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S.E. Wendelaar Bonga

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## Ultrastructure and Histochemistry of Neurosecretory Cells and Neurohaemal Areas in the Pond Snail *Lymnaea stagnalis* (L.)

S. E. WENDELAAR BONGA\*

Department of Zoology, Free University, Amsterdam, The Netherlands

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*Summary.* In the central ganglia of *Lymnaea stagnalis* neurosecretory cell groups have previously been identified by means of chrome-haematoxylin or paraldehyde-fuchsin stains. In the present study seven types have been distinguished within the class of Gomori-positive cells on the basis of different staining reactions with the alcian blue/alcian yellow technique. Five types are located in the cerebral ganglia and in the lateral lobes, whereas two cell types occur in the ganglia of the visceral ring. No neurosecretory cells have been observed in the buccal and pedal ganglia.

The staining technique used proved to be superior to the classic neurosecretory stains, because with this method the secretory substances can easily be distinguished from non-secretory Gomori-positive tissue constituents.

One of the two Gomori-negative neurosecretory cell types of the cerebral ganglia react positively with the alcian blue/alcian yellow technique. In addition, two Gomori-negative neurosecretory cell types, which had not been described before, were identified in the visceral ring.

The ultrastructure of the four neurosecretory cell types in the visceral ring is described. The electron microscope revealed that each of the histochemically distinguished secretory substances consists of elementary granules which differ in size and appearance from each other and from the neurosecretory elementary granules which have been described by other authors in the cerebral ganglia and in the lateral lobes.

The neurohaemal areas of the neurosecretory cells in the visceral ring are very extensive and include the peripheral parts of the nuchal nerves and of the connectives and nerves of the ganglia of the ring. The perineurium and the adjacent parts of the connective tissue which surround the ganglia, the connectives and the nerves are regarded as additional neurohaemal zones, because in these regions many tiny nerves occur, which consist mainly of neurosecretory axons ending non-synaptically near parts of the vascular system. In the perineurium surrounding the cerebral ganglia and their neurohaemal area a similar network of neurosecretory fibres was observed.

Indications of release of the secretory material were regularly observed. Release apparently takes place by exocytosis.

A circadian rhythmicity was observed in the release activity of some of the neurosecretory cell types.

*Key-Words:* Neurosecretion — Neurohaemal areas — Invertebrates — *Lymnaea stagnalis* L. — Cytology.

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Reviews of the literature on neurosecretory phenomena in many species of the Gastropoda have been published by Gabe (1966), Simpson (1966a) and Durchon (1967). In most species investigated several groups of Gomori-positive neurosecretory cells occur. Little attention has been paid so far to the possibility that within the class of Gomori-positive cells in a given species different types may occur. In this respect it is interesting that Cook (1966) distinguished three types of Gomori-positive cells in the stylommatophore *Succinea putris* on the basis of tinctorial differences obtained with the classic neurosecretory stains chrome-haematoxylin (CH) and paraldehyde-fuchsin (PF). Recently similar results have been reported for the colorado beetle *Leptinotarsa decemlineata* (Schooneveld, 1970).

The occurrence of Gomori-negative neurosecretory cells has been established in only a few gastropods (e.g., Joosse, 1964; Boer, 1965; Cook, 1966).

In the cerebral ganglia of the Pulmonata, groups of Gomori-positive neurosecretory cells occur at specific locations. Their products are transported via axonal tracts to large numbers of axon endings localized at the periphery of the intercerebral commissure and of the median lip nerve or the nervus arteriae cerebralis. It has been suggested that in these zones storage and release of neurohormones take place. Accordingly, these zones were called neurohaemal areas (Joosse, 1964; Röhnisch, 1965; Nolte, 1965; Simpson, 1970). Thus, when compared to the compact neurohaemal organs found in arthropods (sinus gland, corpus cardiacum) and vertebrates (neurohypophysis, urophysis) the neurohaemal areas in pulmonates are very extensive.

Detailed morphological studies of the cerebral ganglia of the basommatophoran pond snail *Lymnaea stagnalis* revealed the presence of a Gomori-positive and a Gomori-negative neurosecretory system. Their neurohaemal areas are located in the peripheries of the median lip nerve and of the intercerebral commissure, respectively (Joosse, 1964; Boer *et al.*, 1968a). Little detailed information is available concerning neurosecretory phenomena in the other central ganglia of this snail. Gomori-positive cell groups have been described in the ganglia of the visceral ring, i.e., in the paired pleural and parietal and in the single visceral ganglion (Lever *et al.*, 1961). However, the location of the neurohaemal areas of these cells is not known. No detailed reports have been published concerning Gomori-negative cells in these ganglia.

There are only a few investigations into the ultrastructure of neurosecretory systems in gastropods, and except for some studies on *Aplysia californica* (Rosenbluth, 1963a; Coggeshall, 1967), they are restricted to pulmonates (e.g., Nolte, 1965; Boer *et al.*, 1968a; Simpson *et al.*, 1966b; Simpson, 1970). However, electron microscope studies are rather important, since without detailed physiological information, ultrastructural data, such as the presence of release phenomena in non-synaptically ending axons in neurohaemal areas, are essential for identifying neurones as neurosecretory cells (for discussion see Bern and Knowles, 1966).

The aim of the present study is to examine the neurosecretory cells and their neurohaemal areas in *Lymnaea stagnalis* by using histochemical and electron microscope techniques. Special attention is paid to the question whether different types of Gomori-positive cells occur in this snail. Preliminary investigations (Wendelaar Bonga, 1969) indicated that for this purpose the alcian blue/alcian



yellow staining method (AB/AY), which was introduced for neurosecretion by Peute and van de Kamer (1967), is of great value.

This technique was also useful for identifying Gomori-negative neurosecretory cells. The study of the visceral ring is of particular significance since the information on neurosecretion in these ganglia is limited, whereas experimental studies have indicated that at least some of them exert neuroendocrine activity (Hekstra and Lever, 1960; Lever *et al.*, 1961; Chaisemartin, 1968).

### Materials and Methods

Sexually mature specimens of *Lymnaea stagnalis*, 6–8 months old with a shell height between 30 and 35 mm, were obtained from stock reared in the laboratory for many generations. They were kept in pairs in perforated jars, placed in tanks with continuous water change, as described by van der Steen (1967), at a water temperature of  $20 \pm 1^\circ \text{C}$ . A daily photoperiod of 12 hours (from 8 a.m. till 8 p.m.) was maintained at a light intensity of about 1,000 lux at the water surface. Lettuce was supplied ad libitum. Under these conditions the hermaphroditic snails lay eggs throughout the year at a rate of 1–3 egg capsules per week.

Fixation was normally carried out between 9 and 10 a.m. About 150 animals were used. Preliminary experiments revealed that narcotization of the snails (Joosse and Lever, 1959) caused considerable release of some types of neurosecretory materials. Therefore this treatment was afterwards avoided. For light microscopic purposes the central nervous systems and the proximal parts of the nerves were excised after decapitation of the snails and immersed in freshly prepared Stieve solution for 3 hours. The distal parts of the nerves were studied by using whole animals fixed either in Bouin or in Baker's formalin solution, upgraded in ethanol and amyloacetate and embedded in paraffin (m.p.  $58^\circ \text{C}$ ). Serial sections were cut at  $7 \mu$  thickness. The alternate section technique was used for comparing sections of the same cell stained with different histochemical techniques. The following methods were applied to stain the neurosecretory materials for light microscopy (see Romeis, 1968): CH after Bargmann, PF after Gabe, performic acid-alcian blue (PFAAB) after Adams and Sloper, periodic acid-Schiff (PAS) after McManus, Sudan black B (saturated in 70% ethanol, paraffin sections), phloxin (0.5% aqueous solution), and azocarmin G (0.1% aqueous solution). Furthermore the AB/AY method for neurosecretion (Peute and van de Kamer, 1967) was used in a modified way: the pH of both dye solutions was reversed, and prior to staining acid hydrolysis was applied to improve the specificity of the reactions. Hydrolysis was also frequently applied prior to the other staining procedures. The modified AB/AY technique runs as follows:

1. hydrolyse in 1.0 N HCl solution at  $60^\circ \text{C}$  for 6 min; rinse in tap water;
2. oxydize in acid permanganate solution (0.25%  $\text{KMnO}_4$ ; 0.5%  $\text{H}_2\text{SO}_4$ ) for 2 min; rinse in tap water; bleach in 2%  $\text{NaHSO}_3$ ; rinse in tap water;
3. stain in 0.5% solution of alcian blue 8GX for 30 min at pH 1.0; rinse in buffer (pH 1.0);
4. stain in 0.5% solution of alcian yellow GXS for 30 min at pH 2.5; rinse in buffer (pH 2.5);
5. counterstain in 0.1% solution of nuclear fast red for 1 min; rinse in tap water; dehydrate quickly; mount in DPX.

For electron microscopy the tissues were fixed for 2 hours either in a 1%  $\text{OsO}_4$  veronal buffered (pH 7.4) solution at  $4^\circ \text{C}$  (Pease, 1964), or in a freshly prepared mixture of glutaraldehyde (0.8%) and  $\text{OsO}_4$  (1.0%) with similar buffer, at  $0^\circ \text{C}$ . For postfixation the material was transferred to a solution of 1.0% uranyl nitrate for 30 min. After dehydration in ethanol and propyleneoxyde, embedding followed in Epon 812. Tissue sections were cut on a Reichert ultramicrotome, stained with lead citrate (Reynolds, 1963) for 1 min, and examined in a Zeiss EM-9A electron microscope. For identification in the electron microscope of the histochemically defined cell types, Epon sections (thickness  $1 \mu$ ), treated according to Yensen (1968) and stained with CH and phloxin, were studied prior to cutting ultrathin sections.

For demonstration of bioamines the chromaffin reaction for electron microscopy (Wood, 1963) was applied at pH 5.8, as recommended by Hopwood (1968).



## Observations

### *Morphology of the Central Nervous System*

The anatomy of the nervous system of *Lymnaea stagnalis* is known from descriptions by Elo (1938) and Hekstra and Lever (1960). The cerebral ganglia have been described in detail by Joosse (1964). The terminology used in the present study is adopted from these authors. The eleven ganglia which form the CNS are concentrated around the esophagus, caudally from the buccal mass. A bilateral a-symmetry is pertinent. The left cerebral, pedal and pleural ganglia are slightly smaller than the corresponding nerve centres at the right, whereas the left parietal ganglion is considerably less voluminous than the right one. The latter and also the single visceral ganglion are supposedly formed by fusion with another ganglion during early development of the snail (Régondaud, 1964). The penial nerve and the small right pallial nerve are unpaired.

Two structures, the medio- and latero-dorsal bodies, which consist of small glandular cells, are located on each of the cerebral ganglia. These bodies are endocrine organs which control vitellogenesis (Joosse and Geraerts, 1969).

The neuronal cell bodies lie in several layers at the periphery of the ganglia. The layers are interrupted by commissures, connectives and nerves which have their origin in the centre of the ganglia, the neuropile. Most of the processes of the uni-, bi-, or multipolar neurones are directed towards this neuropile. Here they ramify frequently, giving rise to hundreds of axonal branches, as established in the present study by examining serial sections at the electron microscope level. For the general cytology of the nerve cells in *L. stagnalis* reference is made to light microscope studies of Moussa (1950) and Boer (1965). The latter obtained evidence that the volumes of the cell bodies might be correlated with the degree of polyploidy of the cells.

The ultrastructure of ordinary neurones has been described for many gastropod species (Quattrini, 1963; Simpson *et al.*, 1963; Sakharov *et al.*, 1965; Lane, 1966; Nolte *et al.*, 1965; Schmekel and Wechsler, 1968; Benjamin and Peat, 1969). The ultrastructure of ordinary neurones as well as that of neurosecretory cells in the cerebral ganglia of *L. stagnalis* has been examined by Boer *et al.* (1968a).

In the present study axons were commonly found to project into the cell bodies of ordinary neurones. These axons often possess dense-cored granules of several types with a mean diameter between 800 and 1,400 Å, i.e., within the size range of neurotransmitter granules (Gerschenfeld, 1963). In addition small clear vesicles ( $\varnothing$  350—500 Å) were occasionally observed in the same axons (Fig. 1). Although no differentiations of the cell membranes were found, the presence of these vesicles suggests that synaptic transmission takes place in these areas. The absence of membrane thickenings is consistent with the opinion of Frazier *et al.* (1967), that in gastropod synapses differentiation of the membranes may be absent. The occurrence of axons ending synaptically on cell bodies was also suggested by Nicaise *et al.* (1968), who found granule containing axons which penetrated into the neurones of the opisthobranch *Glossodoris* spec. Moreover, the presence of axo-somatic synapses in *Aplysia californica* is mentioned in the light microscopic work of Ábrahám (1965). These observations are in contrast with the results of Gerschenfeld (1963) who was unable to demonstrate axo-somatic synapses in the pulmonates *Vaginula solea*, *Cryptomphallus aspersa* and *Helix pomatia*.

In the central neuropile as well as in the nerves axo-axonic synapses were commonly found (Fig. 2). They were identified by the occurrence of clusters of clear vesicles and of accumulations of varying amounts of electron dense material on the apposed membranes of two axons. Usually the intercellular space between the membranes is slightly widened in these zones, forming a synaptic cleft. Axo-axonic synapses have been described in many gastropod species (e.g., Gerschen-



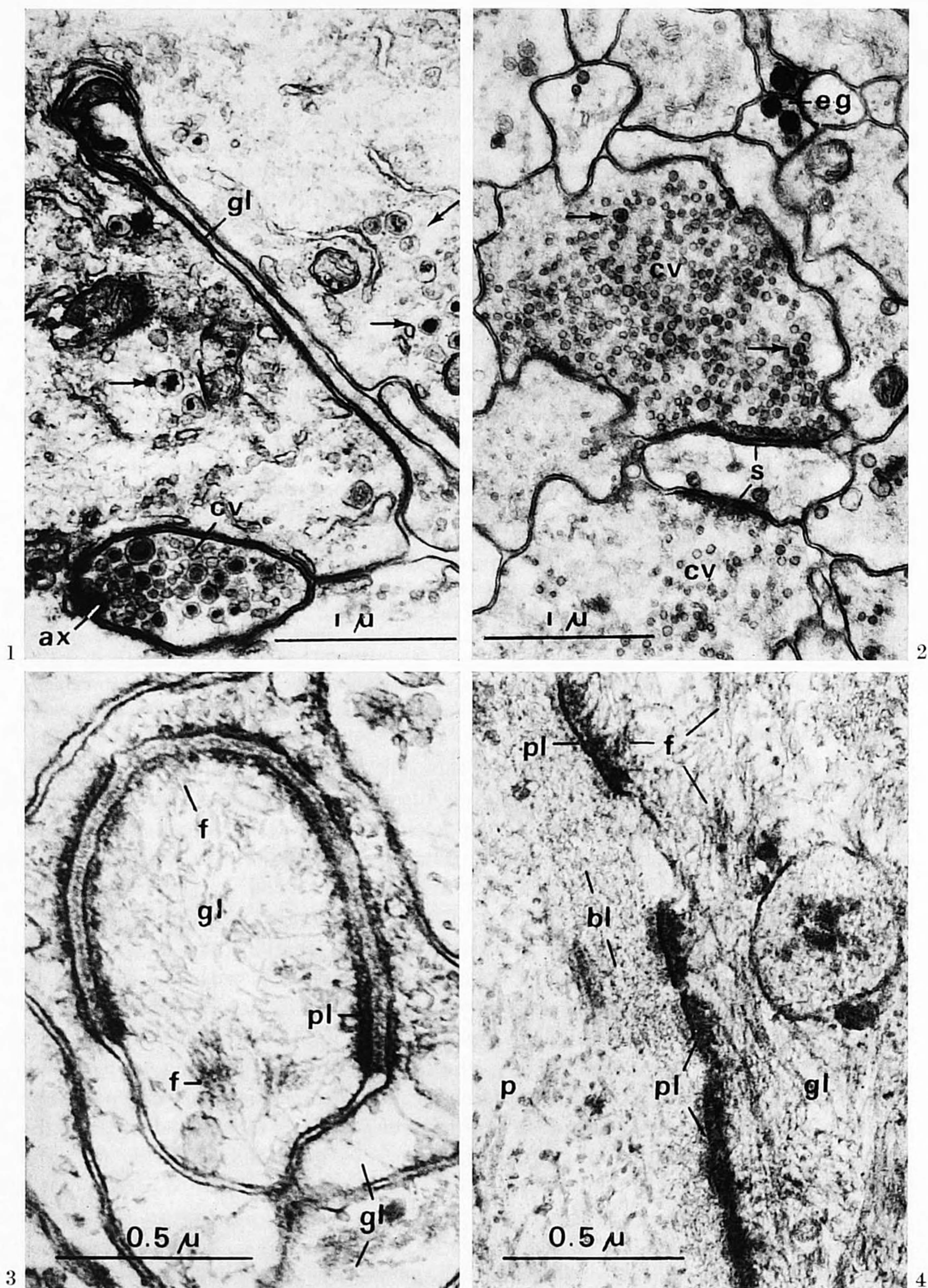


Fig. 1. Part of an ordinary neurone in the right pleural ganglion, containing neurotransmitter like granules (arrows), penetrated by a glial process of the trophospongium (*gl*). An axon profile (*ax*) with dense-cored granules and clear vesicles (*cv*) indicates the presence of an axo-somatic synaptic contact



feld, 1963; Hanneforth, 1965; Coggeshall, 1967; Frazier *et al.*, 1967; Nicaise *et al.*, 1968; Chalazonitis, 1968).

References to glial cells in *L. stagnalis* are lacking. At the electron microscope level the neuronal cell bodies appear to be enclosed by sheath-like arrangements of many glial processes, which in the larger neurones also penetrate into the perikarya (Fig. 1) and into the axons. Except for mitochondria, cell organelles are scarce in the cytoplasm of these glial cells. Usually some glycogen is present. Often the separation of the neurones by glial elements was found to be incomplete.

