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The Morphology of Neurosecretory Neurones in the Pond Snail, Lymnaea stagnalis, by the Injection of Procion Yellow and Horseradish Peroxidase

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THE MORPHOLOGY OF NEUROSECRETORY NEURONES IN THE POND SNAIL, *LYMNAEA STAGNALIS*, BY THE INJECTION OF PROCION YELLOW AND HORSERADISH PEROXIDASE

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[Plates 1–4]

CONTENTS

The morphology of neurosecretory neurones, the Dark Green Cells, Yellow Cells, Yellow-green Cells, Light Green Cells, Caudodorsal Cells and Canopy Cells, in the central nervous system of the snail, *Lymnaea stagnalis*, was investigated by the intracellular injection of Procion Yellow and, for the Yellow Cells only, of horseradish peroxidase.

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The cerebral ganglia neurosecretory cells (Light Green Cells, Caudodorsal Cells and Canopy Cells) had discrete neurohaemal organs and their axons projected exclusively to nerves and connectives close to the central nervous system. The Light Green Cells had single, undividing axons, which projected exclusively to the ipsilateral median lip nerve. Hormone release is thought to take place principally from the lateral edges of axons, at various points along their lengths, within the median lip nerve. The Caudodorsal Cells projected to the cerebral commissure, where their axons often branched before terminating at the edge of the neuropil. The degree of axonal branching and the location of the Caudodorsal Cell terminals varied widely in different cells. Axon terminals penetrated the perineurium and travelled for several hundred micrometres within the connective tissue sheath of the cerebral commissure. Again, release of neurosecretory material at various points along their lengths seems likely. The Canopy Cells (a pair of individually identifiable giant cells) had a single axon, which projected to the contralateral cerebral ganglion via the cerebral commissure. Axons of left and right Canopy Cells were closely apposed in the cerebral commissure and this is the likely site of the electrotonic junction known to connect them. Neurohaemal organs for the Caudodorsal Cells are the ipsilateral lateral lobe, cerebral commissure and contralateral median lip nerve.

Neurosecretory neurones whose cell bodies were located in the pleural, parietal and visceral ganglia (Yellow Cells, Yellow-green Cells and Dark Green Cells) had extensive non-localized neurohaemal areas in the connective tissue sheath surrounding the central ganglia as well as peripheral nerve projections. The Yellow Cells had one or two axons, which, in neurones located in the visceral and right parietal ganglia, projected extraganglionically to the central sheath or to the intestinal and internal right parietal nerves. These nerve projections are appropriate for the innervation of the kidney, the peripheral target organ of the Yellow Cells. Yellow Cells, located in the pleural ganglia, only had axonal projections to the central sheath. Yellow Cells and Yellow-green Cells had well developed dendritic branching terminating in the central neuropil. Yellow-green Cells project mainly to the anal and external right parietal nerves. Pleural ganglia Dark Green Cells had a few terminals located beneath the perineurium of the pleural ganglia but most of their axonal projections were to peripheral nerves. All Dark Green Cells projected to the ipsilateral pedal ganglion and then to pedal nerves. In addition, some pleural Dark Green Cells had further projections to the internal and external right parietal nerves and median lip nerve of the cerebral ganglion. The widespread distribution of Dark Green Cell axons was consistent with their supposed role in regulating ion and water transport across the skin of the foot and mantle. The electrotonic junctions known to connect Dark Green Cells whose cell bodies are close together on the pleural ganglion surface are located in the pleural ganglion, pleuro-pedal connective and pedal ganglion.

Introduction

Joosse (1964) and Wendelaar Bonga (1970*a*) identified a number of types of neurosecretory neurone in the central nervous system (c.n.s.) of the pulmonate snail, *Lymnaea stagnalis*. Particularly useful for identification were the colour reactions of these cells to the neurosecretory stain Alcian Blue–Alcian Yellow (Wendelaar Bonga 1970*a*), but other differences, such as the diameters of elementary neurosecretory granules and the locations of neurohaemal organs, were also found. There is still debate about the precise function of some of these cell types but there is good evidence that the original histological work did delimit distinctive types of neurones with different endocrine functions (reviewed by Boer & Joosse (1975)). In the present study we have carried out intracellular dye injection of six cell types previously investigated by histological techniques, to characterize further *Lymnaea* neurosecretory neurones on the basis of dendritic and axonal architecture. We have examined the morphology of the Dark Green

Cells, Yellow Cells and Yellow-green Cells, for which there is evidence for a role in ion and water regulation (Lever *et al.* 1961; Wendelaar Bonga 1971*a*, 1972; Roubos 1973; Roubos & Moorer van Delft 1976) although the evidence for the Yellow-green Cells is rather preliminary (Soffe *et al.* 1978), the Light Green Cells, which are involved in growth regulation (Geraerts 1976), the Caudodorsal Cells, which cause ovulation (Geraerts & Bohlken 1976), and the Canopy Cells, for which no function is yet certain (Boer & Joosse 1975) although earlier work had suggested that they might also be involved in osmotic regulation (Lever & Joosse 1961).

Two types of anatomical techniques have previously been used to examine the neural geometry of neurosecretory neurones in *Lymnaea*. The first involved light microscopic tracing of neural processes stained with neurosecretory stains (Joosse 1964; Swindale & Benjamin 1976*a*). The second noted that the diameter of elementary neurosecretory granules was characteristic of each cell type and examined the central ganglia and nerves, with the electron microscope, for neurohaemal areas containing granules of similar types to those found in the cell bodies (Wendelaar Bonga 1970*a*; Boer *et al.* 1968). Both these methods have potential disadvantages that were overcome by the techniques of intracellular dye injection used in the present study. Tracing of cells by means of neurosecretory stains relies on the ability to discriminate colour differences at some distances from the cell body, which may be difficult in locations where mixed colour types occur (Swindale & Benjamin 1976*a*), and also assumes that all processes contain stainable material. Electron microscopy of potential neurohaemal organs is laborious in molluscs because of the non-specialized nature of the release sites and depends on the assumption that the diameter of elementary neurosecretory granules does not change as they travel from the cell body to neurohaemal areas. The problems of both these methods were overcome by the intracellular injection of substances that diffuse or are transported to different parts of the cell. In this instance the processes of the cell can be traced directly, in a continuous manner, so that the detailed morphology of individual neurones can be obtained, although only a few cells can be examined in a given preparation. One important problem with *in vivo* intracellular dye injection of neurones is to be sure that neurones impaled with intracellular microelectrodes are the same as those previously identified by histological methods. This was solved by checking the colour reaction of Procion Yellow-filled cells to neurosecretory stains in histological sections after reconstruction of their neural geometry. Other characteristics, such as cell body location, colour and electrical properties, were also used to identify neurosecretory neurones *in vivo*, and the electrophysiological work forms a parallel study to the present anatomical one (Benjamin & Swindale 1975; Benjamin *et al.* 1976; Benjamin 1978).

Our results show that each of the six cell types examined have distinctive dendritic and axonal geometry, which further supports the idea that they are functionally distinct. Many of the neurones have dendritic branching in the central ganglion neuropil, which could represent the site of pre- or postsynaptic release of synaptic or non-synaptic material. Points of close contact occur between cells that are known to be electrotonically coupled. The results also confirm the likely release sites of the neurosecretory neurones in the connective tissue sheath of the central ganglia and peripheral nerve roots and show the peripheral axonal projections of the Yellow Cells and Dark Green Cells, although not their peripheral innervations (but see Swindale & Benjamin (1976*a*, *b*)). The axonal projections and likely neurohaemal organs of the Canopy Cells are revealed for the first time.

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Materials and methods

Adult specimens of *Lymnaea stagnalis* in the 2–6 g mass range were either bred in the laboratory or commercially supplied. They were kept in Brighton tapwater and fed on lettuce. Intracellular recordings were carried out on isolated brains maintained in freshly collected snail blood, phosphate-buffered saline (Winlow & Benjamin 1976) or Hepes-buffered saline (Benjamin 1978). Brains were treated for short periods with a non-specific, fungal protease (Sigma, Type V) to aid penetration of neurones with microelectrodes. Microelectrodes were fibre-filled with 60 g/l aqueous Procion Yellow M-4RS or 4 % horseradish peroxidase (HRP; Miles) made up to 0.2 % K_2SO_4. Electrophysiological recording equipment was conventional. Procion Yellow and HRP were introduced into recorded neurones by iontophoresis. Procion Yellow-injected brains were left in snail saline overnight at 4 °C and processed as described in Benjamin *et al.* (1979). Iontophoresis of HRP was carried out with 500 ms depolarizing pulses of 5–20 nA at 1 Hz for up to 30 min. Brains were left for up to 2 days in Hepes-buffered saline at 4 °C. HRP-injected material was visualized in whole mounts by means of diaminobenzidene, by a method similar to that of Lavail & Lavail (1974). Tissues from both techniques were wax-sectioned at either 10 or 15 μm. Neurones were reconstructed from brains, usually sectioned in the horizontal plane, by means of a *camera lucida*. Photographs of the best material were taken with Gaf 500 colour film or Kodak 2475 black and white film. The best impression of injected material is obtained from drawings of reconstructed material that were produced by methods used previously (Benjamin *et al.* 1979), but examples of photographed material are given in plates 3 and 4.

Identification of neurosecretory neurones

After reconstruction, may of the Procion Yellow-filled cells were stained with neurosecretory stains to confirm their identity. The Alcian Blue–Alcian Yellow technique was used to identify the Yellow Cells, Yellow-green Cells, Dark Green Cells and Light Green Cells, and phloxin to identify the Caudodorsal Cells, which do not stain with Alcian stains but are phloxinophilic. The use of Alcian Blue–Alcian Yellow to identify *Lymnaea* neurones has been discussed in a previous paper (Swindale & Benjamin 1976*a*) and we have followed the same procedure in the present experiments.

Description of plate 1

Figure 1. Reconstruction, from the dorsal view, of a *Lymnaea* c.n.s., showing the distribution of all the Alcian Blue–Alcian Yellow-positive cells from a single preparation. The phloxinophilic Caudodorsal Cells were reconstructed from a second c.n.s. and added to the reconstruction of the Alcian Blue–Alcian Yellow-stained material, to show the distribution of all six neurosecretory cell types investigated. The pedal ganglia were omitted. In some brains Dark Green Cells occur in the left parietal ganglia and a few Yellow or Yellow-green Cells in the right pleural ganglion. The arrow shows the view of the cerebral of the cerebral ganglia used in the drawings shown in figures 37–42.

Description of plate 2

Figure 2. Photographs of a perspex model of the *Lymnaea* c.n.s., showing nine ganglia and the nerves originating from them. (*a*) Dorsal view, showing the nerves originating from the visceral, left and right parietal ganglia and the dorsal lobes of the cerebral ganglia. (*b*) Ventral view, showing the nerves originating from the pedal ganglia and ventral lobes of the cerebral ganglia. Also shown in (*a*) are the Laterodorsal and Mediodorsal bodies, endocrine structures of the cerebral ganglia. Abbreviations in this and subsequent figures are given in the key at the end of the text.

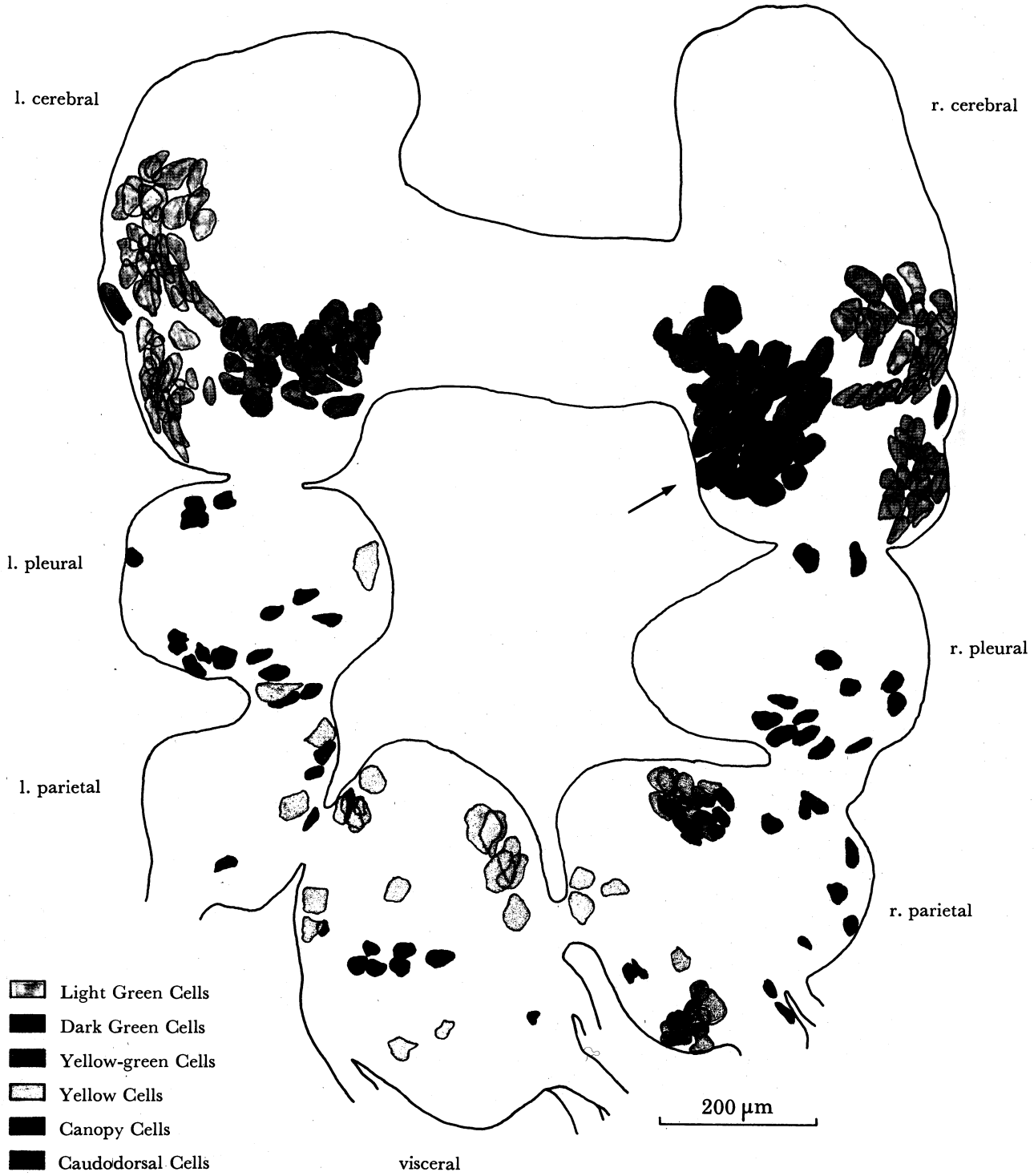

FIGURE 1

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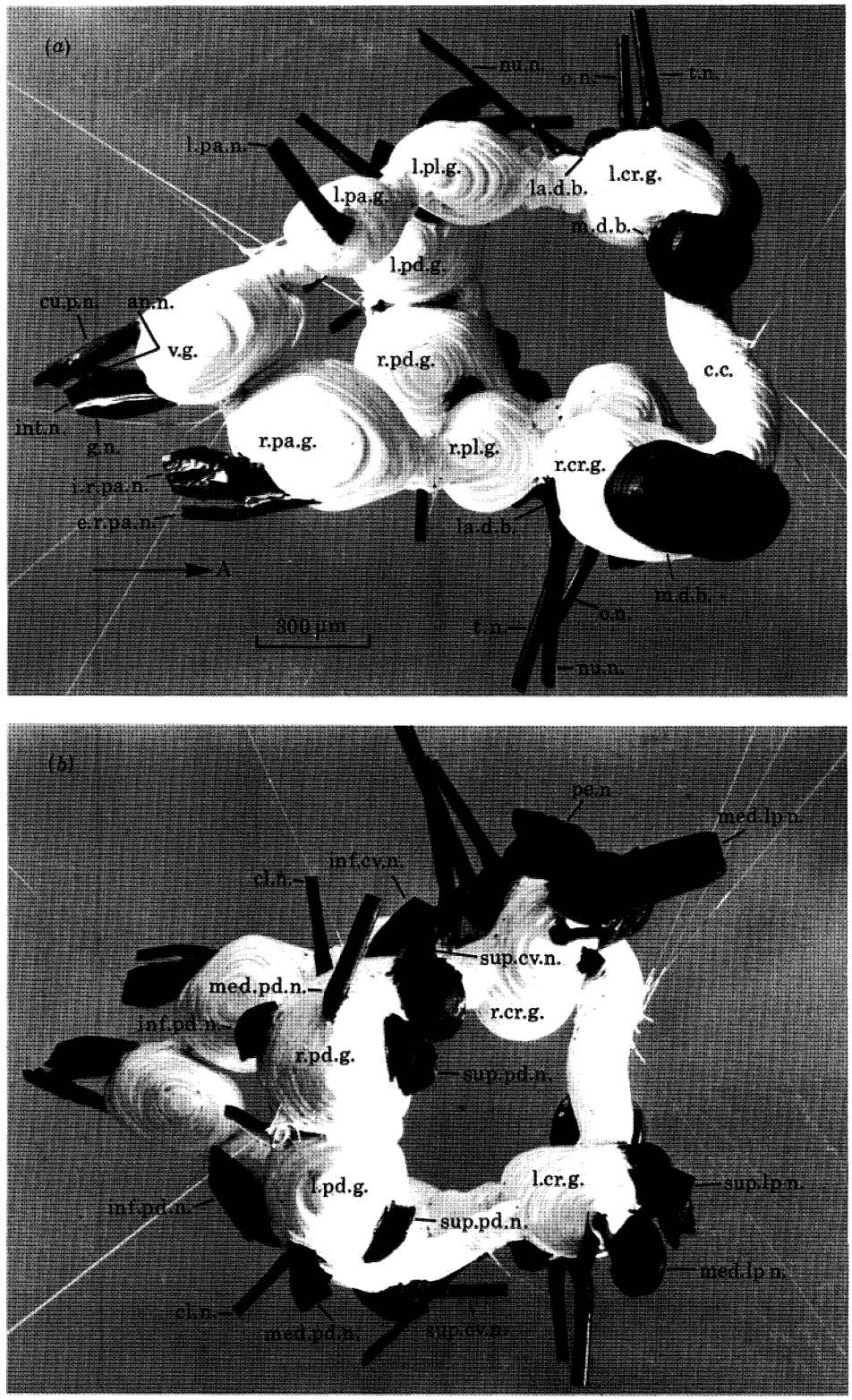
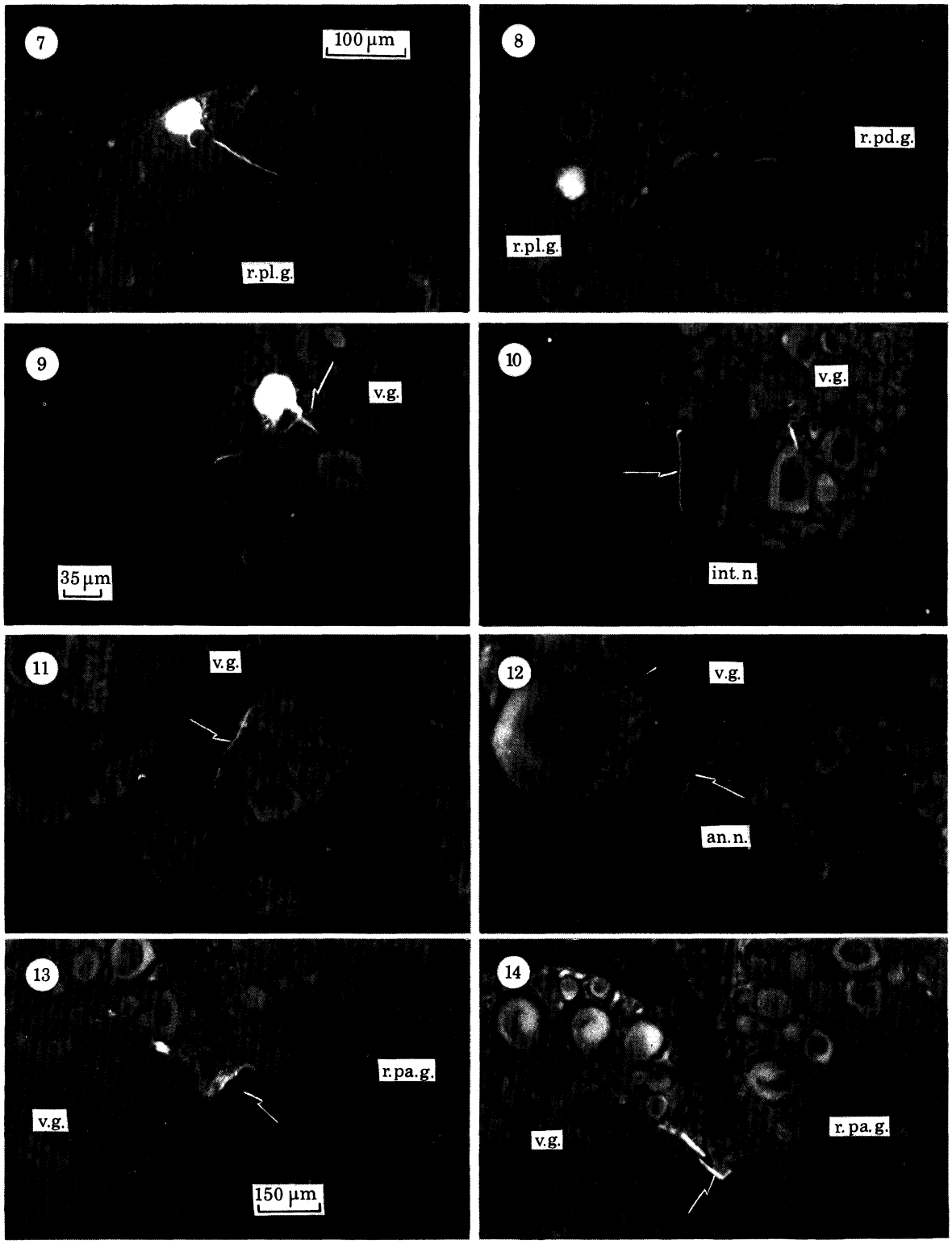


