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THE ANATOMY OF NEUROSECRETORY NEURONES IN THE POND SNAIL *LYMNAEA STAGNALIS* (L.)

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CONTENTS

The anatomy of three neurosecretory cell types in the central nervous system (c.n.s.) of the gastropod mollusc *Lymnaea stagnalis* (L.) – the Dark Green Cells, Yellow Cells and Yellow-green Cells – has been studied by using bright and dark field illumination of material stained for neurosecretion by the Alcian Blue–Alcian Yellow technique. The neuronal geometry of single and groups of neurosecretory cells of the various types has been reconstructed from serial sections, and the likely destination of most of their processes has been determined.

Dark Green Cells are monopolar, occur exclusively within the central nervous system (c.n.s.), have few or no branches terminating in neuropile, and send axons to the

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surface of the pleuro-parietal and pleuro-cerebral connectives. The majority of Dark Green Cell axons however (80–85%), project down nerves which innervate ventral and anterior parts of the head-foot, the neck and the mantle. Dark Green Cell axons can be found in small nerves throughout these areas, and may terminate in a fine plexus of axons on the surfaces of the nerves. Since previous experimental work has shown that the Dark Green Cells are involved in osmotic or ionic regulation, these results suggest that the target organ of the Dark Green Cells may be the skin.

Yellow Cells occur both within and outside the c.n.s. They are usually monopolar, but can be bipolar. They have several axons which normally arise separately from a single pole of the cell body, or close to it. One or more processes leave the cell proximal to the point where separate axons arise, and may run unbranched for some distance through neuropile before terminating in fine branches and blobs of various sizes. These branches may release hormone inside the c.n.s.

Yellow-green Cells are mono-, bi- or multi-polar, and like the Yellow Cells are found both within and outside the c.n.s. Some Yellow-green Cells, though not all, have projections which terminate in neuropile in fine branches and blobs. Yellow-green Cell bodies which occur in nerves can project back along the nerve into the c.n.s.

The axons of Yellow Cells and Yellow-green Cells project to release sites in various ways. Some project into the connective tissue sheath of the c.n.s., which serves as a neurohaemal organ, either directly through the surface of a ganglion, or from the pleuro-cerebral or pleuro-parietal connectives. Other axons leave the c.n.s. via nerves leaving the left and right parietal and visceral ganglia; projections into the intestinal, anal, and internal right parietal nerves being most numerous. Axons which may be from either, or both Yellow Cells and Yellow-green Cells, can be found along the entire unbranched lengths of these nerves, and in subsequent branches which innervate organs lying in the anterior turn of the shell. All of these organs are closely associated with the lung cavity. The pattern of release of hormone which this arrangement implies may have been adopted to ensure a rapid distribution of hormone throughout the circulation following release, or to increase the concentration of hormone in blood flowing through target organs such as the kidney, lung walls or the heart.

Introduction

In this paper we describe the anatomy of three types of neurosecretory neurone found in the central ganglia of the freshwater pulmonate snail *Lymnaea stagnalis* (L.) Several neurosecretory cell types have been identified in the central nervous system (c.n.s.) of *Lymnaea*, by using the Alcian Blue–Alcian Yellow stain for neurosecretion (Wendelaar Bonga 1970*b*). In the electron microscope the different cell types have been shown to contain elementary neurosecretory granules of characteristic size and ultrastructure, and this information has been used to identify processes of the different cell types in neurohaemal areas in the vascular connective tissue sheath surrounding the c.n.s., on the surfaces of connectives and commissures between the ganglia, and on the proximal surfaces of many of the nerves leaving the ganglia (Wendelaar Bonga 1970*b*). The three cell types we have studied are defined on the basis of their reaction to the Alcian Blue–Alcian Yellow stain, and are accordingly known as the Dark Green Cells, Yellow Cells and Yellow-green Cells. There is evidence that the Dark Green Cells and Yellow Cells play a part in the physiology of ion and water exchange, since the secretory activity of these cell types, but not that of the Yellow-green Cells, is increased when animals are kept in de-ionized water, and decreased when animals are kept in hypertonic saline solutions (Wendelaar Bonga 1970*a*, 1971, 1972; Roubos 1973). It has also been suggested that the Yellow Cells are involved in kidney function since axon endings containing elementary neurosecretory granules of the Yellow Cell

type have been found in nerves running adjacent to kidney epithelium (Wendelaar Bonga 1970*a*, 1972).

The work described in this paper was carried out in order to further characterize the Dark Green Cells, Yellow Cells and Yellow-green Cells in terms of their axonal morphology, and by mapping their axons to known or probable neurohaemal areas to provide better evidence than that already available that these cell types are neurosecretory. Staining techniques provide only suggestive evidence that cells are neurosecretory (see, for example, Bern 1966), and even the presence of large numbers of elementary 'neurosecretory' granules in the cell body does not indicate that the cell concerned is functionally neurosecretory i.e. that it releases its secretory product as a hormone into the bloodstream. Identification in the electron microscope of neurohaemal areas of particular cell types on the basis of morphological similarities between elementary neurosecretory granules in the cell body, and in axons in neurohaemal areas, as Wendelaar Bonga (1970*b*) has done for the Dark Green Cells, Yellow Cells and Yellow-green Cells, is problematic, because elementary neurosecretory granules may change in size as they pass down the axon and, secondly, the granules observed in axon endings in neurohaemal areas could originate from other unidentified cell types. By directly tracing the projections of Alcian Blue–Alcian Yellow stained neurones in the light microscope, we hoped to show that the cells project to the neurohaemal areas described by Wendelaar Bonga, and thus to provide strong suggestive evidence that the cells release their secretory products into the bloodstream. Morphological differences between the cell types would also validate the Alcian Blue–Alcian Yellow stain as a method of distinguishing between cell types which are probably also functionally distinct.

Results were obtained by using the Alcian Blue–Alcian Yellow stain, in conjunction with bright and dark field illumination (Swindale & Benjamin 1975). With this technique it has been possible to make detailed reconstructions of cellular geometry from serial sections, and to determine the destination of most of the axonal processes of the cells. The results have shown that there are morphological differences between the cell types, and while they largely confirm the previous description of the neurohaemal areas of the cells given by Wendelaar Bonga, they show that all three cell types additionally project to peripherally located neurohaemal areas, including large regions of the body wall and mantle, and the pallial complex of organs located nside the anterior turn of the shell.

Materials and methods

Specimens of *Lymnaea stagnalis* (L.), supplied by Gerrard & Haig Ltd, were used. They were kept in the laboratory in tanks of aerated tap water at 12–18 °C for up to two months and fed on lettuce. Observations were made on animals varying in mass from 0.3 to 8 g; for reconstructions of neuronal geometry, adult specimens of mass 3–6 g (30–45 mm shell length) were used.

The c.n.s., which is a compact collection of 9 ganglia (figure 1) surrounded by a thin inner capsule and a thick outer vascular connective tissue sheath, was dissected from animals with the shell removed, under a physiological saline solution. Short lengths of nerve (2–5 mm) were left attached to the ganglia. Most of the brains on which anatomical work was carried out had also been examined electrophysiologically. This involved treating the ganglia with a protease enzyme (Type V, Sigma, London) and some dissection of the outer sheath before fixation. The connectives between the ganglia were also subjected to varying degrees of stretch for up to 2 h

during experiments. Although some ganglia were fixed immediately after dissection, the fixation of these was rarely as good as in those treated with protease, and rarely good enough for morphological work. Possibly the protease treatment speeds penetration of the fixative through the sheath (cf. Pease 1964, p. 28).

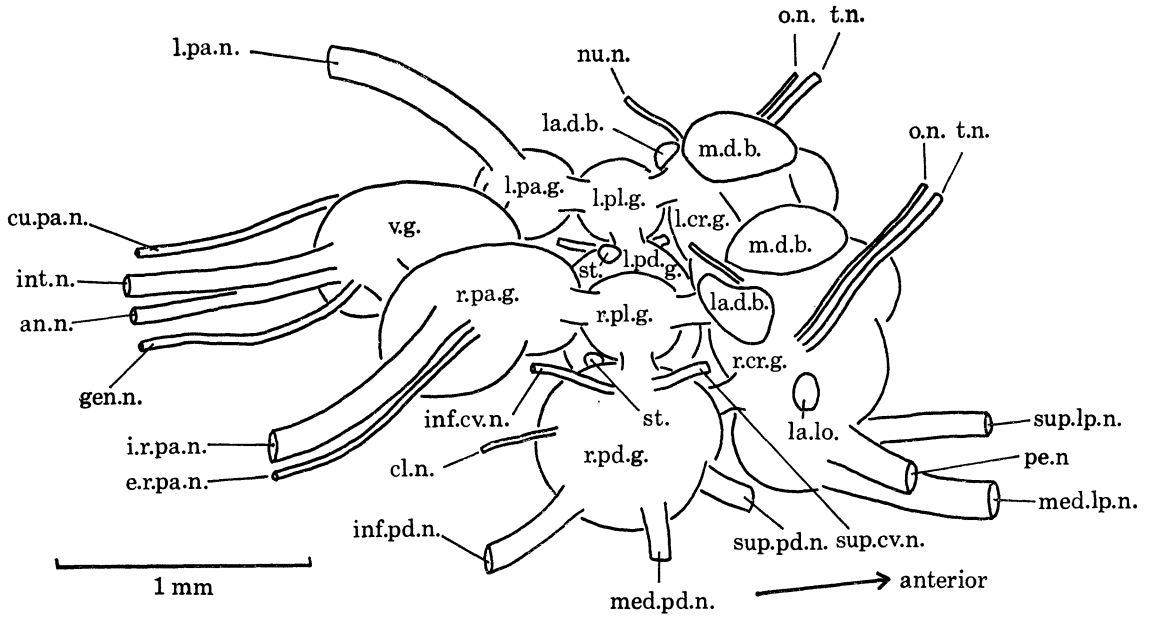


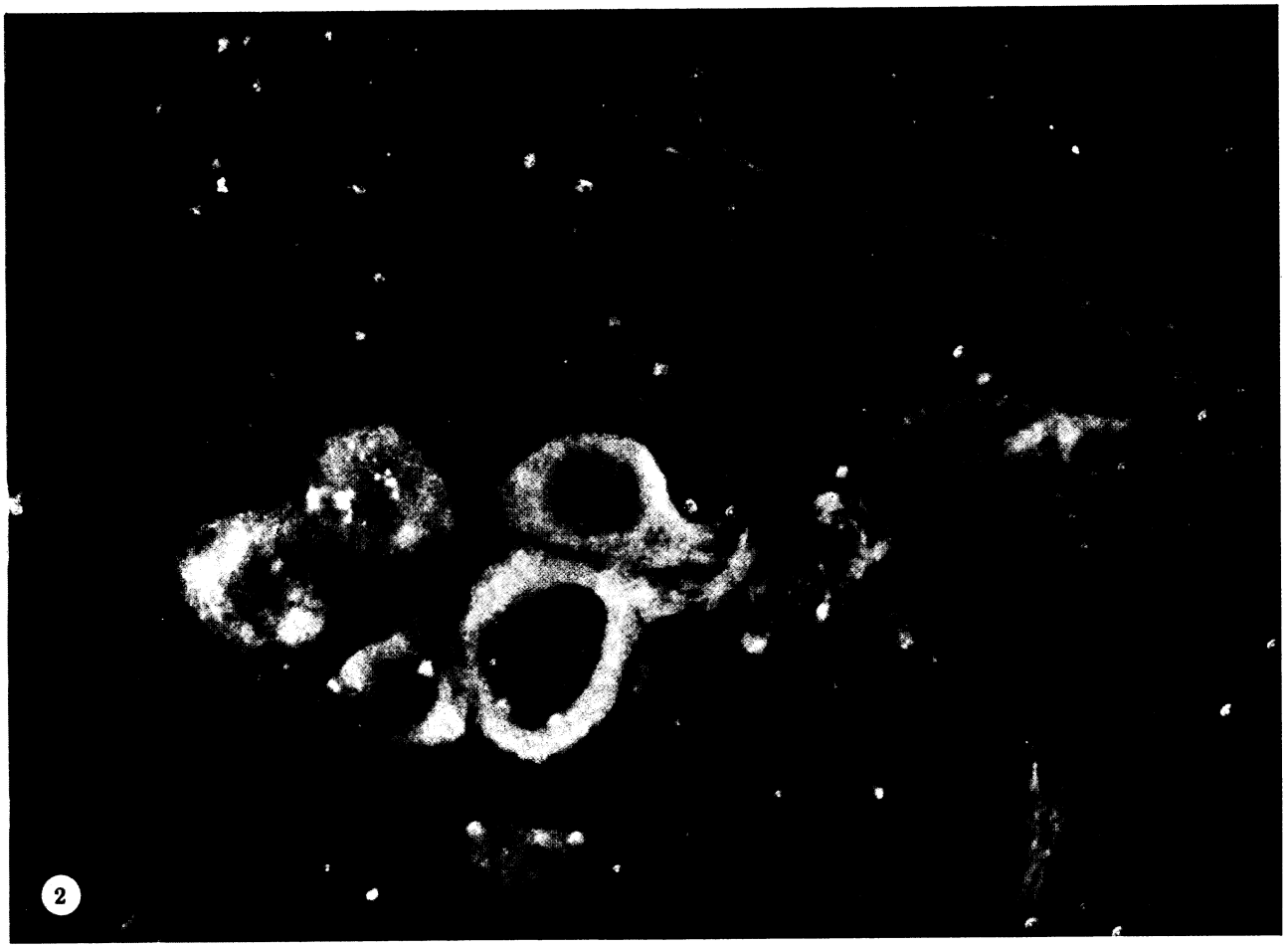
FIGURE 1. The nine ganglia which form the c.n.s. of *Lymnaea stagnalis*, seen from the right hand side. The two pedal ganglia are further apart than in life, when they are joined by a dorsal and a ventral commissure. The nerves leaving the cerebral and pedal ganglia are the same on both sides, with the exception of the penis nerve which only occurs on the right cerebral ganglion. The position of the c.n.s. in the animal is indicated in figure 4. The scale bar is appropriate to a 3–4 g animal. For key to abbreviations see p. 202.

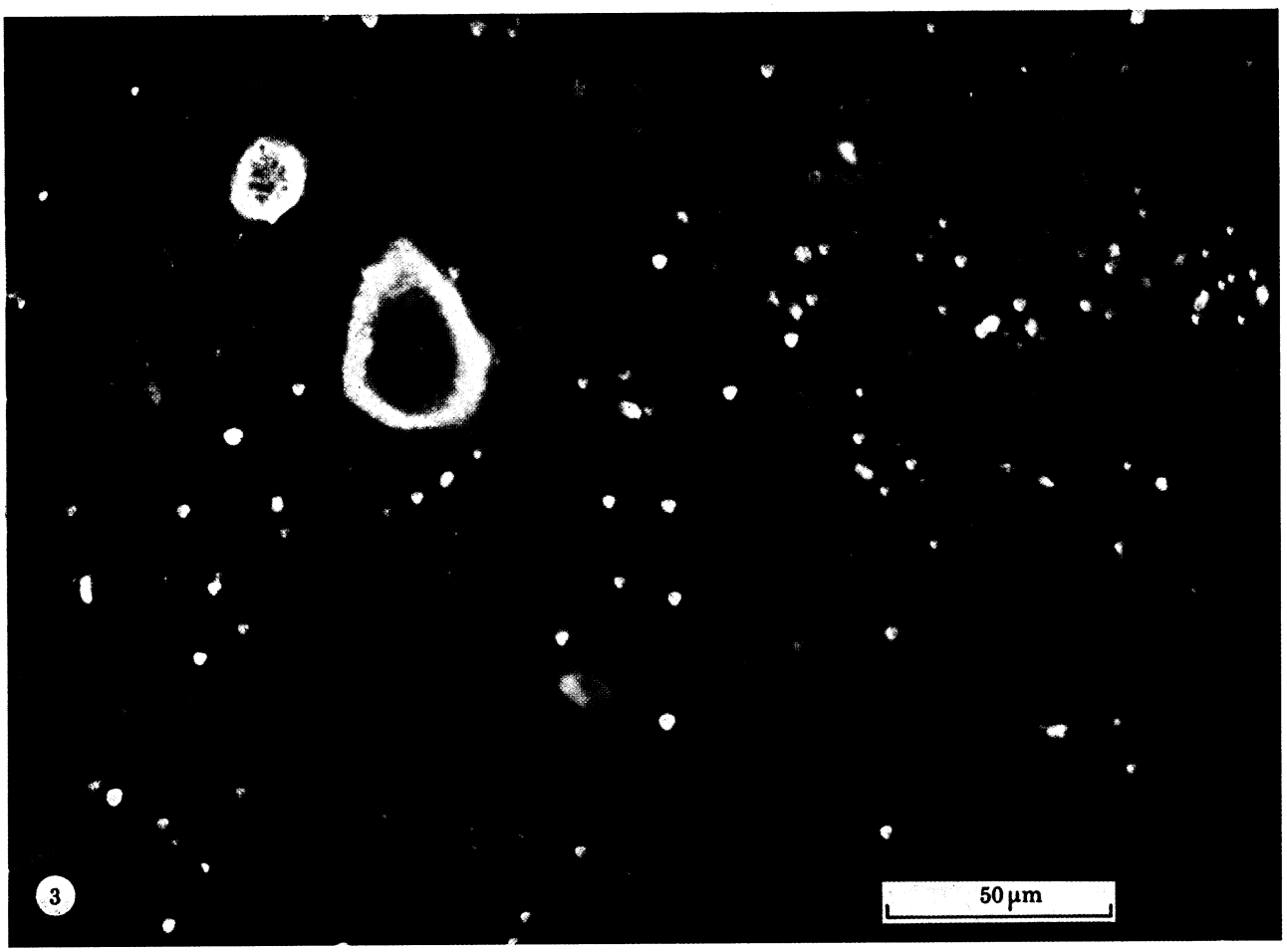
For investigations on the peripheral projections of the Dark Green Cells, portions of tissue of varying size were dissected from the mantle and foot. Tissues from the foot often had the whole c.n.s. or the pedal ganglia left attached. The peripheral projections of the Yellow Cells and Yellow-green Cells were studied by dissecting out the visceral and right parietal ganglia in which Yellow Cell and Yellow-green Cell axons run, to within a few millimetres of their presumed distal terminations. The nerves were cut proximally, close to the c.n.s. Small pieces of innervated tissue were left attached to some of the branches. The nerves, thus isolated from the animal, were pinned flat in a small dissecting dish, and fixed and embedded *in situ*.

DESCRIPTION OF PLATE 1

FIGURE 2. Dark field illumination of a horizontal section through the visceral ganglion stained with Alcian Blue–Alcian Yellow. This shows cell bodies and axons of the large group of Yellow Cells which occur in the anterior part of the ganglion, close to the right parieto-visceral connective. Axons leading to, or from, the left parietal ganglion can be seen in the top half of the picture; two axons enter the right parieto-visceral connective in the lower, right hand part of the picture, while axons running to the right are bound for the intestinal and anal nerves.

FIGURE 3. Dark field illumination of a horizontal section through the c.n.s., showing the cell body of one Dark Green Cell in the visceral ganglion. Axons from it, and other Dark Green Cells in the ganglion, run to the left parieto-visceral connective (on the left of the picture) and to the visceral nerves and to the right parietal ganglion (to the right of the picture). One of the axons has a swelling which is the same turquoise colour as the cell body. The scale bar, which is appropriate to figure 2 also, equals 50 μm.





FIGURES 2 AND 3. For description see opposite.

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After dissection (which was normally carried out between 12h00 and 17h00) tissues were fixed in Stieve's fixative for from ½ to 24 h, dehydrated in ethyl or methyl alcohol, cleared in toluene or chloroform, embedded in paraffin wax and serially sectioned at 15–30 μm. Sections were stained for neurosecretion by the Alcian Blue–Alcian Yellow method, after Wendelaar Bonga (1970*b*). The procedure we used is as follows: (1) bring sections to water; (2) oxidize in acid permanganate (2.5 % $KMnO_4$, 5 % H_2SO_4) for from 1½–4½ min and rinse in tap water; (3) bleach in a 2 % solution of sodium metabisulphite and rinse in tap water; (4) rinse in buffer (KCl–HCl) at pH 0.5–1.0; (5) stain in a 0.5 % solution of Alcian Blue 8GX (Gurr's) in buffer at pH 0.5–1.0, for 30–45 min; (6) rinse twice in buffer at pH 0.5–1.0; (7) stain in a 0.5 % solution of Alcian Yellow (Gurr's) in buffer (Sorensen's Glycine) at pH 2.5, for from 30–45 min; (8) rinse in buffer at pH 2.5, rinse in tap water; (9) counterstain in a 0.1 % solution of nuclear fast red for ½–1 min, rinse in distilled water, dehydrate quickly, clear, and mount in DPX (Gurr's).

Dark Green Cell morphology was studied in brains where the staining of Dark Green Cells was particularly intense. Reconstructions of the morphology of groups of Dark Green Cells in single ganglia were made from serial sections under bright field, using a *camera lucida* attached to a Leitz 'Orthoplan' microscope. Measurements of Dark Green Cell size were made by fitting a major and minor axis to the outline of the cell body under the *camera lucida*. Measurements were made of the largest profile found of each cell. The geometric mean of the two figures obtained was then cubed to give a figure with the dimensions of volume. For photography of Dark Green Cells under bright field, high contrast film (Microneg-Pan, Ilford) was used.

Yellow Cell and Yellow-green Cell anatomy was studied in serial sections of tissue stained with Alcian Blue–Alcian Yellow, using dark field illumination (Swindale & Benjamin 1975). This causes Yellow Cells (figure 2, plate 1) and Yellow-green Cells and their processes to be very clearly differentiated from the surrounding tissue, both cell types appearing bright yellow in colour. Dark Green Cells change colour under dark field and usually have turquoise cell bodies with purple axons (figure 3, plate 1). Stained material in Dark Green Cells, Yellow Cells and Yellow-green Cells consists of densely packed small granules $\leqslant 0.3\,\mu m$ in diameter, which are probably elementary neurosecretory granules or small clumps of them (Swindale & Benjamin 1975).

Because of the difficulty in tracing the processes of particular Yellow Cells and Yellow-green Cells through areas where stained material from many other cells was present, reconstructions of single cell anatomy had to be confined to cells which were isolated to some extent from other Yellow Cells and Yellow-green Cells, which often occur in compact groups. Reconstructions were made using a *camera lucida*, a ×40 objective, and a sub-stage dark field condenser (n.a. = 1.2) with water as the intervening medium between the condenser and the slide. Photography under dark field was with Pan F film (Ilford) or Photomicrography colour film (Kodak).