A second type of glial cells is characterized by large numbers of thin filaments ( $\varnothing$  50 Å) which are comparable to tonofilaments. Moreover, in these cells many mitochondria, a rather extensive granular endoplasmic reticulum, numerous Golgi zones, and lysosome-like structures (cytosomes, *cf.*, Nolte *et al.*, 1965) were observed. Cells of this type — which will be referred to as filamentous glia — are numerous throughout the nervous system. At the surface of the nervous tissue these cells form a continuous layer which encapsulates the ganglia and the nerves. Only cytoplasmic processes and axon endings of neurosecretory cells interrupt this layer. The filamentous glial cells, which are interconnected by extensive desmosomes (Fig. 3) also occur in the neuropile, and furthermore these cells radiate into the nerves where they envelope groups of axons. In the filamentous glial cells at the surface of the nervous system, hemi-desmosomes are found on those parts of the cell membranes which are in contact with the basal lamina (Fig. 4). Bundles of thin filaments can be observed which insert into the desmosomal plaques.

The glial processes penetrating into the neuronal cell bodies are referred to in the literature as Holmgren's canals (*cf.*, Moussa, 1950) or as trophospongium (*cf.*, Sakharov *et al.*, 1965; Coggeshall, 1967). Although the trophospongium seems to be weakly developed in *L. stagnalis*, its ultrastructure is rather similar to that of gastropods like *Archachatina marginata* (Amoroso *et al.*, 1964) and *Aplysia californica* (Rosenbluth, 1963a; Coggeshall, 1967). As in *L. stagnalis*, also in these snails and in other molluscan species, e.g., in the lamellibranch *Anodonta cygnea* (Gupta *et al.*, 1969), filamentous glial cells have been described. According to Amoroso *et al.* (1964) these cells have a mechanical function, *viz.*, the maintenance of the structural integrity of the ganglia. Desmosomes of the type found in *L. stagnalis* have been described to interconnect these cells in *Aplysia californica* (Coggeshall, 1967) and in *Anodonta cygnea* (Gupta *et al.*, 1969). In the latter species also hemi-desmosomes were found.

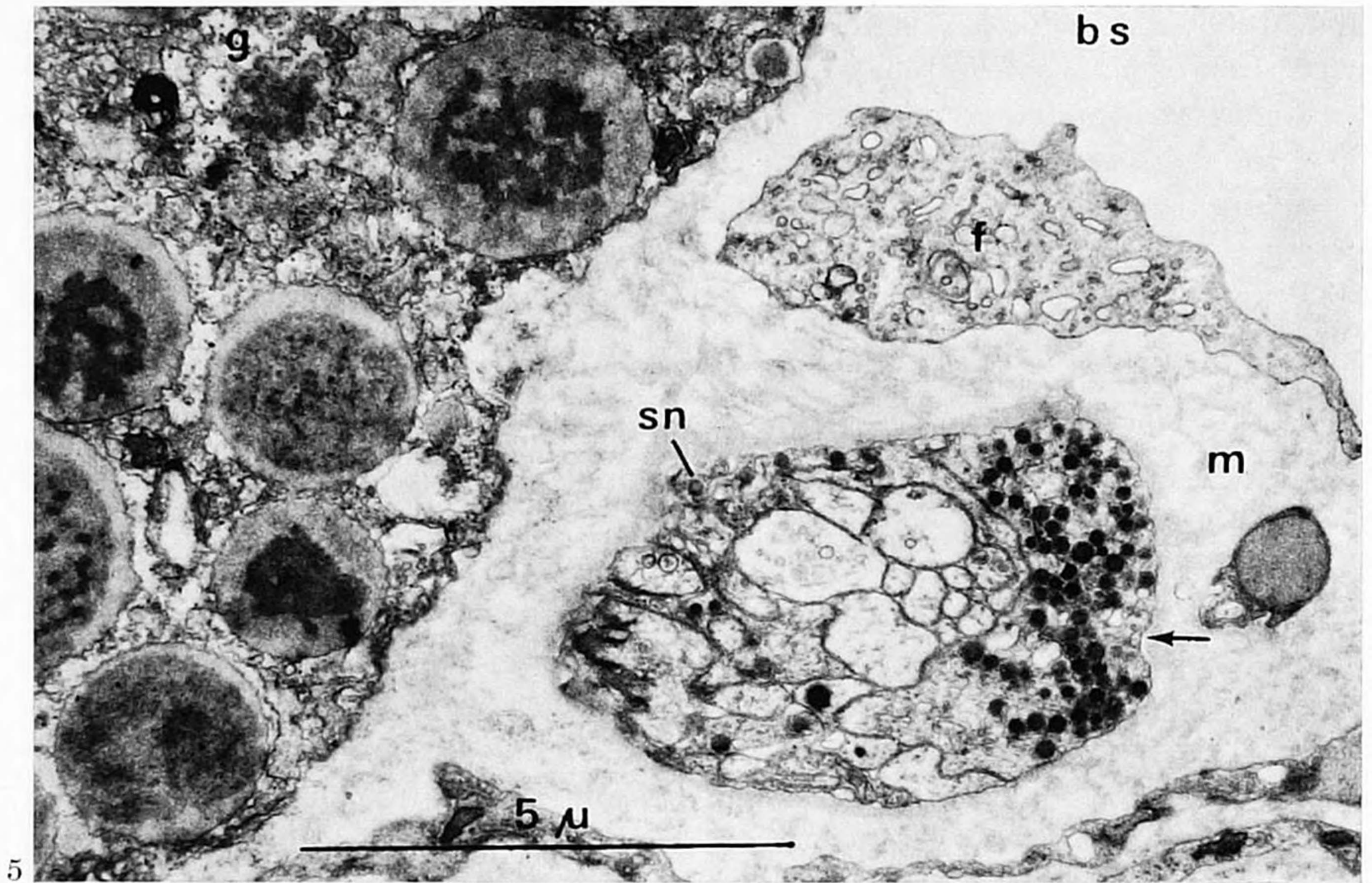
The *perineurium* surrounds the ganglia and nerves, separating the nervous system from the cephalic sinus. The main cellular constituents of this layer are

Fig. 2. Part of the central neuropile of the visceral ganglion. The large axon profile contains some dense-cored granules (arrows) and a large number of clear vesicles (*cv*). *s* synaptic contacts; *eg* elementary granules

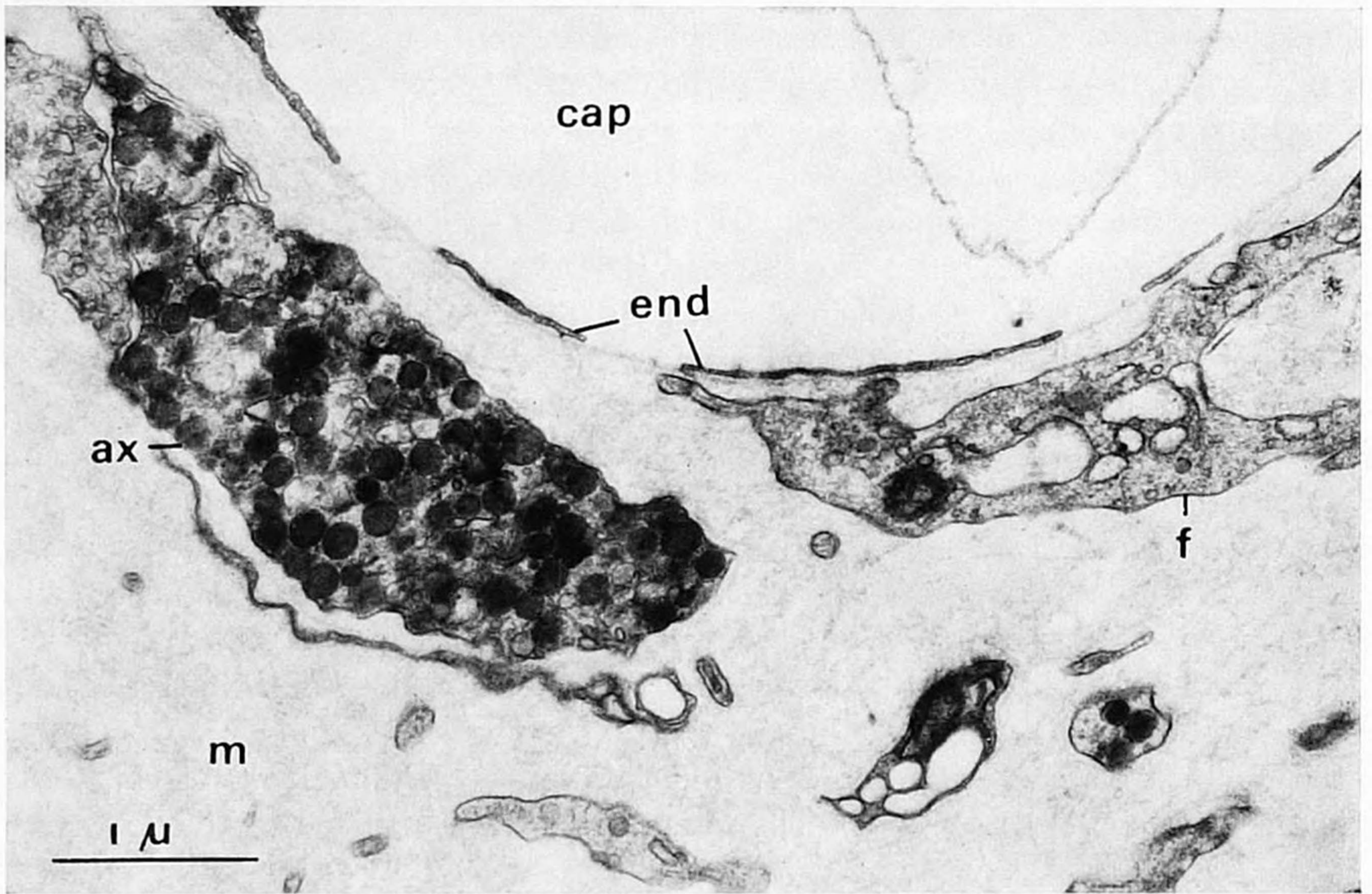
Fig. 3. Desmosome (macula adhaerens) between filamentous glial cell processes (*gl*). *pl* desmosomal plaque; *f* glial filaments which insert into the plaques

Fig. 4. Hemi-desmosomes in a filamentous glial cell lying at the surface of a nerve. *p* perineurium; *bl* basal lamina; *pl* desmosomal plaque; *f* glial filaments which are inserted into the plaques





5



6

Fig. 5. Perineurium around the intercerebral commissure. *g* granular cell; *f* fibrocyte; *bs* blood space; *m* matrix substance; *sn* small nerve containing many small axons and a large axon with neurosecretory elementary granules of the CDC type (arrow)

Fig. 6. Perineurium around the visceral nerves. *cap* blood capillary; *end* fenestrated endothelium; *m* matrix substance; *f* fibrocyte; *ax* axon containing neurosecretory elementary granules of the yellow green type



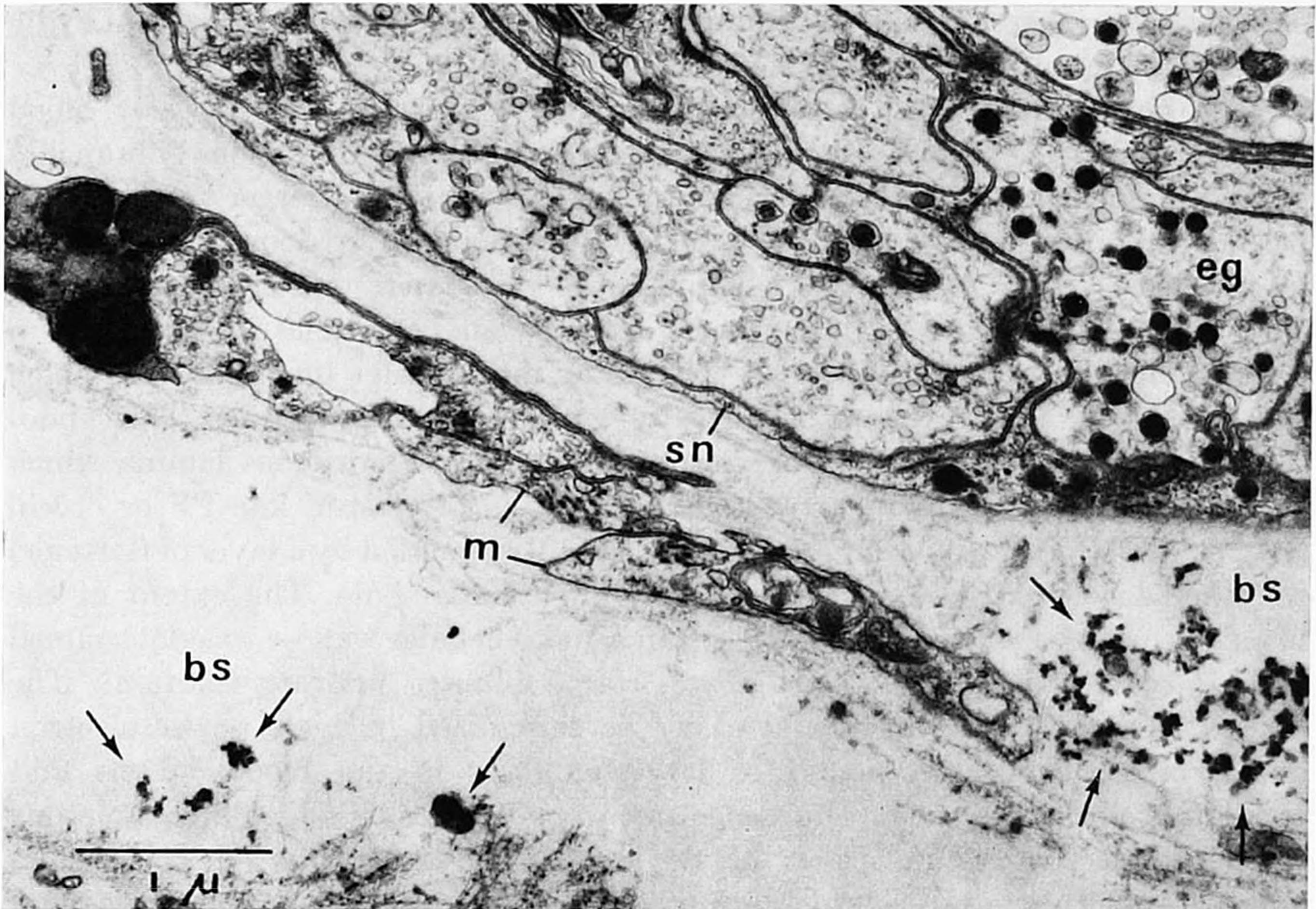


Fig. 7. Perineurium around the visceral ganglion after injection of India ink. *bs* blood space containing carbon particles (arrows); *m* muscle fibres; *sn* small nerve; *eg* neurosecretory elementary granules of the deep green type

fibrocytes, pigment cells, amebocytes, granular cells, and smooth muscle fibres. These cells, together with elements of the vascular system and with a network of many tiny nerves (Figs. 5, 6) are embedded in an electron transparent matrix containing large numbers of collagen fibrils.

The granular cells, also called „cells of Leydig“ (*cf.*, Boer *et al.*, 1967) are fairly prominent in sections stained to study neurosecretion, because they contain large granules of variable size (0.5—4  $\mu$ ) with a strong affinity for basic neurosecretory stains. At the ultrastructural level these moderate electron dense granules can easily be distinguished from the characteristic elementary granules of the neurosecretory cells (Fig. 5).

The smooth muscle cells occur dispersed in the perineurium. In the cytoplasm thick filaments ( $\varnothing$  300—600 Å) occur, which are surrounded by thin filaments ( $\varnothing$  50—100 Å). These filaments have also been described in the muscle fibres of other gastropod species. The thin filaments are supposed to contain actin, the thick filaments consist primarily of paramyosin (Rosenbluth, 1963b; Baccetti, 1967; Sanchiz and Zambrano, 1969). The perineurium is separated from the nervous tissue by a basal lamina.

The general ultrastructure of the perineurium of the gastropods investigated (Rosenbluth, 1963a, b; Coggeshall, 1967; Sanchiz and Zambrano, 1969; Rogers, 1969), is rather similar to that of *L. stagnalis*. However, the granular cells found in this snail are structurally different from those cells, which have been



described in other animals as granular or globular cells or as cells of Leydig (*cf.*, Fernández, 1966; Rogers, 1969).

The perineurium and the adjacent connective tissue is traversed by blood vessels, capillaries and blood spaces. The blood vessels are ramifications of branches arising from the anterior aorta. The anatomical pattern of the larger vessels in the perineurium of the cerebral ganglia was described in detail by Joosse (1964). Only fragmentary records of the ultrastructure of the vascular system of gastropods are available (*cf.*, Coggeshall, 1967). In *L. stagnalis* small arteries are lined by a layer of flattened endothelial cells, which lack intercellular junctions and which are often separated from each other by gaps (width up to  $1\ \mu$ ). This endothelium is joined with a layer of muscle fibres by an amorphous lamina which is stainable with techniques for the demonstration of elastin, like PF or orcein (Pearse, 1961). The capillaries ( $\varnothing$  4—20  $\mu$ ) are only bounded by a layer of flattened endothelial cells (Fig. 6), which may contain myofilaments. The extent of the blood spaces — blood containing clefts which lack a cellular wall — was determined with injection of India ink into the aorta, 5 minutes prior to fixation<sup>1</sup>. The carbon particles of the ink could easily be recognized in high power electron micrographs. They were found in large numbers in the blood vessels and capillaries, and also in completely electron transparent slits which are numerous in the connective tissue matrix of the perineurial layer (Fig. 7). The results indicate that these spaces, which are often in close contact with the nerves and other cellular elements of the perineurium, communicate with the blood vessels and therefore must be regarded as parts of the circulatory system. This conclusion is consistent with the data obtained by Coggeshall (1967) who studied the blood supply of the perineurial sheath of *Aplysia californica* with the same techniques.