FIGURE 2



FIGURES 7–14

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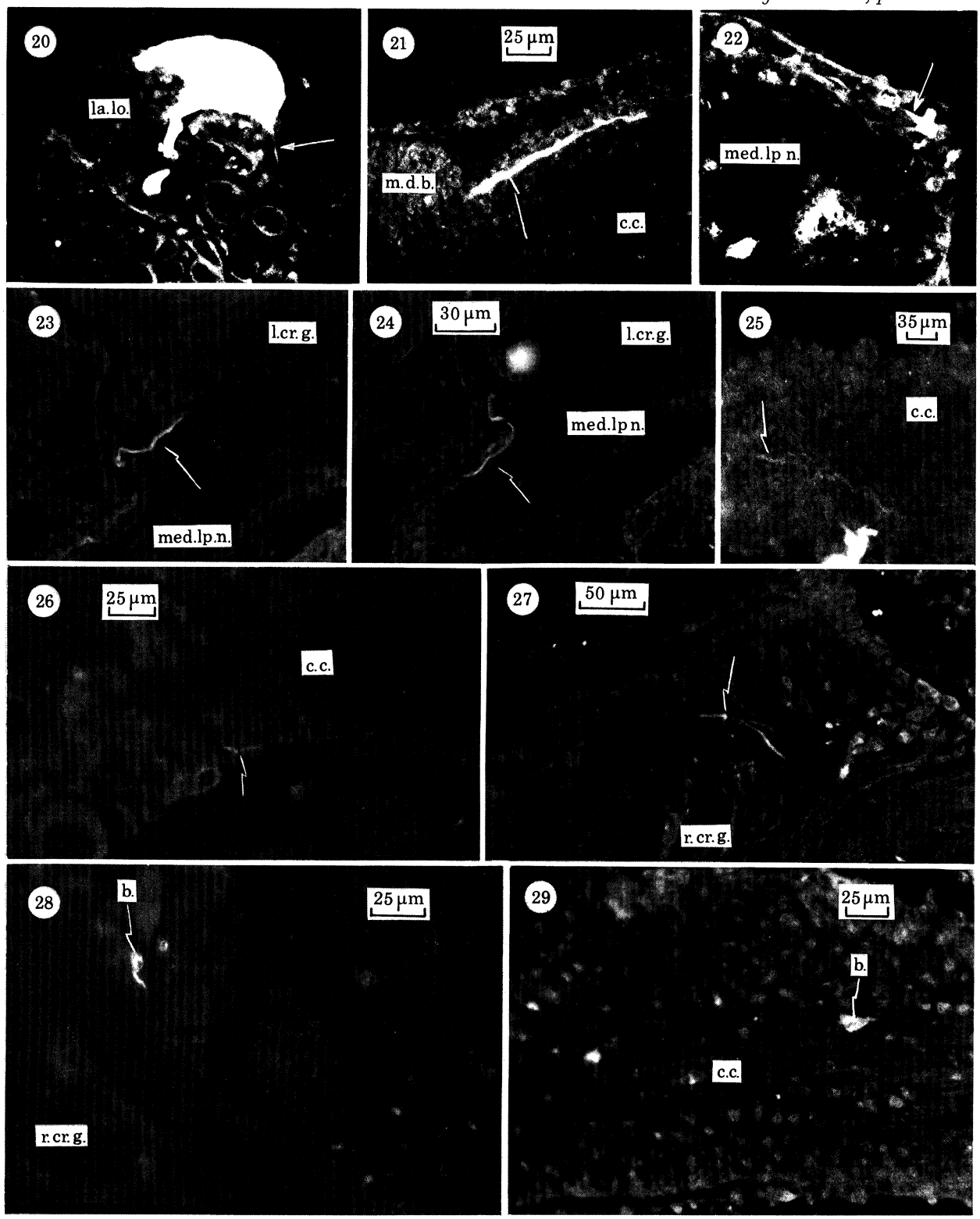

FIGURES 20–29

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The last cell type to be mentioned are the Canopy Cells. These are a pair of giant cells that can be located visually because of their large size and characteristic locations in the lateral lobe of the cerebral ganglia (figure 1, plate 1). It was not usually necessary to confirm their identity by staining although this was done in a few early experiments.

Figure 1 shows the distribution of all the Alcian Blue–Alcian Yellow-stained neurones, from a particular brain reconstructed from serial sections. Caudodorsal Cells from another brain stained with phloxin were added to this drawing to give a complete picture of the numbers and

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Description of plate 3

Figure 7. Section through the right pleural ganglion, showing the bifurcation of a Dark Green Cell axon close to the cell body. Scale bar, 100 μm, also refers to figure 8. All the cells in figures 7–14 were filled with Procion Yellow.

Figure 8. Section through the right pleural ganglion, pleuro-pedal connective and right pedal ganglion, showing a Dark Green axonal projection from the pleural to pedal ganglion.

Figure 9. Section through the visceral ganglion, showing the cell body and proximal axon region of a Yellow-green Cell. Other parts of the same cell are shown in figures 10–12. Two axons originate from different parts of the cell body. Several dendritic processes (one arrowed) arise from these axons. Scale bar, 35 μm, also refers to figures 10–12.

Figure 10. Yellow-green Cell axon in the intestinal nerve.

Figure 11. Yellow-green Cell axon approaching the origin of the anal nerve.

Figure 12. Yellow-green Cell axon in the anal nerve.

Figure 13. Section throughout the anterior part of the visceral and right parietal ganglia, showing part of an axonal process from a cell in the 'visceral Yellow Cell cluster' passing close to the edge of the connective between the visceral and right parietal ganglion. Scale bar, 150 μm, also refers to figure 14.

Figure 14. Adjacent section to figure 13, showing axonal process approaching the viscero-right parietal connective.

Description of plate 4

Figure 20. Section of the right lateral lobe, showing the cell body and proximal axon of the right Canopy Cell reconstructed in figure 18. Other parts of the same cell are shown in figures 21 and 22. A fine process (arrow) projects from one edge of the cell body along the periphery of the lateral lobe. The cell in this section and those shown in figures 21–29 were filled with Procion Yellow.

Figure 21. Section through the cerebral commissure, showing the right Canopy Cell axon (arrow), with short lateral processes that terminate just beneath the perineurium. Scale bar also refer to figures 20 and 22.

Figure 22. Section through the proximal region of the left median lip nerve, showing a terminal of the right Canopy Cell axon (arrow) immediately beneath the perineurium.

Figure 23. Section through the left cerebral ganglion and proximal part of the left median lip nerve, showing the distal part of a left Light Green Cell axon (arrow) just before it enters the nerve.

Figure 24. Adjacent section to figure 23, showing the axon (arrow) of the same Light Green Cells projecting along the lateral edge of the left median lip nerve immediately beneath the perineurium. Scale bar also refers to figure 23.

Figure 25. Section through the perineurium of the cerebral commissure, showing the terminal region of a left Caudodorsal Cell within the perineurium (arrow). Note that this fine process divides and branches at several points.

Figure 26. Fine axonal process of a right Caudodorsal Cell (arrow) within the perineurium of the cerebral commissure.

Figure 27. Section through the neuropil of the right cerebral ganglion, showing the axonal branching point (arrow) of a right Caudodorsal Cell.

Figure 28. Section through the dorsal lobe of the right cerebral ganglion, showing a blob (arrow) on the terminal dendritic process of a right Caudodorsal Cell. Note that the blob is located adjacent to several nerve cell bodies.

Figure 29. Section through the cerebral commissure, showing the axonal blob (arrow) of a left Caudodorsal Cell.

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distribution of the six types of neurosecretory neurone considered in this paper. The reconstruction shows that some of the neurosecretory cell types occur as discrete clusters (e.g. the Light Green Cells and Caudodorsal Cells of the cerebral ganglia) whereas others occur in groups of mixed types or as isolated cell bodies. This makes it easier to identify some neurone types *in vivo* than others. Thus it was only necessary to mark and stain a few examples of the Light Green Cells and Caudodorsal Cells to confirm their identification, whereas the Dark Green Cells, Yellow Cells and Yellow-green Cells were routinely stained after injection. In one location, Yellow Cells occur in a cluster that is free of any other cell type (Swindale & Benjamin 1976*a*). The cluster occurs on the dorsal surface of the visceral ganglion, close to the connective between the visceral and right parietal ganglion (figure 1). This cluster is particularly useful for investigating the morphology of an identified group of Yellow Cells and we will refer to it as the 'visceral Yellow Cell cluster' in later sections. The visceral Yellow Cell cluster has been used for our HRP fills of Yellow Cells where it was impossible to stain with neurosecretory stains after HRP injection. In other locations, Yellow Cells have been identified for HRP injection on the basis of their characteristic double spiking and bursting activity (Benjamin & Swindale 1975; Benjamin 1978). Yellow Cells are the only cell type that have been injected with HRP in an attempt to discover their peripheral release sites, which are far from the injection site in the cell body (Wendelaar Bonga 1970*b*). The advantage of HRP from this point of view is that it is actively transported by neurones (unlike Procion Yellow, which moves passively) and so is likely to reach points distant from the site of injection.

Results

The central nervous system of Lymnaea stagnalis

The nine ganglia of the *Lymnaea* central nervous system are shown in figure 2, plate 2, from the dorsal (figure 2*a*) and ventral (figure 2*b*) aspects. Seven ganglia form a dorsal ring consisting of the paired cerebral, pleural and parietal ganglia and the single visceral ganglion. The paired pedal ganglia are ventral to the others but joined to the rest of the brain on each side by connectives to the pleural and cerebral ganglia. Also labelled in figure 2 are the nerves, which originate from all ganglia except the pleurals. It is important to note that the central ganglia and nerves of *Lymnaea* are surrounded by a connective tissue sheath that consists of a thin inner perineurium and an outer spongy layer of varying thickness (Bekius 1972). Ultrastructural studies show that the perineurium contains blood capillaries and blood spaces, readily penetrated by indian ink particles injected into the anterior aorta, the main blood vessel supplying the c.n.s. (Wendelaar Bonga 1970*a*). The presence of this blood supply to the perineurium is of considerable importance to the present study because many of the processes of the neurosecretory neurones project into or beneath the perineurium and neurosecretory material is likely to be carried into the main circulatory system via the connective tissue capillary system.

The Dark Green Cells

The cell bodies of the Dark Green Cells occur in the pleural, right parietal and visceral ganglia (figure 1) and often in the left parietal ganglion (Swindale & Benjamin 1976*a*, *b*). *In vivo*, they are orange or slightly white-orange in colour and vary in diameter from 30 to 50 μm. The number of Dark Green Cells occurring in particular ganglia varies considerably in different snails (Swindale & Benjamin 1976*b*), but the total number of cells in a brain, that

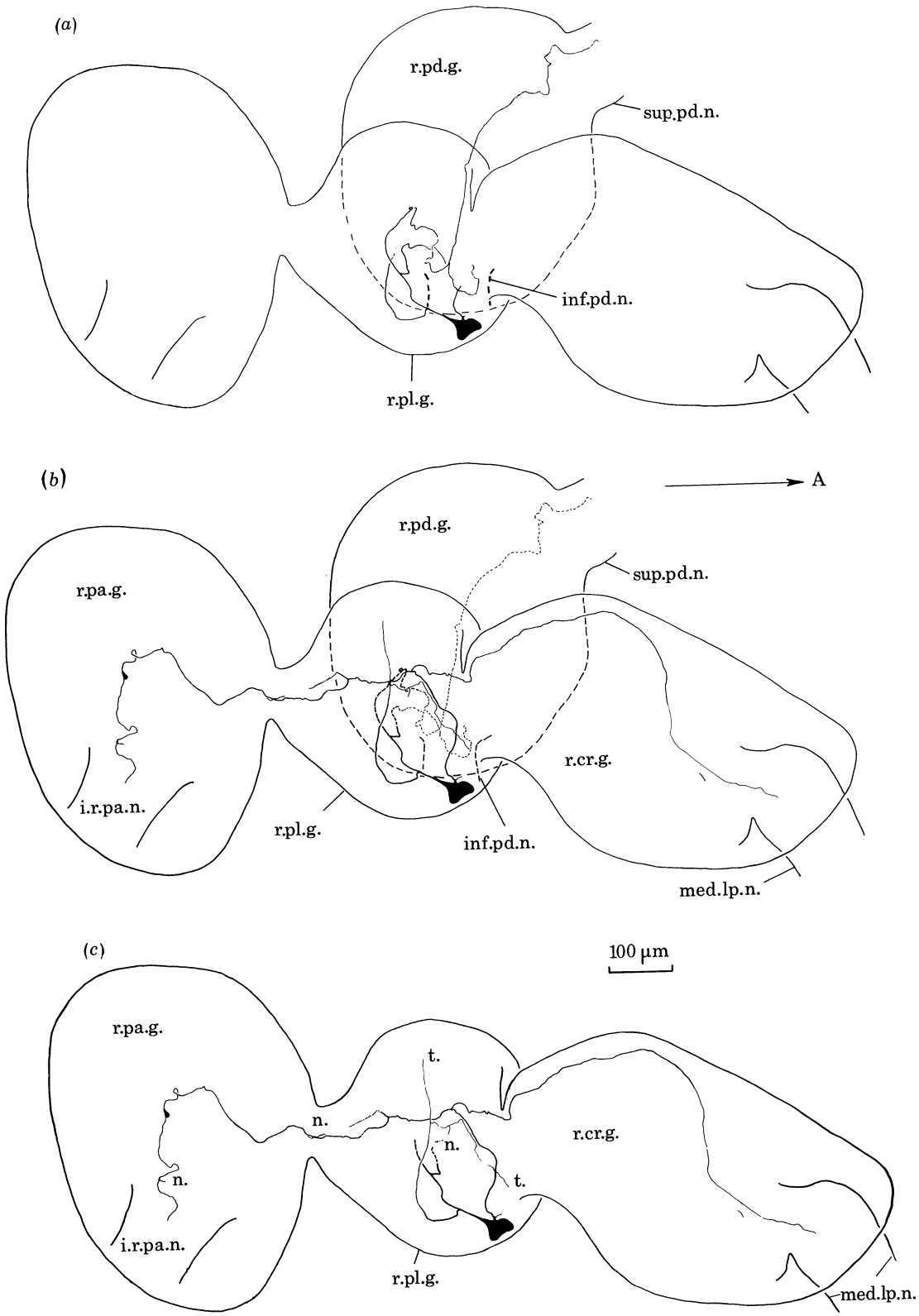

FIGURE 3. Reconstruction of a Procion Yellow-filled Dark Green Cell whose cell body was located on the ventral surface of the right pleural ganglion. The whole cell is shown in (*b*) with the axonal projections to the right pedal ganglion in dashed outline and the more dorsal projections to the cerebral and right parietal ganglia in solid lines. Because of the complexity of the cell and the difficulty of representing it in two dimensions, the pedal ganglion projections are shown separately in (*a*) and the projections to the cerebral and right parietal ganglia in (*c*). Fine axonal branches in the right pleural ganglion, which terminate beneath the perineurium, and short dendritic branches, which terminate in the neuropil of the right pleural and parietal ganglia, are also shown in (*c*).

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can be counted in Alcian Blue–Alcian Yellow-stained material, always averages about 30 when a large number of animals are examined (Soffe *et al.* 1978). Most Dark Green Cells occur in the pleural ganglia (figure 1), chiefly on the ventral surface, and it is these pleural cells that have been injected with Procion Yellow in the present experiments.

In all, 15 Dark Green Cells were reconstructed in detail. They show considerable variability in morphology, particularly in the number of peripheral nerve projections that they possess (compare cells in figures 3–5). This variability was not due to any difficulty in following injected material, because all our cells had plenty of dye in them and we were able to follow fine axonal processes less than 2 μm in diameter.

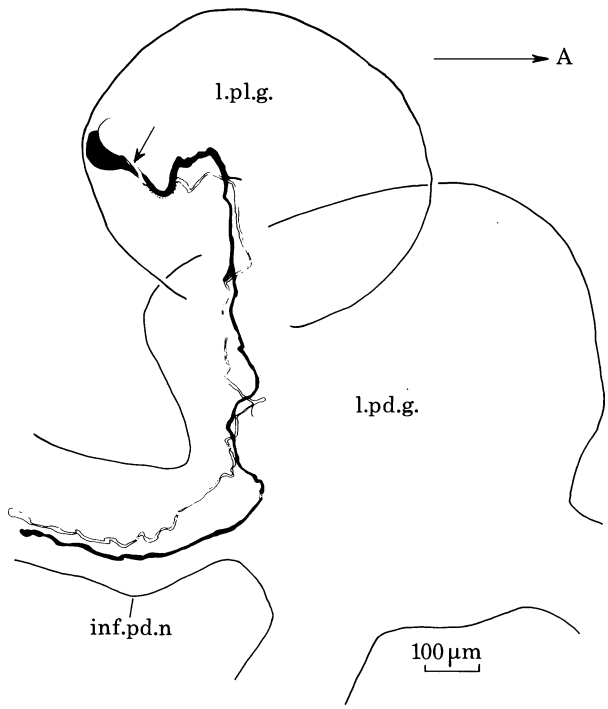

FIGURE 4. Reconstruction of a pair of Procion Yellow-filled Dark Green Cells whose cell bodies occurred adjacent to one another in the left pleural ganglion. The axons from both cells project into the left pedal ganglion and then into the inferior pedal nerve. The axons of the cells run close together in the pleural ganglion neuropil and this is the likely site of the electrotonic junction known to connect Dark Green Cells.

Most of our reconstructed cells were monopolar neurones (see, for example, figure 4) but two cells showed a second finer axonal process originating from the cell body close to the first (figure 7, plate 3). Axons often bifurcated within a few hundred micrometres of the cell body (figures 3*c* and 5, shaded cell), and in the cell shown in figure 3*c* further branching took place after the initial bifurcation. Single short side branches to the neuropil occurred in several cells that terminated in the neuropil. In poorly filled cells this could be due to the problem of tracing distal fine axonal processes, but in the cell of figure 3*c* such fine dendritic branches (marked n.) seemed genuine and occurred at several points along the length of the axon, usually singly but at one point two occurred originating from the same point on the axon but projecting to neuropil on opposite sides of the axon in the pleuro-parietal connective. These rather simple dendritic processes never divided. Swellings occurred in the main axon of some Dark Green

Cells (figure 3*b*, *c*; axon in the right parietal ganglion). These were also seen in Alcian Blue–Alcian Yellow-stained material (Swindale & Benjamin 1976*a*). They could represent the site of accumulation of neurosecretory granules and ressemble the Herring Bodies of vertebrate neurosecretory neurones.

Fine axonal processes projected to the edge of the pleural ganglion in the Dark Green Cell of figure 3. Two processes (marked t. in figure 3*c*) terminated below the connective tissue capsule but did not penetrate it. These axonal terminals are the probable central release sites of the Dark Green Cells observed by Wendelaar Bonga (1970*a*) in the electron microscope. They were rather few in number and did not occur on all Dark Green Cells (figures 4, 5).

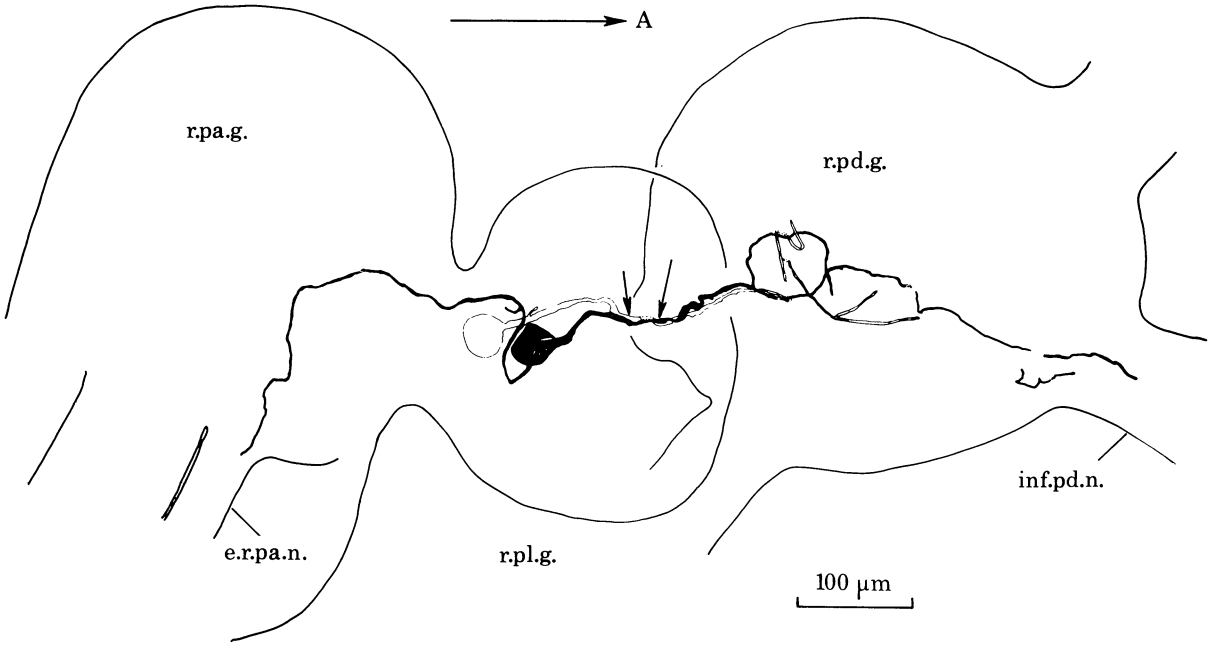

FIGURE 5. Reconstruction of two Procion Yellow-filled Dark Green Cells in the right pleural ganglion. The shaded cell has an axon projecting to the right pedal ganglion and inferior pedal nerve as well as a second finer axon to the external right parietal nerve. The unshaded cell contained less dye and it was not possible to follow its axonal projection beyond the neuropil of the right pedal ganglion. Notice that the axons of the two cells are close together (arrows) at locations within the pleuro-pedal connective (left arrow) and the pedal ganglion.