Our nomenclature of the c.n.s. and nerves is an anglicized version of that used by Hekstra & Lever (1960) and subsequent workers in their laboratory. Details of the anatomy of the peripheral parts of the nervous system have been obtained as a result of our own dissections and have also been verified by reference to papers by Elo (1938), Carriker (1946) and Walter (1969). Some features of the gross anatomy of the animal, relevant to the present work, are indicated in figure 4.

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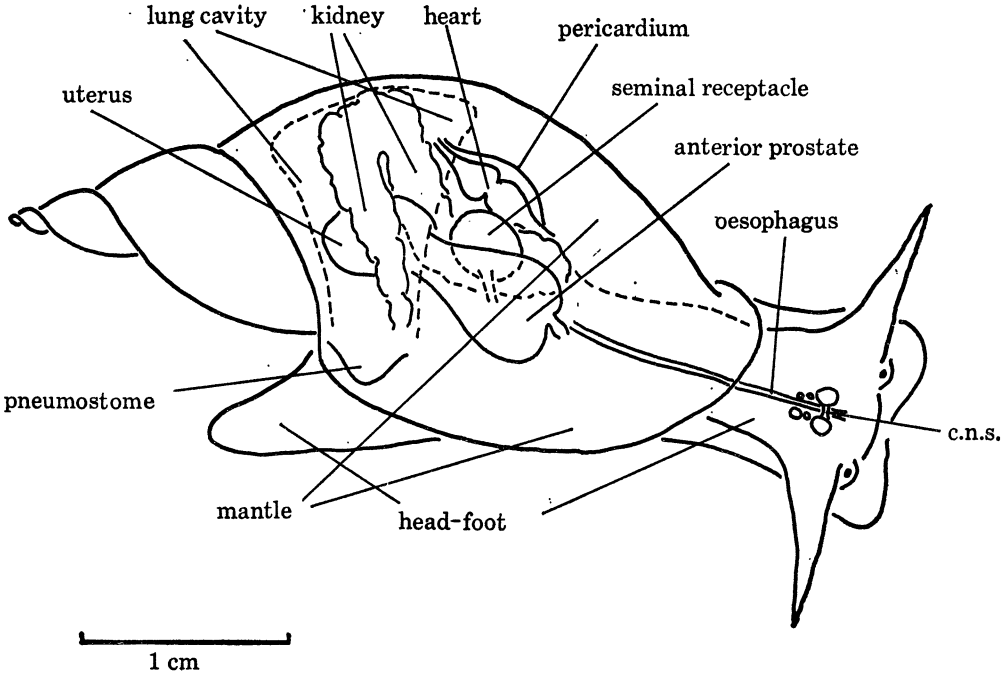

FIGURE 4. Gross anatomical features of *Lymnaea stagnalis*, seen from above. The scale bar is appropriate to an animal weighing 3–4 g.

THE ALCIAN BLUE–ALCIAN YELLOW TECHNIQUE

The Alcian Blue–Alcian Yellow technique was first developed by Ravetto (1964) for the analysis of mucopolysaccharides, and subsequently adopted as a neurosecretory stain by Peute & van de Kamer (1967) and Wendelaar Bonga (1970*b*). It relies on the differing affinities of the Alcian stains at pH 1.0 or less, and at pH 2.5 for strong and weak acid groups respectively. In neurosecretory materials, strong acid groups are presumed (Peute & van de Kamer 1967) to arise from oxidation of S–S bonds and –SH groups in cystine and cysteine to $-SO_3H$ groups, and weak acid groups from oxidation of aldehyde and hydroxyl groups. In the Alcian Blue solution at pH 1.0 or less, only strong acid groups are ionized and only these will bind with the stain. When sections are subsequently stained with Alcian Yellow at the higher pH, the blue stain remains bound to these groups while the weak acid groups are now ionized and can take up the Alcian Yellow stain. Different neurosecretory materials may contain different ratios of strong to weak acid groups after oxidation and thus should be stained different colours. (This implies that the Dark Green Cells, which have a high affinity for Alcian Blue at pH 1.0, contain large amounts of cysteine or cystine, whereas the Yellow Cells must contain little or none.) Whether this theoretical basis is correct or not, empirically the technique promises to be of value as a means of identifying different neurosecretory cell types, particularly among otherwise undifferentiated populations of neurosecretory cells, and possibly in helping to establish (or disestablish) homologies of cell types between related species.

The different colour types which have been identified in *Lymnaea*, and which have also been characterized ultrastructurally (Wendelaar Bonga 1970*b*), are the Dark Green Cells, Light Green Cells, Yellow Cells, Yellow-green Cells and Light Yellow Cells. Because the validity of our results depends on an accurate and reliable identification of cell types, it is essential to

discuss the results we were able to obtain with this technique, and to establish clearly the criteria on which our identification of cell types rests. Unless stated otherwise, the descriptions of colour and staining intensity given below are of material viewed with bright field illumination.

Dark Green Cells were identified without difficulty because of their relatively greater affinity than any other cell type for Alcian Blue. This relative difference enabled us to identify Dark Green Cells, and their axons, unambiguously in every preparation stained.

We were unable to identify, under either bright or dark field, cells of the Light Yellow type in the numbers and positions described by Wendelaar Bonga (30–50 cells, 20–60 μm in diameter, in the ventral lobe of the right parietal ganglion, and a similar number in the dorsal part of the visceral ganglion). This is almost certainly due to the fact that the staining reaction of the Light Yellow Cells is extremely weak, and in fact the cells can apparently only reliably be identified in the electron microscope (E. W. Roubos, personal communication). However, among cells which we classify as Yellow Cells, staining intensity varies from a light yellow, only just distinguishable from the background stain, to nearly brown. This raises the possibility that some of the cells we classify as Yellow Cells could in fact be more heavily stained Light Yellow Cells, and thus that there is a further population of still more faintly stained cells which could include Yellow Cells and Light Yellow Cells. This possibility is ruled out by the effects of dark field illumination, which markedly accentuates the differentiation of Yellow Cells. Under these conditions even lightly stained Yellow Cells are as well differentiated as more heavily stained cells (figure 2, plate 1, and figures 31–34, plate 3, are representative of the reaction of weakly stained cells), and a further population of more weakly stained cells is not revealed. Another possibility is that our class of Yellow Cells could include within its more weakly stained cells, the entire class of Light Yellow Cells. This is unlikely to be the case since (*a*) there are too few weakly stained cells (the class of Light Yellow Cells of Wendelaar Bonga is in fact larger than our entire class of Yellow Cells) and (*b*) the variation in staining intensity among Yellow Cells is gradual and not correlated with any difference in the position of the cells. A few other cells exhibiting a weak affinity for Alcian Yellow have been noted (in particular a small group of 3 cells, 30–50 μm in diameter, which occur on the ventral surface of the left parietal ganglion). The differentiation of these cells is not increased by dark field illumination, and we have excluded them from our class of Yellow Cells. Our class of Yellow Cells therefore includes cells which have two properties: they have an affinity for Alcian Yellow only, and their differentiation is markedly increased by dark field illumination.

Light Green Cells, which are identical to the Medio- and Latero-dorsal Cells of the cerebral ganglia (Joosse 1964) can be recognized as a distinct cell type because of their unique position in two large groups in each cerebral ganglion. We are doubtful whether their colour alone would enable them to be distinguished from the Yellow-green Cells in every instance, though taking each group as a whole, the adjectives 'light green' and 'yellow green-' seem justified. Light Green Cells are not well differentiated under dark field. We are not certain of the reasons for this, but it means that there is no possibility of confusing cells of this type, or their processes, with those of Yellow Cells or Yellow-green Cells under dark field.

The major difficulty we encountered was in distinguishing between the Yellow and Yellow-green Cell types. Frequently we found both types of cell stained in varying green tints of yellow, which made classification into two types of cell impossible. This seemed to be due to the very high affinity of Alcian Blue for Yellow Cell materials, at values of pH greater than 1.0 (Lev & Spicer 1964). Thus even traces of Alcian Blue carried over into the Alcian Yellow solution can

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cause some blue staining of Yellow Cell bodies. We therefore took the following precautions to avoid the Alcian Blue stain coming into contact with the sections at pH values above 1.0: (*a*) keep the pH of the Alcian Blue solution, and the buffer for rinsing below pH 1.0; (*b*) rinse with buffer before Alcian Blue staining and rinse thoroughly in buffer after staining and (*c*) make up fresh Alcian Yellow solutions frequently. When we took these precautions, and provided the tissue was well fixed, we could distinguish cells stained yellow from others stained yellow-green. Amongst these yellow-green stained cells (all of which we classify as Yellow-green Cells) there were variations in colour within animals, colours intermediate between very light green, deep olive and a heavy yellow with only a slight green tint being common. A possible contributing factor to this variation is that glycogen, as well as elementary neurosecretory granules, is present in the cell bodies (Wendelaar Bonga 1970*b*). As glycogen will probably also take up the Alcian stains (cf. Simpson 1969), its presence in varying amounts could also cause differences in the relative amounts of the two stains taken up. However, the Yellow-green Cells vary morphologically (within animals), and possibly more than one cell type is present in this category, though our data at present are insufficient to warrant a sub-division.

Results

The cell bodies of the Dark Green Cells

The numbers and positions of the Dark Green Cells in the various ganglia (figure 5) are similar to those described by Wendelaar Bonga (1970*b*), with the exception that we regularly found a group of 2–7 Dark Green Cells in the visceral ganglion, ventral to the right parieto-visceral connective. A single Dark Green Cell was infrequently found in the left or right cerebral ganglion, close to the pleuro-cerebral connective. Dark Green Cell bodies were never found in the pedal ganglia, or outside the c.n.s. in nerves or in the connective tissue sheath, where Yellow Cells and Yellow-green Cells can be found. The position of the larger groups of Dark Green Cells was constant in different animals, but the number and location of the cells within groups was not.

Using a dye marking technique described elsewhere (Benjamin, Swindale & Slade 1976) we have found that the cell bodies of the Dark Green Cells in the living brain viewed with reflected light, are almost indistinguishable in colour from orange non-neurosecretory neurons – the Dark Green Cells are a slightly paler white-orange colour. Other neurosecretory cell types (e.g. Light Green Cells, Yellow Cells and Yellow-green Cells) are the bright white colour often assumed to be characteristic of living neurosecretory neurons in gastropods.

The cell bodies of the Dark Green Cells increase in size but not in number as the animal grows (figure 6). The average number of Dark Green Cells found in 19 brains from animals of mass from 2 to 8 g, was 29 ± 5, and the average size of the cells after fixation varied from 15 μm × 19 μm to 35 μm × 42 μm, in animals weighing 0.26 and 6.7 g respectively. The cell bodies were not larger in animals where they were less numerous, or *vice versa*. The average size of the Dark Green Cells in the right pleural ganglion was significantly larger than in the left pleural ganglion ($P < 0.05$, Wilcoxon test, one tailed). This parallels a size asymmetry in the brain, the right pleural, pedal, cerebral and parietal ganglia all being larger than their left sided counterparts. Differences in size between cell groups in other ganglia were not significant. There was no significant difference between the number of Dark Green Cells in the left and right pleural ganglia.

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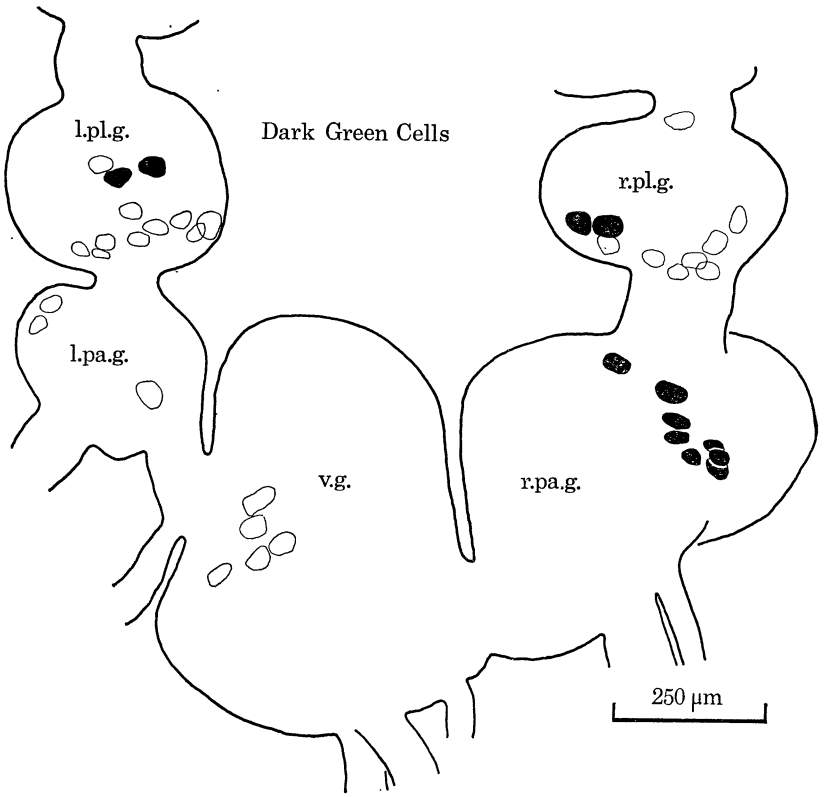

FIGURE 5. The positions of the cell bodies of the Dark Green Cells in one representative c.n.s. of *Lymnaea*. Cells in black lie on the dorsal surfaces of the ganglia; cells shown in outline lie on the ventral surfaces. (This convention does not apply to subsequent figures in this paper.)

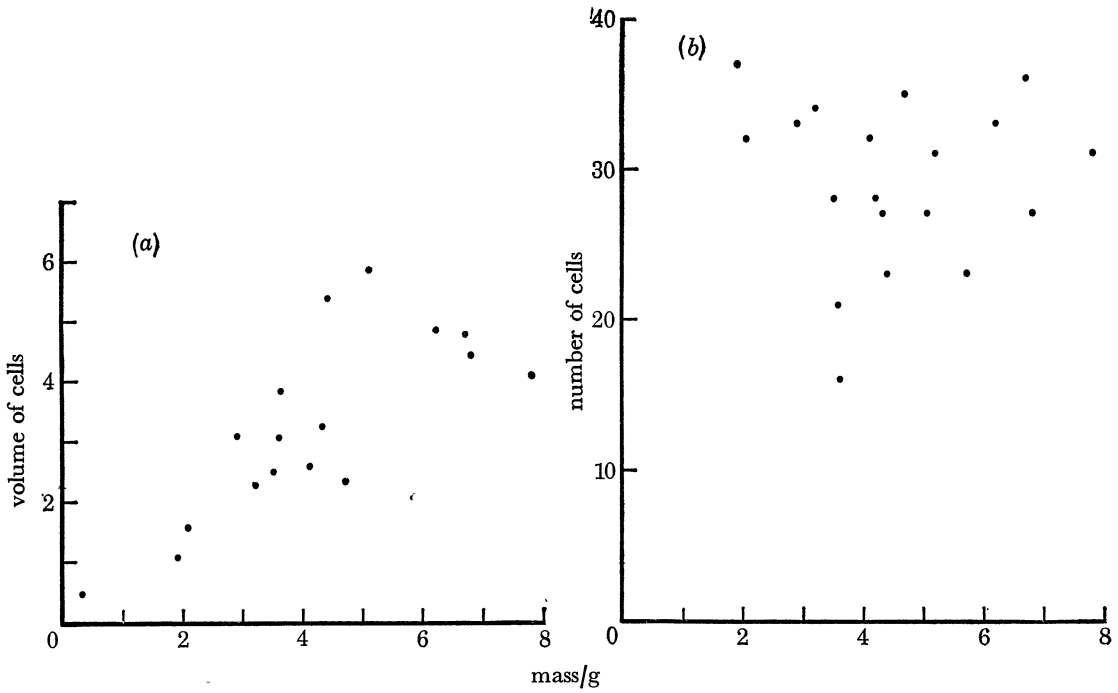

FIGURE 6. Graphs of (*a*) the average volume of Dark Green Cell bodies in animals of different mass; (*b*) the number of Dark Green Cells in animals of different mass. Each point is a measurement from one animal.

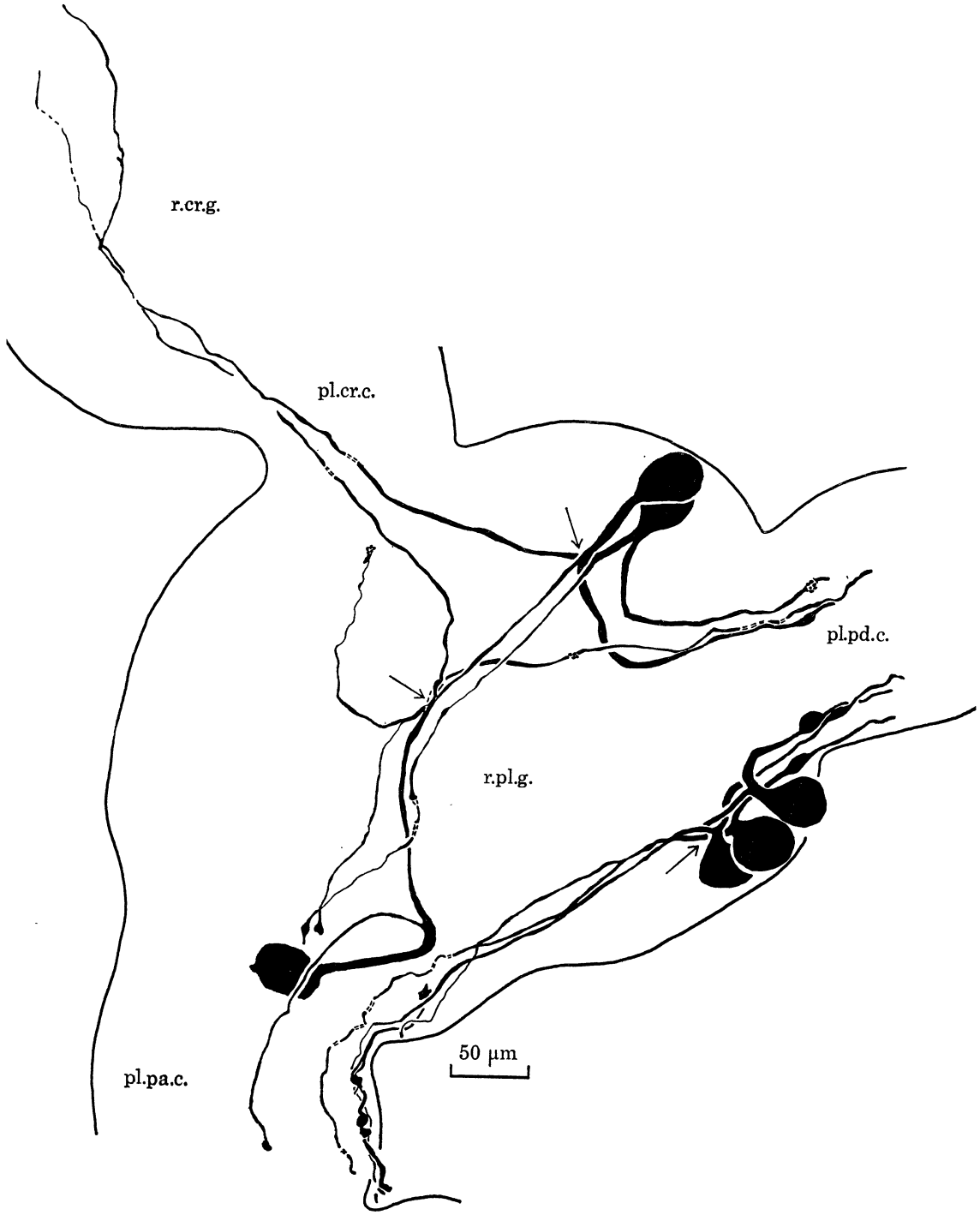

FIGURE 7. A *camera lucida* reconstruction from serial sections stained with Alcian Blue–Alcian Yellow, of all the Dark Green Cells in a right pleural ganglion. Fewer cells than usual are present. Each of three cells near the pleural-pedal connective (pl. pd. c.) sends one axon into the connective and another to the surface of the pleuro-parietal connective (pl. pa. c.) which may terminate there. The other three cells each send one axon into the pleural pedal connective; two other projections from these cells can be followed into the cerebral ganglion and a third towards the pleuro-cerebral connective which cannot be traced beyond a swelling; a third set of projections from these cells runs towards the pleuro-parietal connective, but cannot be traced beyond swellings. It is unlikely that these swellings are terminations. Similar swellings occur *en passant* in the axons in the pleuro-pedal connective. Arrows point to regions of possible contact between Dark Green Cell axons.

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Short, fluffy projections arise from the perimeter of the cell bodies, but do not extend more than a few micrometres (figure 9, plate 2).

The axonal morphology of the Dark Green Cells

The Dark Green Cells are invariably monopolar. Cells usually branch at least once, and often twice, within about 200 μm of the cell body. The axons of different Dark Green Cells sometimes come into close apposition to one another, often at simultaneous branch points (figure 7). Within about 50 μm of the cell body the axons of the Dark Green Cells are about 5 μm in diameter. These narrow gradually, so that by the time the axons leave the ganglia they are usually no more than 1–2 μm in diameter.