#### *Light-Microscopy of the Neurosecretory Cells*

Lever *et al.* (1961), using CH, determined the number and the location of the Gomori-positive cells in the central nervous system of *L. stagnalis*. Two large groups of these cells were described in each of the cerebral ganglia whereas small groups were found in the pleural, parietal and visceral ganglia. No cells were stained in the buccal and pedal ganglia. Axonal discharge pathways were not mentioned. In a detailed study of the cerebral ganglia Joosse (1964) described in each ganglion a prominent tract of axons transporting secretory material from both large groups of about 50 CH-positive cells — he called them, according to their position, the medio-dorsal cells (MDC) and the latero-dorsal cells (LDC) — to the median lip nerve. The axons form bulb-shaped (CH-positive) endings in the periphery of this nerve. Because this type of axon termination is characteristic for neurosecretory cells, he assumed the periphery of the median lip nerve to be a neurohaemal zone.

With the AB/AY method all CH-positive cells in the central nervous system of *L. stagnalis* appear to bind both alcian stains, which results in a green colouration. However, apparently not all cell groups react to the same degree with these stains. Most of the groups were coloured in a different shade of green. This strongly suggests that various CH-positive neurosecretory materials differ in their affinity for the alcian stains.

Due to the selectivity of the AB/AY technique it was possible to detect the areas where the endings of the cell processes are located. The different CH-

<sup>1</sup> These observations were performed in collaboration with Mr. R. Bekius, who is studying the circulatory system of *L. stagnalis*.



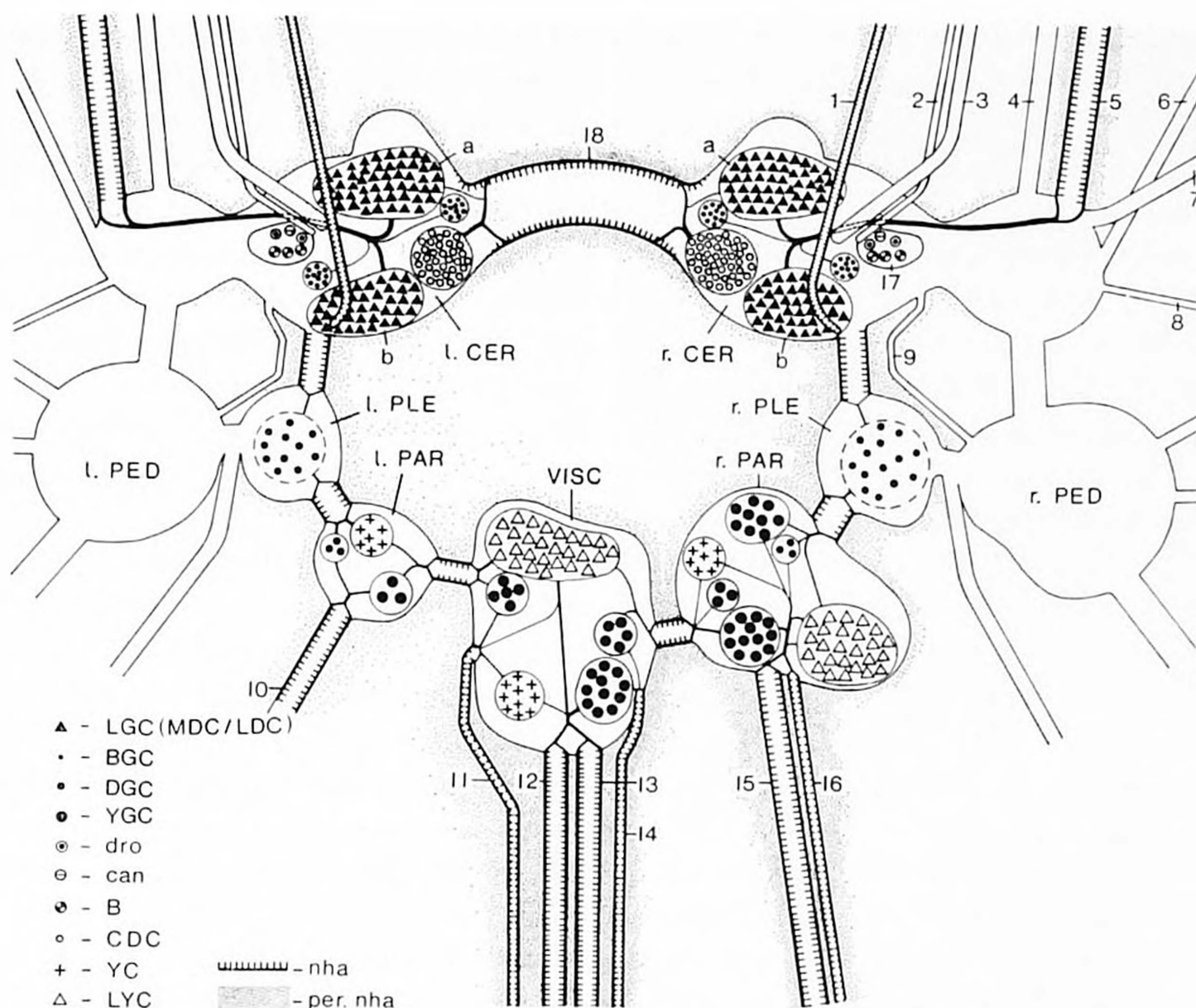


Fig. 8. Diagram of the location of the neurosecretory cell groups and their neurohaemal areas (*nha*) in the central nervous system of *Lymnaea stagnalis* (dorsal view). The mottled areas (*per. nha*) represent parts of the perineurium and of the connective tissue which are traversed by small nerves containing neurosecretory axons. The pedal ganglia and the ventral parts of the cerebral ganglia are turned to the lateral sides. The medio- and latero-dorsal bodies are not indicated. *CER* cerebral ganglia; *PLE* pleural ganglia; *PAR* parietal ganglia; *VISC* visceral ganglion; *PED* pedal ganglia; *LGC* light green cells; *MDC* medio-dorsal cells (*a*); *LDC* latero-dorsal cells (*b*); *BGC* bright green cells; *DGC* deep green cells; *YGC* yellow green cells; *dro* droplet cells; *can* canopy cell; *B* B-cells; *CDC* caudo-dorsal cells; *YC* yellow cells; *LYC* light yellow cells; *1* nervus nuchalis; *2* n. opticus; *3* n. tentacularis; *4* n. fronto-labialis superior; *5* n. labialis medius; *6* cerebro-buccal connective; *7* n. penis; *8* sub-cerebral commissure; *9* n. staticus; *10* n. pallialis sinister; *11* n. cutaneus pallialis; *12* n. analis; *13* n. intestinalis; *14* n. genitalis; *15* n. pallialis dexter internus; *16* n. pallialis dexter externus; *17* lateral lobe (the follicle gland is not indicated); *18* intercerebral commissure

positive cell groups and their axonal pathways will be described on the basis of their reaction with AB/AY. The location of the cells and, as far as known, of their neurohaemal areas, is indicated in Fig. 8.

*Light green* material occurs in the perikarya of the MDC and LDC. The axons of these cells and their endings were stained in the same light green colour. Although most of the axons proceed to the median lip nerve, small numbers of light green axons were observed in the intercerebral commissure and in tiny nerves in the perineurium surrounding this commissure and the median lip nerves. Furthermore, light green stained nerves were found in low numbers in the perineurium around the cerebral ganglia and around the medio- and latero-dorsal bodies.



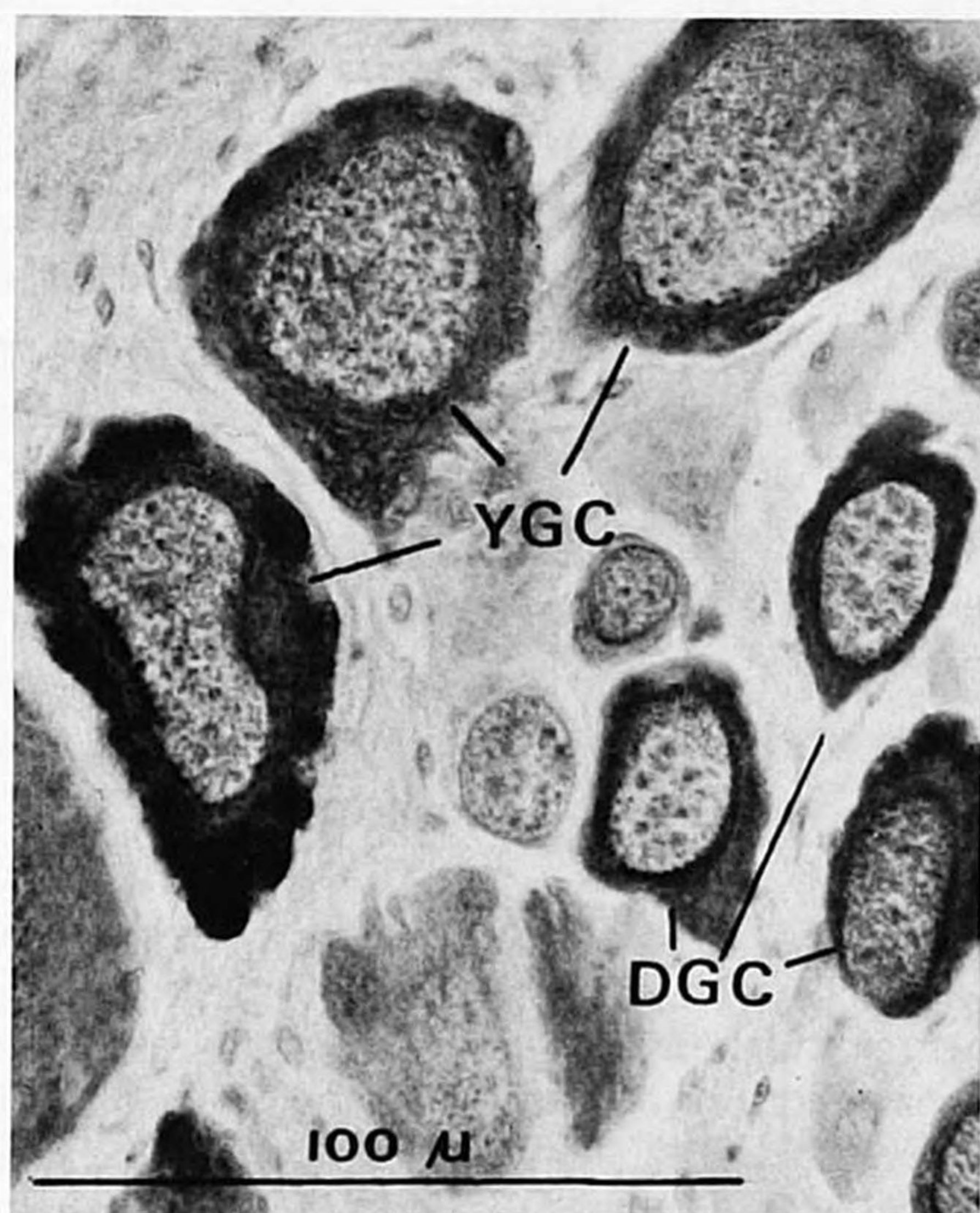
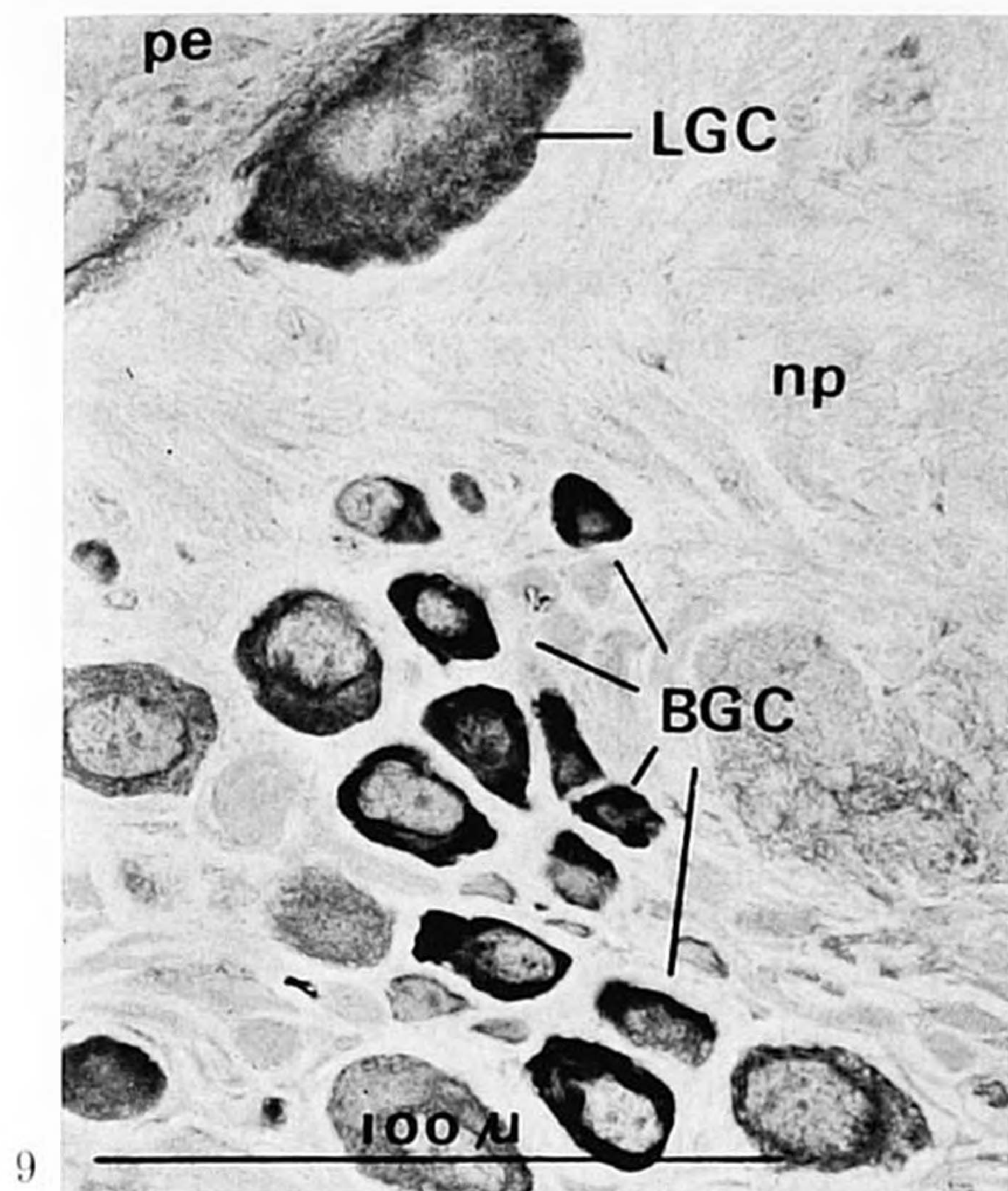
An intense *bright green* colour is displayed by two groups of small cells, which occur in both cerebral ganglia. These groups, each consisting of about 10—20 cells in the left and of 10—25 cells in the right cerebral ganglion, have not been distinguished before. Their secretory material reacts intensely with CH. In each ganglion one group is located near the MDC, between the intercerebral commissure and the anterior lobe (Fig. 9). The other lies adjacent to the neuropile between the MDC and LDC. The length of the perikarya of these neurones, which are often bipolar, varies between 15 and 25  $\mu$ . In some animals only one large cell group of about double size was found in each ganglion. The number of cells in those groups was about twice that of the small groups. The axons, which apparently transport the secretory material, could only be traced over a small distance, because they intermingle with the main tract of the MDC and LDC.

In the lateral lobe — a paired neural structure joined with the cerebral ganglia by two short connectives — some cells containing CH-positive material occur. From differences in location, size and appearance, one canopy cell, two droplet cells and one large and several small B-cells have been distinguished (Lever and Joosse, 1961). With the AB/AY method the canopy cell shows a *brownish green* colour, while the droplet cells stain *faint light green* and the B-cells *brilliant green*. One of the bipolar B-cell axons containing neurosecretory material projects into the lumen of a vesicular structure, the follicle gland (Lever *et al.*, 1959). The contents of this vesicle, which are CH-positive, exhibit with the AB/AY technique a green colour, identical to that of the B-cells. The other axon of the B-cells and also the axons of the other secretory cells of the lateral lobe enter the cerebral ganglion but could only be traced up to the central neuropile. They contain material of the same colour as found in their perikarya.