Much more numerous were the axonal projections to the peripheral nerve roots of the c.n.s., which occurred in all Dark Green Cells (figures 3–5). Nerve projections were always to the centre of the nerve and never to the edge. The number of peripheral nerve projections varied considerably in different Dark Green Cells. Cells could project to one (figure 4), two (figure 5, unshaded cell) or four peripheral nerves (figure 3*b*). All 15 of our reconstructed cells projected to the ipsilateral pedal ganglion (figure 8, plate 3) through the pleuro-pedal connective. From there, axons projected to the inferior pedal nerve (figures 3*a*, 4, 5), the superior pedal nerve (figure 3*a*), medial pedal nerve (cell not shown) and inferior cervical nerve (cell not shown). Some cells also had projections to the nerves of the parietal and cerebral ganglia. The Dark Green Cells of figure 3*c* projected to the median lip nerve and the internal right parietal nerve, and the cell of figure 5 (dark shaded cell) to the external right parietal nerve. Figure 3 shows the most complicated Dark Green Cell that we have reconstructed. This cell had projections, we presume, to many parts of the periphery, since its axons were found in the internal right

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parietal nerve, two pedal nerves (inferior and superior pedal nerves) and the median lip nerve. The best overall impression of this cell can be gained from figure 6, which shows the reconstruction of figure 3 drawn into a standard diagram of the right half of the *Lymnaea* brain. It can easily be seen from this diagram that the Dark Green Cell has projections in both anterior and posterior directions, to the cerebral ganglion and right parietal ganglion, respectively, and a dorsal to ventral projection to the right pedal ganglion.

In previous electrophysiological experiments, we showed that Dark Green Cells were electrotonically coupled if their cell bodies lie close together on the pleural ganglion surface. In the present experiments we injected pairs of Dark Green Cells with Procion Yellow to locate the sites of these junctions (figures 4, 5). We were able to reconstruct confidently the axonal geometry of both members of a pair because of differences in the amount of dye injected and thus differences in the brightness of particular cells under ultraviolet light. The potential problem due to movement of Procion Yellow between electrotonically coupled cells has been discussed elsewhere (Benjamin *et al.* 1979) and, as in this previous study, we think that the amount of dye being transferred is too small to interfere with our interpretation of neural geometry data. Possible sites for the electrotonic junctions occur at points where the axons of two cells are closely apposed (marked with arrows in figures 4 and 5). These occur between pedally projecting axons in the pleural ganglion neuropil close to the cell bodies (figure 4), in the pleuro-pedal connective and in the pedal ganglion (figure 5). These possible sites for electrotonic junctions need to be examined in the electron microscope to confirm the presence of gap junctions.

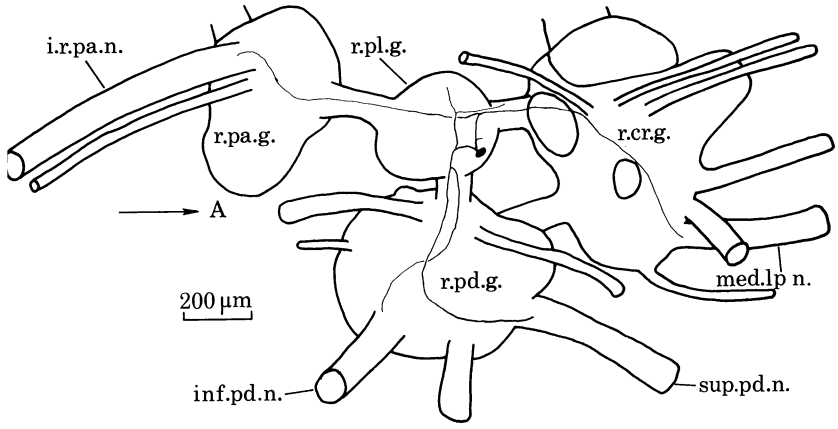

FIGURE 6. The Dark Green Cell of figure 3, drawn diagrammatically into a standard drawing of the right side of the c.n.s. (lateral view). Note the widespread distribution of the Dark Green Cell axons. The lateral view gives a clearer idea of the ventral projections to the pedal ganglion compared with figure 3 as well as showing the extent of the anterior and posterior projections to the right cerebral and right parietal ganglia, respectively.

The overall impression that we have of Dark Green Cells is of neurones with widespread projections to peripheral nerves, with particular cells providing potential distributions of neurosecretory material to widely differing parts of the body. They appear to send rather few projections to central release sites in the pleural ganglia and have only few dendritic projections to the central ganglion neuropil. The pedally projecting axon appears to be present in all Dark Green Cells located in the pleural ganglia and this is the likely site of electrotonic junctions that are known to connect neurones situated close together on the ganglion surface.

The Yellow Cells and Yellow-green Cells

We have found it convenient to consider the Yellow Cells and the Yellow-green Cell in the same section because they occur in the same parts of the c.n.s. and resemble each other in many features of their anatomy.

The cell bodies of these neurosecretory cell types occur mostly in the left and right parietal ganglia and visceral ganglion, although there are usually a few in the pleural ganglia (figure 1). Yellow Cells and Yellow-green Cells often occur in mixed clusters and as both these cell types have a similar appearance *in vivo*, it was necessary to carry out post-experimental staining with Alcian Blue–Alcian Yellow, except for the visceral Yellow Cell cluster. The cell bodies of both types are 40–80 μm in diameter and white or orange-white. The degree of whiteness and thus our ability to separate Yellow Cells and Yellow-green Cells from their orange neighbours is variable in different preparations and depends in Yellow Cells, at least in part, on the environmental osmolarity in which the snails have been kept (Soffe *et al.* 1978). The axons of cells from the mixed Yellow Cell and Yellow-green Cell clusters are white and can often be seen traversing the surface of the ganglion in the outer connective tissue sheath. These superficial fibres were not seen in any other cell types in *Lymnaea*.

We have injected Yellow Cells and Yellow-green Cells with Procion Yellow and, in addition, Yellow Cells with HRP. In general, both methods yielded similar results, although we were able to trace the Yellow Cell axons in the nerves of the visceral and right parietal ganglia further with HRP than with Procion Yellow. Figures 15 and 16 show cells filled with HRP and figure 17 a cell filled with Procion Yellow. These reconstructions show that the fineness of detail of dendritic branching is similar with either technique. We have made detailed reconstructions of nine Yellow Cells (four injected with HRP) and two Yellow-green Cells. Special attention was given to the visceral Yellow Cell cluster, the only identified group of Yellow Cells in the *Lymnaea* c.n.s.

Yellow Cells have generally one (figures 15, 16) but sometimes two (figure 17) main axons originating from the cell body. These axons project to peripheral nerves (figures 15, 16) and/or extraganglionically in particular cells to the connective tissue sheath surrounding the central ganglia (figures 16, 17). Neurones from the visceral Yellow Cell cluster have no main axons projecting into the sheath but finer processes originating from the cell body or proximal axon regions often penetrate the perineurium and travel for tens of micrometres in sheath tissue (figure 15). Yellow Cells in the parietal ganglia have both nerve and extraganglionic sheath projections (see, for example, figure 16), but none of the pleural Yellow Cells appear to have main axonal projections to other than the central connective tissue sheath (figure 17). Yellow-green Cells have nerve projections (figures 10–12, plate 3) and also sheath projections (see Swindale & Benjamin 1976*a*), although the latter have not been examined in the present study.

Fine dendrite-like processes originate from the main axons in Yellow Cells (figures 15–17) and Yellow-green Cells (figure 9, plate 3) and terminate in the central neuropil of the ganglion. These fibres are prominent on the proximal axon regions of cells in the visceral Yellow Cell cluster but also occur on more distal parts of the main axon in the neuropil of the right parietal ganglion (figure 15). All dendritic branching, whether occurring in proximal or distal axonal regions, consists of short processes, often dividing, that terminate in undifferentiated endings in the neuropil local to the axon. Other fine branching processes occur in some Yellow Cells, which instead of terminating in the central ganglion neuropil form endings on the edge of a connective directly below the perineurium (figure 17).

It is likely that central release of Yellow Cell material is taking place from Yellow Cell fibres that penetrate the perineurium and project extraganglionically or from fibres that terminate below the perineurium in a connective. Both these methods of release were described by Wendelaar Bonga (1970*a*) in electron microscopical studies. Release from beneath the perineurium probably occurs from the fine dendrite-like fibres described in the last paragraph but also probably from main axon processes that travel superficially in some of the connectives. This is shown for a cell from the visceral Yellow Cell cluster whose axon was in contact with the sheath in the visceral–right parietal connective (figures 13, 14, plate 3). We have not investigated the central release sites of the Yellow-green Cells in the present study but, according to the work

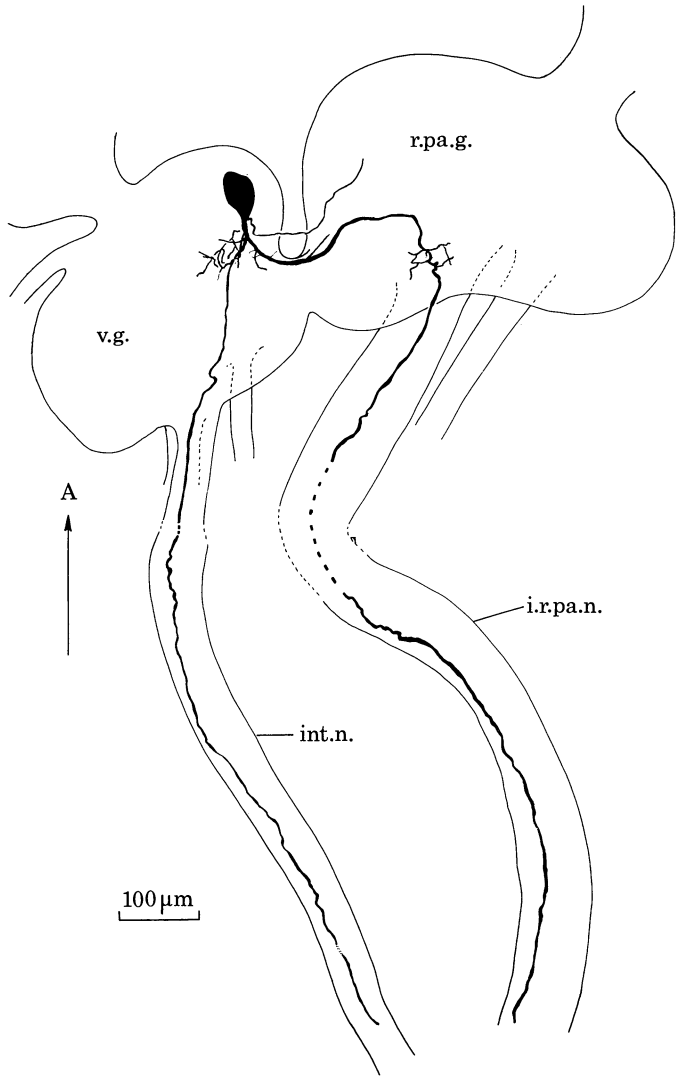

FIGURE 15. Reconstruction of an HRP-filled Yellow Cell from the 'visceral Yellow Cell cluster'. This cell is typical of filled cells from this location. Main axonal processes are to the intestinal and internal right parietal nerves. A fine secondary process leaves the edge of the visceral ganglion and travels, extraganglionically, across the surface of the visceral ganglion in the connective tissue sheath. Note the well developed dendritic branching in the proximal parts of the main axon and further fine branching in the right parietal ganglion. The axon in the intestinal nerve was close to the surface of the nerve just beneath the dorsal perineurium.

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of Wendelaar Bonga (1970*a*) and of Swindale & Benjamin (1976*a*), they are likely to be the same as those of the Yellow Cells described here.

Swindale & Benjamin (1976*a*) traced axons of the Yellow Cells and Yellow-green Cells into the anal, intestinal, internal and external right parietal nerves but it was not possible to say which cell type was responsible for which projection, as both cell types had a similar appearance under the dark field illumination used for the reconstructions. From the present work we can say that Yellow Cells of the visceral Yellow Cell cluster probably all project to the intestinal and internal right parietal nerves (in three cells, including that shown in figure 15). Yellow Cells from the right parietal ganglion also project to these nerves (figure 16). If all the Yellow Cell axons of the right parietal and visceral ganglion project to the intestinal and internal right parietal nerve then most of the fibres in these nerves are likely to be from this type of

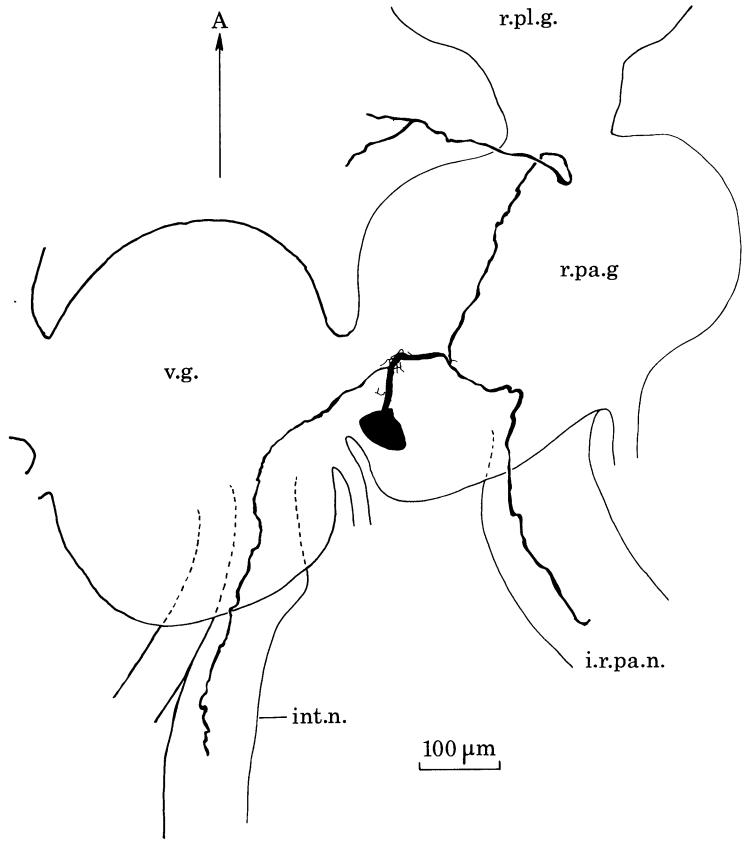

FIGURE 16. Reconstruction of an HRP-filled Yellow Cell from the right parietal ganglion. This monopolar neurone has axonal projections to the intestinal and internal right parietal nerves but also has a projection that leaves the edge of the right parieto-pleural connective and travels, extraganglionically, in the connective tissue sheath.

neurone rather than from the Yellow-green Cells, given the total number of neurosecretory axons in these nerves (Swindale & Benjamin 1976*a*). However, Yellow-green Cells do project to the intestinal nerve, as is shown in figure 10, but their contribution to the total number of neurosecretory axons in this nerve is likely to be small. We think that the small number of neurosecretory fibres in the anal nerve probably mostly come from the Yellow-green Cells because we have not so far found any Yellow Cells that project to the nerve although one is shown in

the Alcian Blue–Alcian Yellow-stained material of Swindale & Benjamin (1975). Direct evidence that Yellow-green Cells project to the anal nerve is shown in figures 11 and 12, plate 3.

Evidence in the last paragraph suggests that Yellow Cells project mostly to the intestinal and internal right parietal nerves. In our HRP-injected Yellow Cells we tried to follow the Yellow Cell axons to their likely sites of peripheral release in the kidney (Wendelaar Bonga 1972) but we were unable to follow them for more than about 2.5 mm along the nerves, well short of this structure (figure 15). For most of its length, the intestinal nerve axon of figure 15 was close to the edge of the nerve beneath its connective tissue sheath and release of neurosecretory material could well be taking place, *en passant*.

In conclusion, we can say that our data show that extensive central release sites for the Yellow Cells occur in the connective tissue sheath surrounding the c.n.s. Release of neurosecretory material probably takes place from axons that project extraganglionically but also from nerve processes within the c.n.s. that project to the edge of connectives. Peripheral nerve

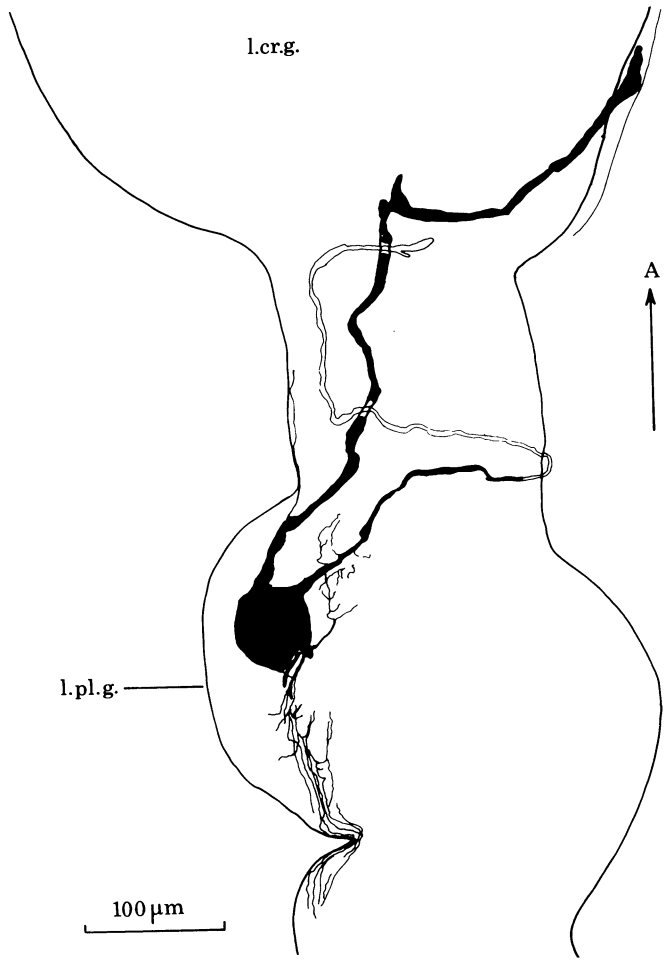

FIGURE 17. Reconstruction of a Procion Yellow-filled Yellow Cell from the left pleural ganglion. Both the main axons of this cell terminated in the connective tissue sheath surrounding the c.n.s. One (shaded axon) projected to the connective tissue of the cerebral ganglion (edge of sheath shown in outline) and the other (unshaded axon) left the c.n.s. at the edge of the left pleuro-cerebral connective and travelled ventrally to the surface of the left pedal ganglion. Fine branching terminates in the left pleural ganglion neuropil but a dense mass of fine fibres also projects to and terminates beneath the edge of the left parieto-pleural connective.

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axons of the Yellow Cells are chiefly to the internal right parietal and intestinal nerves. Neurosecretory axons in the anal nerve are probably chiefly from the Yellow-green Cells rather than the Yellow Cells.