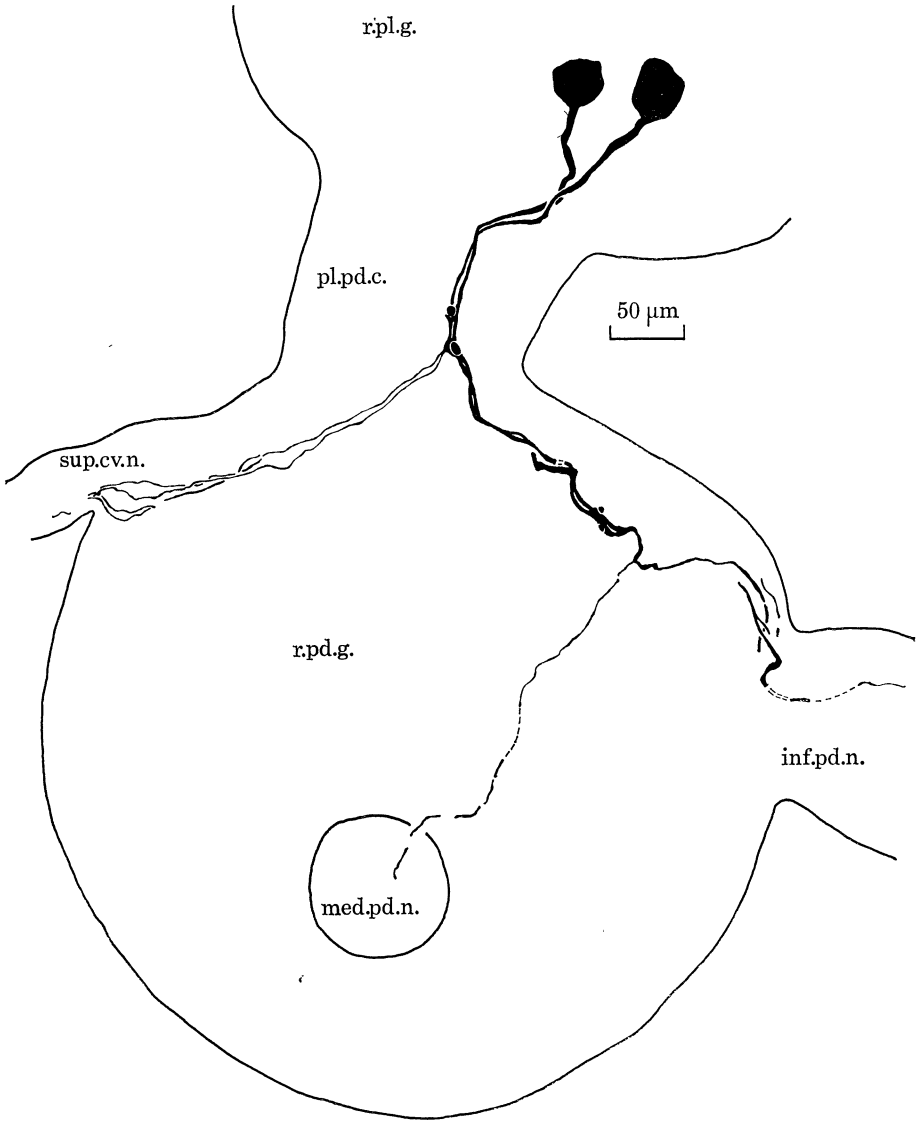

FIGURE 8. *A camera lucida* reconstruction from serial sections, of two Dark Green Cells on the ventral surface of the right pleural ganglion. The cells project through the pedal ganglion and into the pedal nerves. The axons branch in pedal neuropile and project down the superior cervical (sup. cv. n.), inferior pedal (inf. pd. n.) and medial pedal (med. pd. n.) nerves.

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Most and possibly all the Dark Green Cells in the pleural ganglia send one axon into the pedal connective (figures 7 and 8; figure 9, plate 2). These axons run through pedal neuropile, where further branching may take place, and then into the pedal nerves (figure 8; figure 10, plate 2). Other branches of pleural Dark Green Cells can be followed through the pleuro-cerebral connective into medial cerebral neuropile and thence into the superior and medial lip nerves (figure 11, plate 2). Axons can also be traced through the pleuro-parietal connectives into the parietal ganglia, which they leave via the parietal nerves, together with axons from parietal Dark Green Cells (figure 12, plate 2). Axons from Dark Green Cells in the parietal ganglia have also been found to project through the adjacent pleural ganglion into the pleuro-pedal connectives, so that parietal Dark Green Cells, like pleural Dark Green Cells, probably have projections in both ipsilateral pedal and parietal nerves.

One reconstruction was also made of a group of three Dark Green Cells in the visceral ganglion. One projection from these cells was probably to the anal nerve; two others were probably to the right parietal ganglion and the right parietal nerves; another two axons entered the left parietal ganglion, and one of these projections entered the parieto-pleural connective.

Branches of the Dark Green Cells were found which appeared to terminate on the surface of the pleuro-parietal or pleuro-cerebral connectives (figure 7). This confirms the findings of Wendelaar Bonga (1970*b*, 1971, 1972) and Roubos (1973) that these connectives are a neurohaemal area of the Dark Green Cells. Estimates from figure 7 and another similar reconstruction of Dark Green Cells in the pleural ganglia, suggest that 15–20 % of pleural Dark Green Cell projections are to the surface of these connectives, though it is possible that sometimes these projections pass through the connective into the adjacent ganglion. We are not certain what proportion of projections from Dark Green Cells in other ganglia are similarly to the surfaces of connectives.

No firm evidence that the Dark Green Cells have pre- or post-synaptic branches terminating

Description of plate 2

Figure 9. Horizontal section through the left pleural ganglion, stained with Alcian Blue–Alcian Yellow, showing the cell bodies and axons of three Dark Green Cells on the ventral surface of the ganglion. The axons are leading into the pleuro-pedal connective.

Figure 10. Horizontal section through the right pedal ganglion, showing Dark Green Cell axons (arrows) running into the medial pedal nerve.

Figure 11. Horizontal section through the right cerebral ganglion, showing Dark Green Cell axons (arrows) running medially through cerebral neuropile into the superior lip nerve (sup.lp.n.). The compact tract formed by the processes of Light Green Cells as they run to the medial lip nerve, can also be seen on the right of the picture.

Figure 12. Horizontal section through the right parietal ganglion, showing Dark Green Cell axons (arrows) running into the internal right parietal nerve (i.r.pa.n.). Axons from Yellow Cells and Yellow-green Cells can be seen running in the outer regions of the nerve.

Figure 13. Three types of swelling in the axons of the Dark Green Cells: (*a*) conventional, heavily stained; (*b*) broken up and blobbed and (*c*) staining absent, or less intense, in the centre of the swelling.

Figure 14. Dark Green Cell axons in the main branch of the left parietal nerve as it enters the mantle.

Figure 15. Dark green stained fibres running over the surface of a branch of the left parietal nerve in the mantle.

Figure 16. Dark Green Cell axons (arrow) in a small nerve near the mantle epithelium (me). The darkly stained material on the left is mucus.

Figure 17. Dark Green Cell axons (arrows) in a small nerve (probably a branch of the medial pedal nerve) near the sole of the foot.

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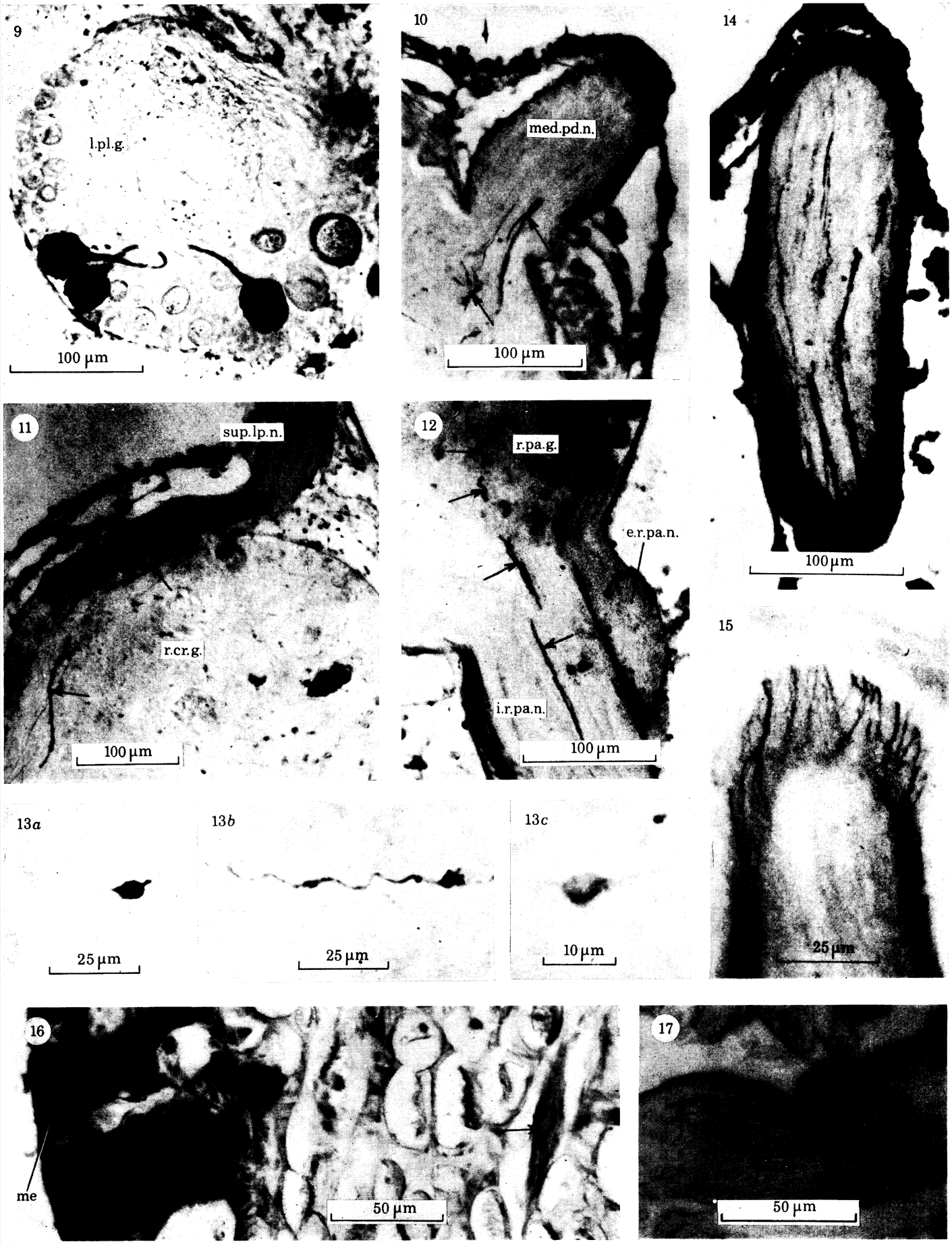

FIGURES 9–17. For description see opposite.

(*Facing p.* 180)

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in neuropile was obtained with either bright or dark field illumination. Very fine and sometimes branching dark green stained processes could be found in neuropile, but these were never numerous and could be merely fine axonal branches. However, our techniques only reveal branches containing stainable material, and such material might well be lacking from dendrites of neurosecretory cells.

TABLE 1. LOWER ESTIMATES OF THE NUMBER OF DARK GREEN CELL AXONS WHICH COULD BE COUNTED IN EACH OF THE NERVES LEAVING THE C.N.S.

Figures from four examples of each nerve, from five different brains, are presented. Complete data from five brains could not be obtained. Where five figures were available for a nerve, the four highest figures were taken.

	projections of the Dark Green Cells		percentage of total Dark Green Cell projections to nerves
pedal ganglia	left	right	
superior cervical nerves	(3, 3, 4, 0)	(4, 5, 3, 0)	7.0
inferior cervical nerves	(3, 5, 1, 0)	(1, 5, 1, 0)	5.1
columellar nerves	(0 1, 0, 1)	(0, 1, 0, 0)	0.9
superior pedal nerves	(9, 6, 5, 5)	(5, 4, 5, 3)	13.4
medial pedal nerves	(10, 5, 1, 3)	(11, 3, 3, 7)	13.7
inferior pedal nerves	(7, 6, 3, 7)	(6, 2, 2, 4)	11.8
cerebral ganglia			
optic nerves	(0, 0, 0, 0)	(0, 0, 0, 0)	—
tentacle nerves	(0, 0, 0, 0)	(0, 0, 0, 0)	—
nuchal nerves	(0, 0, 0, 0)	(0, 0, 0, 0)	—
superior lip nerves	(5, 2, 1, 2)	(4, 1, 2, 2)	6.1
medial lip nerves	(7, 2, 1, 0)	(3, 2, 2, 5)	7.0
penis nerve	—	(0, 0, 1, 1)	0.6
parietal and visceral ganglia			
left parietal nerve	(6, 13, 10, 4)		10.5
cutaneous pallial nerve	(10, 2, 1, 2)		4.8
anal nerve	(3, 5, 1, 4)		4.1
intestinal nerve	(2, 2, 0, 2)		1.9
genital nerve	(1, 2, 1, 1)		1.6
internal right parietal nerve	(8, 13, 5, 5)		9.9
external right parietal nerve	(3, 2, 0, 0)		1.6
percentage of Dark Green Cell axons going to the head-foot			65.6
percentage of Dark Green Cell axons going to the mantle			30.9
percentage of Dark Green Cell axons going to the viscera			3.5

average total number of Dark Green Cell axons counted leaving the c.n.s. $\simeq 80$

Approximately spherical, heavily stained swellings often occur in the axons of the Dark Green Cells (figures 3, 7; figure 13, plate 2). They vary in size from 2–10 times the diameter of the axon. Sometimes the swellings were broken up and blobbed (figure 13*b*), and less commonly staining appeared to be absent or less intense in the centre of the swelling (figure 3 and figure 13*c*, plate 2). They were found most frequently in or proximal to (with respect to the location of the cell body) the connectives of the pleural ganglia, but were also found in other connectives and nerves leaving the ganglia. Often the axon distal to the swelling was less heavily stained than the axon proximal to it. Apart from these large swellings, the axons of the Dark Green Cells are at most only slightly irregular in diameter, and are often quite smooth (figure 3).

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The peripheral projections of the Dark Green Cells

Table 1 shows the number of Dark Green Cell axons which could be counted in each of the nerves leaving the c.n.s. The counts were made in brains where the staining of Dark Green Cell axons was particularly intense. They may well underestimate the number of Dark Green Cell axons present, since the axons are often very fine and difficult to see.

The axons run dispersed throughout cross sections of each nerve (figure 14, plate 2). No indications that the Dark Green Cells terminate or release in the proximal regions of these nerves (i.e. within about 5 mm of the ganglia) were found.

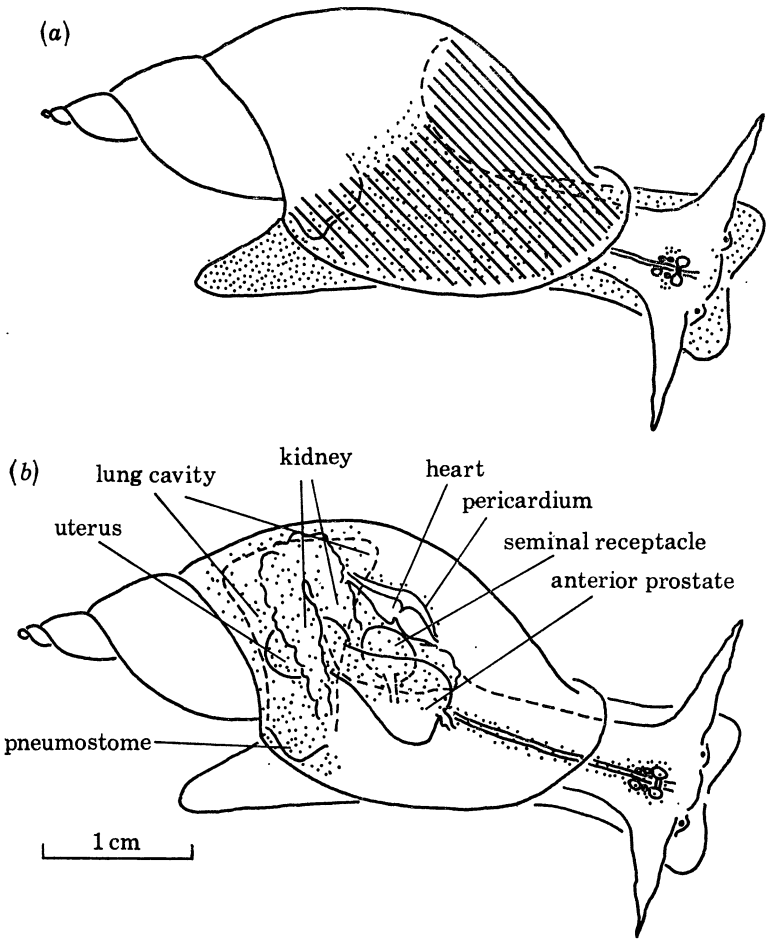

FIGURE 18. Dorsal views of *Lymnaea* showing the probable distribution of release sites of (*a*) the Dark Green Cells (cross hatching indicates release sites in the mantle, dots indicate release sites in the neck and head-foot) and (*b*) the Yellow Cells and Yellow-green Cells.

The largest numbers of Dark Green Cell axons were found in the inferior, medial (figure 10, plate 2) and superior pedal nerves, which innervate the thick layers of muscle and connective tissue which form the sole and lateral walls of the foot. Large numbers of Dark Green Cell axons were also found in the left (figure 14, plate 2) and internal right parietal (figure 12, plate 2) nerves which innervate the mantle (which lines the large inner surface of the last whorl of the shell) and in the superior and medial lip nerves which innervate the lips and surrounding tissues in the most anterior part of the head-foot. Axons were also found in significant numbers

in the superior and inferior cervical nerves, which innervate dorso-lateral parts of the body wall and the neck respectively, and in the cutaneous pallial, anal and external right parietal nerves, which innervate ventral and right hand lateral parts of the mantle not supplied by the left and internal right parietal nerves. Nerves innervating the visceral organs in the shell (the intestinal and genital nerves), the eyes, tentacles, penis and the columellar muscle, contained few or no Dark Green Cell axons. The peripheral areas innervated by nerves containing Dark Green Cell axons are shown diagrammatically in figure 18*a*.

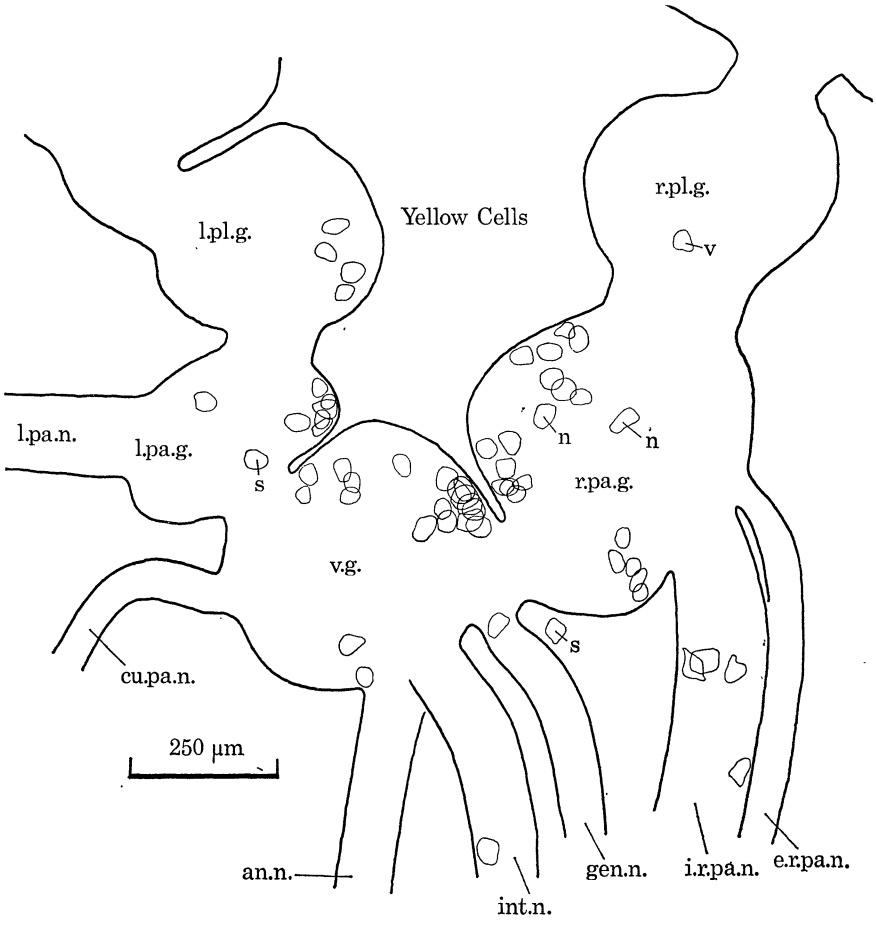

FIGURE 19. The positions of the cell bodies of the Yellow Cells in one particular c.n.s. s, Cells occurring in the sheath; n, cells occurring in the central neuropile of the ganglia; v, cells occurring on the ventral surfaces of the ganglia. All other cells occur on, or near, the dorsal or equatorial surfaces of the ganglia.

As the largest number of Dark Green Cell axons occur in nerves innervating the foot and mantle, we looked for signs of Dark Green Cell axons in Alcian Blue–Alcian Yellow stained sections of tissue from these areas. In preparations where Dark Green Cell axons as a whole were heavily stained, axons could be followed along the pedal, and left and internal right parietal nerves, from the main nerve trunks (figure 14, plate 2) into the smallest branches which could be identified with certainty in layers of unstained muscle and connective tissue (figures 16 and 17, plate 2). Examples of small nerves containing one or more Dark Green Cell axons could be found throughout the mantle and foot. Occasionally we found morphological evidence of hormone release from the surface of nerves in these areas (figure 15, plate 2). Dark green stained fibres were found running along the outside of nerves, branching and forming a

plexus of fibres less than 1 μm in diameter. This arrangement was seen rather infrequently, though a number of factors, including the small diameter of the fibres, frequent poor fixation of small nerves, and varying amounts of Dark Green Cell material present could have prevented identification of the plexus in many cases. Though there is nothing about the appearance of the network which suggests that it is not composed of Dark Green Cell axons, it could be composed of non-neural tissue (perhaps strands of connective tissue) since it lies on the surface of the nerve.

The cell bodies of the Yellow Cells and Yellow-green Cells

The positions of the cell bodies of the Yellow Cell and Yellow-green Cell types of neurosecretory neurone (figures 19, 20 and 25) are similar to those described by Wendelaar Bonga (1970*b*), though our findings differ slightly from his, in that Yellow Cells and Yellow-green Cells were regularly found in the pleural ganglia.