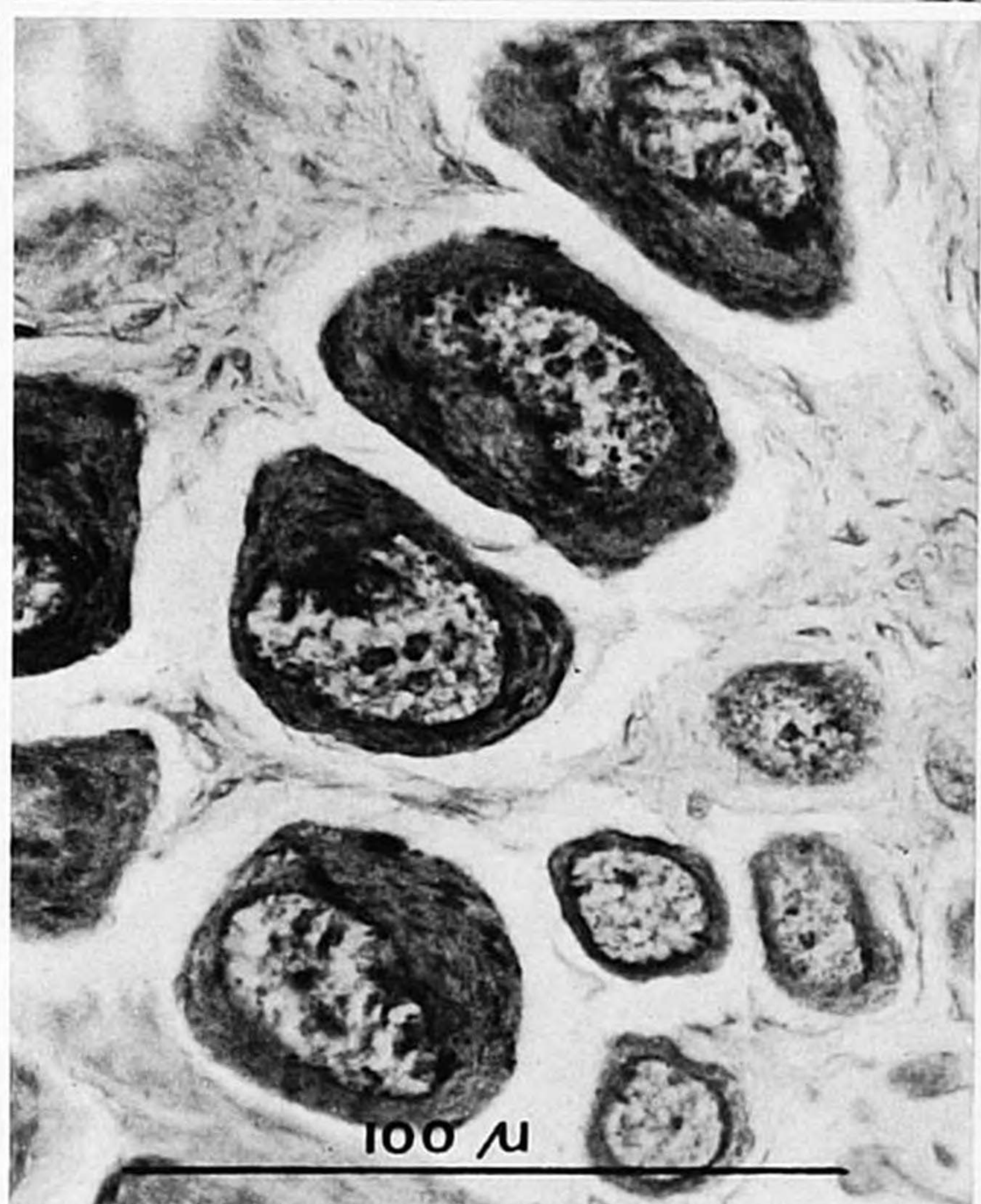
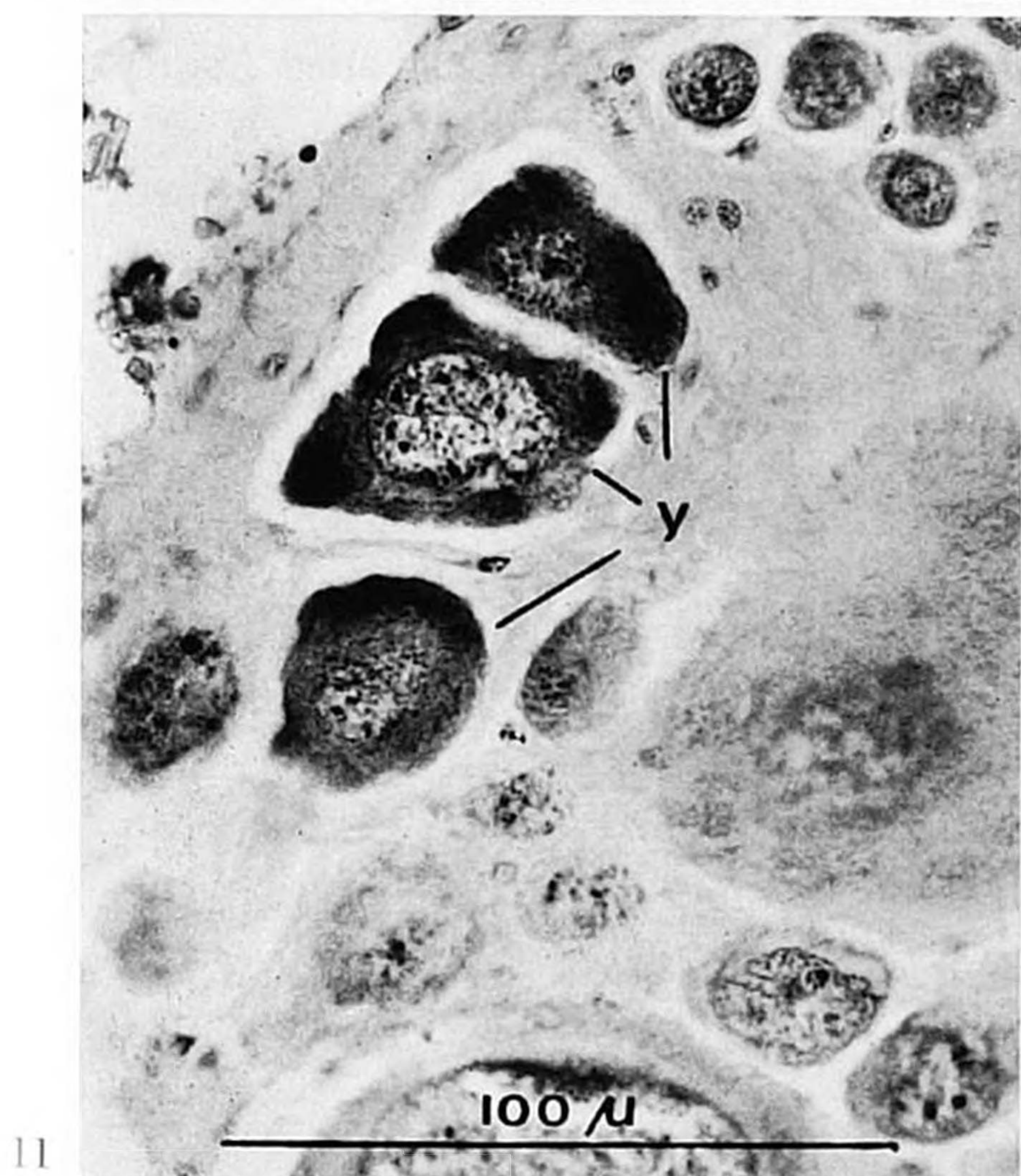
*Dark green* stained cells were observed in the pleural and — in limited numbers — in the parietal ganglia. In the pleural ganglia these cells occur scattered between ordinary neurones throughout the ganglia, with a slight preference for the regions near the cerebro- and parieto-pleural connectives. The cells, 10—15 in the left and 15—20 in the right ganglion, are medium sized. The length of the perikarya varies between 20 and 35  $\mu$ . They are identical to the CH-positive cells described in these ganglia by Lever *et al.* (1961). In both parietal ganglia the number of these dark green cells varies. Approximately 4—10 cells are located in the right ganglion near the parieto-pleural connective (Fig. 10), while 2—5 cells of this type can be observed in the corresponding region in the left ganglion. Occasionally cells of this type occur in the pleural connectives or in the perineurium. Part of the axons of the dark green cells end in the periphery of the cerebro- and parieto-pleural connectives. Dark green axons were, furthermore, found in tiny nerves originating from the pleural ganglia and its connectives. These nerves form a network of deep green fibres between the blood vessels and capillaries in the perineurium and the connective tissue near-by (Fig. 13a).

In addition to some dark green cells, another CH-positive cell type occurs in both parietal ganglia. This stains *yellow green* with AB/AY (Fig. 10). Cells of this type are also present in the visceral ganglion. In the ganglia they are located in the regions near the origins of the connectives and nerves, as established by Lever *et al.* (1961). In the right parietal ganglion 10—20 of these large cells





9 10



11 12

Fig. 9. Group of bright green cells (*BGC*) in the right cerebral ganglion. *LGC* light green cell of the MDC; *np* neuropile; *pe* perineurium; AB/AY staining

Fig. 10. Part of the right parietal ganglion, near the parieto-pleural connective, with deep green (*DGC*) and yellow green (*YGC*) cells. AB/AY staining

Fig. 11. Yellow neurones (*y*) in the left parietal ganglion. AB/AY staining

Fig. 12. Group of light yellow neurones in the dorsal part of the visceral ganglion. AB/AY staining

(length of the perikarya: 25—70  $\mu$ ) are intermingled with the dark green cells near the parieto-pleural connective, while 10—30 are concentrated in the region where the pallial nerves are leaving the ganglion. About 2—5 cells are situated



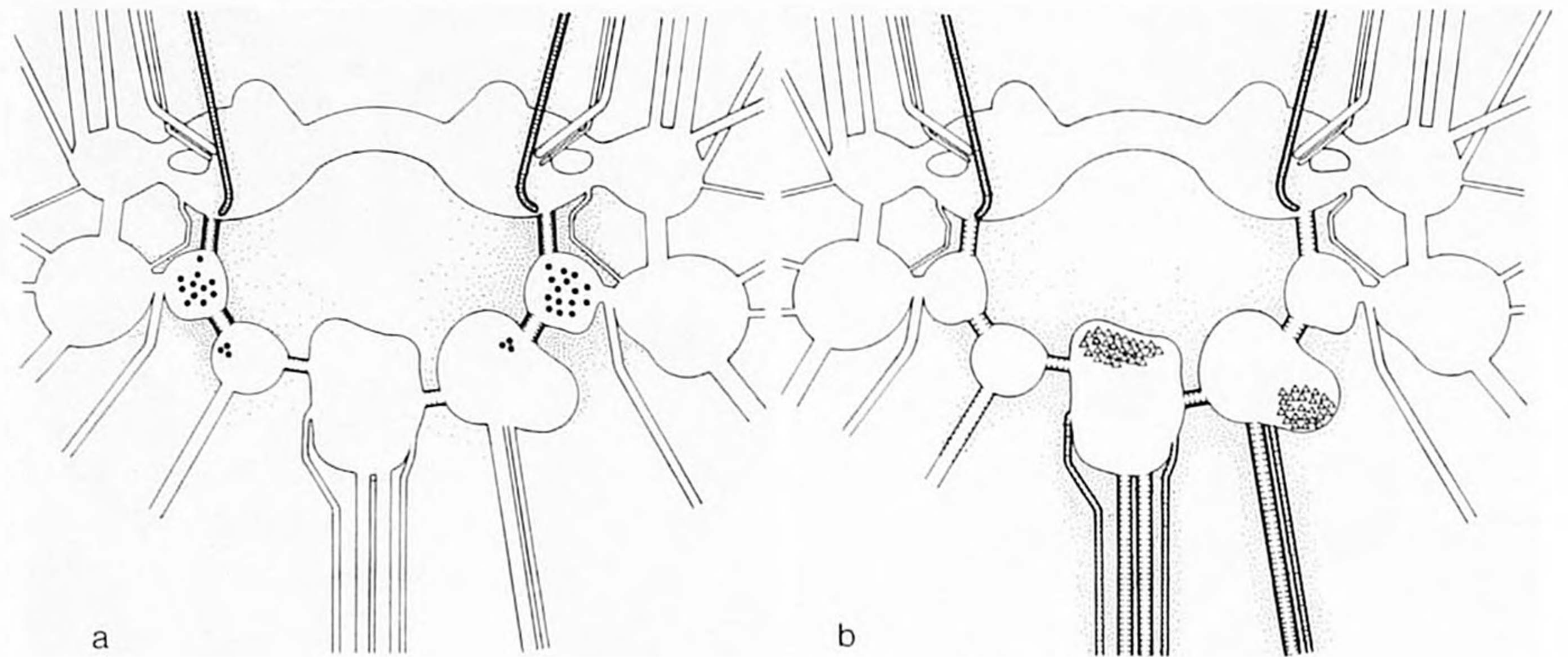
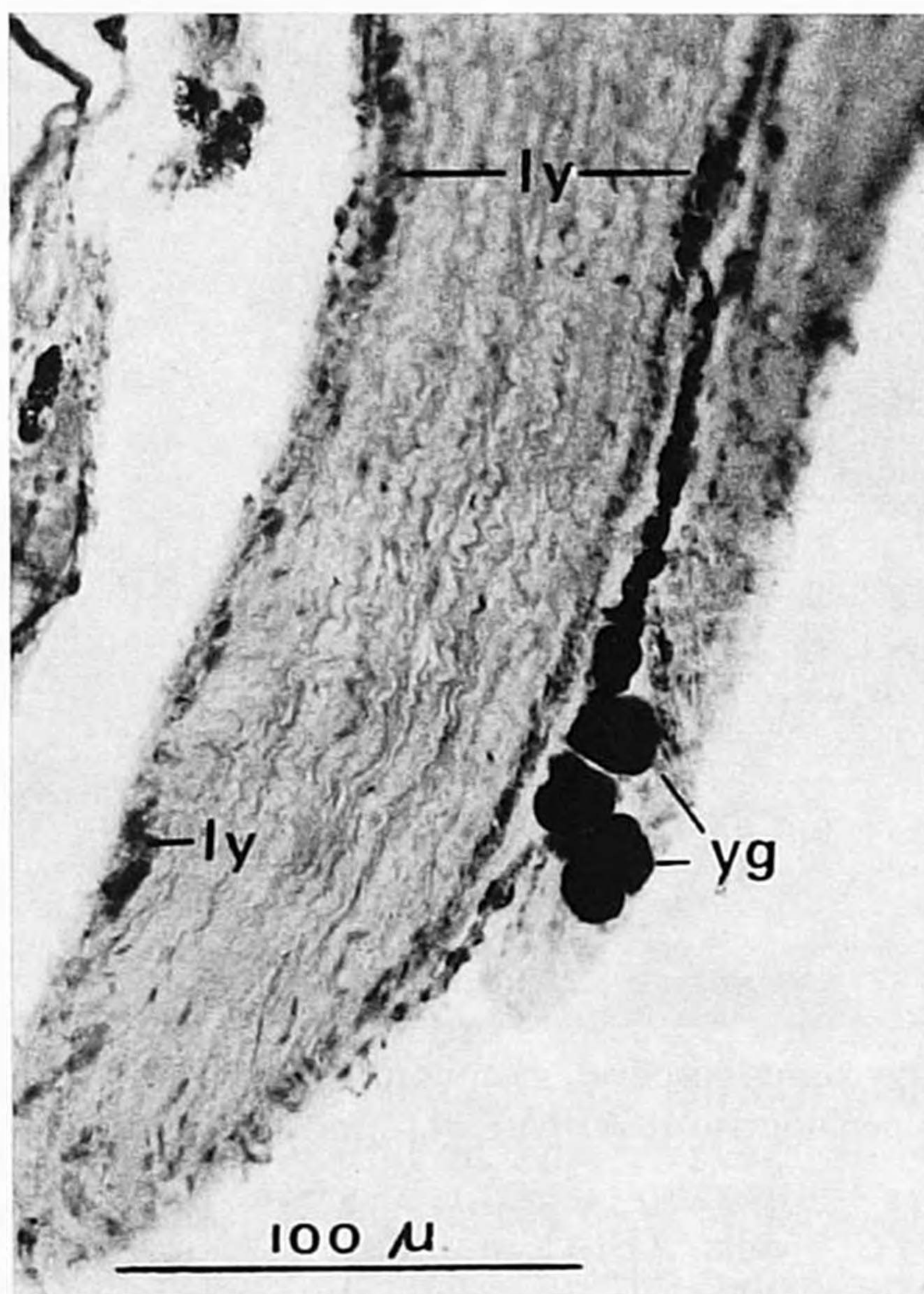
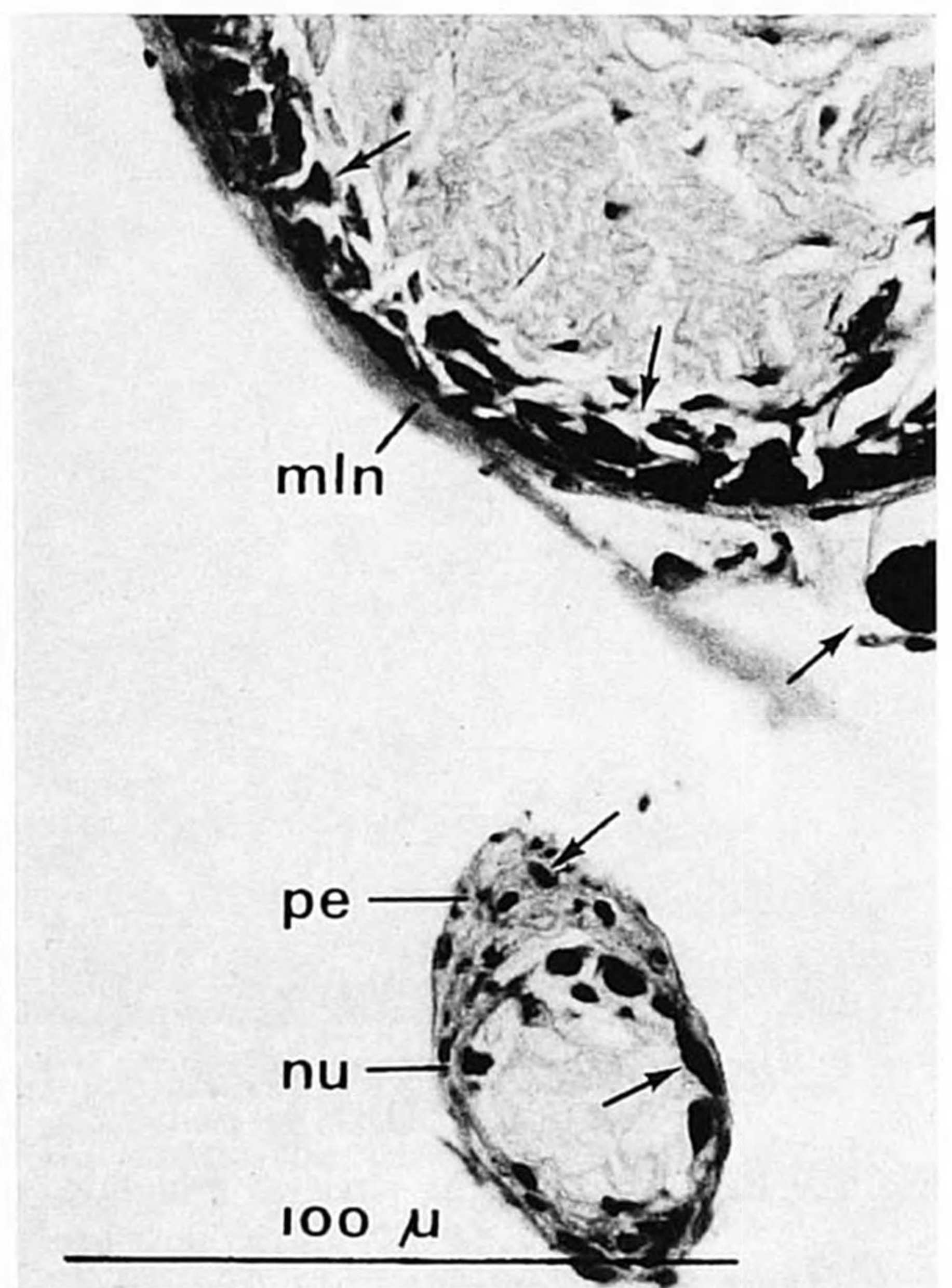