The Canopy Cells

These paired giant cells are unique in the present study in that they are identifiable as individuals in different preparations. Their cell bodies occur in the lateral lobe of the cerebral ganglia (figure 1) and they can usually be recognized *in vivo* on the basis of size and location in

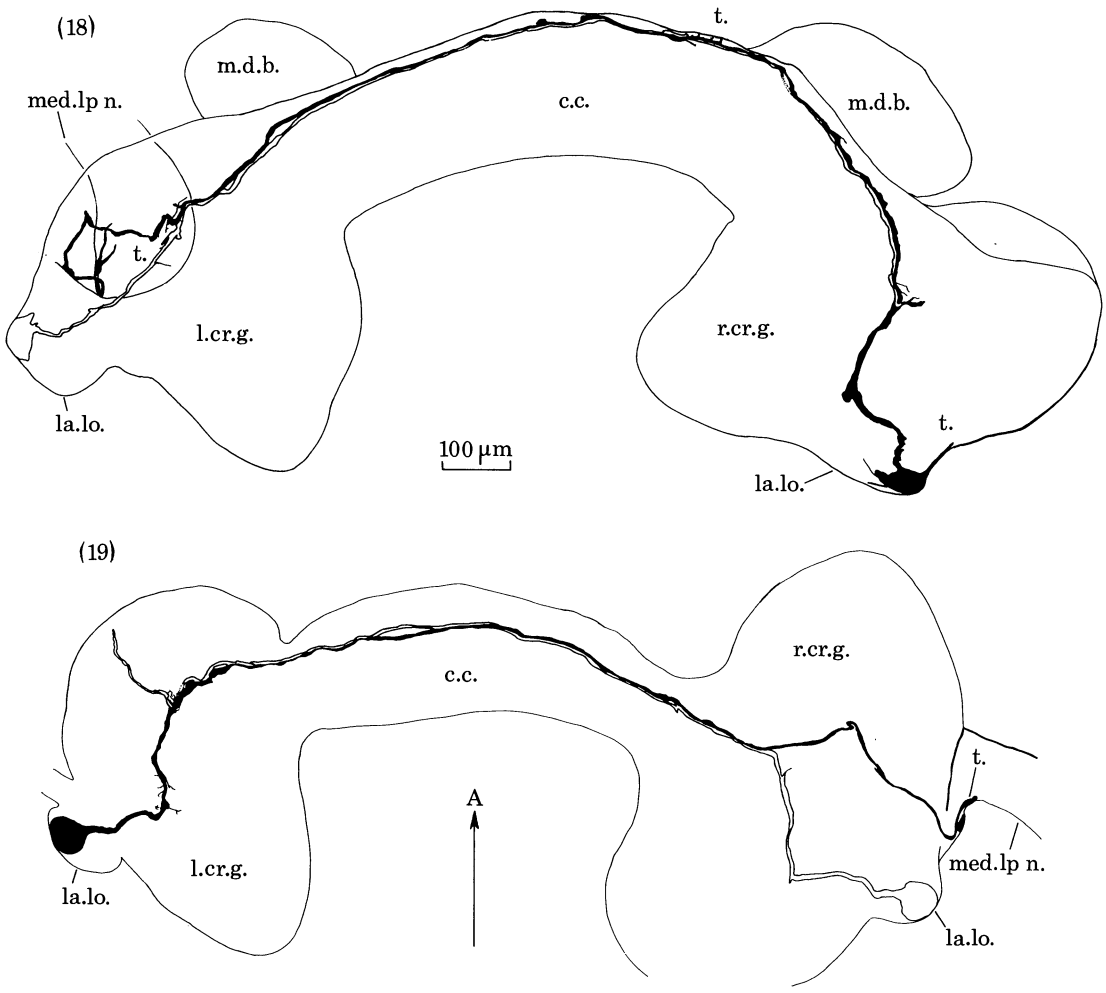

FIGURE 18. Reconstruction of Procion Yellow-filled left and right Canopy Cells. Axons from both cells project across the cerebral commissure to the contralateral cerebral ganglion. The axon of the right Canopy Cell then projects ventrally and terminates in the proximal part of the median lip nerve. The axon of the left Canopy Cell could not be traced beyond the dorsal neuropil of the right cerebral ganglion. Within the cerebral commissure the axons from left and right Canopy Cells are closely apposed, and this is the likely site of the electrotonic junction known to connect them. The right Canopy Cell has fine processes originating from the cell body, which terminate beneath the perineurium of the right lateral lobe. Lateral processes of the right cell originate within the cerebral commissure and terminate below the perineurium local to the axon. At other points the axons of both left and right cells are close to the surface of the cerebral commissure. Scale bar also applies to figure 19.

FIGURE 19. Reconstruction of Procion Yellow-filled left and right Canopy Cells. The main features of their morphology are similar to figure 18 but in this preparation it was possible to follow the axon of the left Canopy Cell to the right median lip nerve. The axons of both cells are very close to the dorsal surface of the commissure, close to the perineurium.

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the dorsal surface of this lobe (Lever & Joosse 1961; Brink & Boer 1967). Their cell bodies are 70–100 μm in diameter and vary in colour from orange through white-orange to pure white. These differences in colour presumably reflect the presence of the varying amount of elementary neurosecretory granules known to be present in their cell bodies (Brink & Boer 1967). In many preparations it was possible to record both left and right Canopy Cells at the same time and figures 18 and 19 both show reconstructions of preparations where left and right Canopy Cells were injected. In all, six Canopy Cells were reconstructed in detail (three left and three right cells). We were impressed by the similarity of the main features of their axonal geometry in different cells and the only difficulty that we had was to find the more distal processes of some cells that were more than 3 mm from the injection site in the cell bodies. The two dark shaded cells of figures 18 and 19 were very bright under ultraviolet light and we believe that we have a complete picture of Canopy Cell anatomy from these fills. The most distal processes of Canopy Cells in the contralateral median lip nerves have since been confirmed by electrophysiological recordings (P. R. Benjamin & R. M. Rose, unpublished data).

Figures 18 and 19 show that Canopy Cells are monopolar neurones with axons that project through the cerebral commissure to the contralateral cerebral ganglion. In the neuropil of the contralateral cerebral ganglion their axons project ventrally and penetrate the proximal parts of the median lip nerves, where they terminate beneath the connective tissue surrounding these nerves (figure 22, plate 4). Figure 18 shows this median lip nerve projection for a right Canopy Cell and figure 19 for a left Canopy Cell. No ipsilateral nerve projections of the Canopy Cells have been found. A few simple dendritic processes terminating in the cerebral neuropil originate from the main Canopy Cell axons and these were seen most clearly on the left Canopy Cell of figure 19.

It was one of the main objectives of this study to identify the possible neurohaemal organs of the Canopy Cells and our data suggest that they could be releasing neurosecretory material from three locations. The projection to the median lip nerve that terminates just below the connective tissue capsule is one possible site for release. The cerebral commissure is a second likely site because in four of the Canopy Cells injected either the axon itself came close to the edge of the commissural neuropil or fine lateral processes left the axon and terminated just below the commissural connective tissue sheath (figure 21, plate 4). Thirdly, we think that neurosecretory material could be released from the cell bodies of the Canopy Cells. This is because blobs of neurosecretory material are often seen *in vivo* in the sheath above the Canopy Cell soma and also the right Canopy Cell of figure 19 had very fine dendrite-like processes actually projecting from the cell body to just beneath the connective tissue sheath of the lateral lobe itself (figure 20, plate 4). These dendritic processes were only seen in this one cell but could have been missed in the other cells, which had less dye in them. It is also possible, in these Canopy Cells with no visible cell body dendrites, that neurosecretory material is released directly from the cell bodies, because no glial tissue appears to separate them from the thin layer of connective tissue that surrounds the lateral lobes (cf. Lever & Joosse 1961).

It was previously shown that the Canopy Cells are electrotonically coupled by a weak non-rectifying junction (Benjamin *et al.* 1976). The site of this junction is likely to be in the cerebral commissure because one of the most striking features of the relationship between left and right Canopy Cells is the close apposition of their axons in this commissure (figures 18, 19). The obvious bilateral symmetry of the geometry of left and right Canopy Cells' axons parallels

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that of other bilaterally symmetrical neurones in the *Lymnaea* c.n.s., such as motoneurones in the buccal ganglia (Benjamin *et al.* 1979) or the cerebral giant neurones (McCrohan & Benjamin 1980).

This study has shown that the Canopy Cells have three possible central sites for the release of neurosecretory material. In this respect, they resemble the Light Green Cells and Caudodorsal Cells (next two sections) rather than the Dark Green Cells, Yellow Cells and Yellow-green Cells, which have both central and peripheral release sites.

The Light Green Cells

About 100 Light Green Cells occur in each cerebral ganglion (Joosse 1964). They form two discrete clusters in each cerebral ganglion (figure 1) and are mostly covered by tissue of the Laterodorsal and Mediodorsal Bodies (figure 2*a*). However, the white or orange-white cell bodies of some Light Green Cells are usually free of this overlying tissue and available for intracellular recording. Sometimes the cell bodies of the Light Green Cell clusters are continuous in their distribution with the Caudodorsal Cells, also shown in figure 1. However, the cell bodies of Caudodorsal Cells are always a paler white than those of Light Green Cells (confirmed by dye marking) and the two cell types can be reliably separated on this criterion. The general features of Light Green Cell morphology were similar in all the nine Light Green Cells that were reconstructed in detail.

The Light Green Cells are the simplest neurones that we have examined in that they are monopolar with a single ventral axon that enters the median lip nerve in the ipsilateral cerebral ganglion. This is shown for two cells in the left ganglion (figures 30, 32) and one in the right cerebral ganglion (figure 31). Dendritic processes originate from the axons of all reconstructed Light Green Cells in the segment of axon 75–200 μm from the cell body. These processes, which sometimes divide (figures 30, 32), terminate in the cerebral ganglion neuropil, close to Light Green Cell axons. The longest processes are about 100 μm (see figure 32) but most are less than 50 μm in length (figures 30–32). These dendrites could be the sites of synaptic interaction between neurones because the Light Green Cells receive an inhibitory synaptic input in many preparations (Benjamin & Rose, unpublished). The irregular outline of the cell bodies of Light Green Cells and their variable shape (figures 30, 32) have been observed by earlier authors (Joosse 1964).

Of particular interest in the present study was the arrangement of the axonal terminals of the Light Green Cells in the median lip nerve. The large number of axons (perhaps 40000; Boer *et al.* 1968) that the Light Green Cells apparently form in the median lip nerve led the previous authors to suppose that the axons must repeatedly divide, given that there are only about 100 cell bodies in each cerebral ganglion. However, none of our cells showed any form of axonal branching either in the cerebral ganglion or in the median lip nerve (figures 30–32). Axons of the Light Green Cells enter the median lip nerve on its lateral edge (figures 23, 24, plate 4) and from there continue along the same part of the nerve (figures 30, 31) or cross over to other parts of the nerve circumference (figure 32). At various points along the length of the nerve the axons of the Light Green Cells flatten against the edge of the nerve immediately adjacent to the sheath (figures 24, 30–32) but never penetrate it. In a particular cell there could be many points of contact between the axon and the adjacent connective tissue (figure 31), so that the axon moves directly adjacent to the sheath, then away from the edge of the nerve and continue to return repeatedly to its furthermost edge. It seems likely that the Light Green

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Cells release neurosecretory material, *en passant*, into the connective tissue sheath of the median lip nerve at the points where their axons come close to the surface of the nerve.

In summary, we can say that the axons of the Light Green Cells project to the ipsilateral median lip nerves. No branching of Light Green Cell axons occurs in the lip nerves and release of neurosecretory material probably takes place from the edge of the nerve, through the connective tissues capsule, from axons that flatten against the perineurium, without penetrating

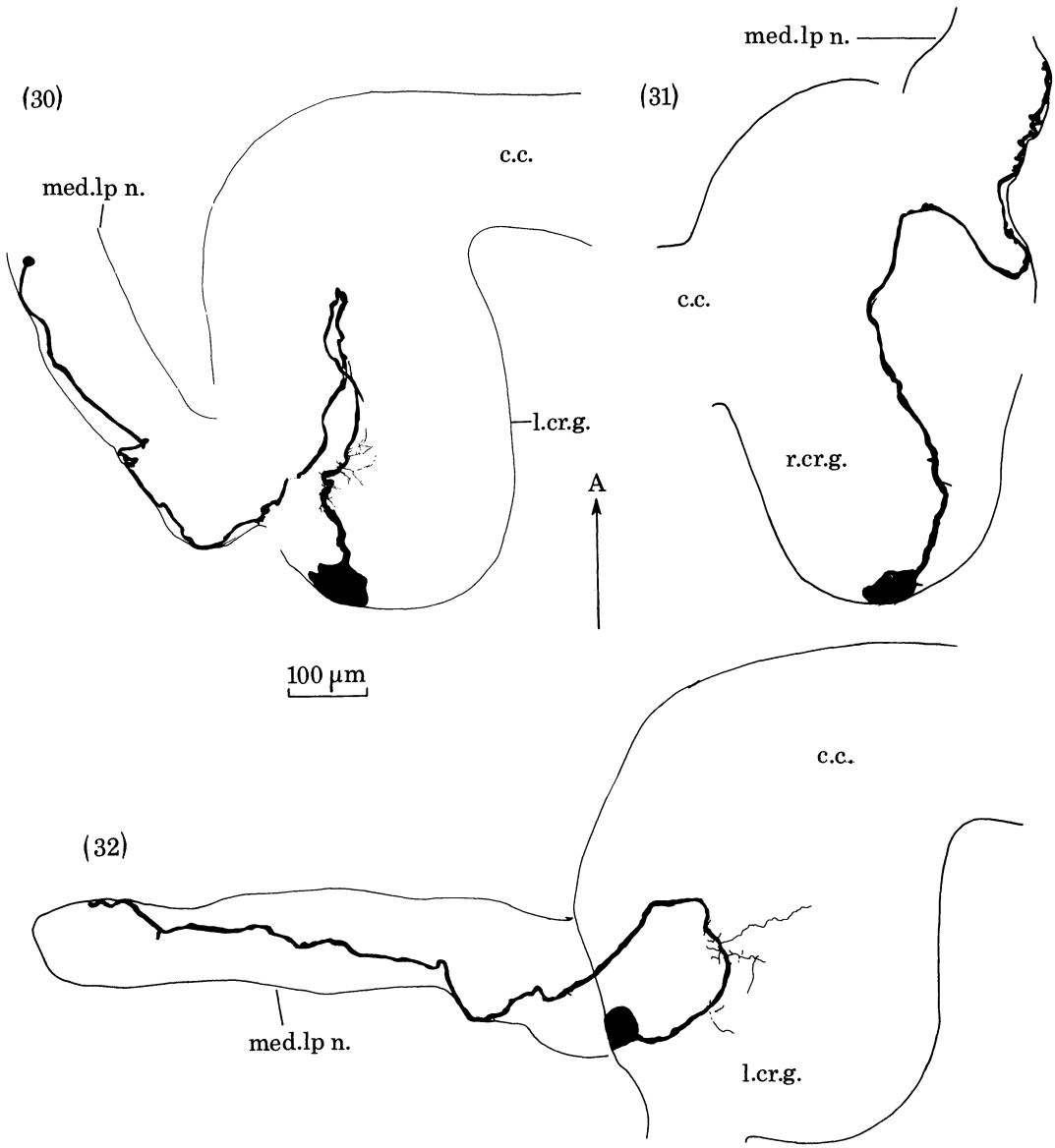

FIGURE 30. Reconstruction of a Light Green Cell filled with Procion Yellow from the left cerebral ganglion. The fine dendritic branching originating from the proximal axonal regions is particularly well developed in this cell. Scale bar also applies to figures 31 and 32.

FIGURE 31. Reconstruction of a Procion Yellow-filled Light Green Cell from the right cerebral ganglion. At many points within the left median lip nerve the axon flattens against the edge of the nerve. (The nerve outline delimits the edge of the nerve neuropil and does not include the connective tissue surrounding the nerve.)

FIGURE 32. Reconstruction of a Procion Yellow-filled Light Green Cell from the left cerebral ganglion. The axon of this cell enters the right median lip nerve close to its dorsal surface, then flattens against its lateral edge and then projects again to the dorsal surface, where it terminates beneath the nerve perineurium.

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it. An *en passant* type of release mechanism is suggested to occur from the lateral edges of the Light Green Cell fibres in the median lip nerves, with release taking place to a lesser extent from nerve endings.

The Caudodorsal Cells

The pale white cell bodies of the Caudodorsal Cells occur in two clusters on the dorso-medial surfaces of the cerebral ganglia (figure 1). According to Joosse (1964) about 75 neurones occur in the right cerebral ganglion and 31 in the left cerebral ganglion. Although almost all (21 out of 22) of our reconstructed neurones projected to the cerebral commissure (figures 34–36), we have been surprised by the structural diversity of the Caudodorsal Cells compared with, for instance, the Light Green Cells. The complexity of axonal branching within the cerebral commissure varied considerably in different cells, as did the part of the commissure to which the axons projected. We do not consider that the variability of form was merely a reflection of the variable amount of dye injected or the difficulty of Procion Yellow penetration of fine

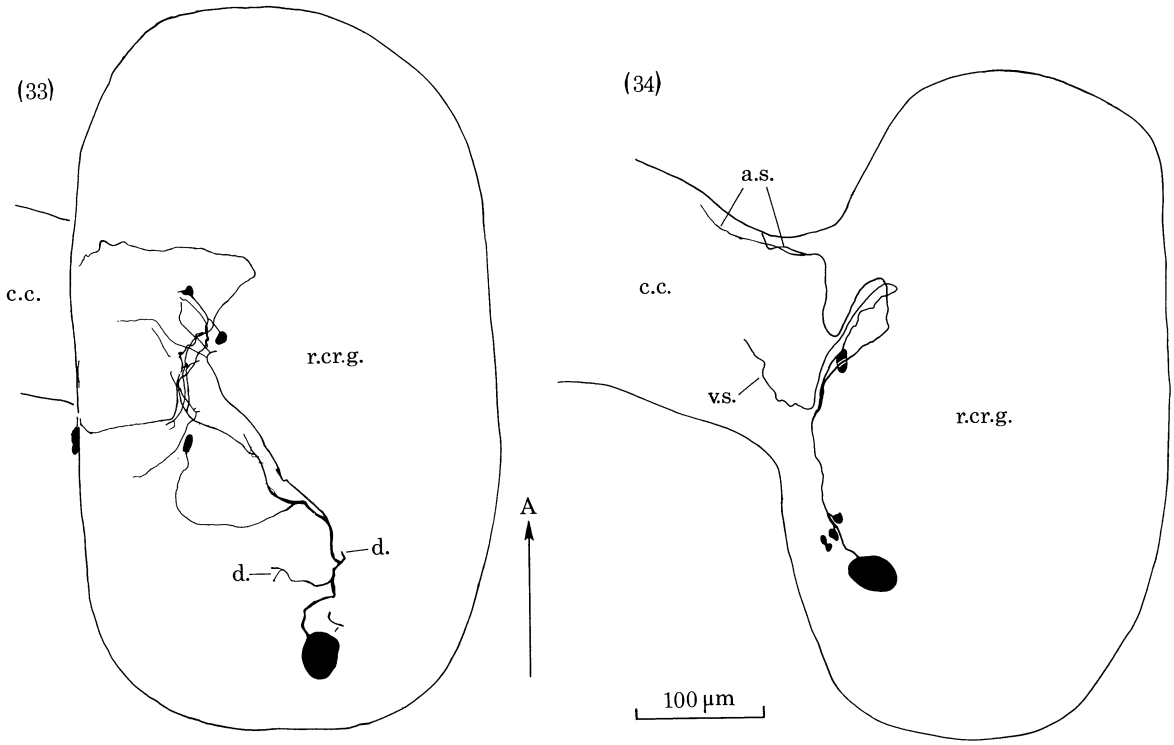

FIGURE 33. Reconstruction of a Procion Yellow-filled Caudodorsal Cell from the right cerebral ganglion, whose axonal projections terminate in the dorsal lobe neuropil of the right cerebral ganglion or in the perineurium surrounding this ganglion (fibre ending in two blobs). This cell was the only one (of 22 reconstructed) whose axon failed to penetrate the cerebral commissure. Note the extensive axonal branching of this cell and the short dendritic branches located on the proximal axon. A particular feature of this cell is the large number of blobs, either terminal blobs or enlargements of the axon just proximal to fine axonal terminals. The pair of blobs in the perineurium of the cerebral ganglion may represent the site of release of the neurosecretory material.

FIGURE 34. Reconstruction of a Procion Yellow-filled Caudodorsal Cell in the right cerebral ganglion, whose axons penetrate the proximal part of the cerebral commissure and terminate on the anterior and ventral surfaces of the commissure. The axonal process on the anterior surface was within the perineurium of the cerebral commissure and is shown, photographed, in figure 26. Blobs were located chiefly on the proximally located dendritic processes, but one occurred more distally as an axonal enlargement. One of the dendritic blobs is shown, photographed, in figure 28. Scale bar also applies to figure 33.

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processes, because the neurones with the simplest axonal branching patterns often had the longest axons, with fine branching processes leaving the main axon (see, for example, figure 35).