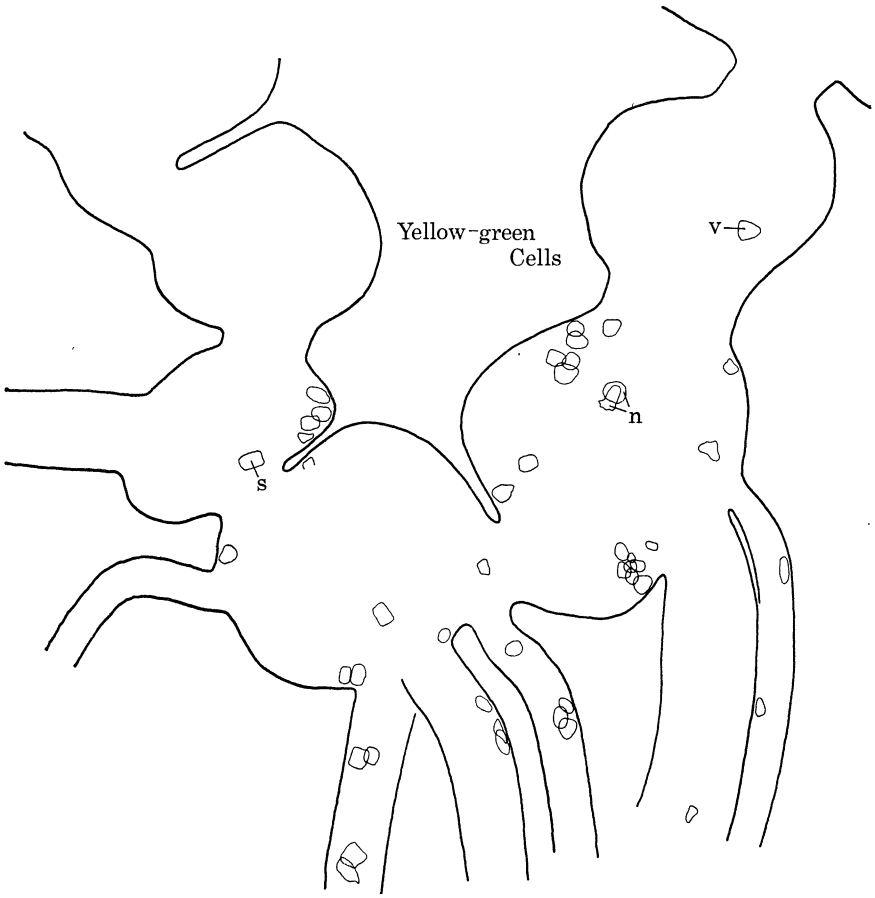

FIGURE 20. The positions of the cell bodies of the Yellow-green Cells in the same c.n.s. as figure 19. The conventions used in figure 19, for showing the locations of the cells in the dorso-ventral plane, also apply to this figure.

The largest single group of Yellow Cells (8–10 cells) occurs in the visceral ganglion, dorsal, anterior and immediately adjacent to the right parieto-visceral connective. Yellow-green Cells are normally absent from this location. Another group of Yellow Cells (4–8 cells) mingled with a similar number of Yellow-green Cells, occurs on the medial surface of the right parietal ganglion, adjacent to the pleuro-parietal connective and extending both ventrally and dorsally to the

connective. Both of these groups are regular in occurrence. Other smaller groups of Yellow Cells and Yellow-green Cells (from 1 to 6 cells of each type) are found in the right parietal ganglion near the right parieto-visceral connective and at the base of the external right parietal nerve; in the left parietal ganglion, usually on the dorsal surface; and on the medio-dorsal aspect of each pleural ganglion, most frequently adjacent to the left or right parietal ganglion. Yellow Cells and Yellow-green Cells also occur singly or in pairs elsewhere in the parietal and visceral ganglia. Cells can often be found deep inside these ganglia, lying within tracts of axons formed by connectives and nerves as they join the ganglia. Both cell types occur in groups varying in size and position on the intestinal nerve, and in lesser numbers on the anal, genital and right parietal nerves. The cell bodies may lie on the surface of these nerves, or sometimes within them. They can be found in the intestinal (figure 31) and genital nerves at large distances from the brain, particularly at the origin of nerve branches. Yellow Cells and Yellow-green Cells sometimes lie outside the c.n.s., in the connective tissue sheath of the ganglia. Rather infrequently, Yellow Cells occur in the cerebral ganglia, close to the cerebro-pleural connective; on the

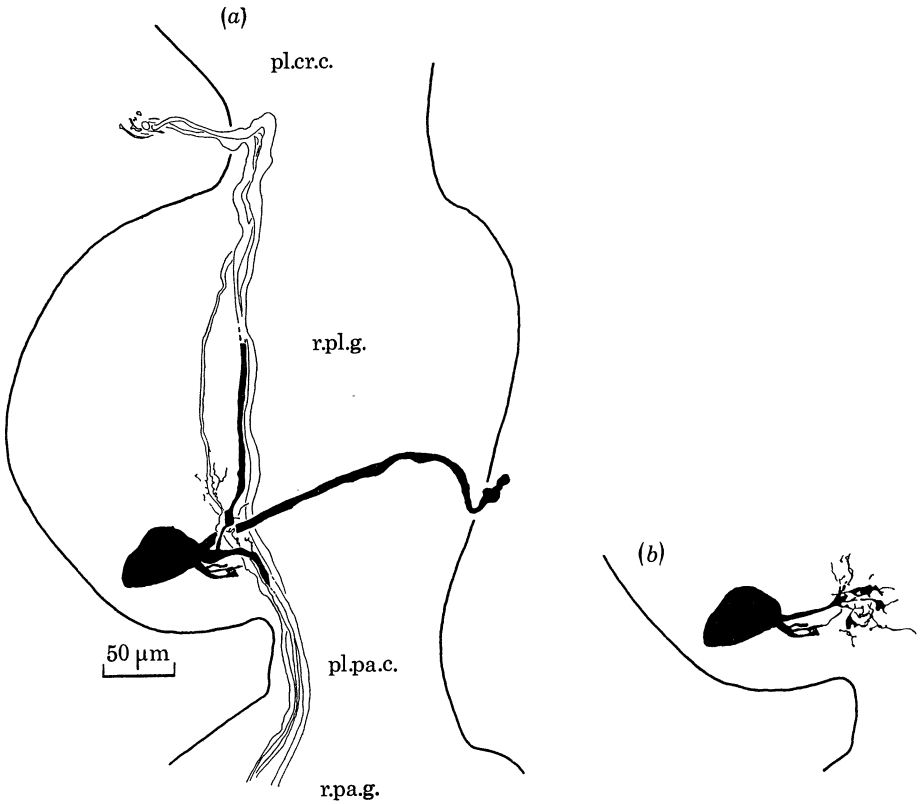

FIGURE 21. Reconstruction from serial sections stained with Alcian Blue–Alcian Yellow, under dark field, of a Yellow Cell in the right pleural ganglion. No other Yellow Cell or Yellow-green Cell bodies were present in the ganglion. (*a*) Two branches of the cell join the tract (drawn in outline) formed by anteriorly projecting axons of Yellow Cells and Yellow-green Cells in the neighbouring right parietal ganglion. One of these branches projects anteriorly with this tract to the pleuro-cerebral connective, and thence into the ganglion sheath; the other branch projects posteriorly down the tract into the right parietal ganglion, which it probably leaves via one of the right parietal nerves. An axon from another cell in the tract makes possible contact with the anteriorly projecting branch close to the cell body, and both these axons give rise to a few short fine branches in this region. A third branch of the cell runs dorsally and laterally across the ganglion, exiting through the ganglion capsule into the sheath. (*b*) The two ventrally projecting branches of the same cell, the shorter of which is also included in (*a*).

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ventral surface of the pleural ganglia, or on the left parietal or cutaneous pallial nerves. Neither type of cell was ever found in the pedal ganglia.

In the living brain viewed by reflected light, both Yellow Cells and Yellow-green Cells vary in colour from a bright crystalline white to a pale translucent orange-white. The whiteness is due to the presence of large numbers of elementary neurosecretory granules acting as Mie scatterers of light (Maynard 1961). The variation in the amount of whiteness probably reflects variations in the density of elementary neurosecretory granules in the cell body (Frazier *et al* 1967).

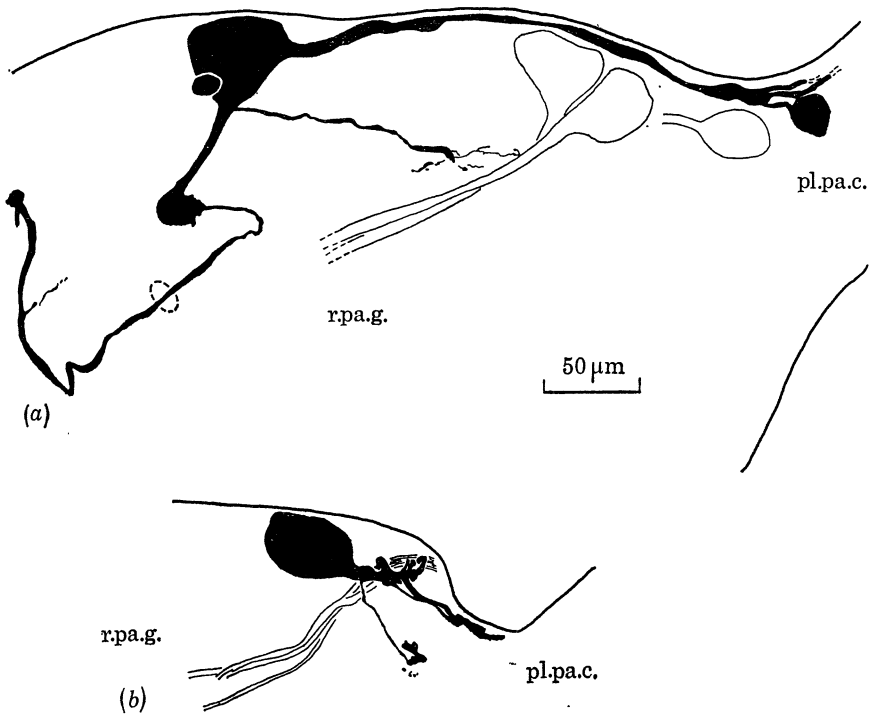

FIGURE 22. Reconstructions of Yellow Cells in the right parietal ganglion. The cell bodies lie on the medial surface of the ganglion. (*a*) One branch of this bipolar cell runs along the inside of the ganglion sheath to the right pleuro-parietal connective, where it divides and forms a large swelling. Further continuation of the axon was uncertain. The other branch of the cell wanders ventrally through the ganglion, forming a large swelling at a bend in the axon, before exiting (dotted circle) through the ventral surface of the ganglion into the sheath. A fine branch leaves this axon at its point of origin from the cell and travels for some distance before branching in a region of neuropile near Yellow Cell and Yellow-green Cell axons (partly drawn in outline). (*b*) Two branches of this cell project to the periphery of the pleuro-parietal connective, and four other branches of the cell join the tract of axons projecting to the right parietal nerves. A fine branch arises close to the cell body and terminates in a blobbed swelling in neuropile.

The cytoplasm of the Yellow Cells and Yellow-green Cells, unlike that of the Dark Green Cells, is normally not evenly stained, but is broken up into large blobs 10–30 μm in diameter, separated by clear canals extending to the perimeter of the cell body. This is only evident under bright field. Short protrusions (figure 22*a*) or lobes of the cell body also occur, most often in cells which occur in the sheath or in tracts of axons.

The axonal morphology of the Yellow Cells

Reconstructions of Yellow Cells from various positions in the c.n.s. are shown in figures 21–24. Cells are either monopolar, as in figures 22*b* or 23*a*, or have two or more axons arising separately

from a single pole of the cell body (figures 21, 23*b*). In extreme cases this may cause the cell to be bipolar in form (figure 22*a*).

Unlike the Dark Green Cells, the Yellow Cells have processes which terminate in neuropile, giving rise to dense fine branches and blobs of varying size (figure 21–24, 32, 33). Normally this material arises from one or two branches, often narrower than the axons, which either leave the cell body from the same pole as the axons (figures 21, 22*a*, 23*b*, 24*a*), or which leave the main

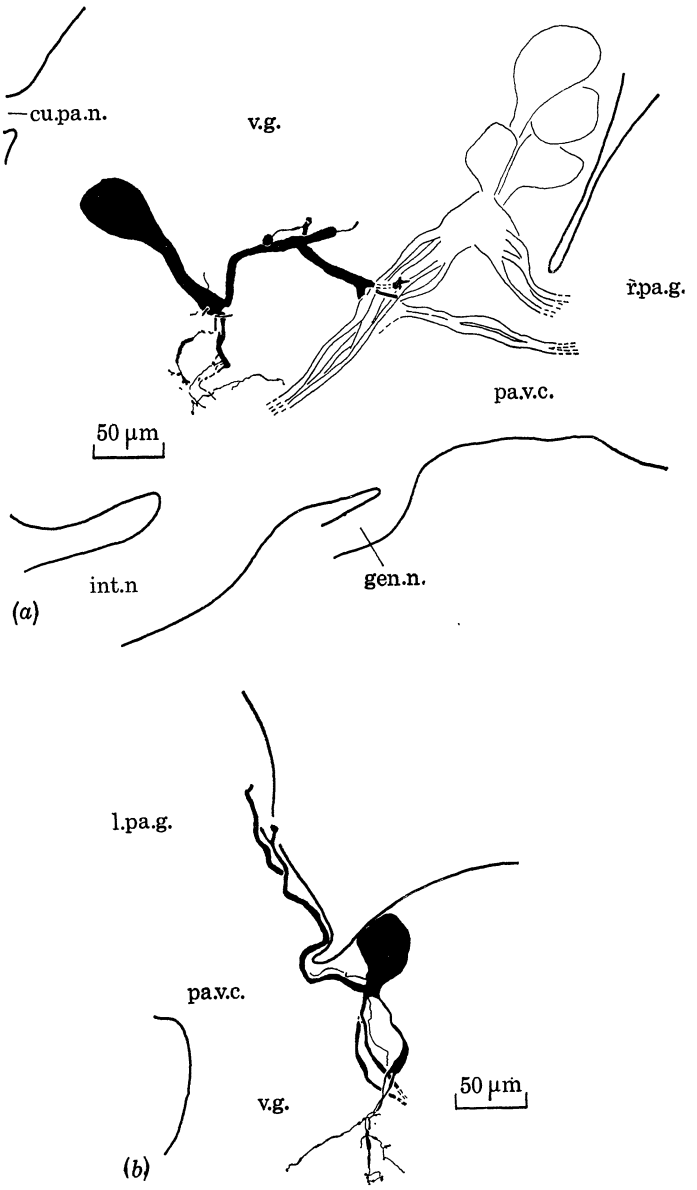

FIGURE 23. Reconstructions of two Yellow Cells in the visceral ganglion. (*a*) The cell body lies deep in the ganglion and has a single stout axon from which a variety of processes arise. The axon joins a tract of Yellow Cell axons (drawn in outline) which project to the intestinal and anal nerves, and may project from this tract to the right parietal ganglion. (*b*) The cell body is adjacent and medial to the left parieto-visceral connective, and six processes arise from a single pole of the cell body. One of these bifurcates, and gives rise to fine blobbed branching in neuropile; another projects to the periphery of the parieto-visceral connective, and two others probably join the tract of axons projecting posteriorly through the visceral ganglion. These two neurones, and those in figure 22, are included in a less detailed reconstruction of the c.n.s. in which they occur, in figure 25.

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axon of the cell before it divides (figures 22*b*, 23*a*). Consequently these projections rarely originate from points more distal to the cell body than that where separate axons arise. After leaving the cell these projections may run undivided through neuropile for some distance before branching again (figures 21, 22, 23*b*; figure 32, plate 3). The fine branches to which these projections give rise are densest in neuropile near the large visceral and right parietal groups of Yellow Cells, where axons and fine branches from many cells intermingle, but they also occur in regions near isolated Yellow Cells where no material from any other Yellow Cell, Yellow-green Cell or Dark Green Cell is present (figures 21, 23). In regions of dense branching, neurosecretory material was sometimes found clustered round small gaps in the neuropile (figure 24; figure 33, plate 3). These might be profiles of large axons, though they do not appear to contain cellular material of any sort.

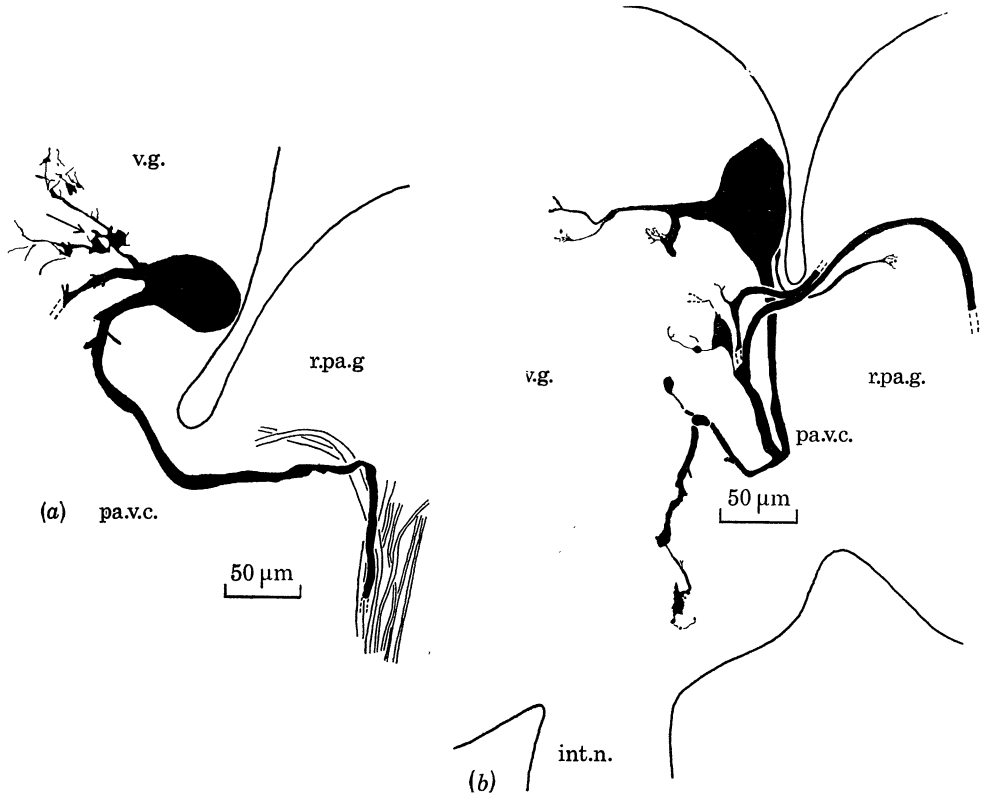

FIGURE 24. Reconstructions of two cells from the large visceral group of Yellow Cells adjacent to the right parieto-visceral connective. (*a*) This cell has one branch which projects into the adjacent right parietal ganglion and joins the tract of Yellow Cell and Yellow-green Cell axons (drawn in outline) going to the internal right parietal nerve; another branch is probably an axon and may project to the intestinal or anal nerve; a third branch ends in fine branching fibres and large blobs. Short stumpy processes arise from each axon. The arrow points to an arrangement of neurosecretory material round a gap in the neuropile which is apparently devoid of cellular material. (*b*) This cell has two axons which project to the right parietal ganglion. None of the other branches of the cell can be followed beyond fine branches and blobs. One of the branches projects into the right parietal ganglion before terminating there.

The axons project to release sites in various ways. Some pass singly through the inner ganglion capsule, apparently at any point on the ganglion surface, and then into the ganglion sheath (figures 21, 22*a*, 25). Others leave the pleuro-cerebral or pleuro-parietal connectives as a group of axons running into the sheath (figures 21, 25; figure 34, plate 3). Once in the sheath the axons run for large distances, branching frequently and forming occasional swellings. Axons proceed-

ing to the surface of the pleuro-parietal or left parieto-visceral connective can form blobbed swellings there, which may be endings (figures 22, 23*b*), though most and possibly all of such axons either enter the sheath or pass through the connective into the adjacent ganglion (figure 21). *En passant* release is a possibility in the latter case since the axons often run over the surface of the connective. The majority of Yellow Cell axons however (figures 22*b*, 23, 24), project into the internal and external right parietal nerves and the intestinal and anal nerves; projections into the internal right parietal nerve and intestinal nerve being most numerous (see figure 25 and the section on the projections of the Yellow Cells and Yellow-green Cells).

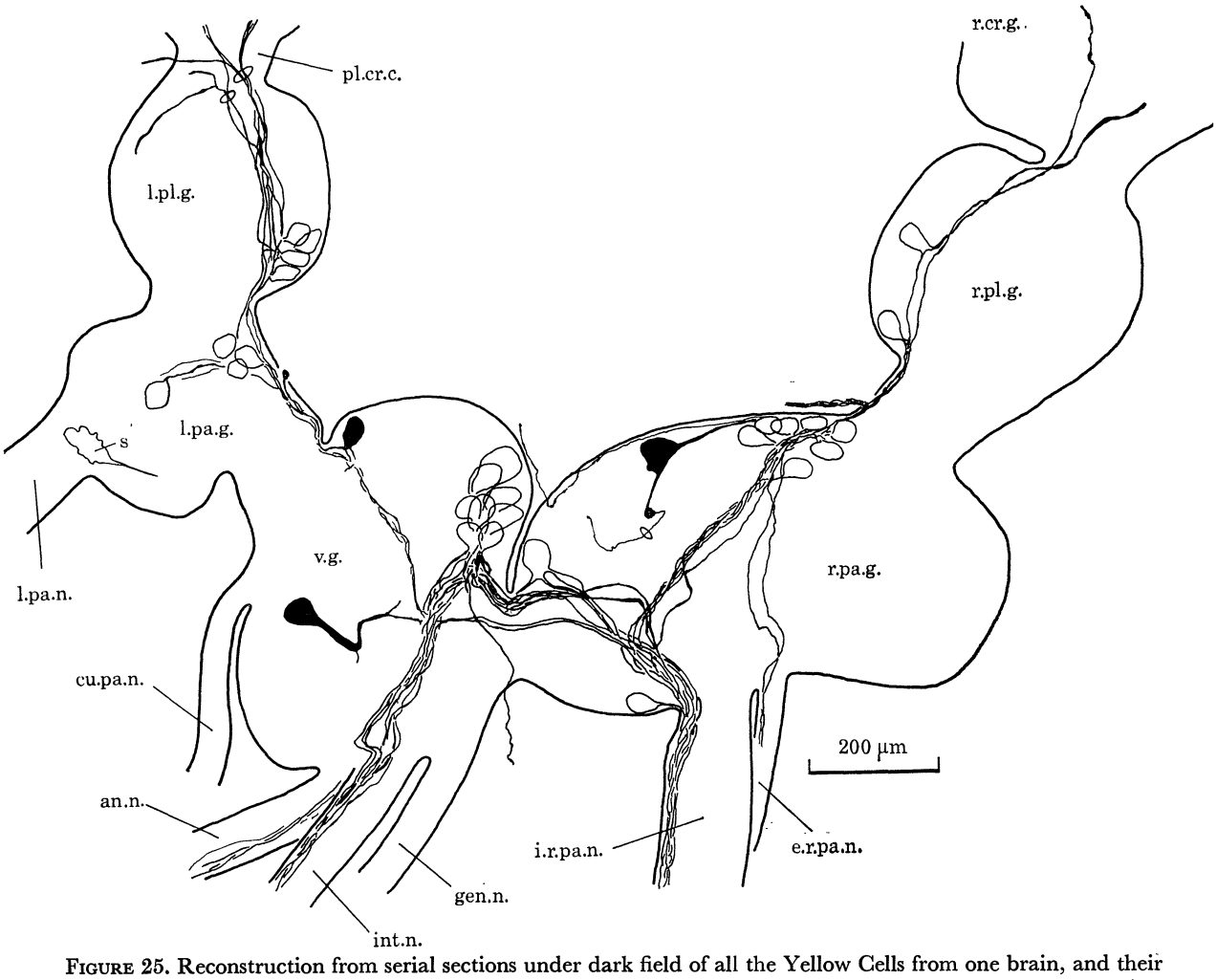

FIGURE 25. Reconstruction from serial sections under dark field of all the Yellow Cells from one brain, and their main axonal tracts through the brain. Yellow-green Cell axons are also present in some of the tracts (see text). Cells filled in black are also shown reconstructed in more detail in figures 22 and 23. The points where axons leave the ganglia and enter the sheath, where not obvious, are marked by small circles. s, Cell in sheath.