Fig. 13. a The location of the deep green neurosecretory cells and of their neurohaemal areas, at the periphery of the connectives of the visceral ring and at the periphery of the nuchal nerves (thick lines), and in the perineurium (mottled areas). b The location of the light yellow neurosecretory cells and their neurohaemal areas, at the periphery of the connectives of the visceral ring and of some nerves (thick lines), and in the perineurium (mottled areas)



14



15

Fig. 14. Oblique section through the nervus pallialis dexter internus. Neurosecretory axon endings of the light yellow type (*ly*) occur at the periphery of the nerve. Distended yellow green axon terminals (*yg*) are present in the perineurium. AB/AY staining

Fig. 15. Cross section through the nuchal nerve (*nu*) and the median lip nerve (*mln*). Gomori-positive neurosecretory material (arrows) occurs in the periphery of the nerves and in the perineurium (*pe*). PF staining



near the parieto-visceral connective. The left parietal ganglion contains only 1—6 cells of this type. In the visceral ganglion yellow green cells occur in 2 groups of 5—10 cells near the connectives with the parietal ganglia. In addition to these two groups approximately 10—20 cells were found dispersed in the region from which the visceral nerves originate. Like those of the dark green cells, the axons of the yellow green cells do not form prominent axonal tracts. In general, the secretion carrying axons proceed to the nearest connective or nerve. In the periphery of these structures, especially of the nerves of the right parietal (Fig. 14) and of the visceral ganglion many axon endings are present. There are also numerous axons traversing the neuropile. These could often be traced to their endings in more distant parts of the nervous system, e.g., in adjoining ganglia and also in the nuchal nerve (Fig. 15). In the perineurium surrounding the visceral ring and the pallial, visceral, and nuchal nerves a network of yellow green axons is present.

Scattered in all visceral and pallial nerves varying numbers of yellow green cells do occur. In three specimens cell counts were made. In each of them about 40 cells of this type were found outside the ganglia.

Apart from the CH-positive cells also Gomori-negative neurones have been described in the nervous system of *L. stagnalis*. A group of neurones, the caudo-dorsal cells (CDC), which contain phloxinophilic secretory material, has been described in each of the cerebral ganglia (Joosse, 1964). The axons of these cell groups, which consist of 20—40 cells in the left and of 50—100 cells in the right ganglion, transport their secretory product to the periphery of the intercerebral commissure. Joosse considered this area a neurohaemal zone. In the same ganglia Boer (1965) established the presence of small numbers of neurones with sudanophilic properties. The Sudan black B positive substance, which was also phloxinophilic, was observed in axons and in axon endings in the median lip nerves.

The CDC show neither affinity for AB nor for AY. The Sudan black B positive cells described by Boer apparently stain intensely with AY. In each cerebral ganglion two small groups of 1—3 of these yellow cells — the first located near the pleural connective, the other near the place of origin of the static nerve — were observed to stain intensely, not only with alcian yellow but also with Sudan black B and with phloxin. They show a moderate affinity for azocarmine. The axons could not be traced to their endings, although stainable material was observed in their proximal parts.

Cells with similarly stained material were observed in groups of 5—10 cells in the dorsal parts of both parietal ganglia (Fig. 11) and in the ventral part of the visceral ganglion. The length of the cell bodies varies between 20 and 40  $\mu$ . The axons of these neurones were often hard to follow. The distribution of the axon endings containing the yellow secretory material of these groups of neurones has the same pattern as that of the yellow green cells.

With the AB/AY technique yet other Gomori-negative cells, viz., two large groups of neurones containing *light yellow* material, were observed. The first group, consisting of 30—50 cells which measure 20—60  $\mu$  in length, is located in a lobe-like protrusion in the ventral part of the right parietal ganglion. The same number of light yellow cells form a group in the dorsal part of the visceral ganglion (Fig. 12). This cell type is absent from the left parietal ganglion. The secretory material



Table. Staining reactions of the secretory substances in the cell bodies as well as in the axons of the neurosecretory cell types in the central ganglia of *Lymnaea stagnalis*. +++ very strong reaction; ++ strong reaction; + moderate reaction; ± weak reaction; — negative reaction

Staining technique	Cerebral ganglia			Pleural ganglia	Parietal and visceral ganglia		
	light green cells (MDC/LDC)	bright green cells	CDC		deep green cells	yellow green cells	light yellow cells
CH	++	+++	—	+++	++	—	—
PFAAB	+	+++	—	++	++	—	—
PF	++	+++	—	+++	++	—	—
AB (pH 1.0; no oxydation)	—	—	—	—	—	—	—
AY (pH 2.5; no oxydation)	±	±	—	±	±	±	+
AB (pH 1.0; oxydation)	+	++	—	++	+	—	—
AY (pH 2.5; oxydation)	+	++	—	+	++	+	+++
Phloxin	—	—	+	—	—	+	+++
Sudan black B	—	—	±	—	—	+	++
Azocarmine	—	—	+	—	—	+	+
PAS	—	—	—	—	—	—	—

of the cells is further characterized by a moderate reaction with phloxin and with Sudan black B. In the cytoplasm of the cells disk-shaped accumulations of ergastoplasm were found. These were, however, less prominent than the Nissl-disks in the CDC (*cf.*, Joosse, 1964). The axons and axon endings of these light yellow cells were encountered in the same areas as described for the yellow green and yellow cells. These regions are indicated in Fig. 13b.

The results of some histochemical techniques, including those reported above, are summarized in the Table. Apart from AB/AY also other neurosecretory stains were applied, like CH, PF and PFAAB. The latter stains were also used by Boer (1965) in a histochemical study of the MDC and LDC. Contrary to the findings reported for other species — e.g., in vertebrates (for discussion see Streefkerk, 1967) and in the pulmonate *Succinea putris* (Cook, 1966) — in *L. stagnalis* only minor differences were observed between CH and PF with regard to their capacity to stain the neurosecretory materials.

The AB/AY technique, originally proposed by Ravetto (1964) for analysing polysaccharides, involves the application of one of the alcian stains at low pH



(0.5—1.0), i.e., at a pH below the pK of the weak acid carboxylic groups (Lev and Spicer, 1964). Accordingly, only strong acid (sulphated) groups can bind this stain. Subsequently, carboxylic groups are stained by the other alcian stain at pH 2.5. Peute and van de Kamer (1967) introduced this method for the demonstration of neurosecretory material by applying oxidation prior to staining. In their opinion, this oxidation leads to the conversion of S-S bonds of cystine and the —SH groups of cysteine — these amino acids are known to occur in fair amounts in Gomori-positive neurosecretory materials — into strong acid — SO<sub>3</sub>H groups. These are stained at the lower pH. Subsequently, carboxylic groups, which can be formed by oxidation of hydroxyl and of aldehyde groups, are stained at pH 2.5.

In the present study the most satisfying results were obtained when AB was used at the lower pH. In this case the reaction of AB would seem to be comparable to that of the neurosecretory stains PF, CH, and especially to that of PFAAB, since these techniques are supposed to be primarily based on staining of strong acid groups formed by oxidation, although the chemical reactions involved are not known in detail (Lev and Spicer, 1964; Hadler *et al.*, 1968).

The different shades of green which resulted from the AB/AY combination in the CH-positive cell groups indicate that the secretory substances concerned contain different proportions of carboxylic and sulphated groups. The different green colours appeared to be reproducible and characteristic, irrespective of the quantities in which the materials were present.

As was emphasized by Solcia *et al.* (1968), basic stains such as the alcian and Gomori stains, often give rise to non-specific staining of the cytoplasm, due to the ability of these dyes to react with RNA. Brief acid hydrolysis prior to staining, as recommended by Solcia and co-workers to destroy RNA, appeared to be sufficient to remove the background staining from the cell bodies.

After adequate hydrolysis no colour differences were found between the secretory materials in the cell bodies and in the nerve endings. This observation is inconsistent with the observations of Peute and van de Kamer (1967) in the frog, and of Gabe (1967) in ten insect species. They concluded from colour differences which they observed with the AB/AY technique, that the neurosecretory substances were chemically altered during transport through the axons.

In *L. stagnalis* all secretory materials proved to be resistant to diastase, an enzyme which breaks down glycogen. Moreover, they all reacted negative to the PAS technique. The PAS-positivity of the yellow green cell bodies, which disappeared after diastase treatment, can be attributed to glycogen, which is present in addition to the yellow green neurosecretory material. Therefore, the possibility that the presence of large amounts of glycogen might have been taken for neurosecretory substances (*cf.*, Simpson *et al.*, 1966b) is not relevant for the cell types described above.

#### *Ultrastructure of the Neurosecretory Cells*

Before cutting ultrathin sections for electron microscopy, the histochemically defined secretory cell groups were identified in 2  $\mu$  thick Epon sections with the phase contrast microscope on the basis of their characteristic location. A further identification of the cells was performed by staining thick sections with CH and phloxin after removal of the Epon (Yensen, 1968).



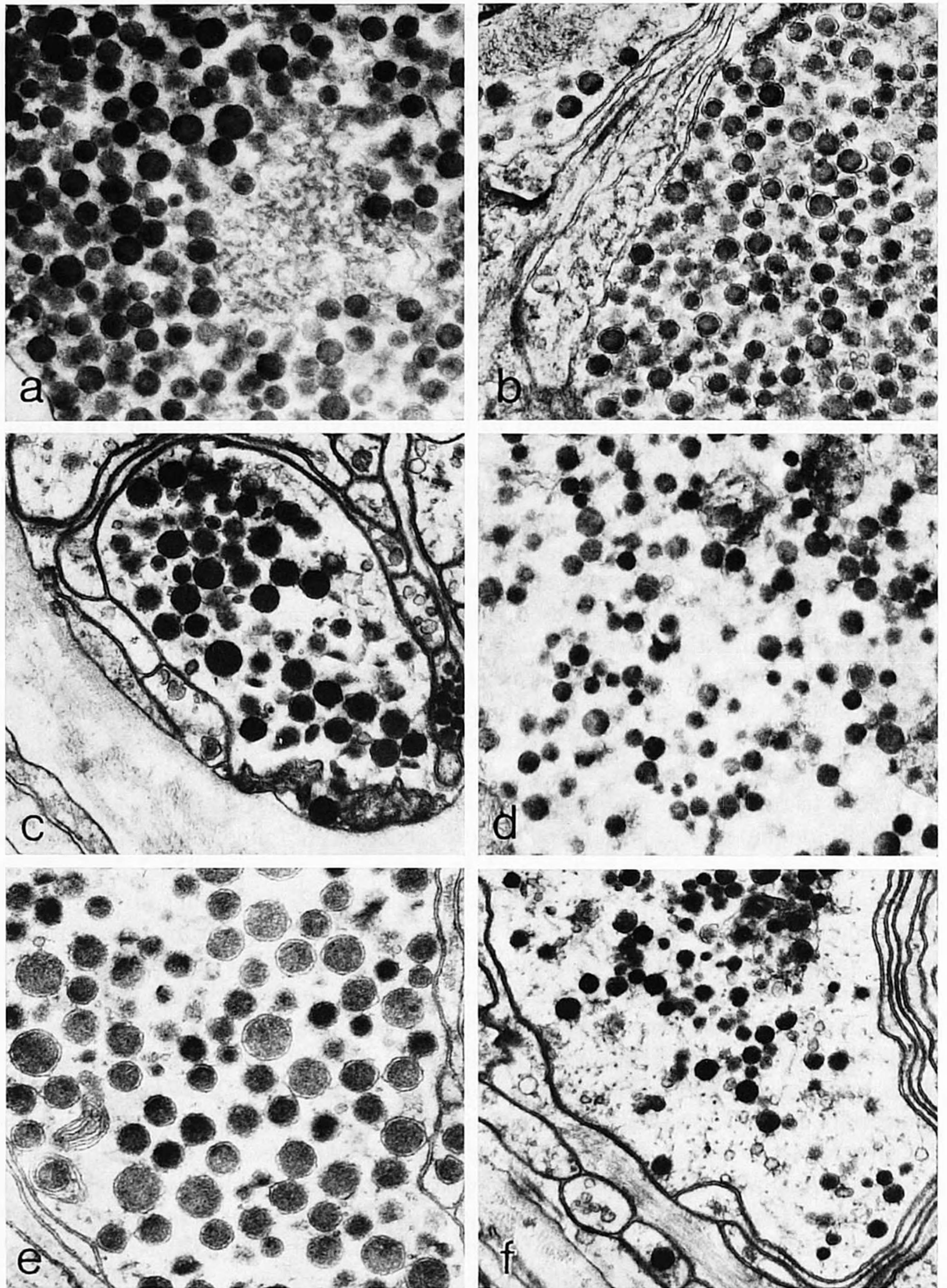


Fig. 16a—f. Neurosecretory elementary granules in axon terminals of six cell types. a light green type; b CDC type; c dark green type; d yellow green type; e light yellow type; f yellow type;  $\times 18,000$

The ultrastructure of the light green cells, the MDC and LDC, and of their neurohaemal areas in the median lip nerves has been described by Boer *et al.* (1968a). The elementary granules in the cell bodies have a mean diameter of



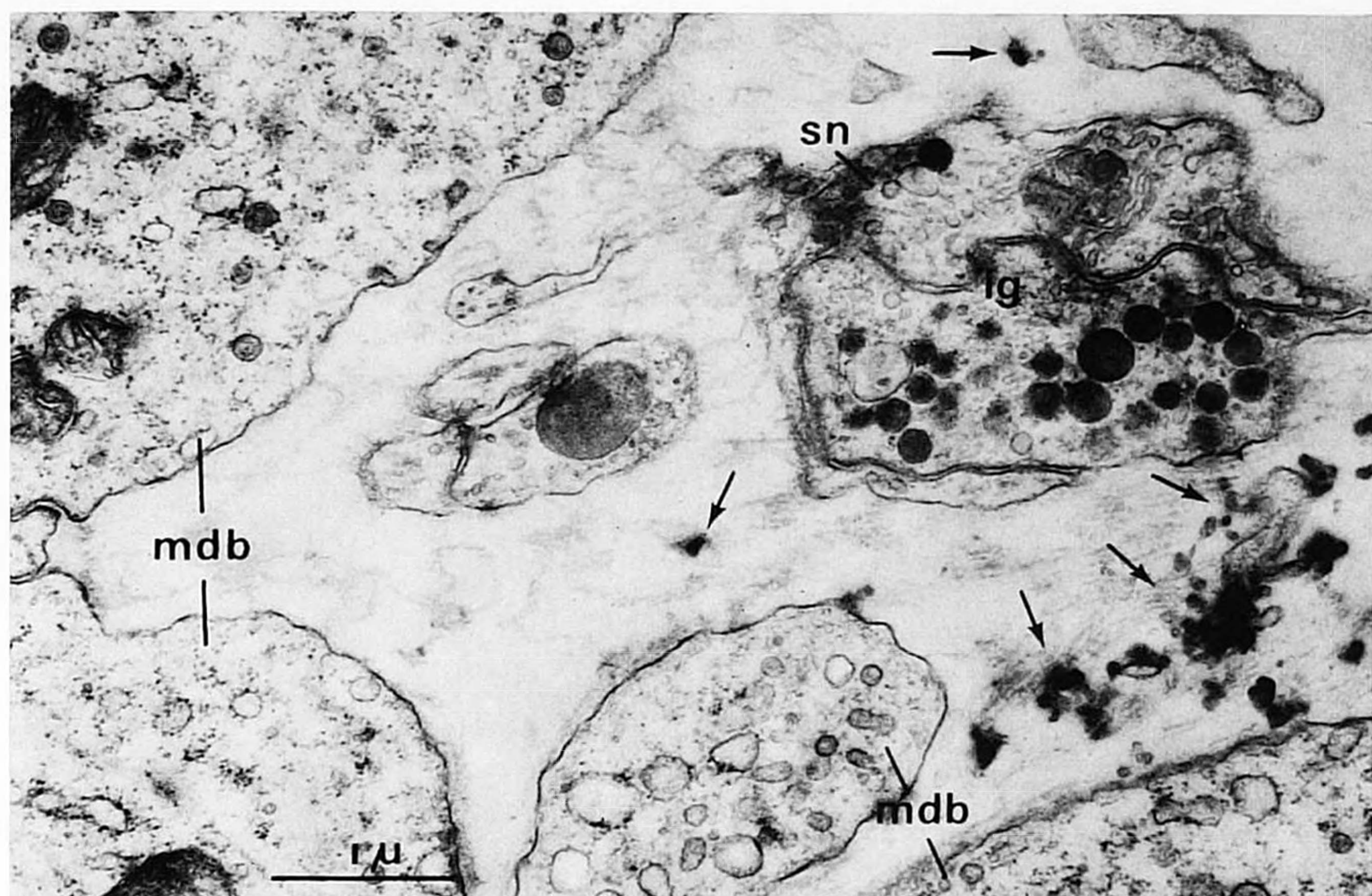


Fig. 17. Small nerve (*sn*) with an axon containing elementary granules of the light green type (*lg*) in the connective tissue between the cells of the medio-dorsal body (*mdb*). India ink injection. The carbon particles (arrows) are found in close contact with the nerves and with the glandular cells

2,000 Å. In the present study, axons carrying the same type of granules (Fig. 16a) were also found in the perineurium surrounding the cerebral ganglia, the median lip nerves and the intercerebral commissure and, albeit in low numbers, in the connective tissue between the groups of endocrine cells of the medio- and latero-dorsal bodies (Fig. 17).