All Caudodorsal Cells are monopolar and their axons form a tract that dips ventrally in the cerebral ganglion neuropil before penetrating the cerebral commissural neuropil (figures 37–42). Axonal branching starts in the cerebral ganglion neuropil (figure 27, plate 4), usually

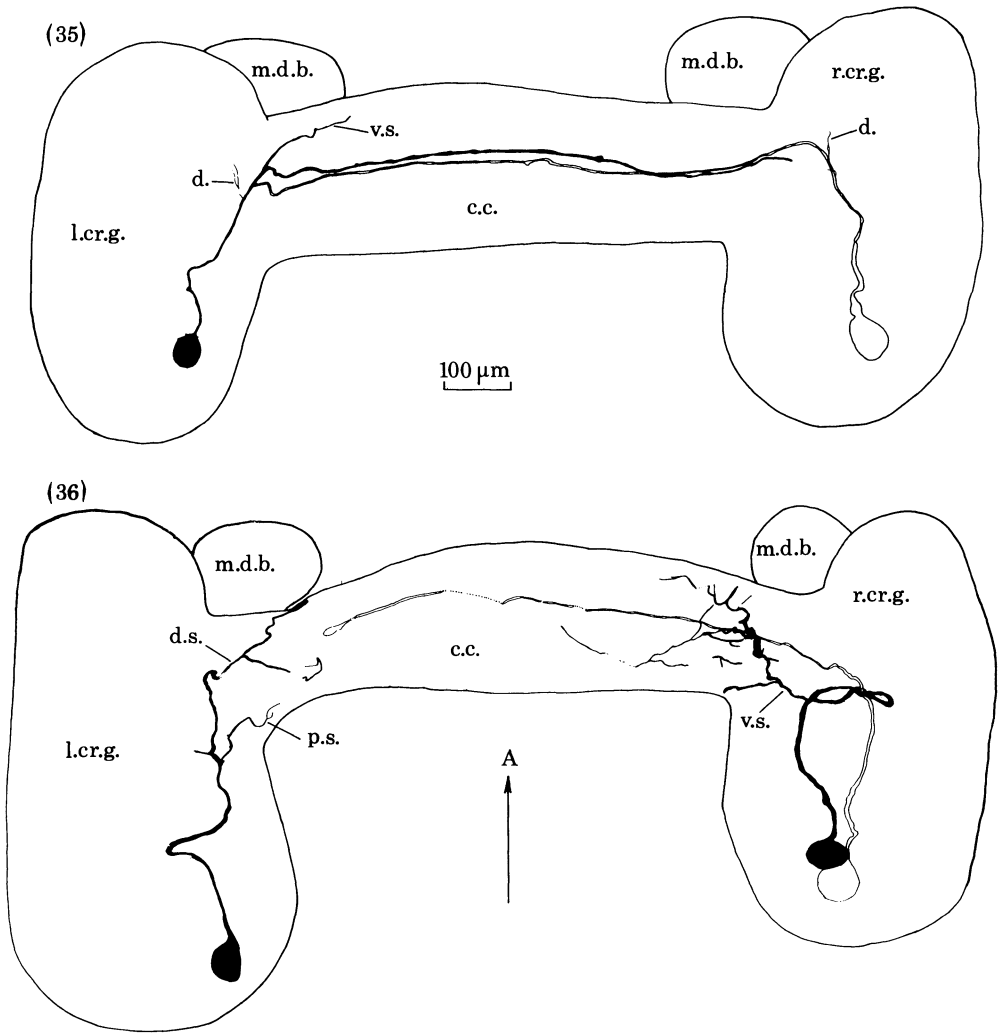

FIGURE 35. Reconstruction of a pair of Procion Yellow-filled Caudodorsal Cells from left and right cerebral ganglia whose axons project contralaterally for long distances within the cerebral commissure but show little branching compared with the Caudodorsal Cells of figures 33 and 34. The left (shaded) cell has an axon branch that terminates on the ventral surface of the cerebral commissure, but no such terminal is present in the right (unshaded) cell. Both neurones have a single dendrite that terminates in the local cerebral ganglion neuropil. At two locations the axons of the left and right cells are closely apposed and this may be the site of the electrotonic junction known to connect contralateral Caudodorsal Cells. The left cell has an enlargement of the axon (axonal blob) in the cerebral commissure. Scale bar also applies to figure 36.

FIGURE 36. Reconstruction of three Procion Yellow-filled Caudodorsal Cells, two in the right and one in the left cerebral ganglion. The reconstruction of the two right cells shows that a Caudodorsal Cell with complex axonal branching (shaded) can lie close to another (unshaded) with a single unbranching contralaterally projecting axon. The shaded Caudodorsal Cell from the right cerebral ganglion has many terminals on the ventral surface of the cerebral commissure, whereas the left cell has fewer axonal processes, which terminate on the posterior and dorsal surfaces of the cerebral commissure.

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at the point where the axons curve dorsally towards the entrance of the commissural neuropil (seen in the reconstructions (figures 33 and 34) but most obvious in the drawings (figures 37–42)). Further axonal branching takes place in the cerebral commissure itself (figure 34), although some cells do not branch at all in this structure (figures 35, 36, unshaded cells).

The simplest types of Caudodorsal Cells had a single axon, which projected along the cerebral commissure as far as the neuropil of the contralateral cerebral ganglion (figure 35). At no point did this axon approach the edge of the connective (figure 35). Sometimes cells of this type had a second shorter branch, which terminated at the edge of the commissure on the same side as the cell body (figure 35, shaded cell). Figures 40 and 42 show that nine of our reconstructed cells had their main axons projecting along the centre of the commissure in this way. The rest of the neurones, apart from one, had axons that projected only part of the way into the cerebral commissure. These axons often branched extensively and terminated close to the edge of the cerebral commissure (see, for example, figures 34, 36, shaded cells). Some of these neurones projected to the ventral surface of the cerebral commissure (figures 37, 38), others to the dorsal surface (figure 41) and a few to both dorsal and ventral surfaces (figure 39). Lastly we mention the single remaining neurone, whose axon never penetrated the cerebral commissure at all. This was the most complicated neurone that we have reconstructed (figure 33). Its axon branched extensively and it had terminals in the cerebral ganglion neuropil as well as at the edge of the ganglion, in the perineurium (figure 33).

Electron microscopy showed that the Caudodorsal Cells release neurosecretory material into the sheath at the edge of the cerebral commissure (Boer *et al.* 1968). We presume that these terminals are formed from the axonal branching processes mentioned in the last paragraph, so that those Caudodorsal Cells with just central axonal processes cannot be contributing to the endings seen in the electron microscope. Wendelaar Bonga (1971 *b*) estimated that each Caudodorsal Cell should, on average, have 800 terminals in the cerebral commissure. This estimate is difficult to reconcile with the number of endings seen in the present study, given that some of the Caudodorsal Cells may not have any endings in the commissure at all. Caudodorsal Cells with three endings are shown in figures 34 and 35 (left side, shaded cell) and one with ten in figure 36 (right side, shaded cell). Even the most complicated cells could only provide a number of endings about two orders of magnitude lower than Wendelaar Bonga's estimate. The arrangement of the fibres in the cerebral commissure suggests that neurosecretory material could be released from fibres arranged *en passant* with respect to the edge of the commissure as well as from nerve terminals. Fibres often run for distances of up to 100 μm along the edge of the cerebral commissure (figure 34, along the anterior surface and ventral surface; figure 36, right hand shaded cell, along the ventral surface).

The electron microscopic data of Boer *et al.* (1968) suggested that release of Caudodorsal neurosecretory material takes place from endings located beneath the perineurium of the cerebral commissure. In our material shown in figures 25 and 26, plate 4, the fine terminal parts of the Caudodorsal Cell axons actually penetrate and travel within the perineurium, where they may divide (figure 25). This suggests that neurosecretory material may be released directly into the perineurium as well as from the edge of the commissural neuropil.

The branching processes described in the previous paragraphs were axonal in nature in that they arose from the division of the single main axon of the Caudodorsal Cells. Other shorter processes originated from the main axon in its proximal regions and terminated in the local cerebral ganglion neuropil (figures 33–35). One or two of these dendrite-like processes occurred

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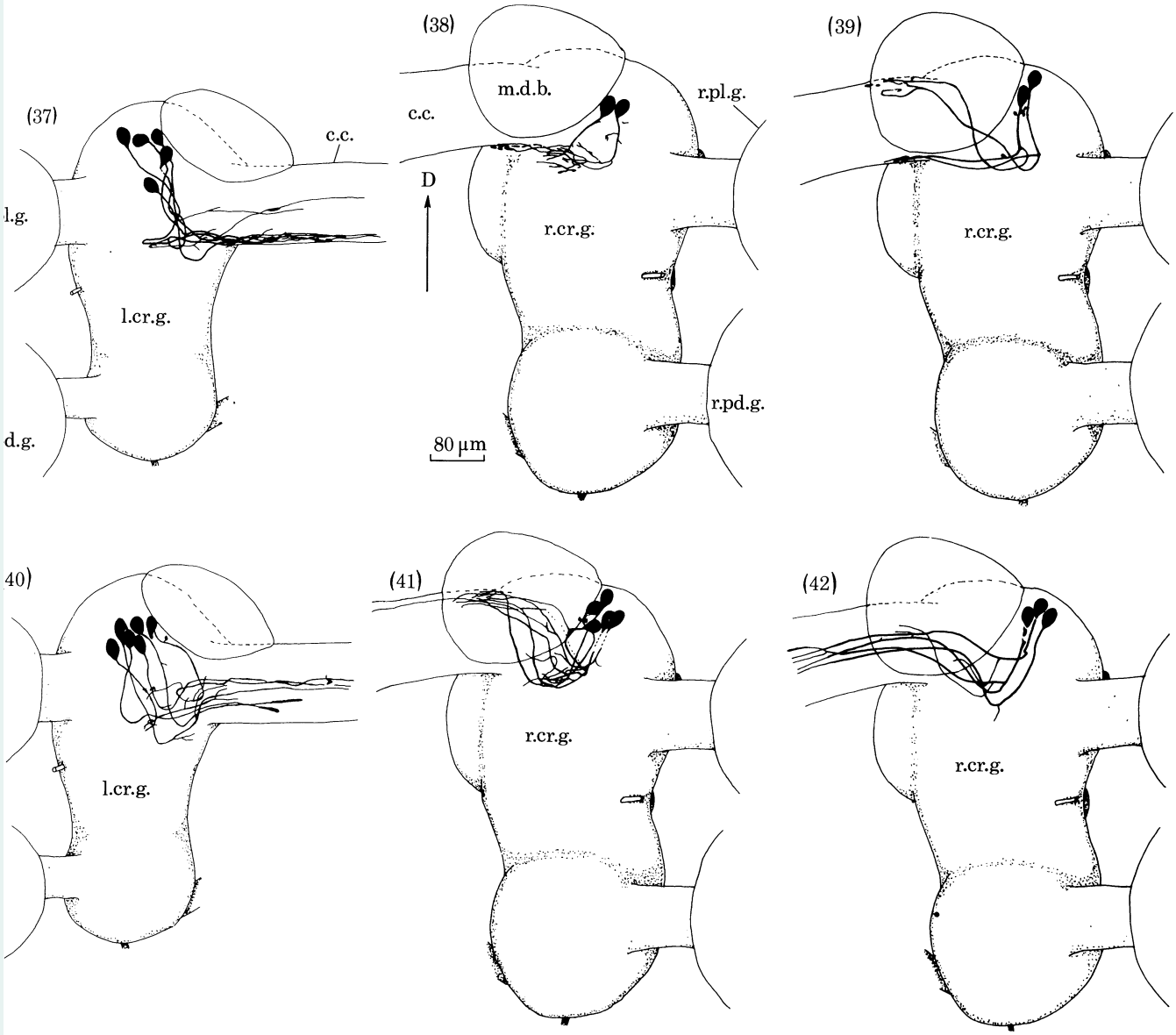

FIGURE 37. Reconstruction of Caudodorsal Cells from the left cerebral ganglion that project to the ventral surface of the cerebral commissure. Two of the cells had additional projections to the central core of the neuropil in the cerebral commissure. In figures 37–42 all 22 Procion Yellow-filled Caudodorsal Cells have been classified according to which part of the cerebral commissure their axons project and then drawn diagrammatically into standard diagrams of the cerebral ganglia viewed from the medio-posterior direction (shown by an arrow in figure 1). These figures thus summarize our data on the overall distribution of Caudodorsal axons, although they do not give, in a statistical sense, an accurate idea of the number of each cell type occurring in the population of Caudodorsal Cells from a particular c.n.s.

FIGURE 38. Two Caudodorsal Cells from the right cerebral ganglion that projected to the ventral part of the cerebral commissure or close to it. The axons of one of these cells (right cell body) never penetrated the cerebral commissure but terminated in neuropil or at the edge of the cerebral ganglion just ventral to the origin of the cerebral commissure (see figure 33 for a detailed reconstruction of this cell). Scale bar applies to figures 37–42.

FIGURE 39. Two Caudodorsal Cells from the right cerebral ganglion that had axonal projections to both the dorsal and the ventral surfaces of the proximal cerebral commissure.

FIGURE 40. Left Caudodorsal Cells with axonal projections to the central core of the cerebral commissure.

FIGURE 41. Caudodorsal Cells from the right cerebral ganglion with axonal projections to the dorsal surface of the cerebral commissure.

FIGURE 42. Right cerebral ganglion Caudodorsal Cells with axonal projections to the central core of the cerebral commissure.

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in a particular cell (marked d. in figures 33–35). They sometimes divided once (figure 33) but always terminated in undifferentiated endings within about 100 μm of the axon. In some instances these proximal processes ended in blobs (figures 34), whose diameter was four to six times that of the axon. Figure 28, plate 4, shows that these blobs can occur in the cell body layer of the cerebral ganglia. It is interesting that similar blobs occurred in the axonal processes of seven (our of 22) reconstructed cells, either as terminals (figure 33) or as swellings of the axon (figures 33, 34). These blobs were occasionally seen as swellings of the main axon within the cerebral commissure (figure 29, plate 4) but were more commonly located on axons before they reached the cerebral commissure (figure 34). In one cell only (figure 33), blobs were seen on the edge of the cerebral ganglion within the perineurium. This suggests that terminal blobs could represent the site of release of neurosecretory material from Caudodorsal Cells into the perineurium. A final point concerns the close apposition of Caudodorsal Cell axons within the cerebral commissure. These points of contact between neurones with contralaterally projecting axons (figure 35) could be the sites of the electrotonic junctions known to connect Caudodorsal Cells from electrophysiological experiments (Benjamin & Rose, unpublished).

In summary, our results show that the cerebral commissure is the main neurohaemal organ for the Caudodorsal Cells, although a few cells may terminate outside this structure. The extent to which axons branch within the cerebral commissure varies in different cells, as does the part of the cerebral commissure to which the axons project. The number of terminals that the Caudodorsal Cells form in or close to the perineurium of the cerebral commissure is not nearly enough to account for the numbers of axonal profiles containing elementary neurosecretory granules seen in the electron microscope (Wendelaar Bonga 1971 *b*). *En passant* release of neurosecretory material may be a common feature of the Caudodorsal Cells.

Discussion

Comparison of morphology of neurosecretory cell types in Lymnaea

Table 1 summarizes the main features of neurosecretory cell morphology revealed in the present study. Each of the six cell types has a distinctive combination of morphological characteristics, which provides further evidence for the previous classification of *Lymnaea* neurosecretory neurones into cell types based on their staining reaction to Alcian Blue–Alcian Yellow (Wendelaar Bonga 1970*a*).

Obvious differences between the cell types in *Lymnaea* divide the cells that occur in the cerebral ganglia from those of the pleural, parietal and visceral ganglia. The cell bodies of the cerebral cells (Caudodorsal Cells, Light Green Cells, Canopy Cells) occur in characteristic locations on the ganglion surface, form discrete clusters (Caudodorsal Cells, Light Green Cells) and have well defined neurohaemal organs in particular nerves or connectives. Neurones in the pleural, parietal and visceral ganglia (Dark Green Cells, Yellow Cells and Yellow-green Cells) are scattered in different locations and rarely form identified groups in particular locations. Neither do they have neurohaemal areas in discrete central locations. A further major difference between the two groups is that, while the cerebral cells have only central release sites, cells in the other ganglia have both central and peripheral sites of release. The reasons for having peripheral release sites for neurosecretory substances have been previously discussed by Swindale & Benjamin (1976*a*).

Further characterization of cell types can be carried out on the basis of differences in neural

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TABLE 1. SUMMARY OF MORPHOLOGICAL FEATURES OF NEUROSECRETORY NEURONES IN *LYMNAEA*

	Dark green Cells	Yellow Cells	Yellow-green Cells	Canopy Cells	Light Green Cells	Caudodorsal Cells
cell body locations	pleural ganglia	pleural, parietal and visceral ganglia		cerebral ganglia	cerebral ganglia	cerebral ganglia
numbers in c.n.s.	30	25	25	2	200	100
axons	monopolar	mono- or bipolar	mono-, bi- or multipolar	monopolar	monopolar	monopolar
main projections	ipsilateral pedal ganglia also to cerebral and parietal ganglia in some cells	intestinal and internal right parietal nerves or extraganglionic	anal, few fibres in intestinal and external right parietal nerves	contralateral median lip nerves	ipsilateral median lip nerves	cerebral commissure
dendritic branching in c.n.s. neuropil	little, single undividing processes	complex	complex	a few simple processes	several fibres which may divide	one or two fibres which may divide
site of electrotonic junctions	pleural ganglia pleuro-pedal connectives or pedal ganglia	—	—	cerebral commissure	tract in cerebral ganglion	cerebral commissure
blobs and swellings	few, as axonal swellings	none	none	none	none	many blobs on dendrites, axonal swellings
central release sites	pleural ganglia	many parts of central sheath, proximal parts of visceral and parietal ganglia nerves	?	lateral lobes, cerebral commissure, contralateral median lip nerves	ipsilateral median lip nerves	cerebral commissure
mode of release	nerve endings, beneath perineurium	nerve endings, *en passant* from beneath and within perineurium	?	nerve endings, lateral axonal branches, *en passant* from main axon, all from beneath perineurium	nerve endings, *en passant* from beneath perineurium	nerve endings, *en passant* from beneath and within perineurium
peripheral release sites	distal to c.n.s. from fibres in pedal, cerebral parietal ganglia nerves	distal to c.n.s. in intestinal and internal right parietal nerves	distal to c.n.s. in intestinal, anal and external right parietal nerves	none	none	none

geometry. The Light Green Cells were the simplest cell type that we investigated. They were monopolar cells with a single, undividing axon that projected to the ipsilateral median lip nerve. In contrast, many Caudodorsal Cells had complicated axonal branching patterns within the cerebral commissure and a surprisingly variable pattern of terminal distribution. A characteristic feature of the third type of cerebral neurosecretory cell, the Canopy Cell, was the

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contralateral axonal projection to the median lip nerve. No other cell type had such obvious bilateral symmetry (see figures 18, 19), while the axonal projections to the opposite side of the brain allowed the axons of the left and right Canopy Cells to meet in the cerebral commissure, resulting in electrotonic coupling (Benjamin *et al.* 1976). The Yellow Cells and Yellow-green Cells differed from other cell types in often having more than one axon originating from the cell body, with Yellow-green Cells showing this tendency to a greater extent than the Yellow Cells (see also Swindale & Benjamin 1976*a*). Both of these cell types had more highly developed dendritic branching processes than other neurosecretory cells. The most distinct feature of the Dark Green Cells was their large number of peripheral nerve projections, which were widely distributed to nerves of the pedal, parietal and cerebral ganglia. This widespread distribution reflects their presumed function in controlling some aspect of ion and water balance via the skin of the foot and mantle (discussed by Swindale & Benjamin 1976*b*).

Location of central neurohaemal areas

By tracing processes of dye-filled neurones, we were able to confirm previous electron microscopical studies that showed that the connective tissue surrounding the c.n.s. and nerves of *Lymnaea* is a major site of release of neurosecretory material (Wendelaar Bonga 1970*a*).

As mentioned previously, three of our cell types, the Light Green Cells, Caudodorsal Cells and Canopy Cells, had well defined neurohaemal areas in specific central structures, whereas the Yellow Cells, Yellow-green Cells and Dark Green Cells had no single structure that could be called a neurohaemal organ.

Projections of the Light Green Cells to the ipsilateral median lip nerve and the Caudodorsal Cells to the cerebral commissure confirmed the results of Joosse (1964). For the first time we showed that the Canopy Cells project to the contralateral median lip nerves, where their axons terminate below the perineurium. This suggests that the median lip nerve serves as neurohaemal organ for both the Canopy Cells and the Light Green Cells, as well as containing axons of the Dark Green Cells, which continue to the periphery. Further possible release sites of the Canopy Cells are the cerebral commissure and the lateral lobe.

The release sites of the Yellow Cells are much more diffuse than those of the cerebral ganglion neurosecretory neurones. Axonal processes of the Yellow Cells project through the ganglion sheath to various parts of the surface of the visceral, parietal and pleural ganglia and can even terminate in the connective tissue sheath surrounding the pedal ganglia. Release of neurosecretory material also probably occurs from beneath the perineurium of the connectives joining the visceral–parietal and parietal–pleural ganglia as well as from the edges of nerves leaving the central ganglia, particularly the intestinal nerve. Neurosecretory material from all these sites would presumably collect in the blood system supplying the c.n.s., where it would be distributed generally to different parts of the body. No specific target organ is indicated from studies of these central release sites. We have not investigated in any detail the central release sites of the Yellow-green Cells but, from the work of Swindale & Benjamin (1976*a*), they are probably similar to those of the Yellow Cells.

Axons of the Dark Green Cells projected to the edge of the pleural ganglia close to the connective tissue sheath. These processes were few in number and did not occur in all cells. This was rather unexpected in view of the electron microscopy of Wendelaar Bonga (1970*a*), who described many Dark Green Cell terminals in the pleural ganglia and connectives leaving these ganglia but did confirm the results of Swindale & Benjamin (1976*a*), using Alcian

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Blue–Alcian Yellow-stained material, who estimated that only about 15–20 % of Dark Green Cell processes terminated in the c.n.s.

Arrangement of terminals in the central neurohaemal areas

Neurosecretory fibres in the *Lymnaea* neurohaemal areas either penetrate the connective tissue capsule surrounding the c.n.s. or project to the edge of a nerve of connective beneath the perineurium. Extraganglionically projecting fibres occur in Yellow Cells and Caudodorsal Cells (shown in the present study) as well as the Yellow-green Cells (Swindale & Benjamin 1976*a*). No such fibres were seen in the Dark Green Cells. We presume that neurosecretory material can be released from neurosecretory fibres into the perineurium from both locations and this is supported by the electron microscopical evidence of Wendelaar Bonga (1970*a*). One point of difference between this study and the previous one by Wendelaar Bonga (1970*a*) concerns the Dark Green Cells. They were reported to have axons that run through the connective tissue sheath, but in the present study no Dark Green Cells axons were found that project beyond the edge of the neuropil.

It is not clear why the location of neurosecretory fibres in *Lymnaea* varies so much. At first sight it would seem an advantage to have extraganglionically projecting fibres because they would be closer to the blood vessels of the perineurium. However, none of the neurosecretory fibres within the perineurium directly innervate the blood vessels (Wendelaar Bonga 1970*a*) and neurosecretory material would have to travel through the amorphous ground substances of the connective tissue capsule whether fibres were located in the perineurium or beneath it. From this point of view both sites of release would be equivalent.