The axonal morphology of the Yellow-green Cells

Reconstructions of Yellow-green Cells from various ganglia are shown in figures 26–30. These cells comprise a greater variety of morphological types than either Yellow Cells or Dark Green Cells. Monopolar, bipolar and multipolar types are found. The simplest type of Yellow-green Cell has only a single axon which projects from an adjacent connective into the sheath (figure 27) or which runs to the surface of a nearby nerve. Other Yellow-green Cells appear similar to

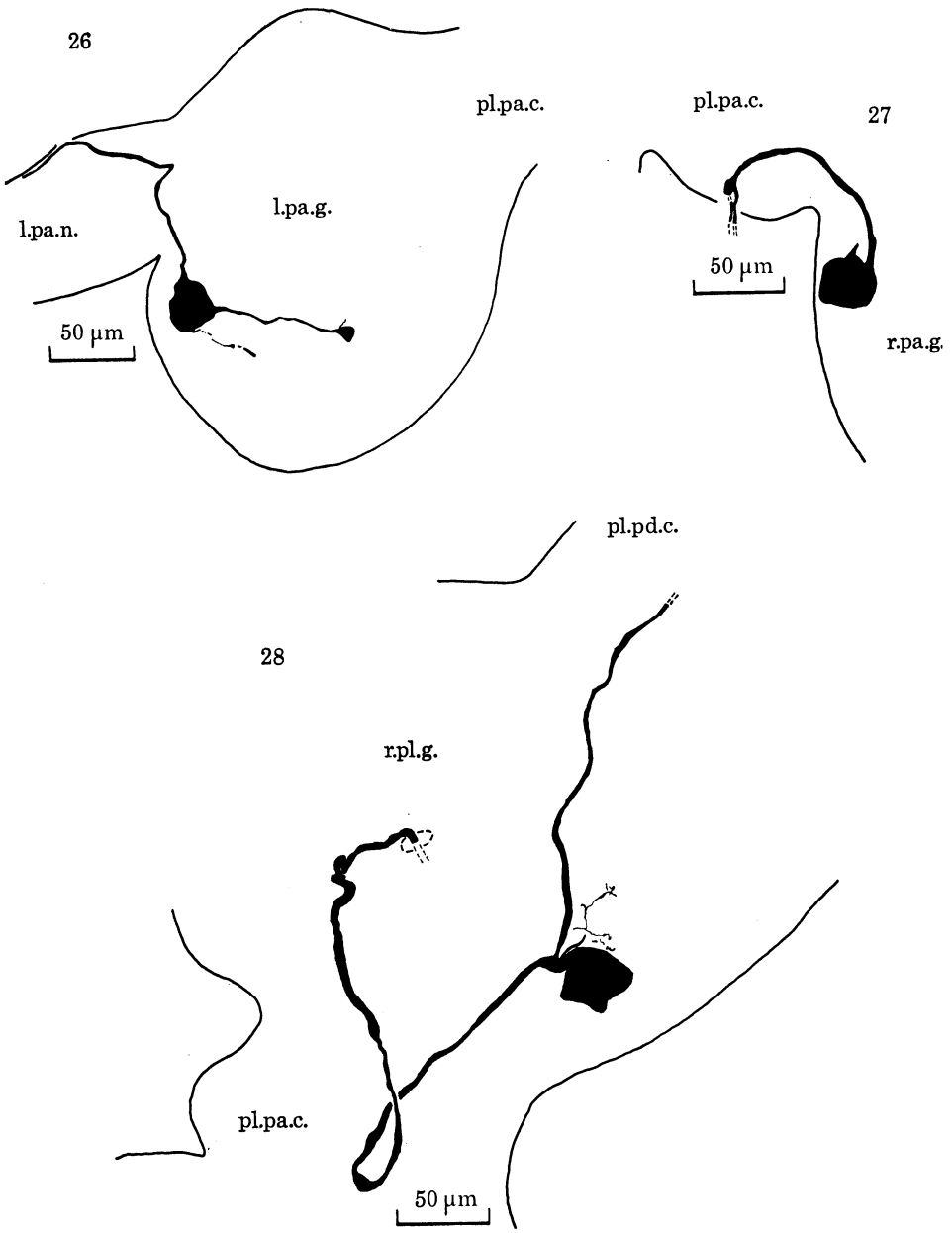

FIGURE 26. Reconstruction from serial sections of a Yellow-green Cell in the left parietal ganglion. Three processes leave the cell body; one of these runs to the surface of the left parietal nerve; another descends ventrally through the ganglion and cannot be followed beyond a blob inside the ganglion.

FIGURE 27. Reconstruction of a Yellow-green Cell in the right parietal ganglion. This cell has one axon which enters the sheath via the pleuro-parietal connective.

FIGURE 28. Reconstruction of a Yellow-green Cell in an unusual location on the ventral surface of the right pleural ganglion. This cell is also shown in Fig. 20. The cell has two axons; one of these projects to the pleuro-pedal connective, but cannot be followed further; the other axon enters the pleuro-parietal connective, but turns back sharply and runs dorsally and anteriorly to leave the ganglion (dotted circle) from the dorsal surface of the pleuro-cerebral connective. A fine branch leaves the main axon before it divides. This branch terminates in neuropile.

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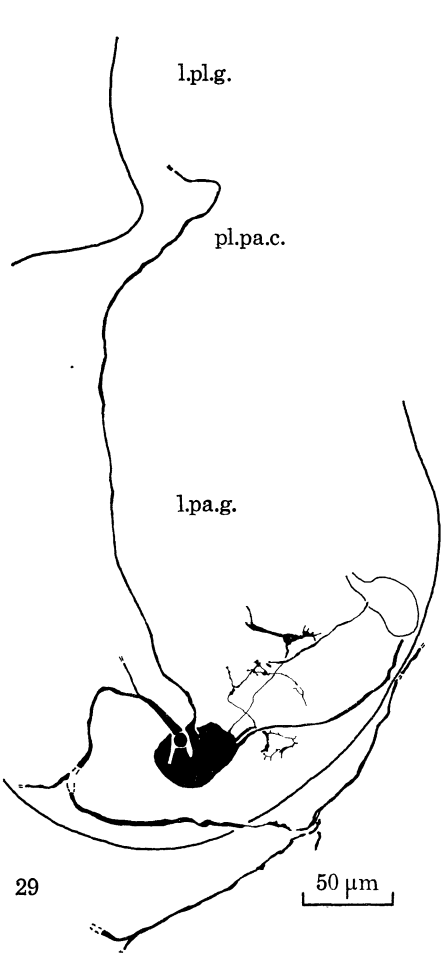
Yellow Cells in their morphology and give rise to fine branches proximal to the point where separate axons arise (figure 28). Some Yellow-green Cells are bipolar or multipolar (figures 26, 29, 30) and have axons which may project through the ganglion capsule to the sheath (figure 29) or to the surfaces of nerves and connectives (figures 26–28, 30). Often these axons run separately from the tracts formed by the axons of Yellow Cells, though some Yellow-green Cell axons do join these tracts (see following section).

Some, but not all, Yellow-green Cells have branches which terminate in neuropile (figures 28, 29), forming fine branches and blobs of varying size. This material usually originates from one or two branches which leave the cell body.

Yellow-green Cells which occur in nerves (and Yellow Cells also) appear to project in both directions along the nerve. In one case (figure 30) we were able to confirm this and show that



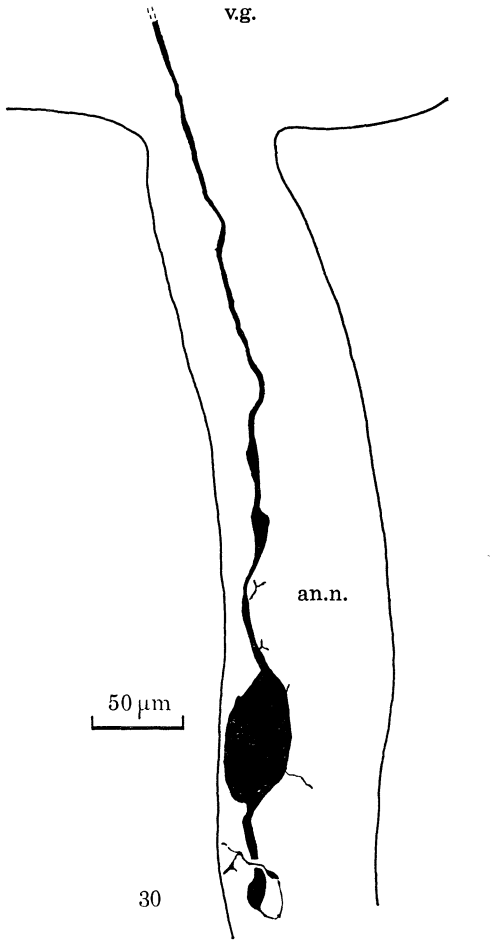


FIGURE 29. Reconstruction of a Yellow-green Cell in the left parietal ganglion. This cell was stained a light green colour. Five processes leave the cell body. The largest of them rises dorsally and leaves the ganglion near the parieto-visceral connective to enter the sheath, where it branches. A second process extends into the adjacent pleural ganglion, and a third runs medially to the surface of the left parietal ganglion. Neither of these branches could be followed further; they both probably enter the sheath. Two other processes run medially and terminate in neuropile, forming fine branches and blobs. Some of the branching processes may also have arisen from another Yellow-green Cell (drawn in outline), which sends a fine branch to the same area.

FIGURE 30. Reconstruction of a Yellow-green Cell in the anal nerve. One branch projects back into the visceral ganglion, and another terminates close to the cell on the surface of the nerve. Short fine branches arise from the cell body and one of the axons.

the centrally directed axon can enter the c.n.s., though whether the axon terminates within the c.n.s., or (more probably) whether it subsequently projects into the sheath, could not be determined.

The projections of the Yellow Cells and Yellow-green Cells

The projections of the Yellow Cells and Yellow-green Cells form prominent tracts through the ganglia (figure 25). The large group of Yellow Cells in the visceral ganglion project to the anal, intestinal and internal right parietal nerves (e.g. figure 24). Few, if any, of these cells project into the left parietal or pleural ganglia. Yellow Cells from the group in the right parietal ganglion which lies near the right pleuro-parietal connective (e.g. figure 22) project together with Yellow-green Cells in the same group, into the right parietal nerves and into the right pleuro-parietal connective, from which they may either enter the sheath or pass into the right pleural ganglion. It is not certain whether any of the cells in this group project into the visceral ganglion, though Yellow Cells and Yellow-green Cells located elsewhere in the right parietal ganglion may do so. Yellow Cells and Yellow-green Cells in the left parietal and pleural ganglia, and those in the visceral ganglion near the left parietal ganglion, project both anteriorly into the pleuro-cerebral connective and sometimes into the cerebral ganglion, and posteriorly into the visceral ganglion, from which they appear to project into the internal right parietal nerve, but possibly also into the intestinal and anal nerves. Yellow Cells (e.g. figure 21) and Yellow-green Cells in the right pleural ganglion project anteriorly to the pleuro-cerebral connective, and posteriorly into the right parietal ganglion, where they join the tract of Yellow Cell and Yellow-green Cell axons which project to the right parietal nerves. The anteriorly directed projections of the left and right pleural and parietal Yellow Cells and Yellow-green Cells may enter the sheath directly from the pleuro-cerebral or pleuro-parietal connectives, or they may pass through the cerebral ganglia for a variable distance before eventually projecting into the sheath. When Yellow Cells occur in the cerebral ganglia they behave similarly to cells in the pleural ganglia, projecting into the sheath directly and projecting posteriorly to join the main tracts into the parietal and visceral ganglia.

Description of plate 3

Figure 31. Yellow Cells and Yellow-green Cells lying within the intestinal nerve.

Figure 32. Dark field illumination of a horizontal section through the right parietal ganglion, showing the cell body of a Yellow Cell ventral and adjacent to the parieto-pleural connective. A branch leaves the cell body and runs undivided through neuropile for a short distance before dividing into further fine branches, some of which are arrowed.

Figure 33. Dark field illumination of a section through the right parietal ganglion, showing the cell body of a Yellow Cell near the pleuro-parietal connective. Axons and fine branching processes from Yellow Cells and Yellow-green Cells, and a peculiar arrangement of neurosecretory material round a small spherical gap in the neuropile can be seen (arrow).

Figure 34. Dark field illumination of a section through the right parietal ganglion, showing axons from a group of Yellow Cells leaving the pleuro-parietal connective and entering the sheath. The arrow shows the point where the axons leave the connective.

Figure 35. Neurosecretory axons running along the surface of the external right parietal nerve before it joins with the anal nerve.

Figure 36. Blobs of neurosecretory material (arrows) in the outer surface of a small branch of the combined anal and right parietal nerves, which runs through tissue in the wall of the lung near the pneumostome.

Figure 37. Neurosecretory material in a branch of the intestinal nerve which runs across muscle lining the posterior wall of the lung cavity.

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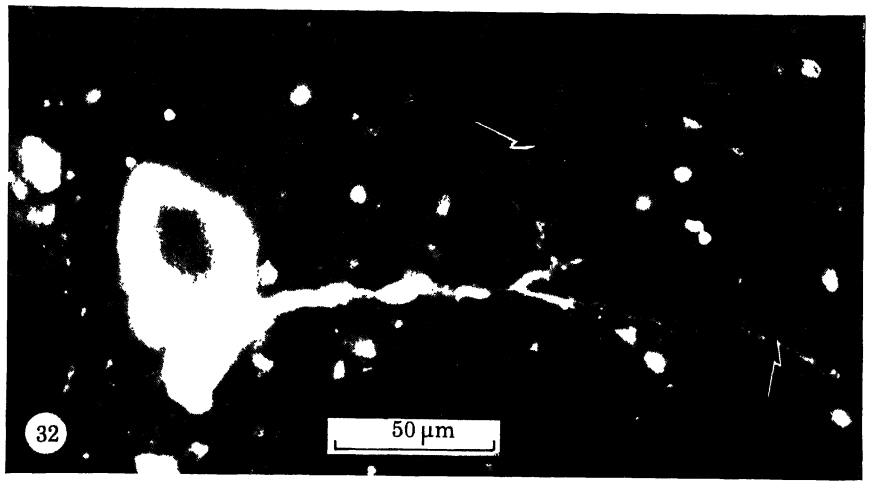

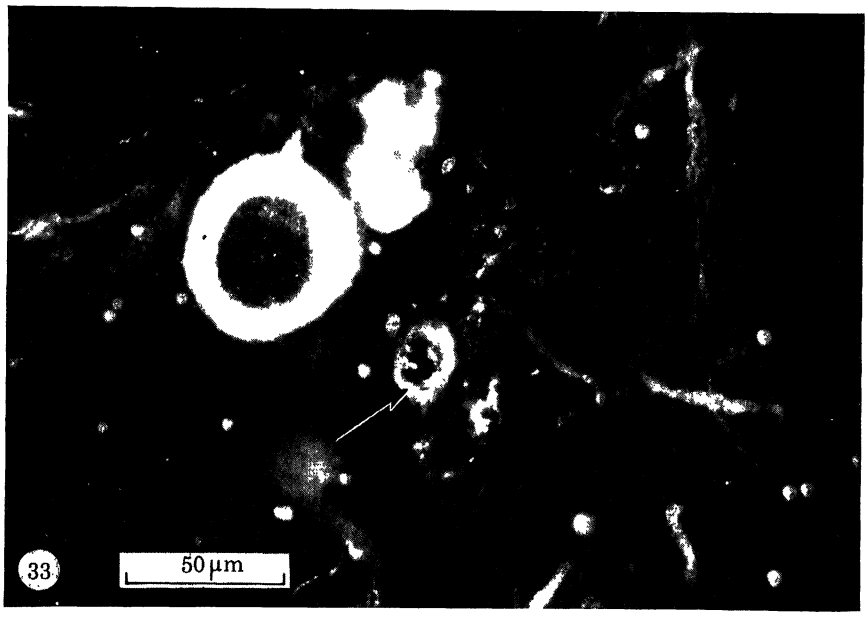

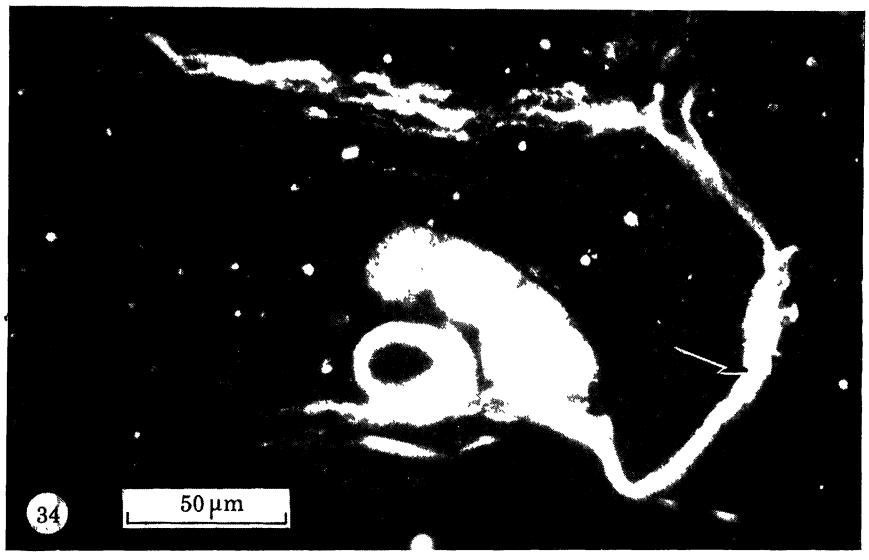

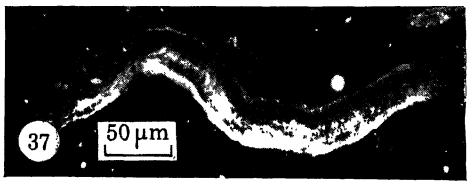

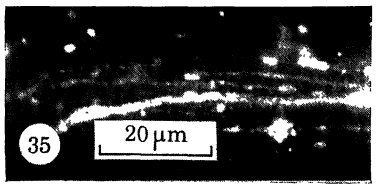

FIGURES 31–37. For description see opposite.

(*Facing p.* 192)

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Although release of Yellow Cell and Yellow-green Cell neurosecretory material is known to occur in the proximal regions (i.e. within about 5 mm of the c.n.s.) of all the visceral and parietal nerves (Wendelaar Bonga 1970*b*), neurosecretory axons extend beyond these regions, and can be found in the main unbranched trunks of the internal and external right parietal, anal, intestinal and genital nerves, and in many of their subsequent branches, at distances of more than 1 cm from the c.n.s. (figures 35–37, plate 3). As it is not possible to distinguish Yellow Cell from Yellow-green Cell axons under dark field, except by tracing them to their cells of origin, we cannot be certain how many of the neurosecretory axons found in the distal parts of these nerves are Yellow Cell axons and how many are Yellow-green Cell axons. However, since both types of cell project into these nerves from the c.n.s., and the cell bodies of both Yellow Cells and Yellow-green Cells occur in the nerves, it seems simplest to assume that the axon endings of both cell types are similarly distributed.

The internal right parietal nerve runs unbranched towards the right hand region of the mantle where the osphradium is situated. No neurosecretory material was found in the branch of this nerve which innervates the osphradium (or in the osphradium itself, cf. Benjamin 1971), but axons were found in branches which run through the mantle in a region dorsal to the pneumostome, and in a branch which innervates the kidney. This is in agreement with Wendelaar Bonga's (1970*a*, 1972) finding of axon terminals containing the Yellow Cell type of elementary neurosecretory granule in nerves running adjacent to kidney epithelium.

The external right parietal nerve and the anal nerve join in the head cavity, and the combined nerve runs in the floor of the lung to the extreme right hand corner of the mantle, where the pneumostome, rectum and urinary pore are located (see figure 4). Neurosecretory axons were found in all the larger branches of this nerve which run through these areas (figures 35, 36, plate 3).

The intestinal nerve runs posteriorly, embedded in thick layers of connective tissue which surround the cephalic artery, through the cervical septum into the visceral cavity. One large branch innervates a part of the columellar muscle which winds dorsally over the posterior surface of the last 180° turn of the shell (the muscle tract of the angle, Walter 1969), and which forms the posterior wall of the lung. Other branches innervate some of the genitalia (the prostate and the uterus) which lie ventral to the lung cavity. Further along this nerve, the cardiac branch (Elo 1938; Carriker 1946) innervates the pericardium and probably also the base of the kidney. Beyond this branch the nerve runs along the ventro-visceral artery to the digestive organs. Neurosecretory axons were found in the main trunk of the intestinal nerve, as far as the cardiac branch, beyond which very little material was present. Axons were also found in large numbers in the branch innervating the columellar muscle (figure 37, plate 3), and in branches innervating the genitalia. A few axons were present in the cardiac branch.

The genital nerve runs posteriorly in the connective tissue wall of the cephalic artery into the left hand side of the visceral cavity, where it divides once, and subsequently innervates the seminal receptacle and probably also the uterus. Large quantities of neurosecretory material were found in the main trunk of this nerve and in its two subsequent branches, but it is not known whether the material extends into subsequent branches within the innervated organs. Few of the Yellow Cells and Yellow-green Cells in the c.n.s. project into this nerve and most of the neurosecretory axons found in it arise from neurosecretory neurones with cell bodies in the genital nerve itself.