No ultrastructural data of the small bright green cells in the cerebral ganglia are presented, because these cells could not be recognized beyond doubt in the electron microscope. In a study of the lateral lobe, the ultrastructure of the brownish green canopy cell, the light green droplet cells and the brilliant green B cells — which contain elementary granules with a mean diameter of 2,050, 1,350 and 1,100 Å, respectively — has been described by Brink and Boer (1967).

At the EM-level the dark green cells appeared to contain large numbers of elementary granules with a mean diameter of 2,000 Å (Fig. 16c). These granules were also found in axon endings in the periphery of the pleural connectives and in the perineurium (Fig. 18). In the cell bodies numerous Golgi fields were found to be evenly distributed in the cytoplasm. The Golgi membranes enclose dark material of the same electron density as that of the core of the granules (Fig. 19). An extensive rough endoplasmic reticulum and a low number of cytosomes (Fig. 20) is present. The dark green cells lying near the perineurium possess short cytoplasmic processes which reach the basal lamina around the ganglion.

Examination of the groups of neurones with yellow green material revealed that all cells of this type show identical ultrastructural characteristics. They contain an extensive granular endoplasmic reticulum, many Golgi fields (Fig. 21),

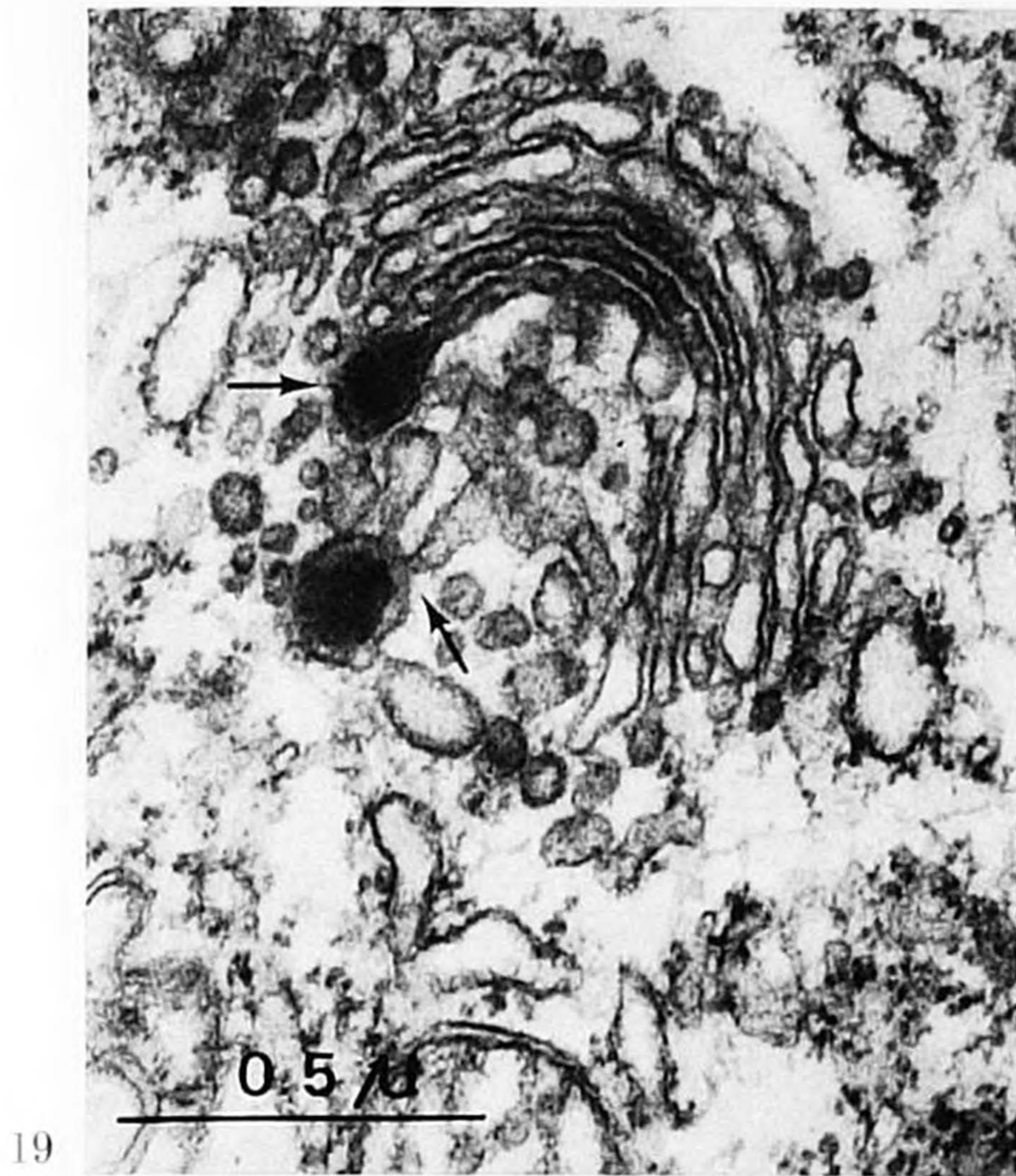




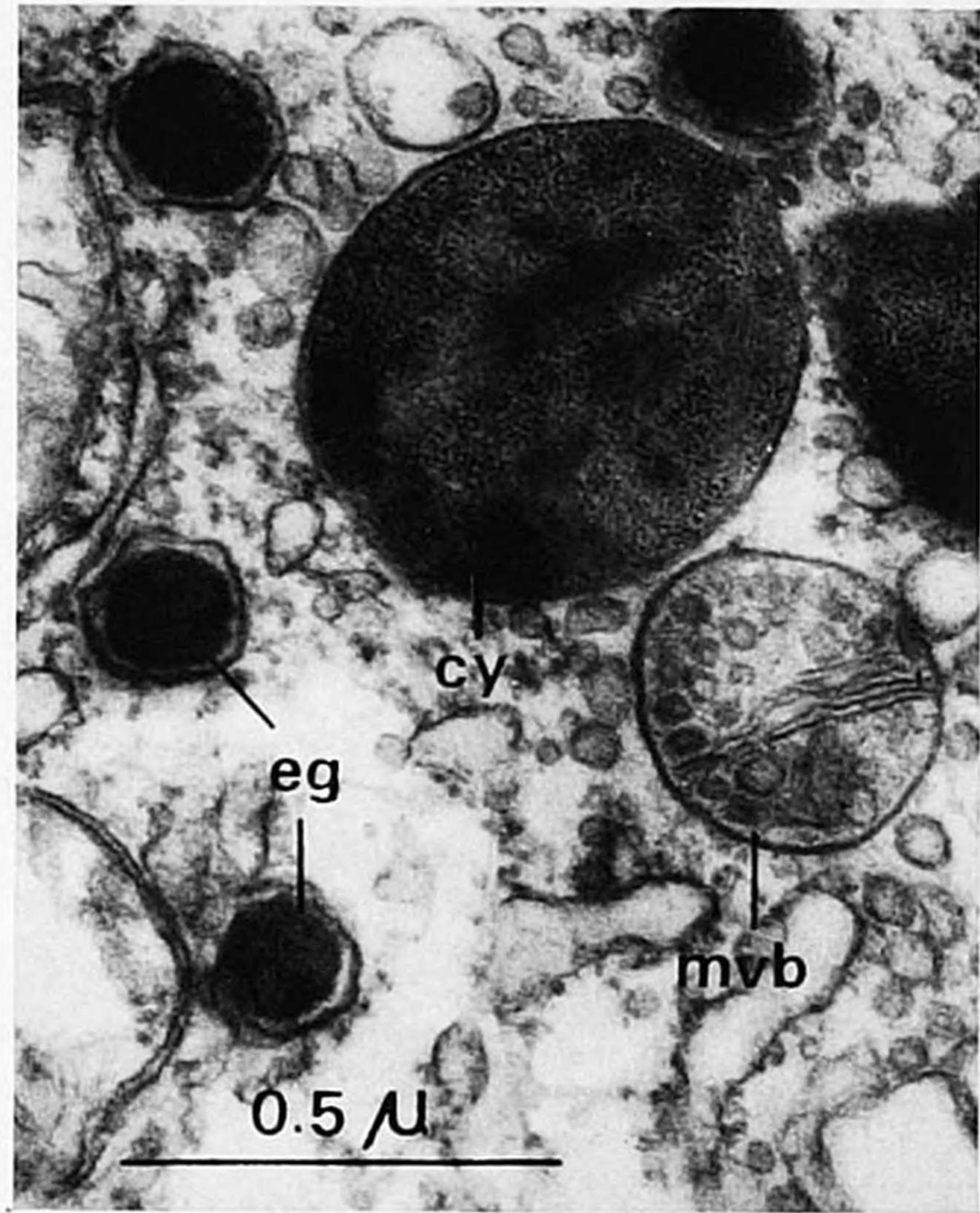
Fig. 18. Cross section of part of a small nerve in the perineurium near the pleural ganglion, containing many neurosecretory axons. *y* axon containing the yellow type of neurosecretory elementary granules; *dg* axon containing the dark green type of elementary granules; *gl* filamentous glial cell processes; *m* muscle fibre; *cy* cytosome

large amounts of glycogen and many secretory granules ( $\varnothing 1,650 \text{ \AA}$ ) consisting of a core of moderate electron-dense material surrounded by a small transparent zone (Fig. 16d). Between the Golgi lamellae only little material — with low electron density — was seen. The cell bodies are not completely separated

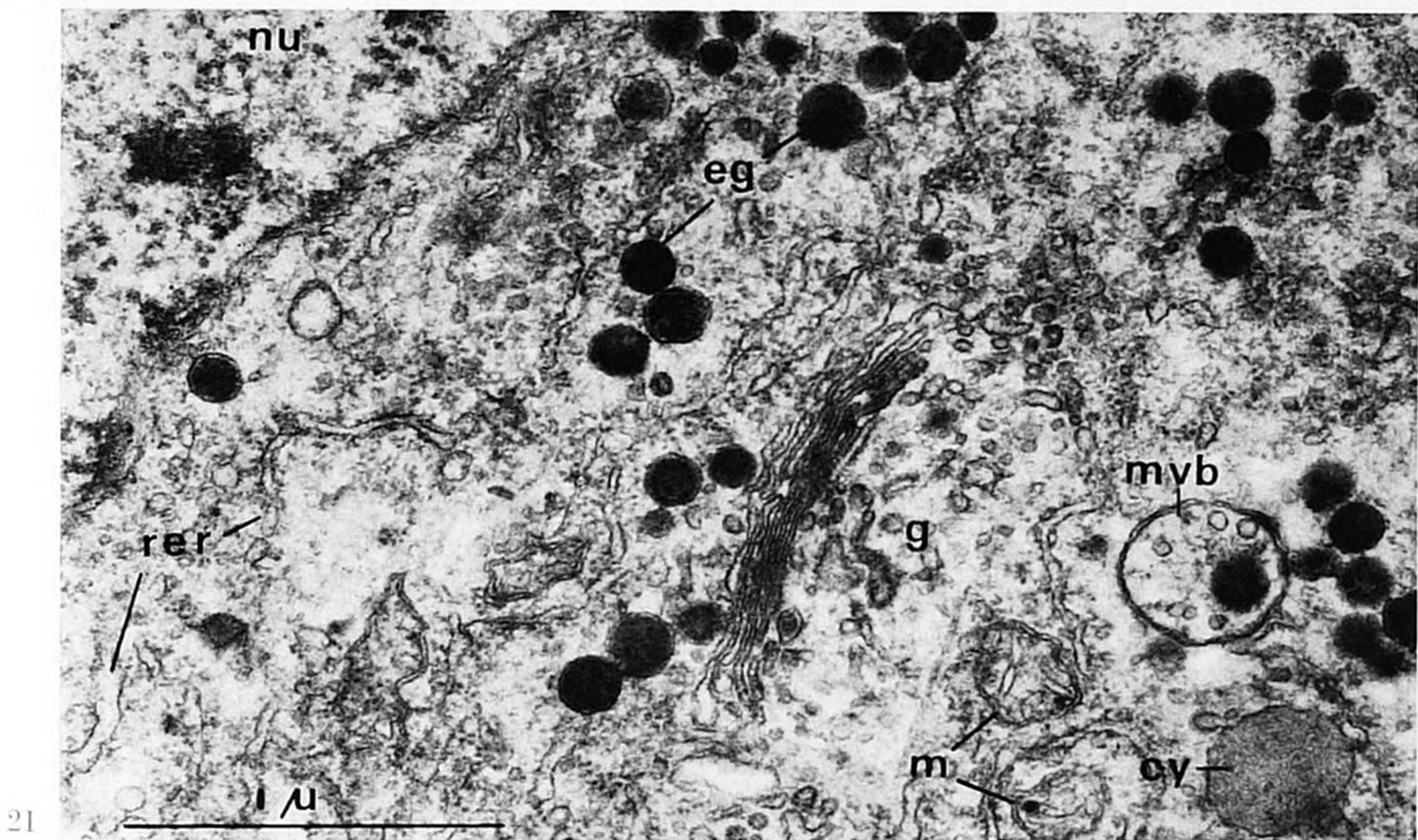




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Fig. 19. Golgi apparatus in the cell body of a dark green cell, with accumulations of electron-dense material (arrows) suggesting the formation of the elementary granules by budding from the Golgi lamellae

Fig. 20. Cell body of a dark green cell with elementary granules (*eg*), a cytosome (*cy*) and a multivesicular body (*mvb*)

Fig. 21. Cell body of a yellow green cell. *eg* elementary granules; *g* Golgi field; *mvb* multivesicular body; *cy* cytosome; *ger* granular endoplasmic reticulum; *m* mitochondrion; *nu* nucleus



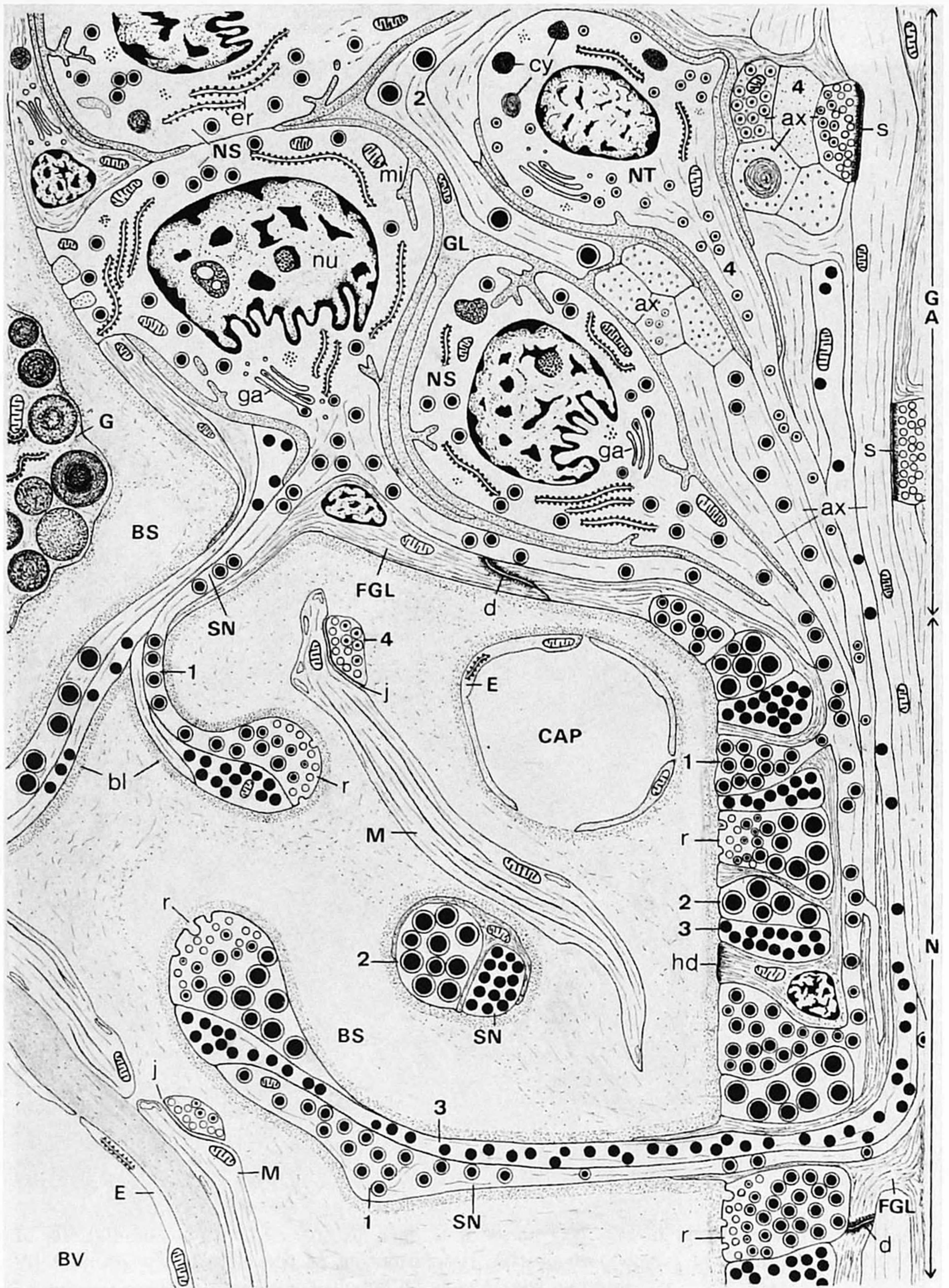


Fig. 22. Diagrammatic representation of neurosecretory cells and their neurohaemal areas, in particular of the group of yellow green neurones near the origin of the right pallial nerves. *GA* ganglion; *NS* neurosecretory neurones; *NT* neurotransmitter neurone; *N* nerve with neurosecretory axon terminals at the periphery; *SN* small nerve in the perineurium; *FGL* filamentous glial cell; *GL* glial cell forming the trophospongium; *CAP* blood capillary; *BS* blood space; *BV* large blood vessel; *E* endothelium; *M* muscle fibre; *G* granular cell; *nu* nucleus; *ga* Golgi apparatus; *cy* cytosomes; *er* granular endoplasmic reticulum; *mi* mitochondrion; *s* synaps; *j* neuromuscular junction; *d* desmosome; *hd* hemi-desmosome; *r* release phenomena in neurosecretory axon terminal; *bl* basal lamina; *ax* axons; *1* axon of the yellow green type; *2* axon of the light yellow type; *3* neurosecretory axon of the yellow type; *4* axon with neurotransmitter granules



