that the pneumostome can continue to open and close at a normal rate after the anal nerve has been cut, although certain parameters such as amplitude and the length of time the pneumostome remains open, are affected. From this it can be concluded that pneumostomal movements are not wholly dependent on a connection with the CNS through the anal nerve.

When both the anal and right pallial nerves were cut (complete central denervation), a cessation of normal activity occurred. This indicates that peripheral pathways alone cannot mediate the movements of the pneumostome and also that the right pallial nerve must play a necessary role in the control of these movements. The results of electrically stimulating the distal cut end of this nerve would seem to indicate a lack of involvement of this nerve in pneumostomal control. Further work is needed to elucidate the role of this nerve in the control of pneumostomal movements. For a start, it would be advantageous to cut the right pallial nerve to see what effect this operation has on the movement of the pneumostome.

Further work is also needed to show whether the activity recorded from the CNS is endogenous. This would involve the use of intracellular recording techniques. Experiments should be done to see if the burst pattern is modulated by sensory inputs. It has been shown that electrical stimulation of the proximal end of the intestinal nerve induced the pneumostome to open. The effect of such stimulation on the burst pattern should be elucidated. Also the nature of the stimulant or stimulants which trigger the pneumostome to open and the sites at which they act should be examined.

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