Neurosecretory axons are distributed for the most part on the outer surfaces of all of these

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nerves (figures 35–37, plate 3). Blobs, suggesting release are also frequent (figure 36). The areas of the animal where axons from Yellow Cells and/or Yellow-green Cells have been found, and where release from either cell type could potentially take place, are shown diagrammatically in figure 18*b*.

Discussion

Are the Dark Green Cells, Yellow Cells and Yellow-green Cells neurosecretory?

The results presented in this paper strengthen the evidence that the Dark Green Cells, Yellow Cells and Yellow-green Cells are neurosecretory. Electron microscopic work (Wendelaar Bonga 1970*b*) has already shown that the cell bodies are the sites of intense secretory activity, and neurohaemal areas for all three cell types (and for two other cell types – the Light Yellow Cells and Light Green Cells) have been identified on the basis of ultrastructural similarities between elementary neurosecretory granules observed in cell bodies and in axons in neurohaemal areas. Although the latter technique is potentially unreliable – there is no assurance that granules may not alter morphologically as they pass down the axon, or that the granules observed in axon endings do not originate from other unidentified cell types – our results confirm Wendelaar Bonga's findings: Dark Green Cells have projections to the surface regions of the pleuro-parietal and pleuro-cerebral connectives; Yellow Cells and Yellow-green Cells project into the vascular connective tissue sheath which surrounds the c.n.s., and have projections running over the surfaces of nerves leaving the right parietal and visceral ganglia. Wendelaar Bonga's finding of axon terminals containing the Yellow Cell type of elementary neurosecretory granule in nerves in the kidney is also consistent with our results (figure 18*b*). One difference has been noted: Dark Green Cells have not been found to project into the outer connective tissue sheath, though Wendelaar Bonga described axons containing the Dark Green Cell type of elementary neurosecretory granule in small nerves which run through the sheath. Possibly these axons are too fine for us to trace, but they might originate from some other cell type containing elementary neurosecretory granules similar in morphology to those of the Dark Green Cells.

Further evidence that the Dark Green Cells are neurosecretory comes from their ability to respond in a sustained fashion to a specific stimulus. When animals are exposed to de-ionized water, the Dark Green Cells show an increase in release activity which can be detected 24 h after exposure, and which continues for a further 14 days after this (Wendelaar Bonga 1971). Secretory activity within the cell bodies also increases during this period. Conversely, both release activity, and secretory activity within the cell bodies, are reduced when animals are kept in hypertonic saline. Yellow Cells show similar increases and decreases in release activity following 24 hours exposure to de-ionized water and hypertonic saline respectively (Wendelaar Bonga 1970*a*, 1972). These responses are unlikely to be aspecific or pathological, because the Light Yellow and Yellow-green Cell types show no changes in release activity under the same conditions.

Functional significance of the release sites of the Dark Green Cells

Fifteen to twenty per cent of the projections of the pleural Dark Green Cells are to the surfaces of the pleuro-parietal and pleuro-cerebral connectives, while the remainder of Dark Green Cell projections are to nerves innervating tissues underlying the major epithelial surfaces of the animal: the mantle, the neck and the sole and lateral walls of the head-foot (table 1). In view of this distribution of projections it is interesting that the secretory activity of the Dark Green Cells

is altered by changes in the composition of the external medium. The responses to the different media might involve hormonally mediated changes in the properties of the skin (Greenaway 1970), and thus the skin could be the target organ of the Dark Green Cells. Although Wendelaar Bonga (1971, 1972) interpreted the results of his experiments to suggest that the Dark Green Cells secrete a diuretic hormone, we believe this interpetation to be in itself, untenable, for reasons we give elsewhere (Swindale & Benjamin 1976). One possible function of the Dark Green Cells could be to stimulate active transport of ions across the animal's skin. Stimulation of active transport of sodium ions across the skin of *Lymnaea* is known to follow exposure to de-ionized water (Greenaway 1970). Another possibility is that the Dark Green Cells act to reduce the permeability of the skin to water. This is suggested by the results of Hekstra & Lever (1960) and Lever, Jansen & de Vlieger (1961) who found that removal of the pleural ganglia, which contain the majority of Dark Green Cells in the animal, caused animals to swell, while re-implantation of the ganglia, or injection of homogenates of pleural ganglia, reversed this effect, or caused animals to shrink. Thus the swelling caused by removal of the pleural ganglia could be due to an increase in the permeability of the skin to water (rather than a decrease in urine production rate, as Wendelaar Bonga (1971, 1972) suggests), and the shrinkage produced by injection of homogenates of pleural ganglia, or reimplantation of whole ganglia, could be caused by reduction of water inflow (rather than an increase in urine production rate). Reduction of skin permeability to water would be an adaptive response to de-ionized water if passive loss of ions was minimized as a result. Possibly the Dark Green Cells stimulate active transport of ions across the skin as well as decrease the permeability of the skin to water, both effects serving to maintain the osmotic pressure of the blood at the expense of a decrease in blood volume.

Functional significance of the release sites of the Yellow Cells and Yellow-green Cells

The proportion of Yellow Cell and Yellow-green Cell axons which project to the neurohaemal areas surrounding the c.n.s. is probably higher than in the Dark Green Cells. Perhaps about half of the projections from Yellow Cells and Yellow-green Cells in the pleural and parietal ganglia enter the sheath, either directly through the ganglion surface or via the cerebro- or parieto-pleural connectives. Once in the sheath the axons probably release into blood spaces or into small capillaries (Wendelaar Bonga 1970*b*). In the present study however, neurosecretory material was found along the entire lengths of the main unbranched trunks of the internal and external right parietal nerves and the intestinal, anal and genital nerves and in many subsequent branches occurring within peripheral tissues. Exceptions to this were the branch of the right parietal nerve which innervates the osphradium, and only small quantities of neurosecretory material were found in the branch of the intestinal nerve which runs posterior to the cardiac branch. Axons are also contributed by peripherally occurring Yellow Cell and Yellow-green Cell bodies.

Further electron microscopic studies are now needed to establish the detailed distribution of the release sites of each type of cell. Though it may turn out that the Yellow Cells and Yellow-green Cells have different release sites in the periphery of the animal, at present there are no anatomical reasons for supposing this to be so. Release could potentially occur along the entire lengths of the nerves in which Yellow Cell and Yellow-green Cell axons run. Both types of cell are known to release in the proximal regions of the nerves in which they run (Wendelaar Bonga 1970*b*), and the appearance of the neurosecretory material in these parts of the nerves in the light microscope is no different from the appearance of more distal regions. It also seems safe to

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assume that most of the neurosecretory axons found in the nerves will release in regions distal to those where they are found. If these assumptions are justified, then our observations imply that the Yellow Cells and Yellow-green Cells release in a variety of peripheral tissues (figure 18*b*) which include (*a*) the most lateral portion of mantle on the right hand side of the animal, including and surrounding the pneumostome; (*b*) the kidney and complex of veins running through it; (*c*) the part of the columellar muscle which forms the posterior wall of the lung and (*d*) connective tissues on the surfaces of the pericardium, seminal receptacle, anterior prostate and uterus, which protrude into the ventral part of the lung. Release might also occur within the latter three organs, though we have no evidence for or against this.

A possible clue to the significance of this wide distribution of release sites is given by the blood supply to these regions, which is rather complexly organized (Bekius 1972; Walter 1969). Venous blood from the visceral sinus forms a portal system supplying the mantle with blood. There is no other blood supply to the mantle. In the region (*a*) of the mantle mentioned above, blood draining from the mantle joins with venous blood returning from the head-foot. Nearly the entire venous return of the animal is concentrated at this point. This blood then forms a second portal system supplying the lung walls and kidney before finally joining the single large vein in the kidney going to the heart. Thus peripherally released Yellow Cell and Yellow-green Cell hormone in regions (*a*), (*b*) and (*c*) above will probably be transported rather rapidly through the lung and kidney and then to the heart and the arterial side of the circulation. Release of neurosecretory material from branches of the intestinal and genital nerves in region (*d*) above, may also fit into this pattern, since these nerves run for large distances over the surfaces of organs which protrude into the ventral parts of the lung, increasing its surface area. Release from the surfaces of these nerves may thus also be into venous channels in the walls of the lung.

Different functional interpretations of this pattern of release are possible. If the Yellow Cell and Yellow-green Cell release sites are as widely distributed as they appear to be, then the most likely explanation of the arrangement is that it is a means of ensuring a rapid distribution of the hormones throughout the circulation following release. Alternatively, one or both cell types may be concentrating release within target organs such as the kidney, lung walls or the heart.

Both the intestinal nerve and the genital nerve run embedded in the cephalic artery for a large part of their length, and many of the axons running in the sheath of the c.n.s. also run in the walls of this artery, the tissues of which are continuous with the sheath. Thus release into the arterial side of the circulation seems possible. However, electron microscopic work (Wendelaar Bonga 1970*b*) has not shown that neurosecretory axons penetrate the arterial endothelium, so that whether any Yellow Cell or Yellow-green Cell release occurs directly into arterial blood is uncertain.

Reasons for peripheral release of neurohormones

Various factors may contribute to the development of terminals located in the periphery of the animal. There will be a saving in energy needed for synthesis of hormone if its concentration can be kept higher in blood surrounding target tissue than elsewhere in the circulation. Since this saving is dependent on the maintenance of a concentration gradient of the hormone in the bloodstream, a highly localized target tissue, a low rate of blood flow to the target tissue, and a short half life of the hormone in the circulation, will all favour the existence of terminals located close to the target tissue. Peripheral release might also occur to overcome the limitations

on the rapidity of offset and onset of action of the hormone, set respectively by the half life of the hormone in the blood and the circulation time between releasing terminals and target tissue.

Some of these factors may well have contributed to the development of peripherally releasing terminals in the Dark Green Cells. Blood pressure in *Lymnaea* is low (Dale 1974) and although the dynamics of blood flow through the various tissues is unknown, circulation through the tissues of the mantle and the foot is likely to be sluggish, particularly in the mantle, which has no arterial supply (Bekius 1972; Walter 1969). Release into the central neurohaemal areas surrounding the brain would be inefficient if a rapid onset of action of the hormone was necessary, since blood from the head sinus returns to the heart, via the kidney–lung portal system, before circulating to the foot, and to reach the mantle would have to circulate first through the heart and viscera into the visceral sinus, which is the only source of blood to the mantle. Osmoregulatory responses may have to occur rapidly when the animal is exposed to sudden changes in its external environment, since about 20 % of blood volume per hour (9 % of body mass) is excreted (van Aardt 1968). The function of the centrally ending projections of the Dark Green Cells may be to supply the skin and tentacles on the dorsal surface of the head-foot since, unlike other tissues in the head-foot, these are supplied with blood directly from the head sinus (Bekius 1972). This may explain the absence of Dark Green Cell projections in the tentacle and nuchal nerves (see table 1) which innervate these areas.

Peripherally located neurosecretory terminals are common in insects (Maddrell 1967), where they are located in proximity to a variety of tissues, including epithelia specialized for active transport (see, for example, Jarial & Scudder 1970). In molluscs other than *Lymnaea* there have been only a few reports of possible peripheral neurosecretion. Cottrell & Osborne (1969) describe a neurohaemal complex in the heart of *Helix pomatia*, though similar structures observed in the hearts of *Archachatina* (Baxter & Nisbet 1963; Nisbet & Plummer 1966) and *Helisoma* and *Helix aspersa* (Simpson 1969) are regarded by these authors as neuromotor rather than neurosecretory. An equivalent structure in *Lymnaea* heart has not yet been identified.

Intraganglionic morphology of the Dark Green Cells, Yellow Cells and Yellow-green Cells

The fact that axons of Dark Green Cells often make apparent contact with one another, within a few hundred micrometres of the cell body (figure 7), suggests that direct synaptic interaction between the Dark Green Cells might occur. This has been confirmed by intracellular electrophysiological recording from pairs of Dark Green Cell bodies, which has shown that some Dark Green Cells, though not all, are electrotonically coupled (Benjamin *et al.* 1976). Intracellular injection of Procion Yellow (P. R. Benjamin, unpublished results) has also confirmed the present finding that the Dark Green Cells have few or no fine branches terminating in neuropile.

The fine branches of the Yellow Cells and Yellow-green Cells which terminate in neuropile could be pre- or post-synaptic to other neurones, or both. They are unlikely to represent only sites of direct synaptic interaction with other Yellow Cells, Yellow-green Cells or Dark Green Cells, since branching could occur in regions of neuropile where material from no other known neurosecretory cell type was present. If the branches are only post-synaptic then the presence of secretory material in them has to be explained. If the branches are pre-synaptic, then either Dale's principle is violated (Finlayson & Osborne 1975) (in which case the presence of secretory material still has to be explained) or the neurosecretory material has a duel role as transmitter and hormone. The latter hypothesis is made more plausible by the observation in a number of

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invertebrate species, of granules similar to elementary neurosecretory granules at synapse-like structures in neuropile (Gerschenfeld 1963; Schlote 1963; Shih-Kai & Zapf 1965; Golding 1967) and by the observations of Kandel (1964) and Nicoll & Barker (1971) which show that hypothalamic neurosecretory neurones have recurrent inhibitory collaterals to other neurosecretory cells. The function of intraganglionic release of hormone, if it occurs, is a matter for speculation. It could provide the c.n.s. with information about the amount of hormone being released elsewhere, as part of a feed-forward control system regulating release activity. Alternatively, intraganglionic release might mediate behavioural changes appropriate to the peripheral effects of the hormone, or the stimuli causing its release.

Morphological studies on neurosecretory neurones in other gastropods have revealed some features similar to those found in the Dark Green Cells, Yellow Cells and Yellow-green Cells. Projections of single cells through the ganglion capsule to the sheath, as well as to nerves leaving the ganglia, are found in the rostral white cells of *Aplysia* (Coggeshall, Kandel, Kupfermann & Waziri 1966; Frazier *et al.* 1967; Winlow 1975) and in the RPaD cells of the right parietal ganglion of *Helix pomatia* (Sakharov & Salánki 1971). In the case of the rostral white cells, the projections to the nerves almost certainly end peripherally (Jahan-Parwar, Smith & von Baumgarten, 1969), but it is not known where. In both *Helix* and *Aplysia* the projections into the sheath arise as numerous fine hairlike processes from the cell body, whereas in *Lymnaea* the processes are thicker, less numerous and clearly axonal in character.

Another feature common to the rostral white cells, the Dark Green Cells, Yellow Cells and Yellow-green Cells is the occurrence in ganglia, connectives and nerves, of approximately spherical swellings about 2–10 times greater in diameter than the axons. Those in the Dark Green Cells, Yellow Cells and Yellow-green Cells stain heavily, while those in the rostral white cells were found to be densely packed with elementary neurosecretory granules (Frazier *et al.* 1967). These are rather infrequent features however, and many single rostral white cells, Dark Green Cells, Yellow Cells and Yellow-green Cells appear to lack them altogether. This suggests that they do not represent the form in which neurosecretory materials are transported along the axon. They could perhaps be the result of some obstruction to the passage of neurosecretory material along the axon. This is suggested by the observation that swellings often occur at bends in the axon (e.g. figure 22*a*); that swellings in Dark Green Cell axons often occur proximal to, or within, connective which were stretched for some time before fixation (see Materials and Methods), and that the axon distal to the swelling is frequently less heavily stained than the axon proximal to it.

Although there are some similarities between the Dark Green Cells, Yellow Cells and Yellow-green Cells, these cell types have many different features (summarized in table 2) which suggest that the cell types are functionally distinct, and that the classification which can be effected with the Alcian Blue–Alcian Yellow technique is a reliable one. Many of the anatomical differences suggest that either in ontogeny or in evolution, or both, the Dark Green Cells derive from elements of the c.n.s., whereas the Yellow Cells and Yellow-green Cells derive from elements of the peripheral nervous system which have become only partly incorporated into the c.n.s. Thus the cell bodies of the Dark Green Cells are never found outside the c.n.s. and always occur in the conventional location for nerve cell bodies on or near the surface of the ganglion. Yellow Cells and Yellow-green Cells on the other hand, occur close to the connectives and nerves of the ganglia, and are frequently found outside the c.n.s., in the thick outer connective tissue sheath, or lying on, or within, nerves leaving the parietal and visceral ganglia. Mor-

TABLE 2. A SUMMARY OF THE DIFFERENCES IN THE PROPERTIES OF THE DARK GREEN CELLS, YELLOW CELLS AND YELLOW-GREEN CELLS

Dark Green Cells	Yellow Cells	Yellow-green Cells
histochemical		
strong affinity for Alcian Blue at pH 1·0	no affinity for Alcian Blue at pH 1·0	varying affinity for Alcian Blue at pH 1·0
ultrastructural		
elementary granules 150–240 nm in diameter	elementary granules 100–160 nm in diameter	elementary granules 130–230 nm in diameter (Wendelaar Bonga 1970*b*)
elementary granule refractive index $\simeq$ 1·53	elementary granule refractive index $\neq$ 1·53 (Swindale & Benjamin 1975)	
cell bodies		
pale white-orange in colour in living ganglia	often bright white in living ganglia	
found on the ventral surface of the pleural ganglia, where Yellow Cells and Yellow-green Cells are normally absent	yellow cells are found in the visceral ganglion near the right parieto-visceral connective, where Yellow-green Cells are normally absent	
never found outside the c.n.s.	often found outside the c.n.s. in nerves, and in the connective tissue sheath of the c.n.s.	
cytoplasm evenly stained	cytoplasmic staining separated by clear canals into large blobs	
axonal morphology		
always monopolar	monopolar, or sometimes bipolar	can be mono-, bi-, or multi-polar
pre- or post-synaptic branches not demonstrated	fine blobbed and branching processes terminating in neuropile: some Yellow-green Cells lack these processes	
projections entering the sheath not demonstrated	project into the sheath from the ganglion surface and from the pleuro-parietal and pleuro-cerebral connectives	
numerous projections to the foot and mantle	projections few or absent in nerves innervating the foot and the left hand part of the mantle	
projections run dispersed within cross sections of nerves and connectives	projections run over the surfaces of nerves and connectives	
several axons, branching at varying distances from the cell body	several axons, normally diverging from one pole of the cell body, or close to it	one or more axons, often diverging separately from the cell body
functional		
release activity increases when animals are placed in de-ionized water, and decreases when animals are placed in hypertonic saline		release activity unaffected by either of these treatments (Wendelaar Bonga 1972)
differences in the electrophysiological properties of all three cell types (Benjamin & Swindale 1975 and in preparation)		

phologically the Dark Green Cells are much more like ordinary neurones in the c.n.s. than the Yellow Cells or Yellow-green Cells. The axons of the Dark Green Cells always run parallel to, and within, tracts formed by non-neurosecretory axons in ganglia and nerves, whereas Yellow Cell and Yellow-green Cell axons can cut across these tracts and run as isolated axons, sometimes through layers of cell bodies, to reach the sheath. They can also run outside the cell body layer immediately under the thin inner sheath (cf. Rosenbluth 1963). In these respects the Yellow Cells and Yellow-green Cells behave quite differently from the majority of neurones in the c.n.s. (Benjamin & Ings 1972). When Yellow Cell and Yellow-green Cell axons do run parallel to axon tracts, they normally run along the outside of them, so that when they leave the ganglia they run on the surfaces of nerves and connectives, thus preserving their 'peripheral' relationship to the rest of the c.n.s.