It is usually assumed in electron microscopy that release of neurosecretory material takes place from the terminals or nerve endings of neurosecretory cells in *Lymnaea* (Wendelaar Bonga 1970*a*; Boer *et al.* 1968). However, the longitudinal orientation of Light Green Cell and Caudodorsal Cell fibres in their neurohaemal areas has led us to suggest that an additional *en passant* type of release was occurring. A similar type of release could also be taking place from the lateral edges of the Yellow Cell and Yellow-green Cell axons within the connective tissue of the central sheath or from beneath the perineurium in the intestinal and other nerves of the parietal and visceral ganglia. That this type of mechanism is occurring in *Lymnaea* neurosecretory axons needs to be confirmed by electron microscopy.

Estimates of the numbers of Light Green Cell axons in the median lip nerve of *Lymnaea* have been made by Boer *et al.* (1968). The number of axons exceeded the number of cell bodies; so these authors suggested that the Light Green Cell axons must repeatedly divide in the lip nerve. However, we have found no branching of Light Green Cells in our Procion-injected material and, from the present work, we are unable to explain the data of Boer *et al.* A similar problem arises from another estimate made from electron microscopy. Wendelaar Bonga (1971*b*) estimated that each Caudodorsal Cell had on average 800 terminals in the cerebral commissure. The maximum number of terminals that we have found for a particular Caudodorsal Cell is ten, approximately two orders of magnitude lower than the estimates from electron microscopy. We think it possible that Wendelaar Bonga's estimate is exaggerated because of the *en passant* arrangement of the Caudodorsal Cell axons close to the connective tissue of the commissure. Transverse sections through the commissure would lead to an overestimate of the number of 'endings' if the same axon were repeatedly sectioned at various points along its length.

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Peripheral projections of the Yellow Cells and Yellow-green Cells

Figure 25 of Swindale & Benjamin (1976*a*) summarized the peripheral nerve projections of all the Yellow Cells from a single brain, based on serial section reconstructions of Alcian Blue–Alcian Yellow. While this technique gave a very good idea of the total distributions of axons, it did not allow the projections of Yellow Cells from particular locations to be determined with absolute confidence. A particular problem was the likely occurrence of Yellow-green Cells in the same nerves and the fact that Yellow Cell and Yellow-green Cell Alcian Blue–Alcian Yellow-stained axons have a similar appearance under the dark field illumination used for reconstructions. This meant that some of the fibres in the nerves of the visceral and parietal ganglia were probably from Yellow-green Cells as well as Yellow Cells. By injecting Yellow Cells with Procion Yellow, we could follow axons from Yellow Cells to peripheral nerves with confidence.

Neurones from the visceral Yellow Cell cluster projected to the intestinal nerve and internal right parietal nerve. Swindale & Benjamin (1976*a*) thought that Yellow Cells from this cluster also projected to the anal nerve, but we have no evidence for this either from the present morphological data or from electrophysiology (S. R. Soffe, unpublished results). Yellow Cells from the right parietal ganglia also project to the internal right parietal and intestinal nerves, but we have no data that suggest that pleural ganglia Yellow Cells have such nerve projections and their axons probably terminate exclusively in the central sheath. We have presented evidence that Yellow-green cells from the visceral ganglion project into the anal nerve, and it may well be that the small number of Alcian Blue–Alcian Yellow-positive fibres in this nerve are all Yellow-green axons. The Yellow Cells are known to have terminals in the ureter of the kidney (Wendelaar Bonga 1970*b*) and our data are entirely consistent with this electron microscope data, as the kidney is innervated by branches of the intestinal and internal right parietal nerves. Our attempts to follow the axons in these nerves all the way to the periphery with HRP have failed, although we did show axons from the visceral Yellow cluster projecting for long distances into the nerves.

Peripheral projections of the Dark Green Cells

Swindale & Benjamin (1976*a*) showed that the Dark Green Cells of *Lymnaea* had widespread peripheral projections along nerves that innervated the foot and mantle. These projections could be traced to the skin, where neurosecretory material is released non-synaptically from the edges of small nerves (Swindale & Benjamin 1976*b*). In contrast to the Yellow Cells, there is no evidence from the study of Swindale & Benjamin or the present work that the Dark Green Cells release neurosecretory material from nerves close to the c.n.s.

We found that the pleural ganglia Dark Green Cells always projected to the ipsilateral pedal ganglia and nerves with, in some cells, additional projections to the nerves of the parietal and cerebral ganglia. This confirmed the results of Swindale & Benjamin (1976*a*), using the Alcian Blue–Alcian Yellow technique, but allowed us, in addition, to show the extent to which a particular cell contributed to the overall pattern of Dark Green Cell projections shown previously. Our results showed that the number of peripheral projections that the Dark Green Cells have varies considerably in different pleural ganglion cells. Some project to just the pedal ganglia and a single pedal nerve, and others project to the pedal ganglia and a parietal nerve, whilst the most complicated cell that we have reconstructed had axons in two pedal nerves, one parietal nerve and one cerebral nerve.

Intraganglionic morphology and relationships to electrophysiology

We have used the work 'dendrite' as a convenient descriptive term for the fine lateral branching that originated from main axons and terminated in the neuropil of the central ganglia Such processes were found in the proximal axonal regions of all six neurosecretory cell type. in *Lymnaea* and occurred on more distal axonal segments in the Yellow Cells and Dark Green Cells. We emphasize that there is no conclusive proof in gastropod molluscs that such fine processes are the sites of synaptic interaction between neurones, although there is some evidence from electron microscopy (Gorman & Mirolli 1970), Golgi studies (Benjamin & Ings 1972) and cobalt chloride injections (Winlow & Kandel 1976) indicating that the proximal dendritic branches are likely sites for the location of synapses. Roubos (1973, 1975) has examined several types of *Lymnaea* neurosecretory neurone in the electron microscope and found synapses on the Light Green Cells and Caudodorsal Cells but not on the Dark Green Cells. The location of the presynaptic terminal in the Caudodorsal Cells and Light Green Cells was on the cell bodies and proximal parts of the axons, but it was not clear in the latter case whether they were present on the main axon or dendritic processes. The small size of the fibres illustrated suggests that synapses may well have been on the dendrites, but this requires further confirmation. The terminals described by Roubos were all presynaptic on the neurosecretory neurones and this fits in with electrophysiological data, which show that a number of the cell types are modulated by synaptic inputs from outside the population of neurosecretory neurones. Benjamin & Rose (unpublished data) have recorded inhibition of long duration in the Light Green Cells and a similar type of input has also been seen in the Canopy Cells (Benjamin *et al.* 1976). Excitatory synaptic input has been recorded on the Yellow Cells and Dark Green Cells (Benjamin 1978; Benjamin *et al.* 1976). The last-mentioned input contrasts with the apparent absence of morphological synaptic structures on the Dark Green Cells (Roubos 1973), but such synapses might well be difficult to find if they are located on the dendritic processes described in the present study, which were some distance from the cell body.

The synaptic inputs described in the last paragraph were all probably of the conventional chemical type arising from non-neurosecretory interneurones in the c.n.s. Synaptic junctions of the electrotonic type were found to connect neurosecretory neurones in *Lymnaea*. These always occur between neurosecretory neurones of the same type, e.g. the Canopy Cells, Light Green Cells and Dark Green Cells (Benjamin *et al.* 1976). By injecting two cells at a time with Procion Yellow, we have discovered the likely sites for these electrotonic junctions between Canopy Cells and between Dark Green Cells. Thus the axons of the Canopy Cells are closely apposed within the cerebral commissure and the axons of the Dark Green Cells in the pleural ganglia, pleuro-pedal connectives and pedal ganglia. Other recently obtained electrophysiological data (Benjamin & Rose, unpublished) showed that the Caudodorsal Cells are also electrotonically coupled and our present results show that the axons of these cells can have points of contact in the cerebral commissure.

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Key to symbols and abbreviations used on figures

A	anterior	l.pd.g.	left pedal ganglion
an.n.	anal nerve	l.pl.g.	left pleural ganglion
a.s.	anterior surface	m.d.b.	mediodorsal body
b.	blob	med.lp n.	median lip nerve
c.c.	cerebral commissure	med.pd.n.	medial pedal nerve
cl.n.	columellar nerve	n.	fine dendritic branch
cu.pa.n.	cutaneous pallial nerve	nu.n.	nuccal nerve
D	dorsal	o.n.	optic nerve
d.	dendrite	pe.n.	penis nerve
d.s.	dorsal surface	p.s.	posterior surface
e.r.pa.n.	external right parietal nerve	r.cr.g.	right cerebral ganglion
g.n.	genetal nerve	r.pa.g.	right parietal ganglion
inf.cv.n.	inferior cervical nerve	r.pd.g.	right pedal ganglion
inf.pd.n.	inferior pedal nerve	r.pl.g.	right pleural ganglion
int.n.	intestinal nerve	sup.cv.n.	superior cervical nerve
i.r.pa.n.	internal right parietal nerve	sup.lp n.	superior lip nerve
la.d.b.	laterodorsal body	sup.pd.n.	superior pedal nerve
la.lo.	lateral lobe	t.	terminal
l.cr.g.	left cerebral ganglion	t.n.	tentacle nerve
l.pa.g.	left parietal ganglion	v.g.	visceral ganglion
l.pa.n.	left parietal nerve	v.s.	ventral surface

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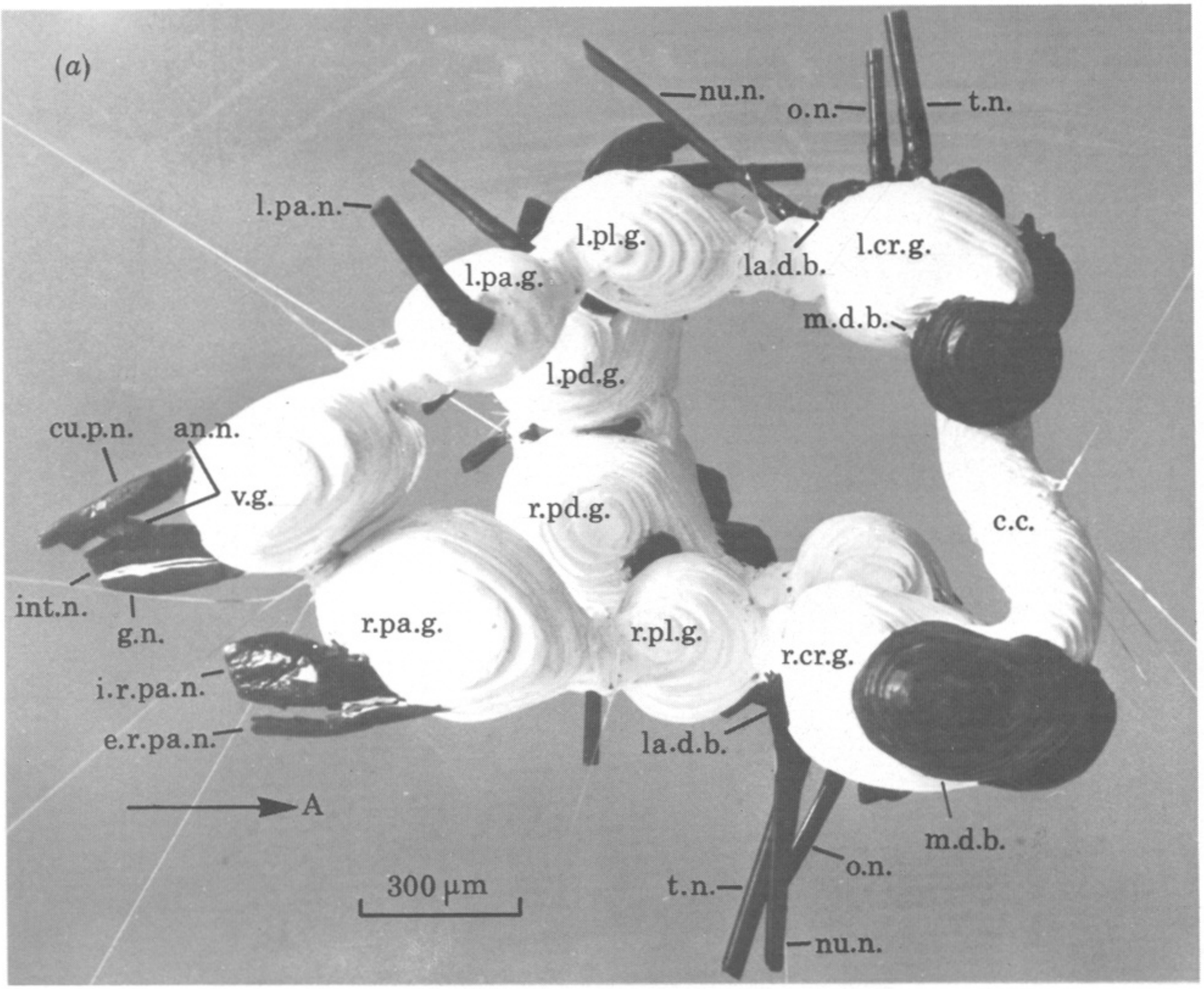
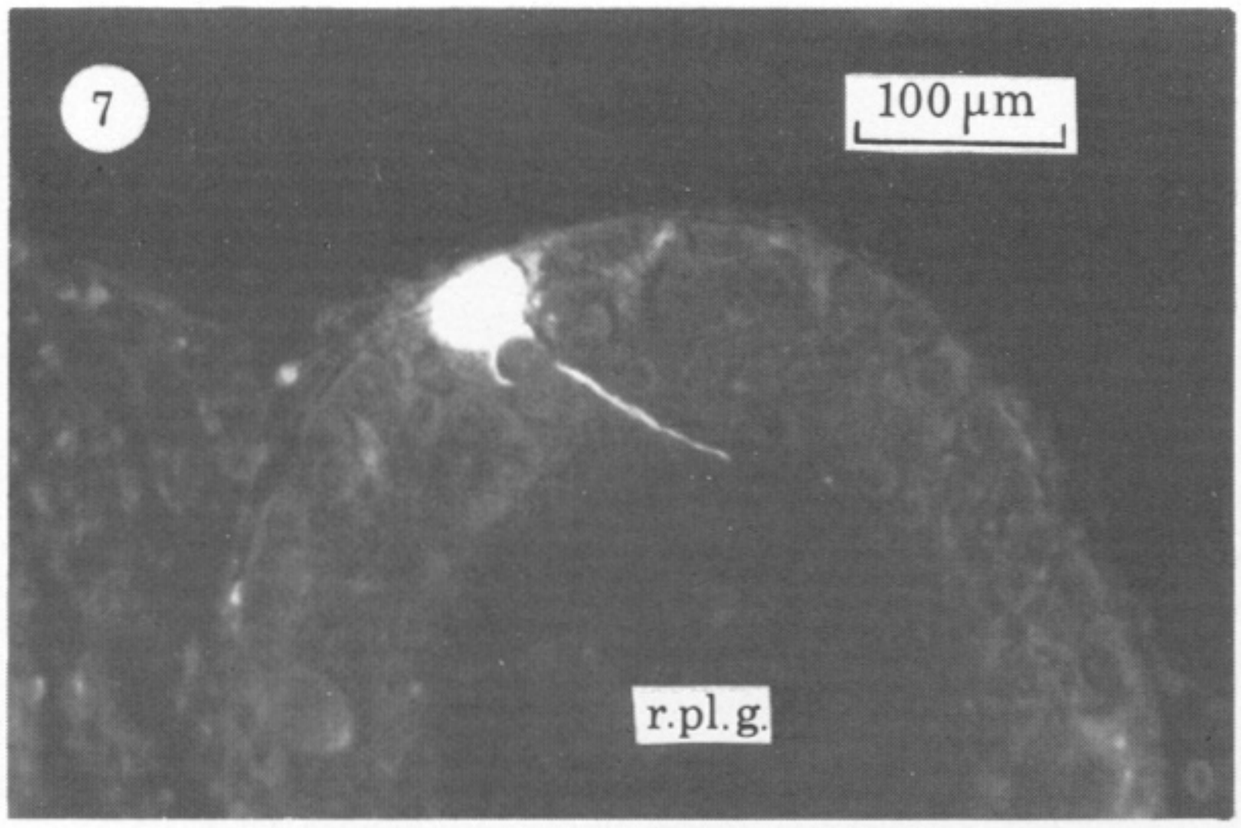
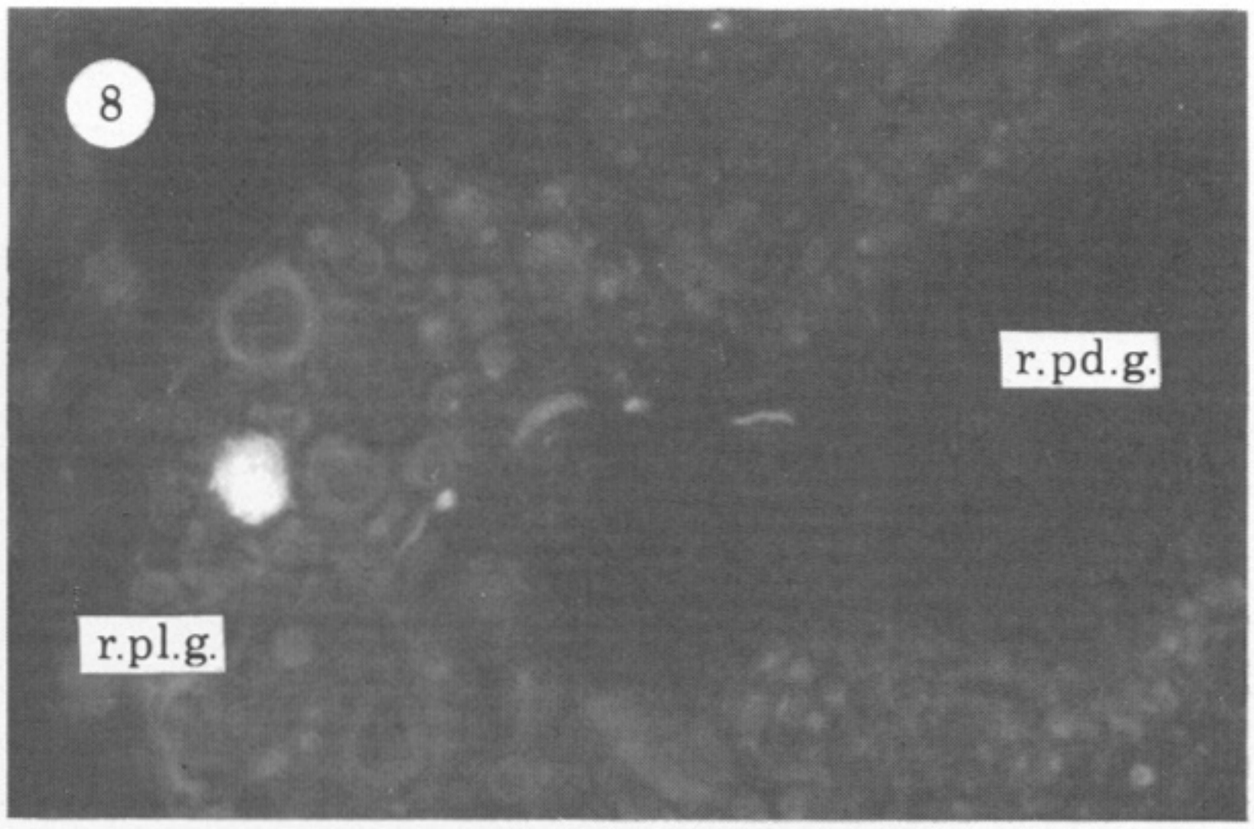
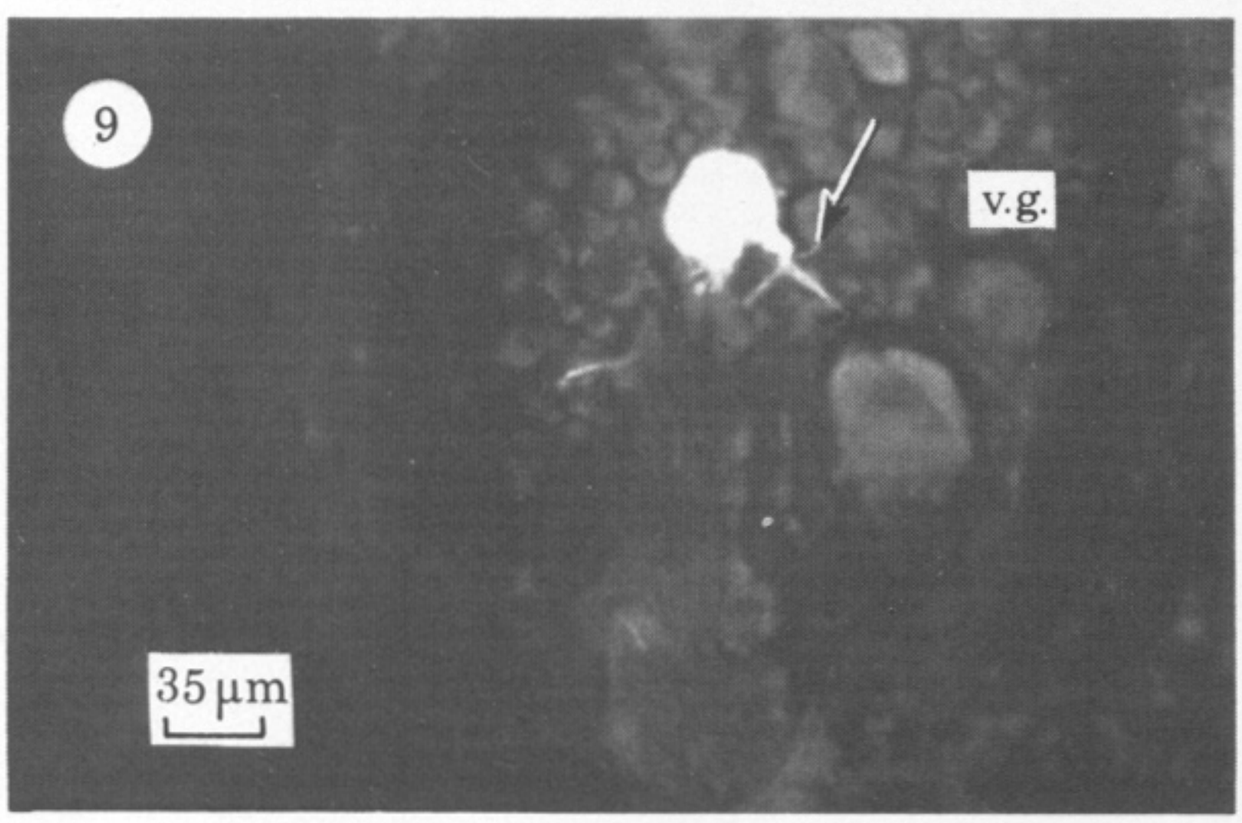
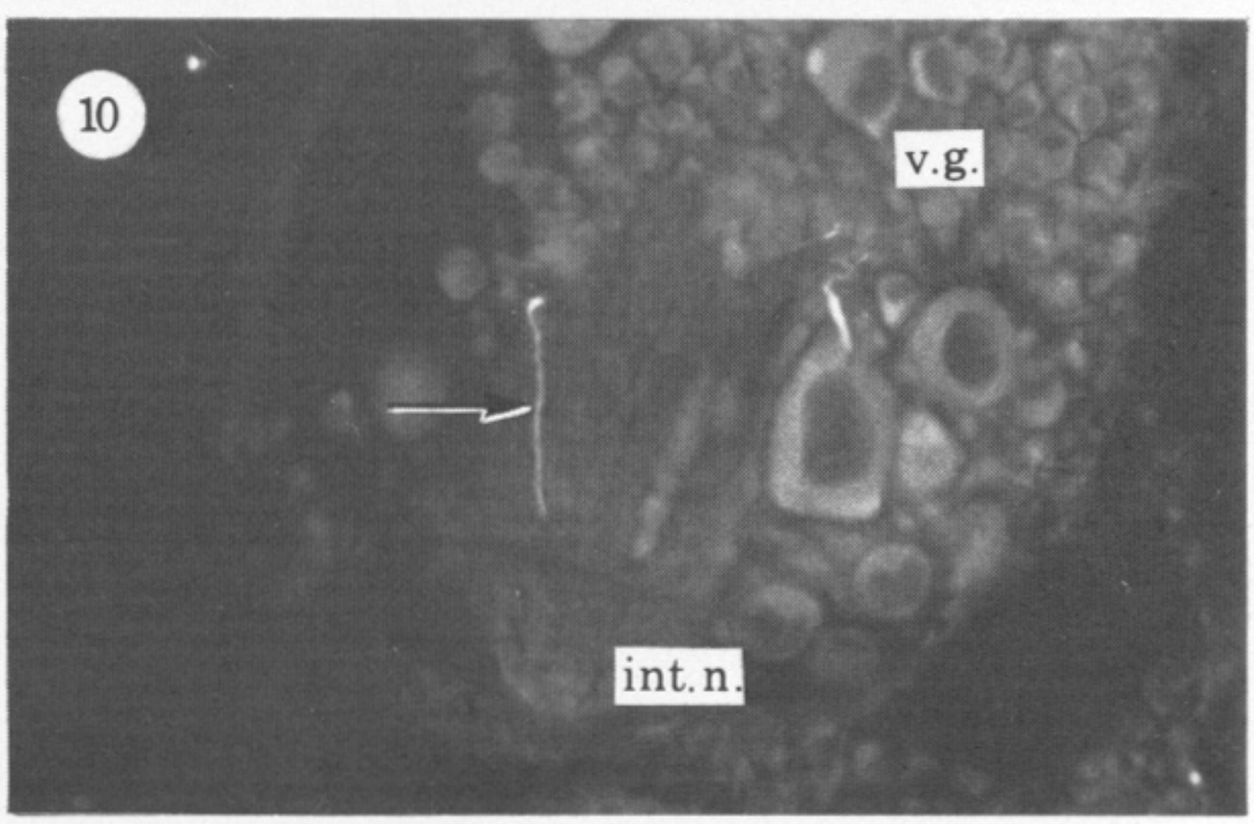