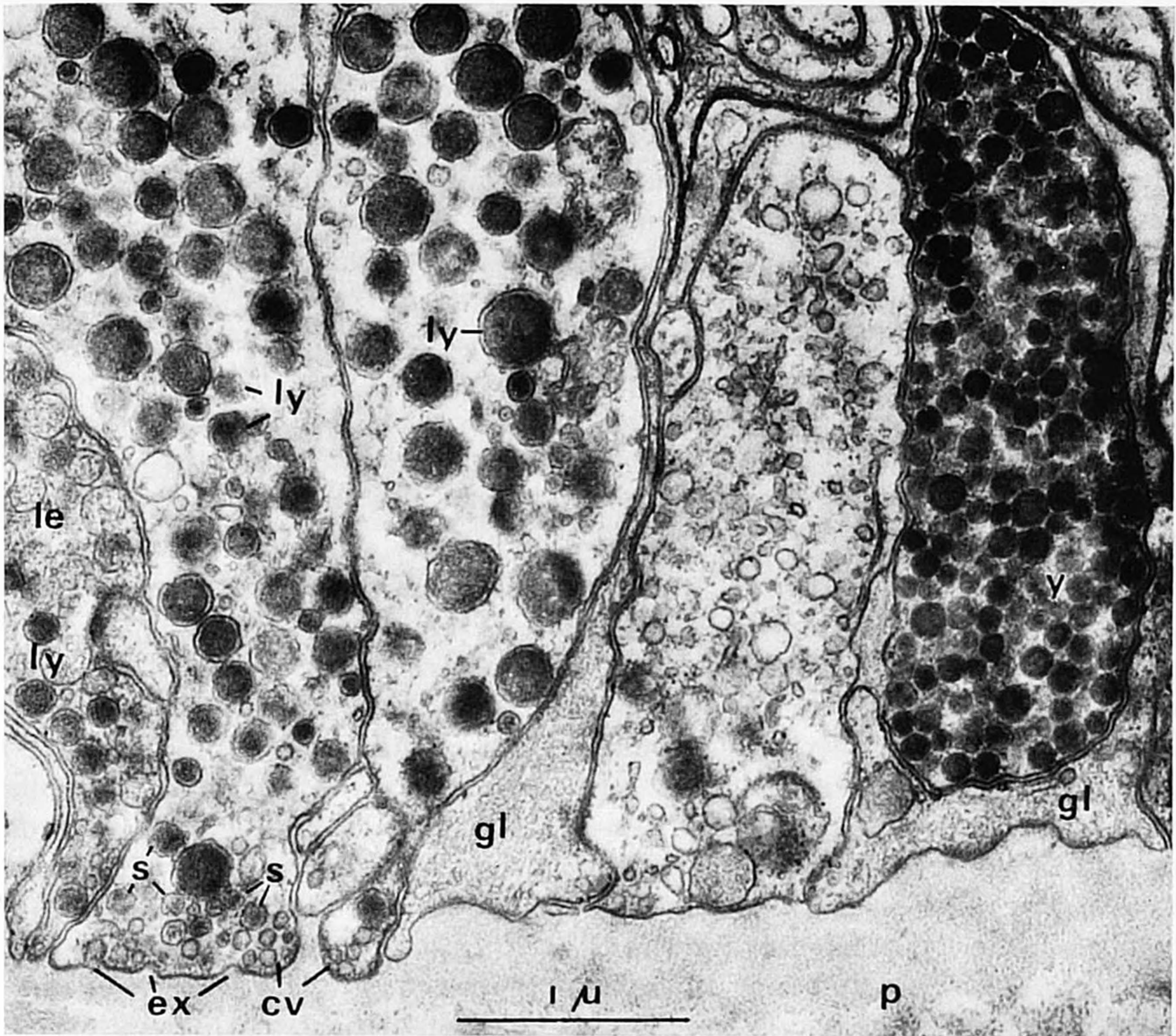


Fig. 23. Axon terminals in the periphery of the nervus pallialis dexter internus. Release phenomena can be observed in the left axons. *ly* neurosecretory elementary granules of the light yellow type; *y* neurosecretory elementary granules of the yellow type; *gl* filamentous glial cell processes; *p* perineurium; *s* small elementary granules; *cv* clear vesicles; *ex* membrane invaginations indicating exocytosis; *le* elementary granules of low electron density

by trophic glial cell processes: extensive areas of contact between neighbouring cells were found. However, at these places no junction-like differentiations of the cell membranes were observed. As mentioned for the dark green cells, the yellow green neurones lying adjacent to the perineurium show cytoplasmic processes which are bounded by the perineurium (Fig. 22). The ultrastructural characteristics were found in all groups of yellow green cells which occur in the central nervous system. Furthermore, in all areas in which yellow green axons are distributed (see section on light microscopy) the elementary granules typical of the yellow green cells were observed.

Also the Gomori-negative cell groups were studied at the ultrastructural level. The phloxinophilic CDC are known to contain elementary granules with a mean diameter of 1,500 Å (Fig. 16b; see Boer *et al.*, 1968a). The two groups of light yellow cells in the parietal and visceral ganglia appeared to possess secretory granules which are fairly different from those in the CDC. These granules which consist of an electron dense core surrounded by a transparent zone, are the largest



secretion granules (mean diameter: 2,300 Å) met with in the central nervous system (Fig. 16e). The cell bodies are separated from each other and from the perineurium by relatively thick layers of glial cell processes. The cells have an extensive granular endoplasmic reticulum and numerous Golgi lamellae, which often contain electron dense material. The yellow cells in the cerebral ganglion could not be identified with certainty in Epon sections, due to the fact that only a few of these cells are present. In the visceral and parietal ganglia the yellow cells are more numerous. They contain small secretory granules (mean diameter 1,400 Å; Fig. 16f) which lack a distinct electron transparent zone between the limiting membrane and the very dense core. In these cells granular endoplasmic reticulum prevails, although also smooth membranes occur, especially near the Golgi zones. Usually accumulations of electron dense material were observed between the Golgi membranes. These small cells are completely separated from each other by glial cell processes.

In the peripheral parts of the connectives and nerves of the pleural, parietal and visceral ganglia large numbers of distended axon endings containing secretory granules were observed (Fig. 23). They penetrate the layer of filamentous glial cells, which covers the nervous system. The structure of these regions (Fig. 22) is similar to that of the neurohaemal zones of the CDC and of the MDC and LDC (Boer *et al.*, 1968a). It was possible to identify the elementary granules within the axon endings on the basis of their size, of the specific electron opacity of the granular core and of the width of the electron transparent zone surrounding the core. If the size distributions of the various types of granules are compared (Fig. 24) differences in mean size and their spread are apparent for most of them. Although only minor differences between the granules of the CDC and those of the yellow green cells were found on the basis of these parameters, these two granule types are easily distinguished morphologically (Fig. 16b, d).

If the size of the granules within the perikarya of the various cell types is compared to that of the granules in the axon endings, it is evident that granules of small size are more numerous in the cell bodies (Fig. 24). Additional measurements were carried out in the proximal parts of the axons of the CDC and of the light yellow cells. The histograms of the granules in these areas appeared to be similar to that of the granules in the axon endings. These observations suggest that the granules increase in size in the period between their formation in the Golgi apparatus and their transit into the axons. No evidence was obtained with regard to the mechanism which causes the growth of the elementary granules after their budding from the Golgi lamellae. Fusion of elementary granules with small vesicles, as has been observed in the MDC/LDC and in the CDC of *L. stagnalis* (Boer *et al.*, 1968a), was not established in the present study for the secretory cells in the visceral ring.

Studies of serial sections revealed that axons of the neurosecretory cells often end blindly in the perineurium. The distended endings were found in the vicinity of the blood spaces, capillaries, small blood vessels, and in the muscular wall surrounding the larger blood vessels (Figs. 5—7, 22).

The morphological evidence for the way in which the axons of the neurosecretory cells terminate, seems to be in accordance with the supposition that



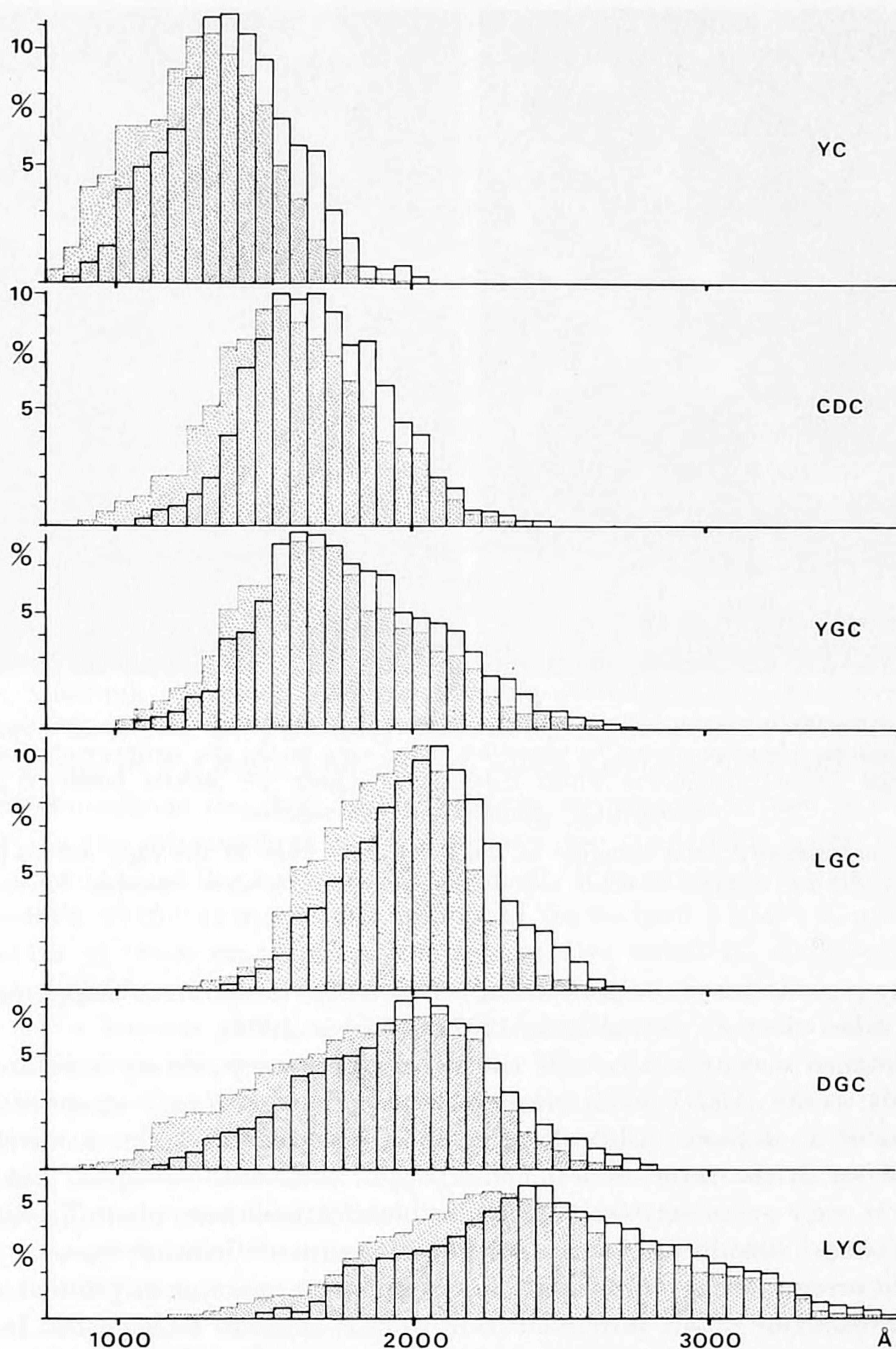


Fig. 24. The size-distribution of the diameters of the neurosecretory elementary granules located in the axon terminals (solid lines) and in the neuronal cell bodies (mottled areas). Each histogram refers to 1,000 measurements. *YC* yellow cells; *CDC* caudo-dorsal cells; *YGC* yellow green cells; *LGC* light green cells; *DGC* dark green cells; *LYC* light yellow cells

these cells produce neurohormones, which are released into the blood. To obtain further evidence on this subject, the release mechanism was studied.

No agreement exists about the details of the mechanism of release of neurosecretory materials from the axon endings. However, in vertebrates as well as in invertebrates the presence in the axon terminals of small clear vesicles with a diameter of about 350–500 Å, similar to those encountered in synapses, is



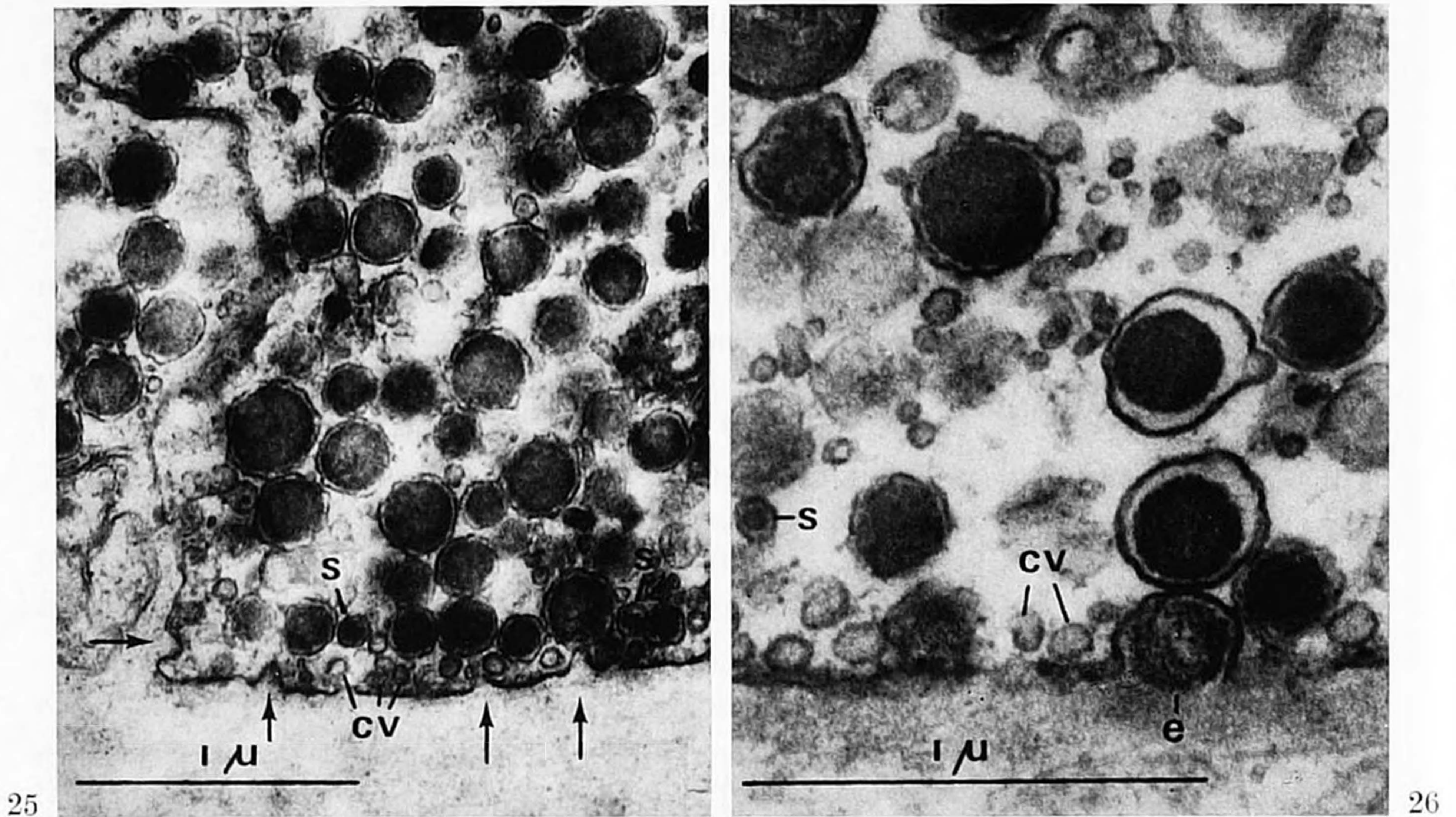


Fig. 25. Neurosecretory axon terminals of the light yellow cells in the nervus pallialis dexter internus. Release phenomena can be observed in the area facing the perineurium. Indentations of the axonal membrane which indicate exocytosis are present (arrows). *s* small elementary granules; *cv* clear vesicles

Fig. 26. Neurosecretory axon terminal of the deep green type in the right pleuro-parietal connective, during release. *s* small elementary granule; *cv* clear vesicles; *e* exocytosis

commonly regarded as an indication that release of the content of the elementary granules takes place (*cf.*, Streefkerk, 1967; Scharrer, 1968).