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Key to abbreviations used on figures

an.n.	anal nerve	med.pd.n.	medial pedal nerve
cl.n.	columellar nerve	nu.n.	nuchal nerve
cu.pa.n.	cutaneous pallial nerve	o.n.	optic nerve
e.r.pa.n.	external right parietal nerve	pa.v.c.	parieto-visceral connective
gen.n.	genital nerve	pe.n.	penis nerve
inf.cv.n.	inferior cervical nerve	pl.cr.c.	pleuro-cerebral connective
inf.pd.n.	inferior pedal nerve	pl.pa.c.	pleuro-parietal connective
int.n.	intestinal nerve	pl.pd.c.	pleuro-pedal connective
i.r.pa.n.	internal right parietal nerve	r.cr.g.	right cerebral ganglion
la.d.b.	latero-dorsal body	r.pa.g.	right parietal ganglion
la.lo.	lateral lobe	r.pd.g.	right pedal ganglion
l.cr.g.	left cerebral ganglion	r.pl.g.	right pleural ganglion
l.pa.g.	left parietal ganglion	st.	statocyst
l.pa.n.	left parietal nerve	sup.cv.n.	superior cervical nerve
l.pd.g.	left pedal ganglion	sup.lp.n.	superior lip nerve
l.pl.g.	left pleural ganglion	sup.pd.n.	superior pedal nerve
m.d.b.	medio-dorsal body	t.n.	tentacle nerve
med.lp.n.	medial lip nerve	v.g.	visceral ganglion

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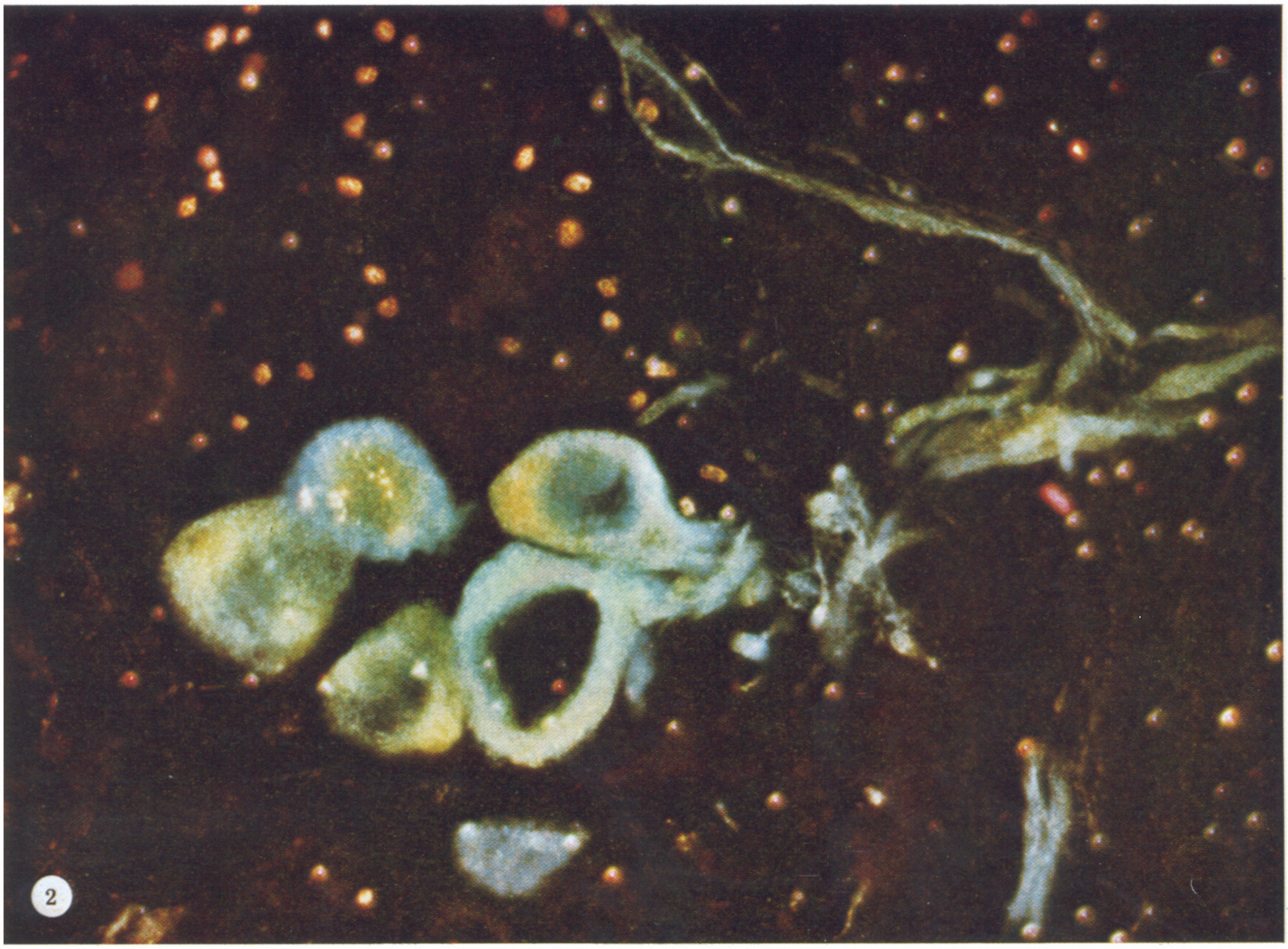

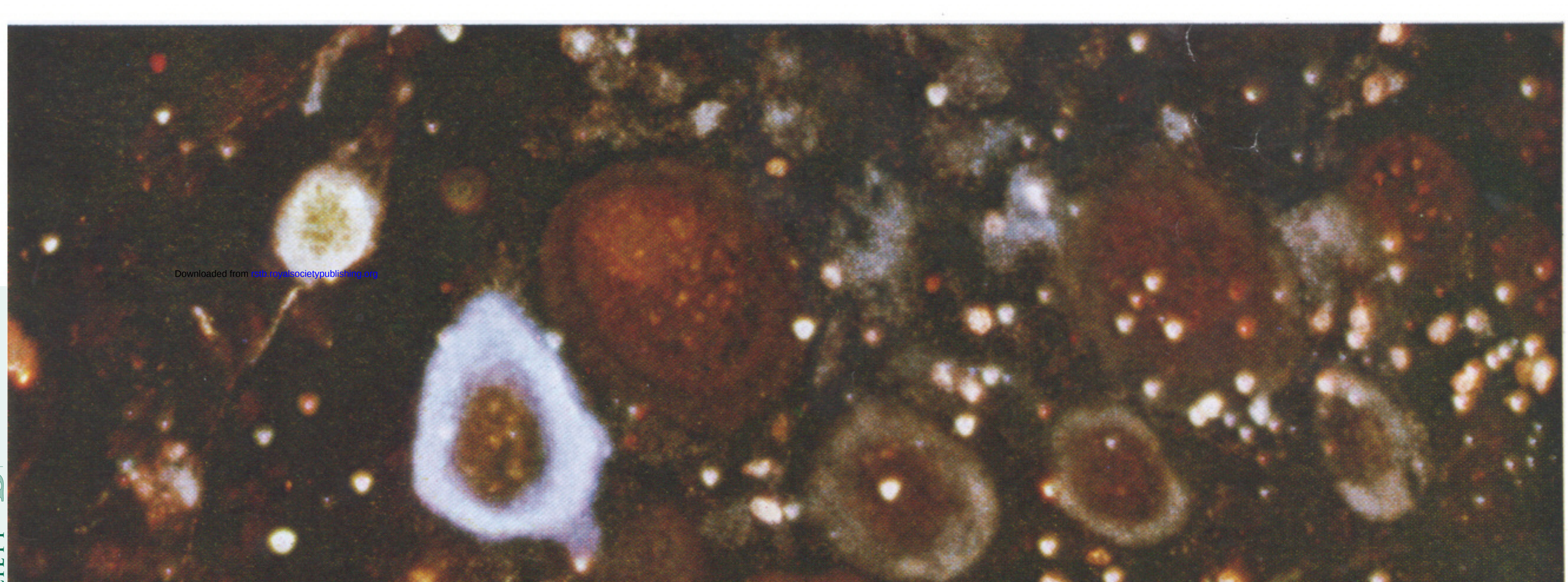

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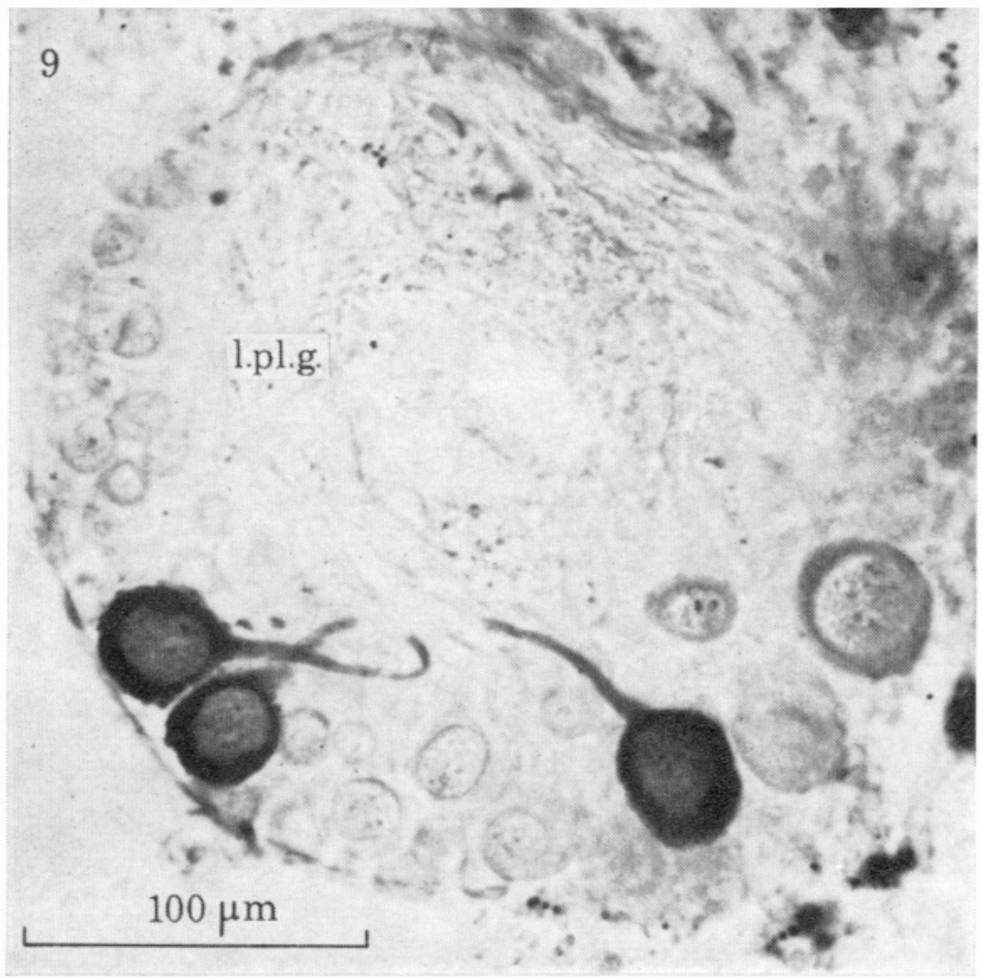
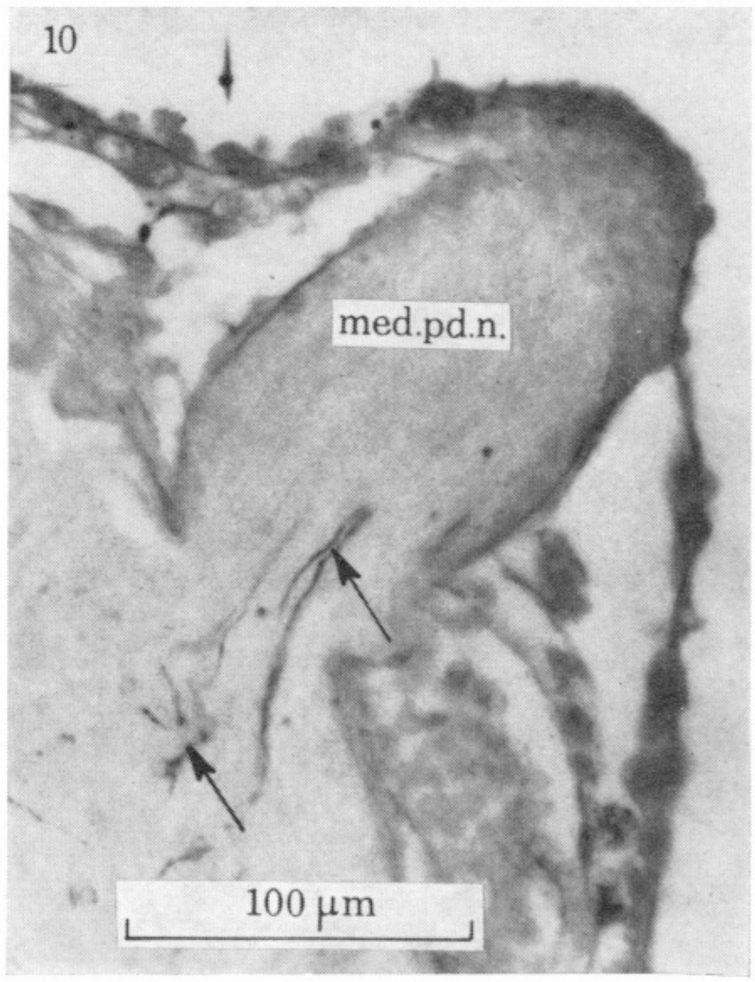
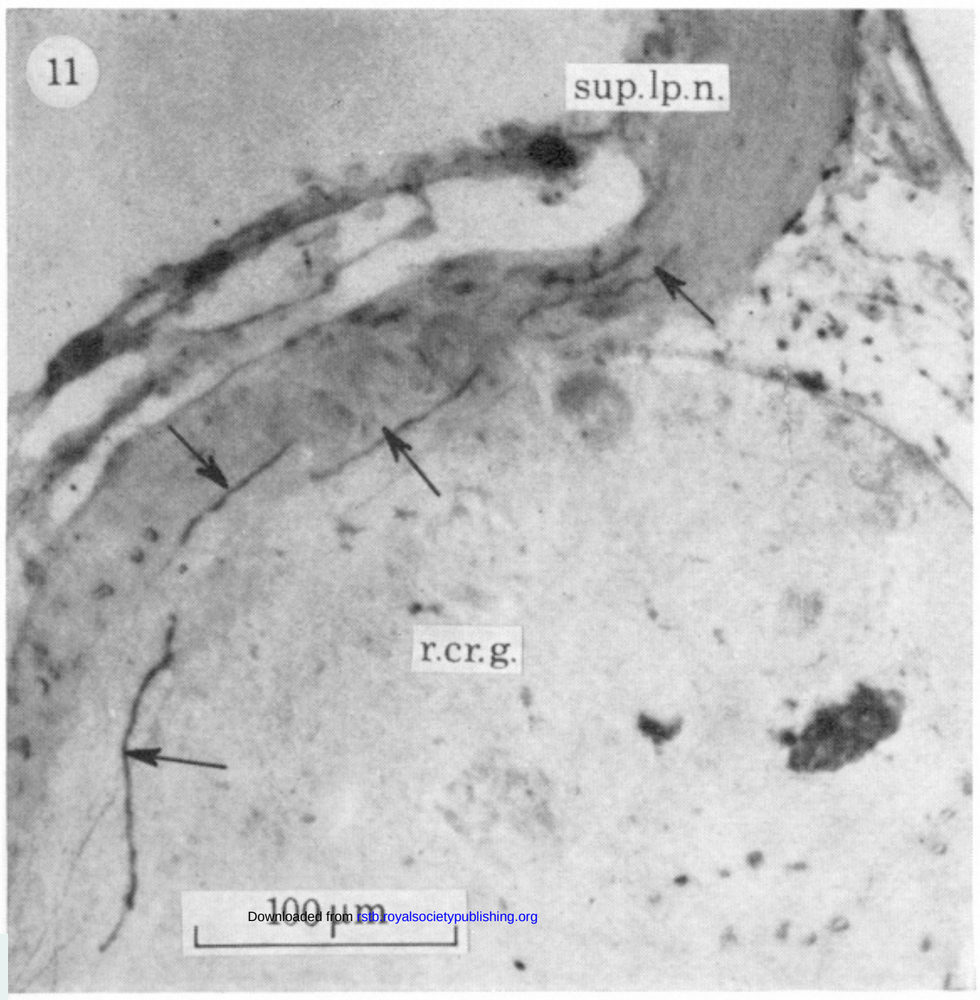
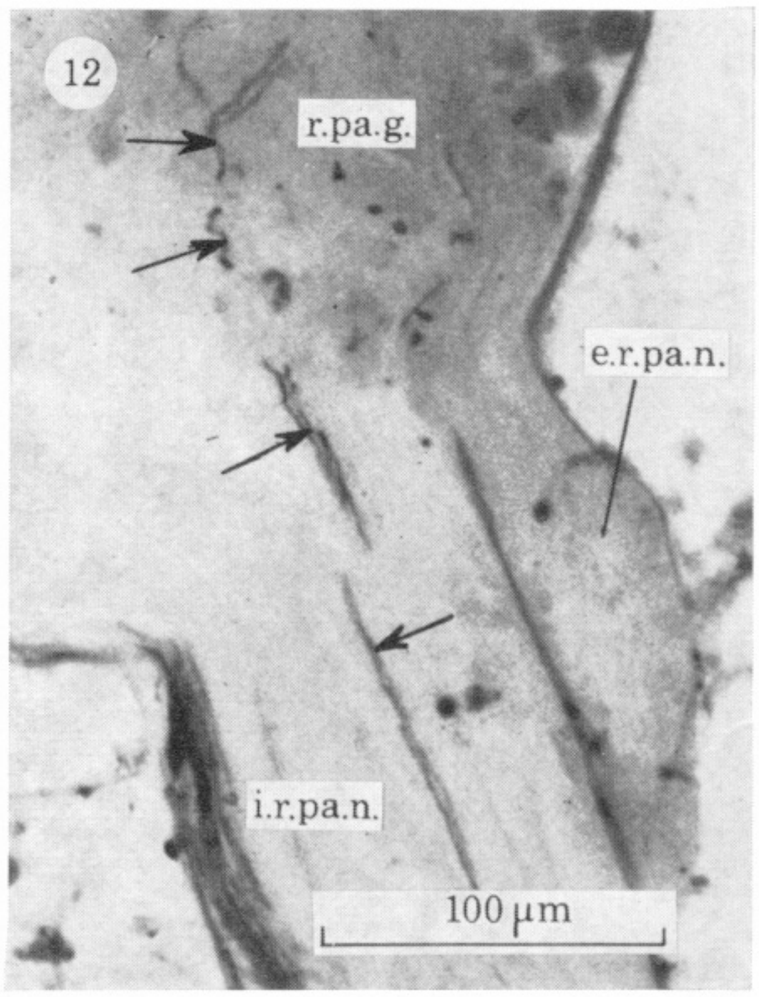
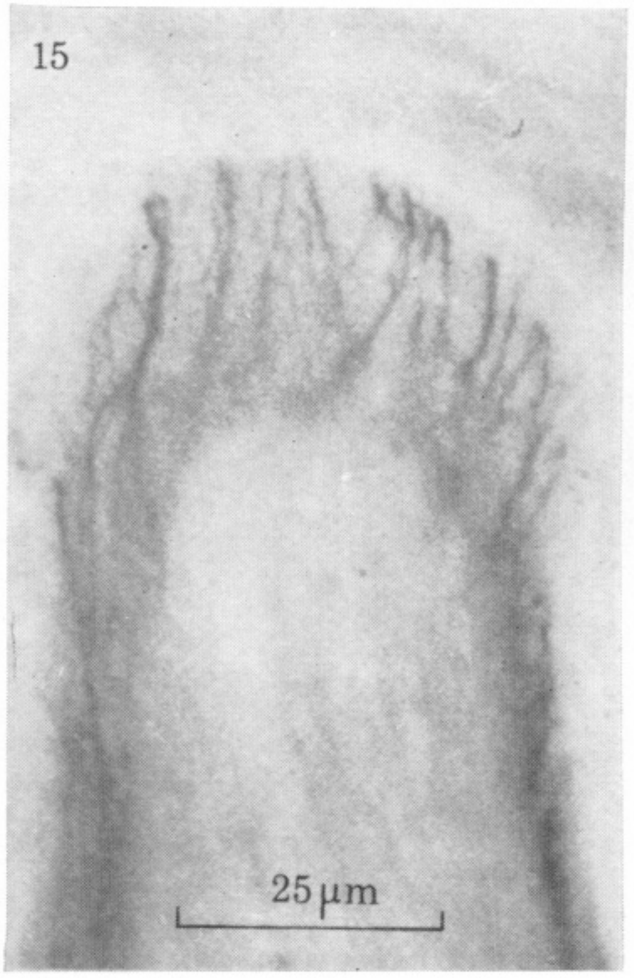
FIGURES 2 AND 3. For description see opposite.