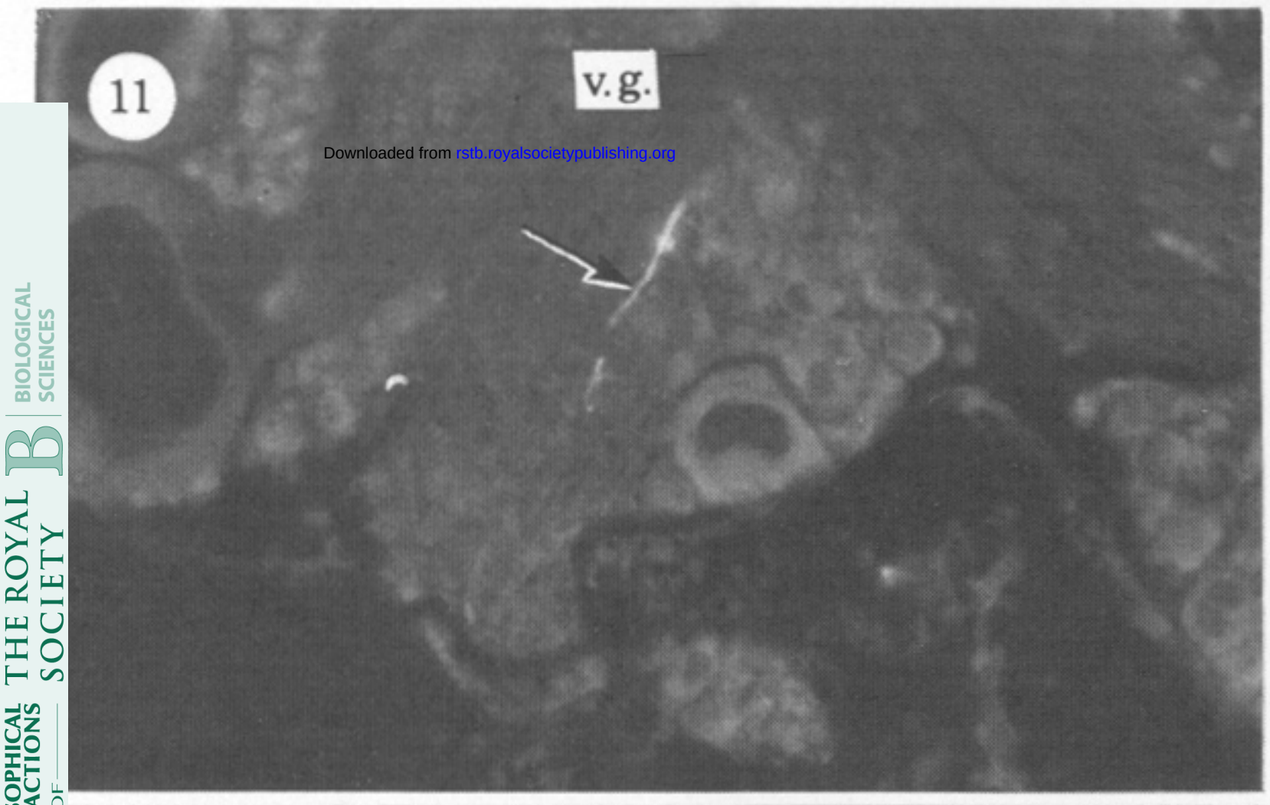
FIGURE 2

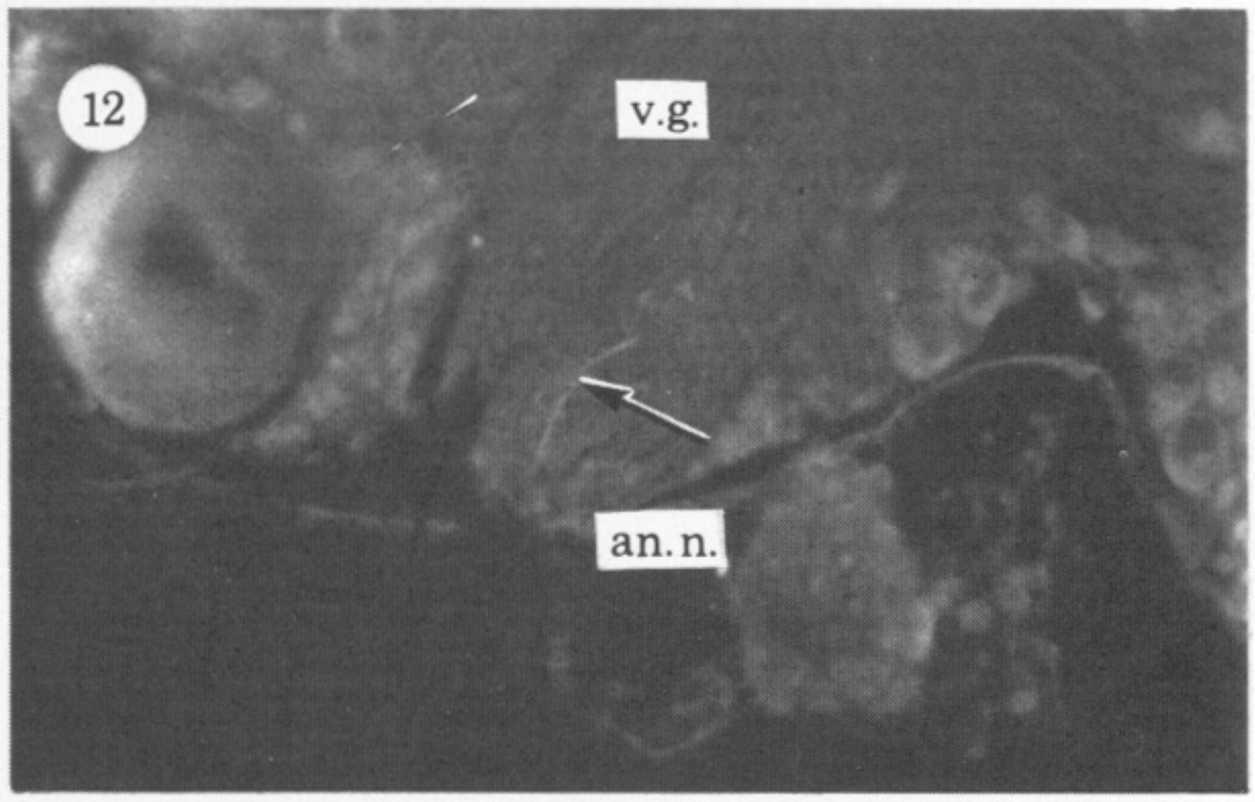


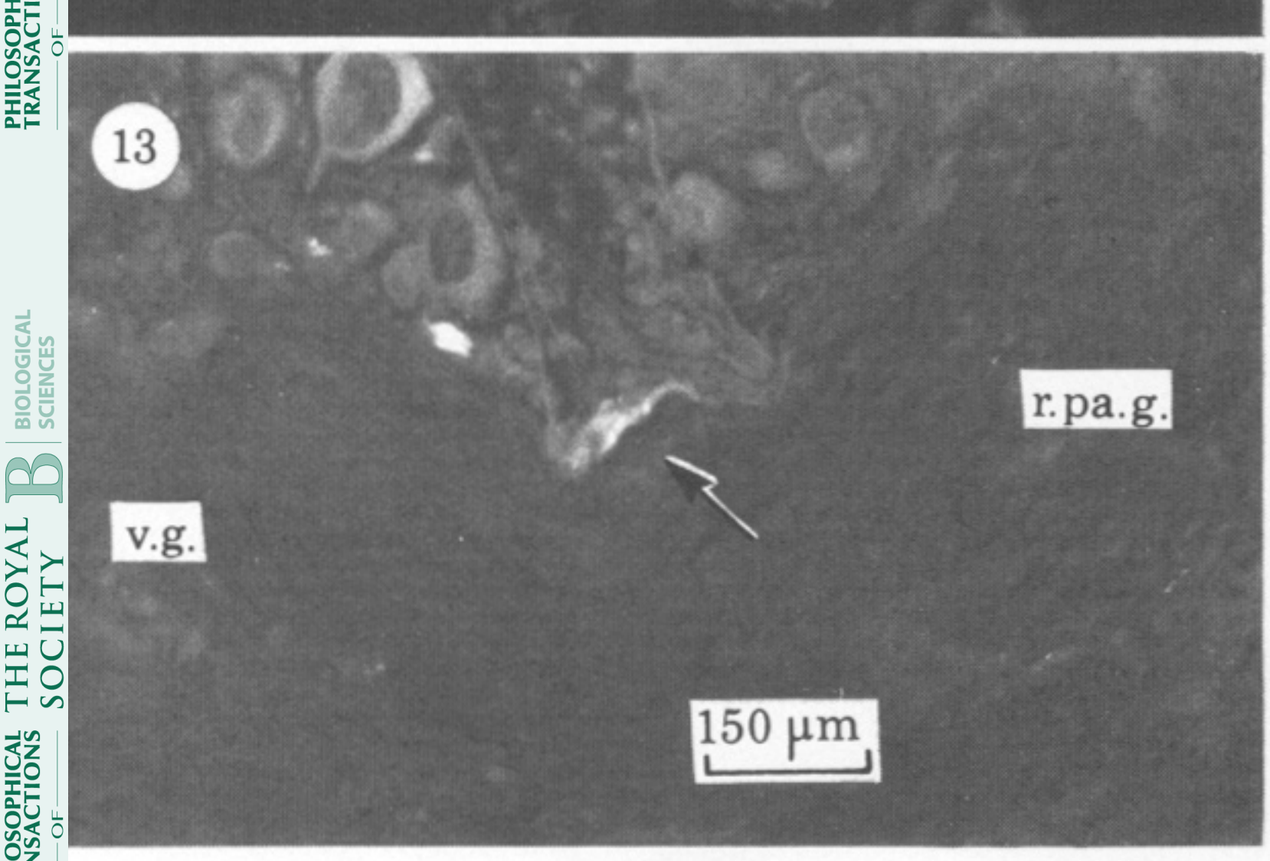


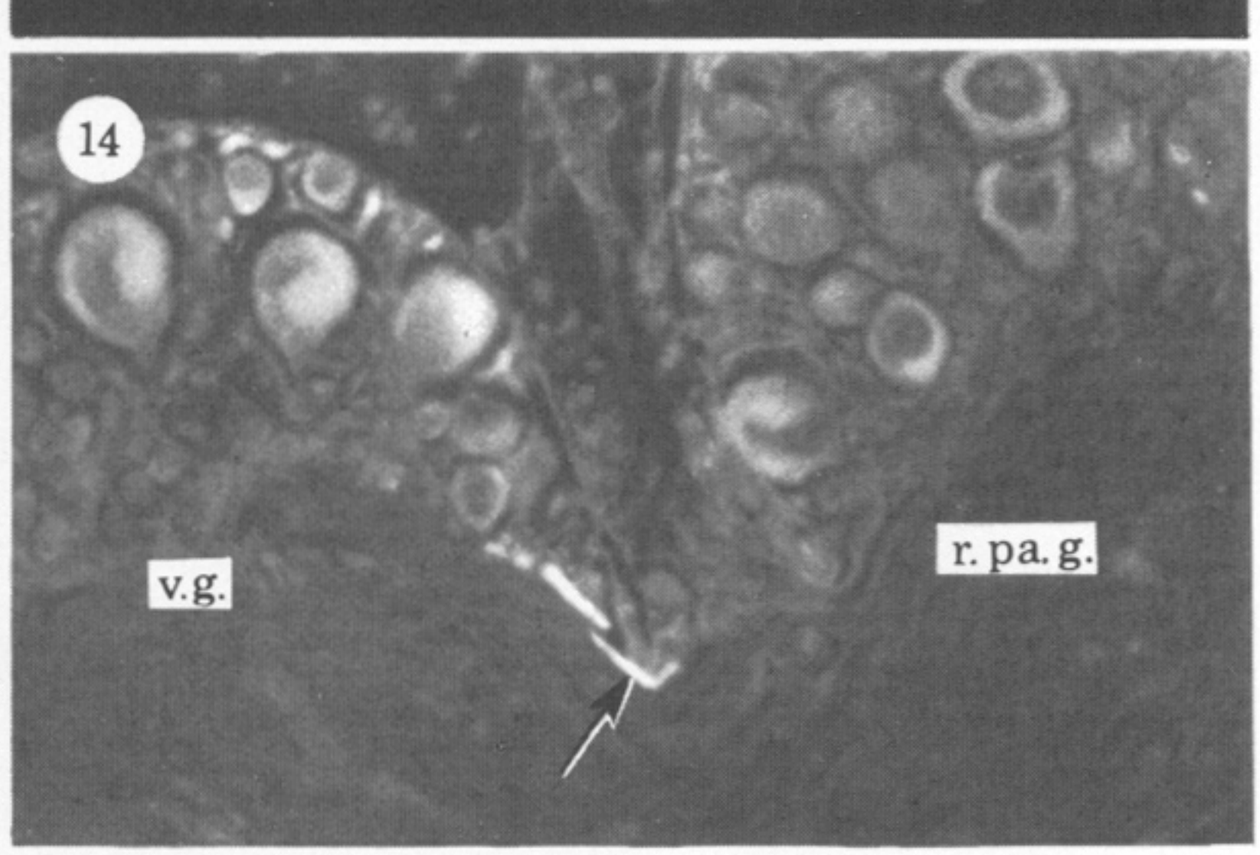




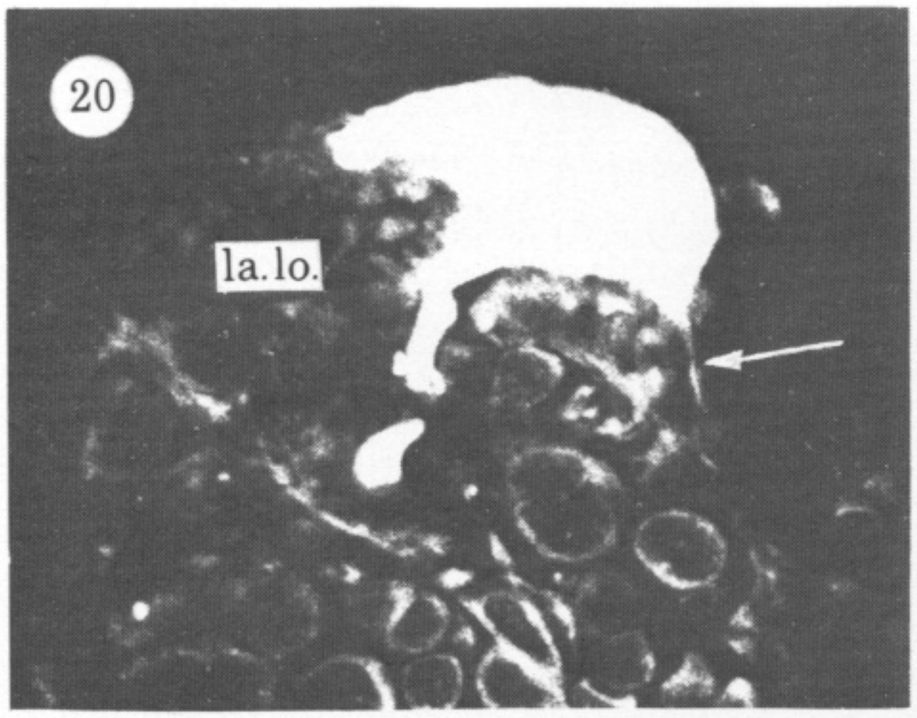


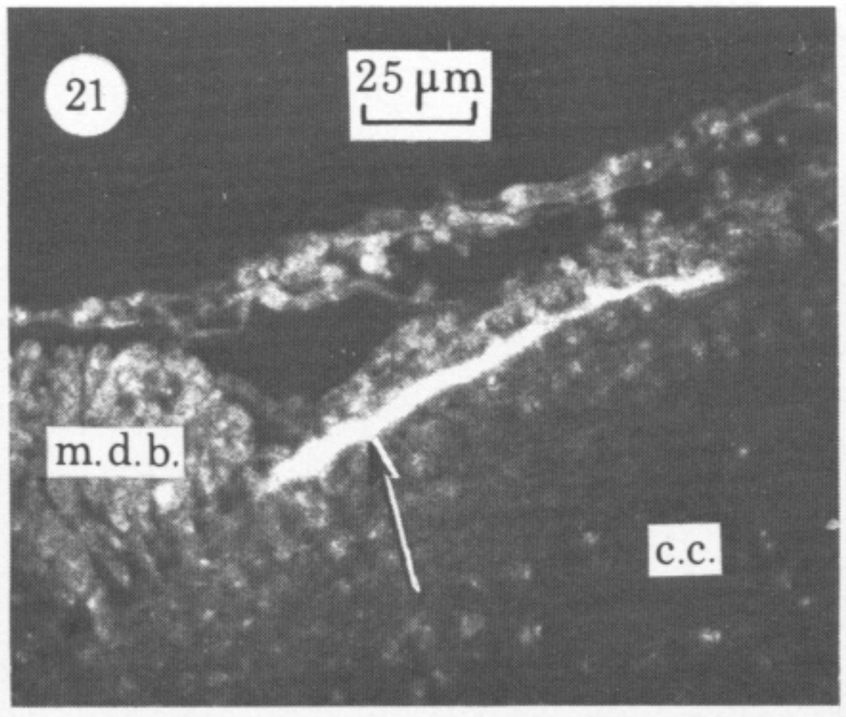


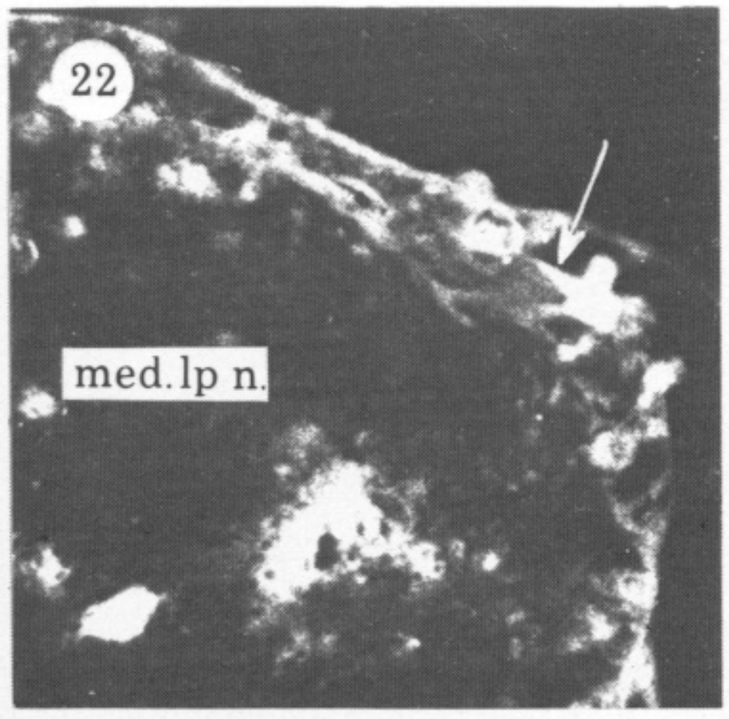