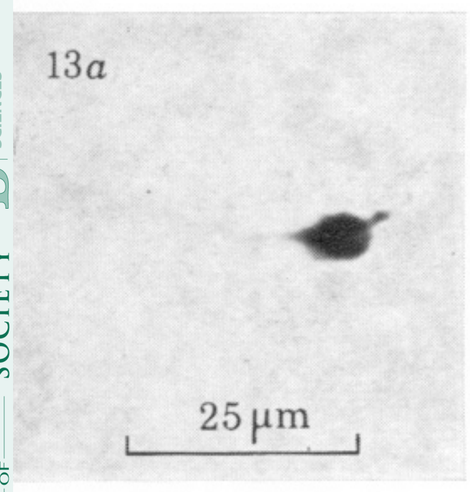


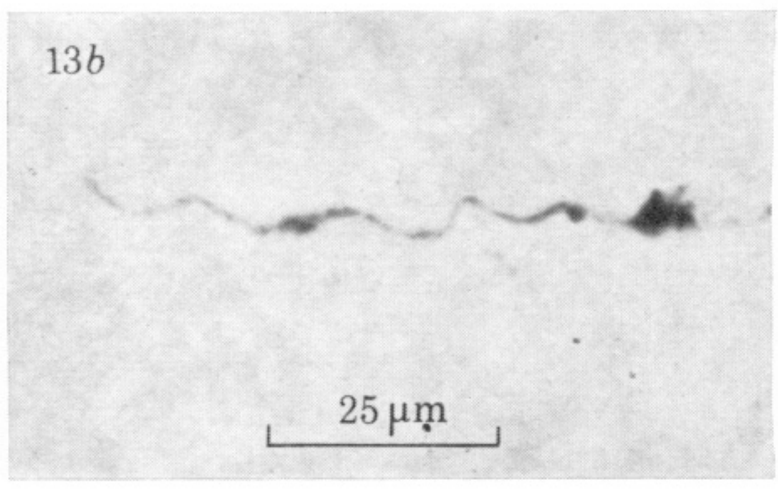


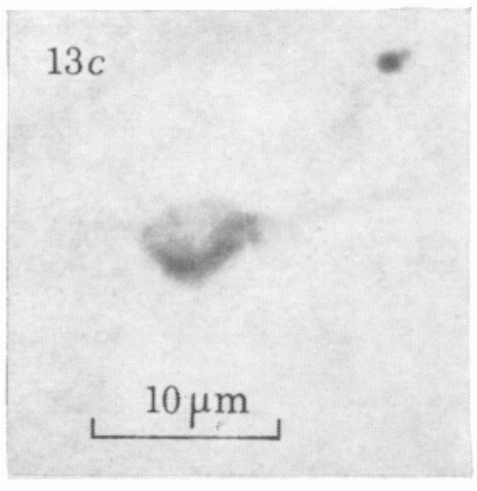


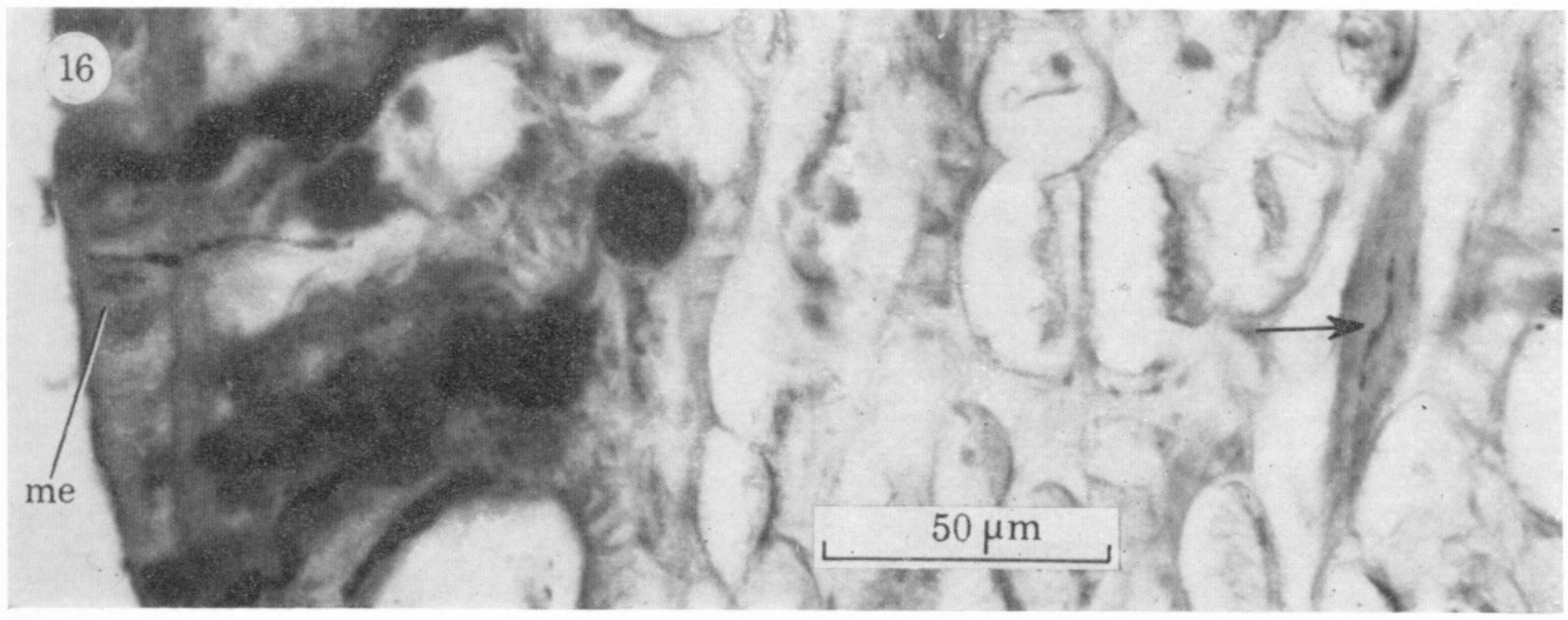


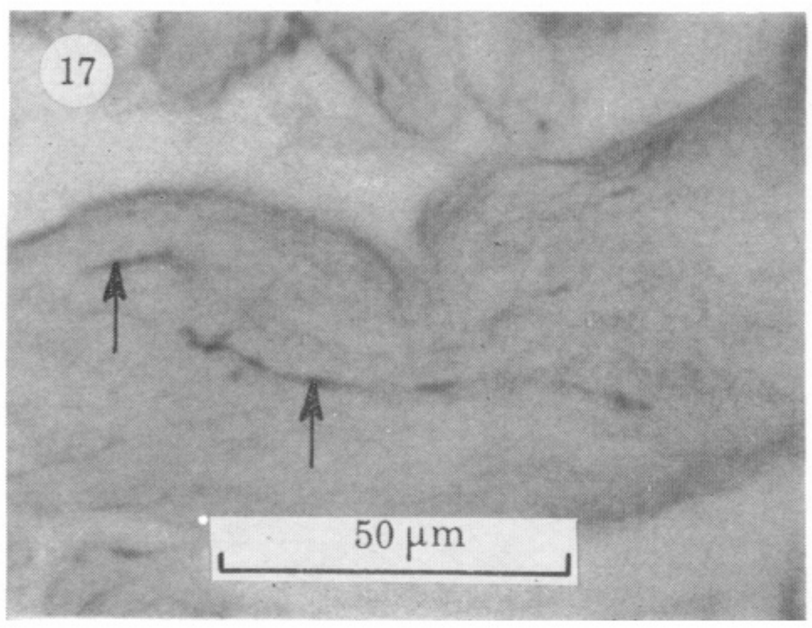




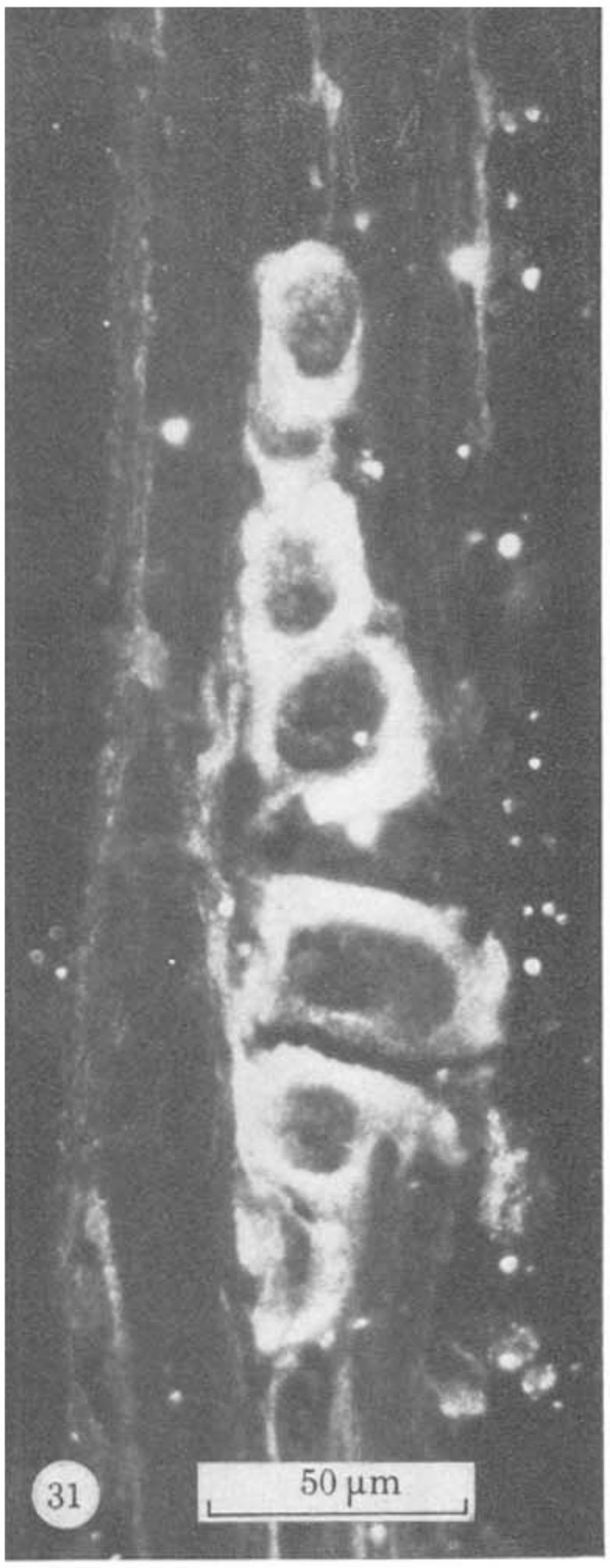


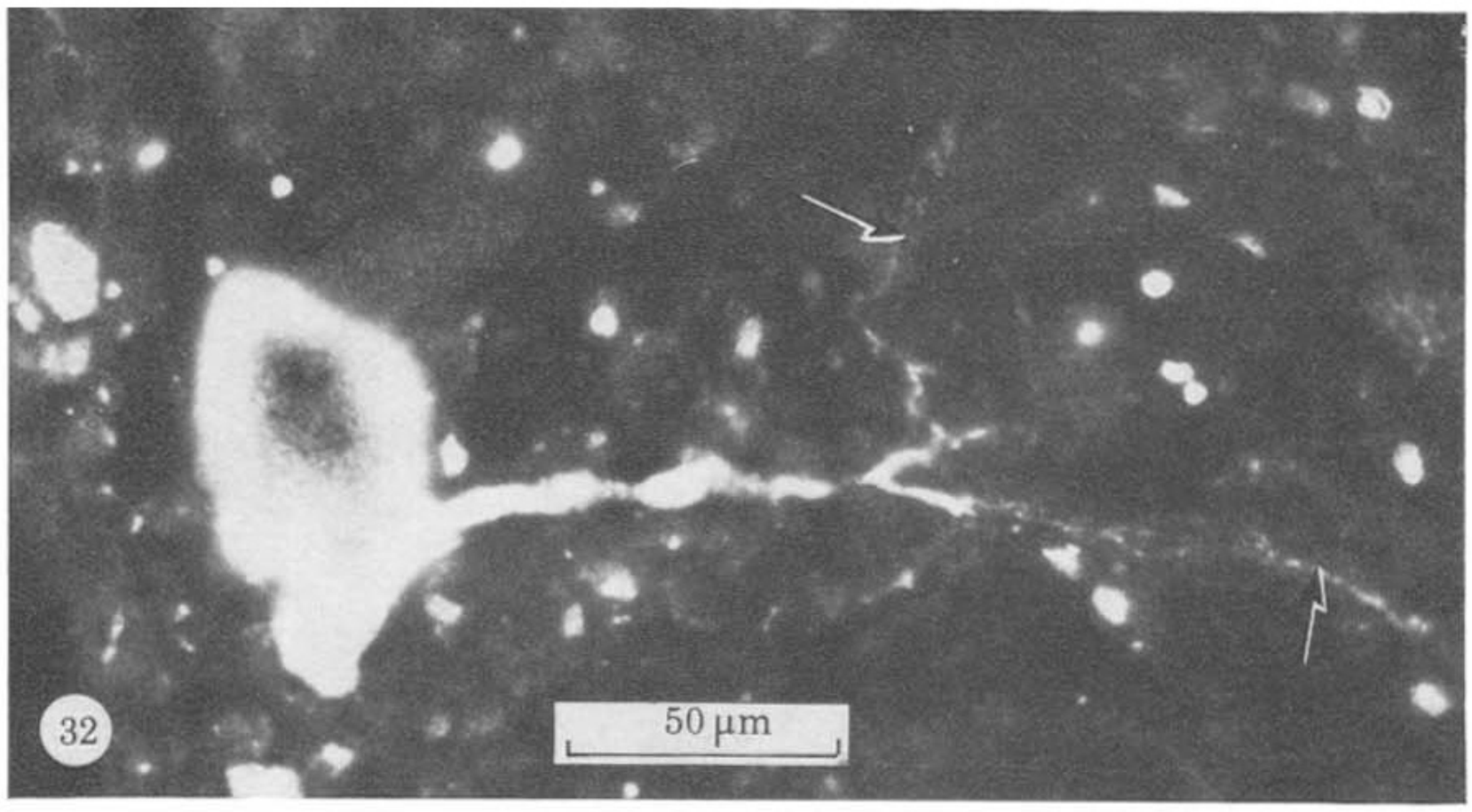


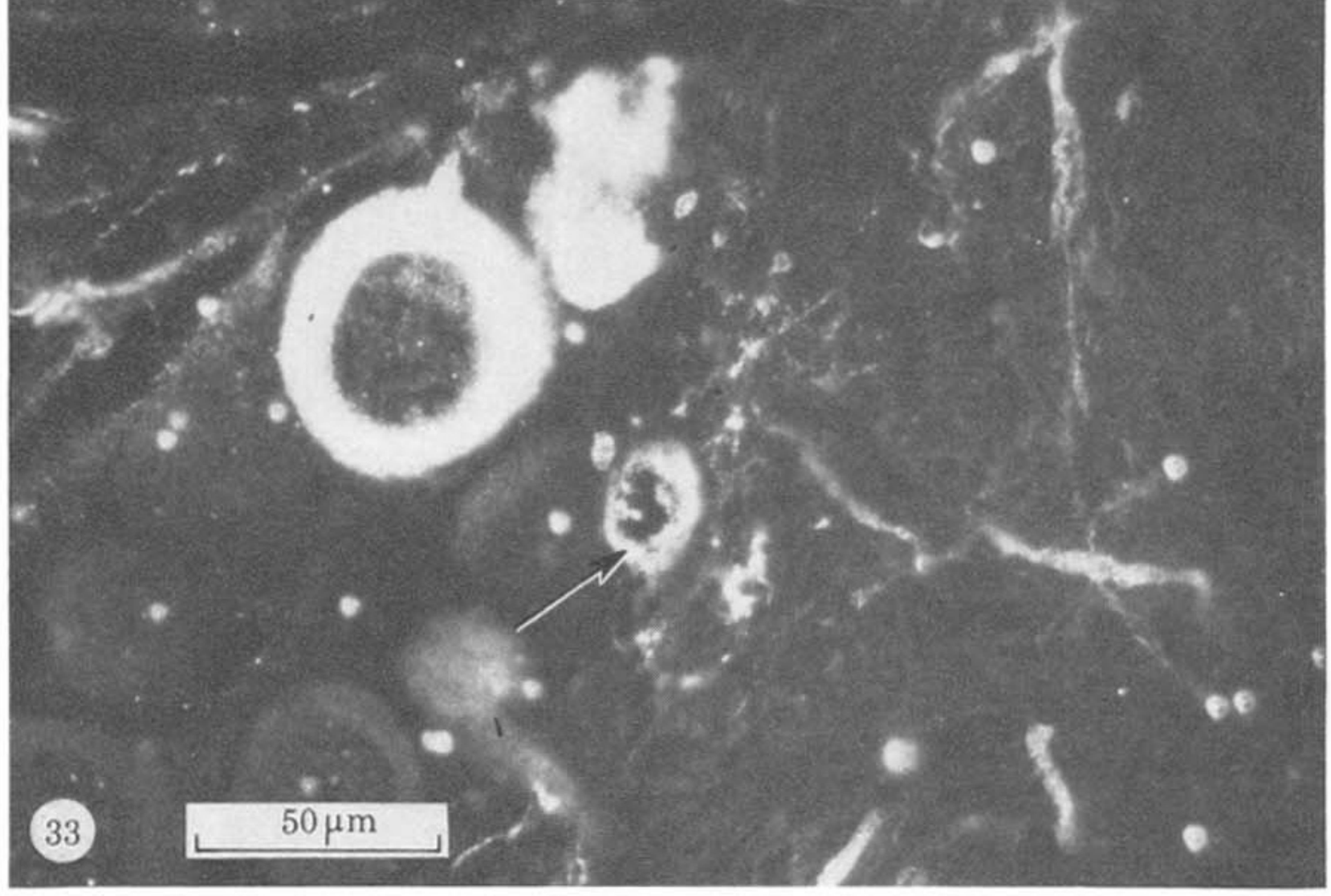


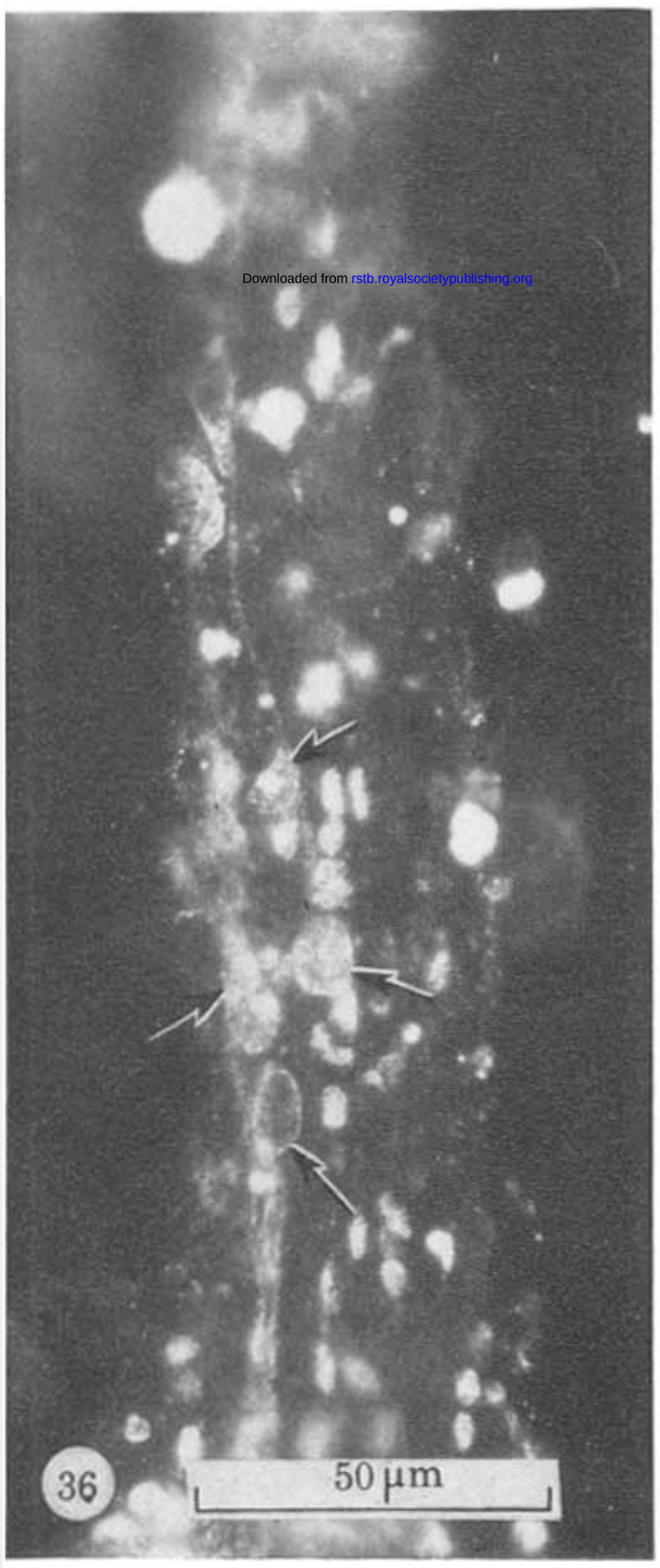


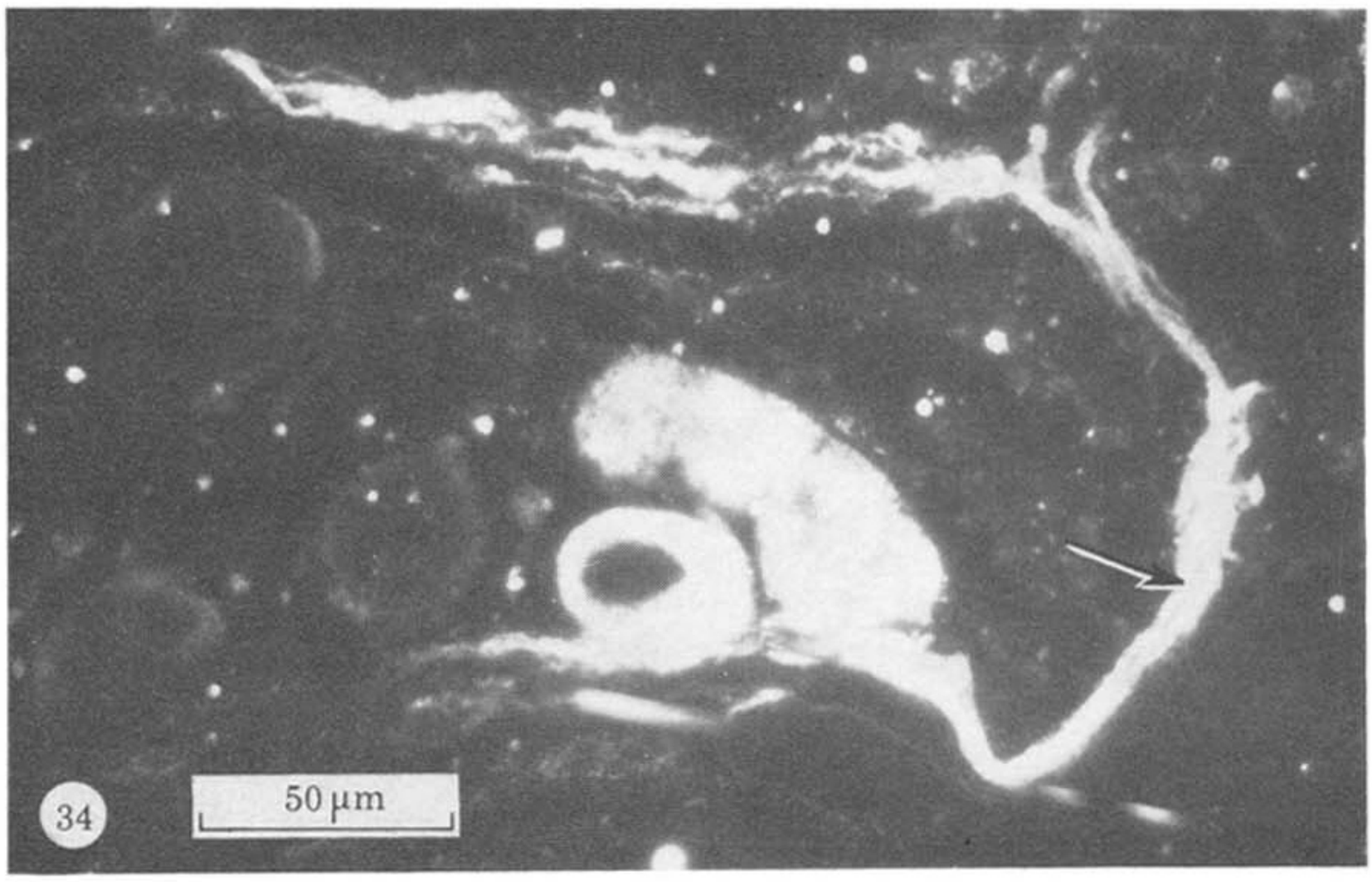
FIGURES 9–17. For description see opposite.

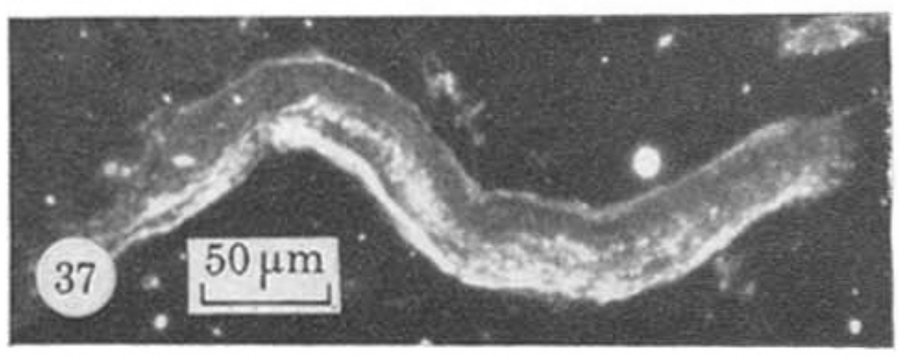


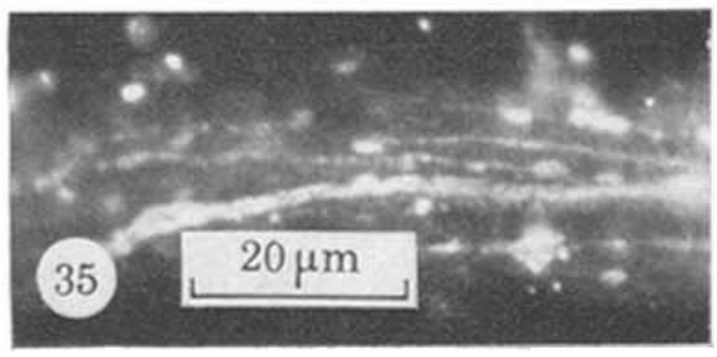












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FIGURES 31–37. For description see opposite.