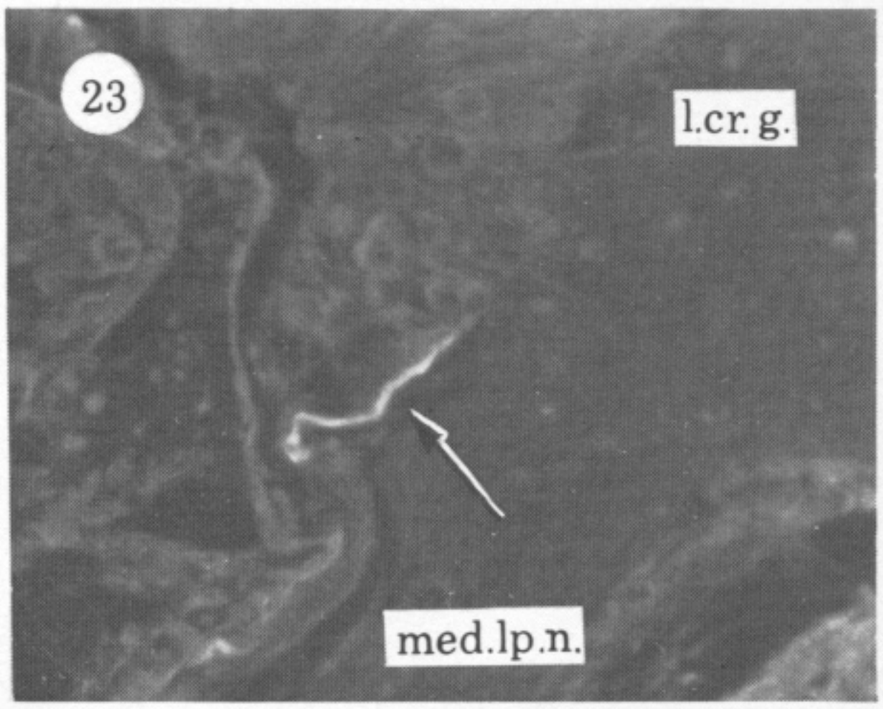


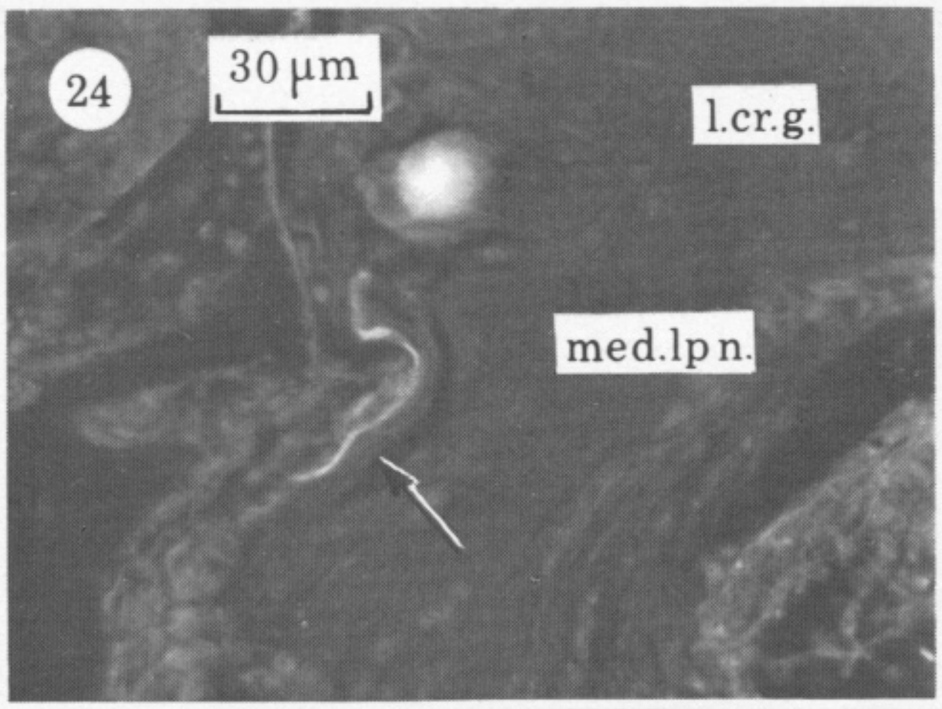
FIGURES 7–14

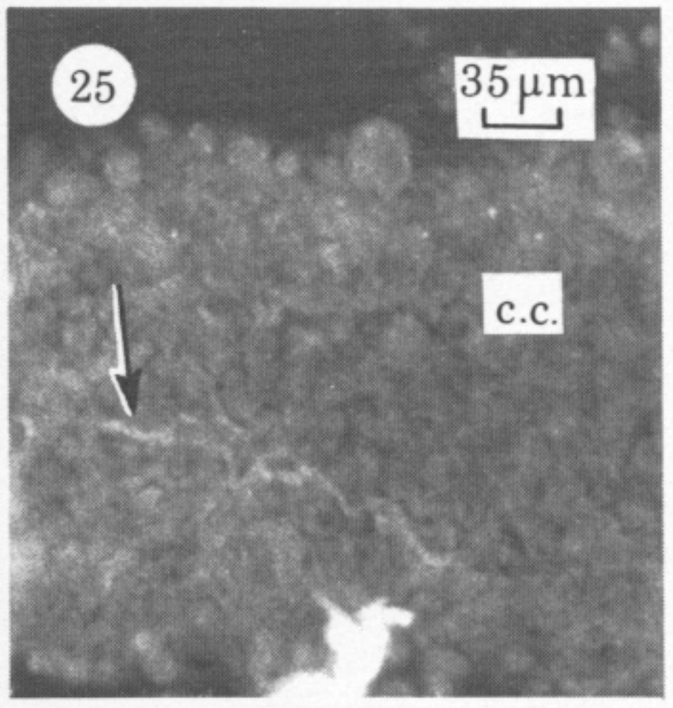


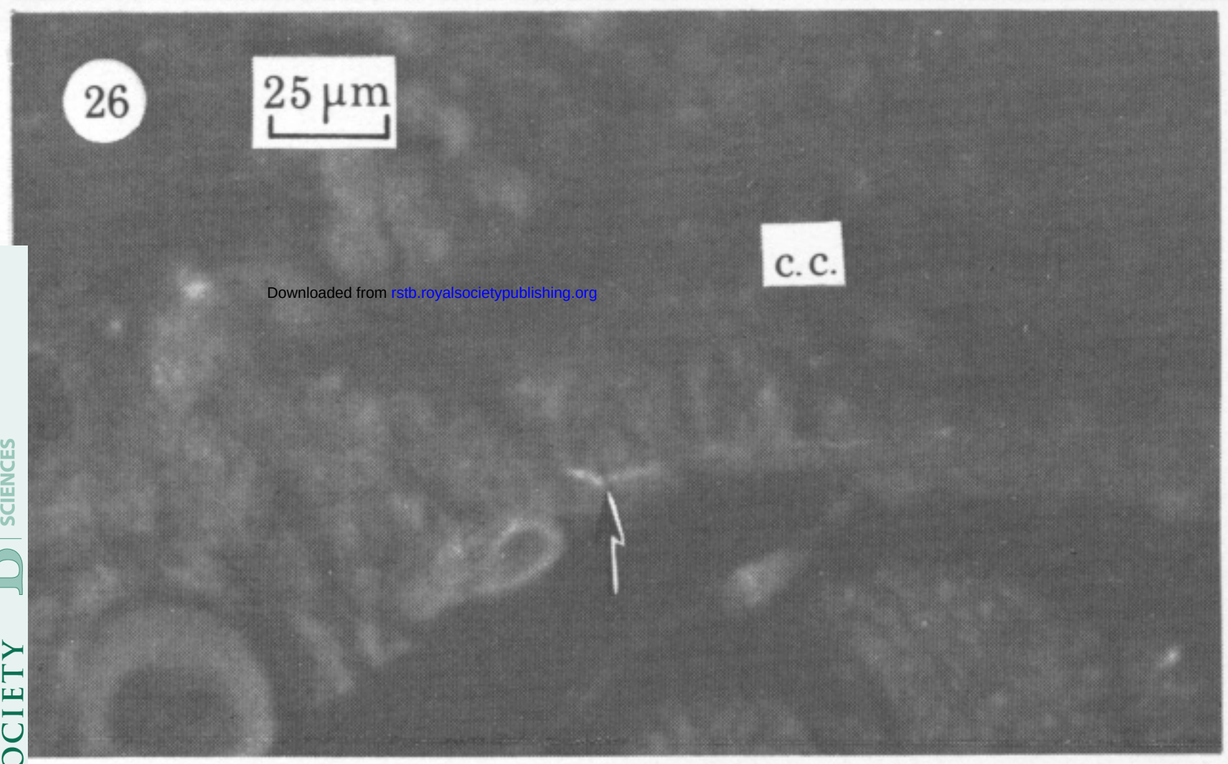


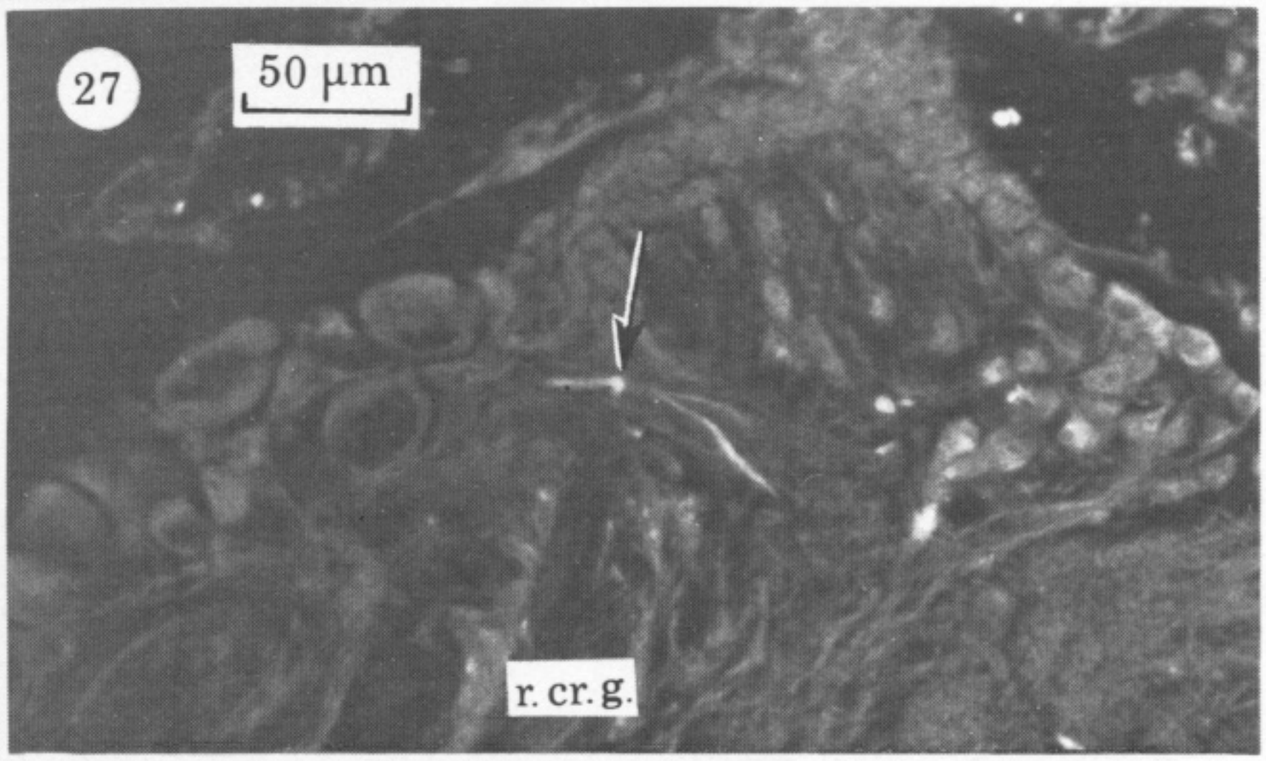


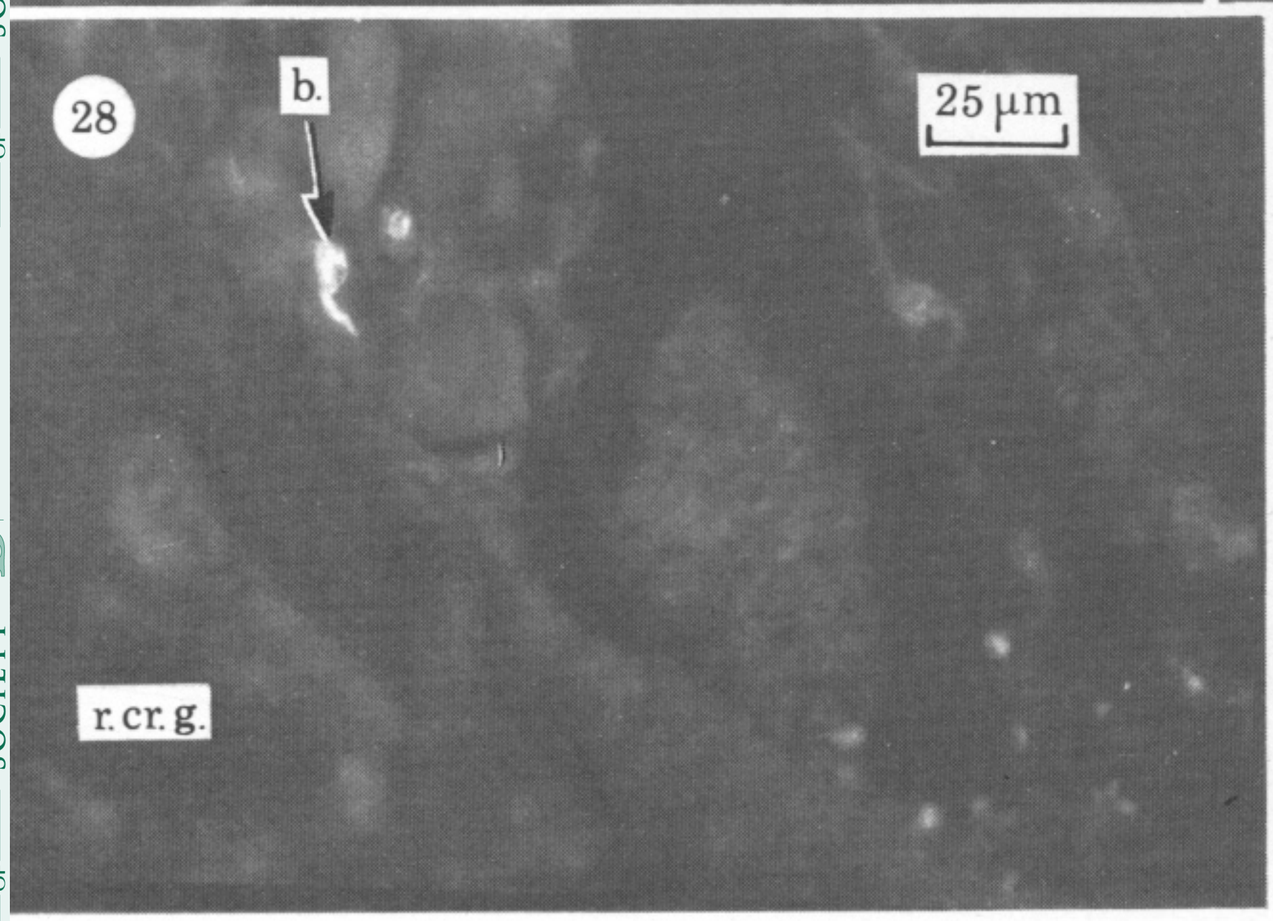


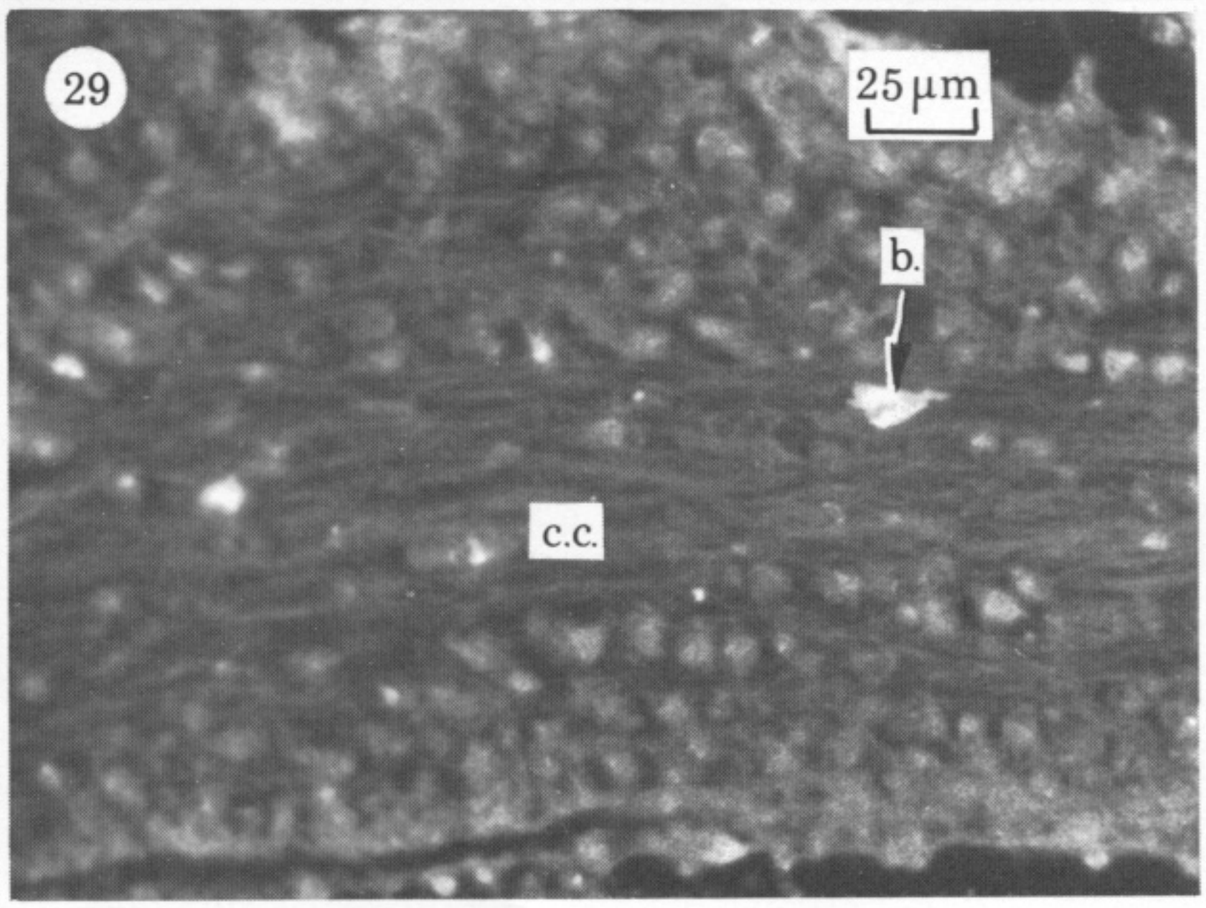












FIGURES 20–29