No detailed accounts occur on the release of neurosecretory substances in gastropods. Nolte (1967) noted clear vesicles and membrane fragments in the lip nerves of *L. stagnalis*, while Boer *et al.* (1968a) reported the occurrence of clear vesicles in the intercerebral commissure. Both authors agree that these phenomena were extremely scarce. Similar observations were made by Simpson (1970) in some axons in the intercerebral commissure of *Helisoma tenue*.

In the present study clusters of clear vesicles were commonly found within the axon terminals in the presumed neurohaemal areas in *L. stagnalis*. In these axon endings the appearance of the elementary granules varied according to the fixation procedure used. With  $\text{OsO}_4$  alone the membranes of the granules had ruptured. Occasionally flocculent electron-dense material was observed near the axonal membrane. By adding glutaraldehyde in low concentrations (0.5–1.0%) to the  $\text{OsO}_4$  fixative, fragmentation of the membranes was considerably reduced. Therefore, rupture of the granule membrane is regarded as an artifact due to inadequate fixation. However, the occurrence of this artifact in these particular axons — in non-releasing axon terminals broken elementary granules were not found — indicates that the membranes of the granules are weakened prior to release (see also Nolte, 1967). After fixation involving glutaraldehyde small elementary granules appeared to be present in addition to normal sized granules



and clear vesicles (Figs. 23, 25). Occasionally, a gradient of size stages towards the axonal membrane was observed. Accumulations of electron-dense material outside the granules was not observed. Irrespective of the fixation procedure applied, small invaginations of the cell membrane were found in the areas near the clear vesicles (Figs. 23, 25). These indentations are regarded as being formed after fusion of the small elementary granules with the axonal membrane, resulting in the release of the contents of the granules (exocytosis; Weitzmann, 1969; Normann, 1969). The occasional observation of electron-dense material within the invaginations (Fig. 26) is consistent with this hypothesis. The origin of the clear vesicles remains obscure.

In specimens sacrificed between 9 and 10 a.m. — for routine studies, fixation was carried out at this time of the day — release phenomena were observed in approximately 15% of the axon terminals of the secretory cell groups located in the pleural, parietal and visceral ganglia. In these animals release phenomena were only encountered in 0—3% of the axon terminals in the neurohaemal areas of the CDC (the intercerebral commissure) and of the MDC/LDC (the median lip nerve). This finding is in agreement with the results of Boer *et al.* (1968a) who established in animals fixed during the day that clear vesicles were extremely scarce, although they met with considerable activity within the corresponding perikarya. To verify the possibility that release might occur at other times of the day, 7 groups of 5 animals were studied, which had been fixed with intervals of 4 hours throughout the day. In the evening, an impressive rise of the release activity in the intercerebral commissure and the median lip nerve was found, which started after the light was switched off. This increased activity, observed in 70—90% of the axon endings, prolonged for at least 4 hours. A quantitative evaluation of these phenomena, which were also found in snails living under natural conditions in ponds outside the laboratory, is in progress.

In a low number of axons in the neurohaemal zones granules were observed which differ from the types described so far. Most of them occur in the lip nerve. In addition to the elementary granules of the MDC/LDC at least three more types were found. Release phenomena were observed in the axon endings containing these granules. Therefore, they are regarded as neurosecretory elementary granules. The corresponding cell bodies were not detected.

In all neurohaemal areas occasionally axons with granules dissimilar to the neurosecretory elementary granules were found. These granules are usually smaller (800—1,400 Å) than those of the neurosecretory cells and may contain neurotransmitters (*cf.*, Gerschenfeld, 1963). This supposition was substantiated by demonstrating the presence of bioamines in some of these axons with the chromaffin reaction at the ultrastructural level after Wood (1963). The neurosecretory elementary granules reacted negative to this technique. Synaptic contacts between neurotransmitter containing axons and axons or cell bodies of neurosecretory neurones were not observed.

### Discussion

Cook (1966), using the PF and CH method, distinguished three types of Gomori-positive cells in the stylommatophore *Succinea putris*, on the basis of different affinities for these stains. Simpson (1970) demonstrated that the fuchsino-



philic cells in the visceral ganglion of *Helisoma tenue* were victoria blue-positive, unlike those in the cerebral ganglia. The tinctorial differences observed indicate that within a given species the class of Gomori-positive neurosecretory substances is chemically inhomogeneous, as was also demonstrated in the nemertinean worm *Cerebratulus marginatus* (Bianchi, 1969) and in the colorado beetle *Leptinotarsa decemlineata* (Schooneveld, 1970).

In *Lymnaea stagnalis* only minor differences between the Gomori-positive cell groups are observed when the results of PF, CH, and PFAAB are compared. However, with the AB/AY technique, it is possible to distinguish between seven types of Gomori-positive neurones. The colour differences obtained with this method are supposed to reflect different ratios of strong and weak acid groups in the secretory materials (Peute and van de Kamer, 1967). This indicates that the various secretory products are of distinct chemical nature in *L. stagnalis*.

These results are substantiated by the ultrastructural findings. In *L. stagnalis* the CH-positive cell types investigated ultrastructurally — the bright green cells of the cerebral ganglia were not examined so far — appear to contain elementary granules of characteristic size and appearance. This is evident from the present study and, as regards the cells located in the cerebral ganglia and in the lateral lobe, from observations by Boer *et al.* (1968a) and by Brink and Boer (1967), respectively.

With the classic methods for demonstrating neurosecretory material also cytosomes, like lipofuscin pigments, are stained. For this reason CH and PF are rather unreliable as diagnostic methods for secretory material, as has been discussed by Boer (1965), Nolte *et al.* (1965), and Simpson *et al.* (1966a, b). The cytosomes are also stained with AB/AY. However, they can be easily distinguished from secretory substances, even in the same cell, because of their characteristic bluish colour.

In vertebrates (*cf.*, Barry, 1967) as well as in invertebrates (*cf.*, Herlant-Meewis *et al.*, 1967) Gomori-negative neurosecretory cells do occur. For their demonstration a number of stains have been used, e.g., phloxin (Joosse, 1964; Bianchi, 1969; Schooneveld, 1970), azocarmine (Raabe, 1965), and Sudan black B (Boer, 1965).

In gastropods reports on Gomori-negative cells are scarce (Joosse, 1964; Quattrini, 1964; Boer, 1965; Cook, 1966). In *L. stagnalis* three types of Gomori-negative neurosecretory cells occur which are all phloxinophilic, although to a different degree. Only two of them (the yellow, and light yellow cell types) stained with the yellow component of AB/AY. Their secretory materials apparently contain a lipid component, since they show affinity for Sudan black B. Just as within the group of Gomori-positive cells, it was possible to correlate the histochemical differences with the presence of distinct types of elementary granules.

#### *Location of the Neurosecretory Cells*

In the *cerebral ganglia* of *L. stagnalis* the neurosecretory cells occur in distinct groups in well localized areas. The same holds true for other pulmonates (*cf.*, Simpson *et al.*, 1966a) although the number of cell groups is in most species smaller than in *L. stagnalis*. As in this species, two large cell groups can be



distinguished in the medio- and latero-dorsal parts of the cerebral ganglia in the basommatophore *Planorbarius corneus* (Röhnisch, 1964) and in the stylommatophore *Succinea putris* (Cook, 1966). In the latter snail tinctorial differences were found between these groups. Therefore, as Cook pointed out, it is doubtful whether both groups are homologous to the cell groups in *L. stagnalis*. In the basommatophores *Australorbis glabratus* (Lever *et al.*, 1965) and *Helisoma tenue* (Simpson *et al.*, 1966b) only one group of Gomori-positive cells is present in the cerebral ganglia. Small cells comparable to the bright green cells in *L. stagnalis* have not been reported for other basommatophores.

In the cerebral ganglia of pulmonates investigated so far Gomori-negative cells were only reported for *L. stagnalis*.

The Gomori-positive cells in the *pleural* and *parietal* ganglia and in the *visceral* ganglion are located in less defined groups than in the cerebral ganglia. The deep green cells, which lie primarily in the pleural ganglion, occur dispersed between ordinary neurones, whereas the yellow green cells, which form many small groups, are found throughout the parietal and visceral ganglia and also scattered in the perineurium and in the pallial and visceral nerves.

Hekstra and Lever (1960) and Lever *et al.* (1961) observed in *L. stagnalis* a statistically significant increase in bodyweight due to water uptake after removal of the pleural ganglia. The swelling decreased after reimplantation as well as after injection of homogenates of these ganglia. They concluded from these experiments that these effects are caused by a hormonal factor, probably produced by neurosecretory cells. The presence of a distinct type of neurosecretory cells — the deep green variety — in the pleural ganglia supports this hypothesis. The fact that these cells occur also — albeit in low numbers — in the parietal ganglia corroborates this view, since injection of homogenates of right parietal ganglia had, according to Lever *et al.* (1961), a small effect.

The presence of Gomori-positive neurosecretory cells in the pleural ganglia has, as regards pulmonates, only been reported for *Vaginula* sp. (A-cells, Nagabushanam and Swaranamayye, 1963) and *Succinea putris* (Cook, 1966). As in *L. stagnalis*, in *S. putris* these cells differ histochemically from Gomori-positive cells in the other ganglia. When compared to *L. stagnalis* the number of Gomori-positive cells in the parietal ganglia of *Ferrissia shimckii* (B-cells, Lever, 1957), *Planorbarius corneus* (Röhnisch, 1964), *Australorbis glabratus* (Lever *et al.*, 1965), *Helisoma tenue* (Simpson *et al.*, 1966b) and of *Succinea putris* (Cook, 1966) is rather limited; in *Vaginula* sp. and in species of the Helicidae (Type II cells, Kuhlmann, 1963) Gomori-positive neurosecretory cells are absent in the parietal ganglia. On the other hand, cells of this type are present in the visceral ganglion of all species mentioned. However, except for *Australorbis glabratus*, the numbers are low when compared to *L. stagnalis*.

As regards other pulmonates Gomori-negative cells have only been described in the parietal ganglia of *Milax gagates* (Quattrini, 1964) and *Succinea putris* (Cook, 1966). In the latter species these cells are located in two groups, one in the lateral part of the right parietal ganglion and the other in the transitional area between the left parietal and the visceral ganglion. These cells can be compared with the light yellow cells of *L. stagnalis*, because of their typical asymmetrical position. Moreover, in both species the cells contain Nissl disks.



*Neurohaemal Areas*

As determined by Joosse (1964), the axons of the MDC and LDC of *L. stagnalis* run in a distinct tract to the neurohaemal regions in the median lip nerve, whereas those of the CDC terminate in the intercerebral commissure. In the present study it was impossible to detect the axonal terminations of the other neurosecretory cells of the cerebral ganglion and those of the lateral lobe. However, it is possible that these cells transport their secretory products also to the lip nerve, because in the periphery of this nerve three types of elementary granules occur in addition to those of the MDC/LDC. This supposition is supported by the presence in this nerve of elementary granules which are comparable in size and morphology to those described in the canopy cell and in the droplet cells of the lateral lobe (Brink and Boer, 1967) and by the finding of axon endings which react positive to phloxin and Sudan black B (Boer, 1965).

Of the other cerebral nerves only the nuchal nerves contain considerable amounts of secretory substances. From light as well as from electron microscopy it is evident that these materials are produced by neurosecretory cells in the pleural and parietal ganglia and in the visceral ganglion. Furthermore, most of the non-secretory axons of this nerve, which originates from the rostral part of the cerebro-pleural connective (Joosse, 1964), come from the pleural region. These observations show that the nuchal nerve is non-cerebral.

In the intercerebral commissure a few axon endings containing elementary granules of the MDC/LDC type occur in addition to nerve endings of the CDC. This is in agreement with the light microscope observation that in this area light green axons are present. Gomori-positive axons were also found in the intercerebral commissure of *Planorbarius corneus* (Röhnisch, 1964). However, in Basommatophora the main neurohaemal area of the Gomori-positive cells in the cerebral ganglia is located in the lip nerve. This appeared from light and electron microscope studies of the following species: *Lymnaea stagnalis* (Joosse, 1964; Nolte, 1965; Boer *et al.*, 1968a), *Planorbis planorbis*, *Planorbarius corneus*, *Bathyomphalus contortus* (Nolte, 1965) and *Helisoma tenue* (Simpson, 1970). The same holds true for the stylommatophores *Strophocheilus oblongus* (Kuhlmann, 1963) and, probably, *Succinea putris*. In the latter species Cook (1966) observed that the neurosecretory axonal tracts of the large cerebral cell groups run to the median and internal lip nerve. In other stylommatophores the axons of the cerebral Gomori-positive cells end in the nervus arteriae cerebralis (van Mol, 1960; Kuhlmann, 1963) and in small nerves arising from the intercerebral commissure (Kuhlmann, 1963). The ultrastructure of the periphery of this commissure in the basommatophores *Planorbarius corneus* (Nolte, 1965) and *Helisoma tenue* (Simpson, 1970), is essentially similar to that of *L. stagnalis*. Large numbers of elementary granules were encountered within distended axon endings. In *L. stagnalis* these granules represent the phloxinophilic material produced by the CDC (Boer *et al.*, 1968a). In *Planorbarius corneus* and in *Helisoma tenue* histochemical methods including staining with phloxin, failed to demonstrate this neurosecretory material at the light microscope level. It may be concluded from these observations that at least in the basommatophores studied so far the intercerebral commissure serves as a neurohaemal area for Gomori-negative secretion.



In other pulmonates little is known with regard to the neurohaemal zones of the neurosecretory cells outside the cerebral ganglia. Ultrastructural investigations have only been carried out by Simpson (1970) who observed neurosecretory axon terminals in the periphery of the proximal part of the intestinal nerve of *Helisoma tenue*. No terminations were found in the other nerves of the visceral ganglion or in the pallial nerves. In *Planorbarius corneus* Röhnisch detected fuchsinophilic secretory tracts ending in the pallial nerves. In *L. stagnalis*, on the other hand, the neurohaemal zones of the neurosecretory cells in the pleural and parietal ganglia and in the visceral ganglion are very extensive and include all connectives and nerves of these ganglia. The cell groups have, in contrast to those in the cerebral ganglia, no distinct neurosecretory tracts to the neurohaemal zones. Although most of the axons of a cell group extend into the most nearby connective or nerve, a large number also proceeds in various directions and terminates in more distant neurohaemal zones. Consequently, in all these areas axon terminals of the four cell types occurring in these ganglia can be found, although in varying proportions.

The present results show that the neurohaemal zones are not limited to the peripheries of the intercerebral commissure and many connectives and nerves. Large regions of the perineurium and of the surrounding connective tissue are regarded as parts of the neurohaemal system. Along the entire length of many small nerves occurring in these regions release of elementary granules was observed. Similarly, in a number of gastropods neurosecretory fibres have been reported to occur in the perineurium (Lemche, 1955; Kuhlmann, 1963; Röhnisch, 1965; Coggeshall, 1967; Sanchiz and Zambrano, 1968; Simpson, 1970). In this respect *L. stagnalis* has been investigated by Altmann and Kuhnen-Clausen (1959) who established fuchsinophilic cells and traces of fuchsinophilic material in the perineurium. However, release phenomena have not been mentioned in the species investigated.

In the vicinity of the neurosecretory nerves in the perineurium in *L. stagnalis* an extensive network of capillaries and blood spaces is present, which is connected with the arterial system as was determined by injecting India ink. This suggests that discharge of neurosecretory substances into the blood can easily take place. Part of the neurosecretory fibres were found in the vicinity of the large blood vessels. With the light microscope similar observations were made in *Arion rufus* and *Arion subfuscus* (Herlant-Meewis and van Mol, 1959) and in *Helisoma tenue* (Simpson *et al.*, 1966b). In the vena cava of *Octopus vulgaris* neurosecretory axons penetrate through the muscular wall around this vein and form a layer of axon terminals which face the blood (Martin, 1969). The electron microscope observations in *L. stagnalis* revealed that the neurosecretory axons in the wall of the large blood vessels are located within the muscular layer and terminate at a distance from the endothelium.

#### *Neurosecretory Cells — Dorsal Bodies*

Joosse and Geraerts (1969) demonstrated in *L. stagnalis* that the dorsal bodies lying upon the cerebral ganglia are endocrine organs which control vitellogenesis. Anatomically, the bodies are closely related to the dorsal neurosecretory cells, as established for several basommatophoran species. Lever (1958) assumed on the



basis of light microscope observations, especially of ancyloid snails, that axons of the neurosecretory cells penetrate into the bodies. He suggested that they might be neurohaemal organs. Boer *et al.* (1968 b) demonstrated with histochemical and electron microscopical techniques that in four different species, including *L. stagnalis*, no direct contact exists between the neurosecretory cells and the dorsal bodies (see also Joosse, 1964; Simpson, 1970). However, in the present study neurosecretory axons of the light green type (MDC/LDC) were encountered in the medio- and latero-dorsal bodies. In the latter also deep green, yellow and light yellow axons were found. Although the significance of these fibres is not clear, it seems unlikely that they play a role in the control of the dorsal body activity, since their arrangement (often near capillaries and blood spaces) in the narrow strands of connective tissue which separate the cell groups of the dorsal bodies suggests that they are merely part of the perineurial neurohaemal system.

#### *Release*

In the present study the occurrence of clear ("synaptic-like") vesicles in the neurosecretory axon terminals is regarded as a criterion for release of neurosecretory material. This seems to be valid since it has been possible in some vertebrates (*cf.*, Streefkerk, 1967) as well as in some invertebrates (Scharrer and Kater, 1969; Normann and Duve, 1969) to correlate experimentally induced release of neurohormones with the occurrence of these vesicles. Moreover, it has frequently been reported that a decrease in the number of elementary granules is associated with a rise in the number of clear vesicles (*cf.*, Streefkerk, 1967).

In the literature various interpretations of the origin and significance of the small clear vesicles have been forwarded. They have been regarded as a special class of granules containing a transmitter substance (Gerschenfeld *et al.*, 1960), but also as structures arising from microtubules (Vollrath, 1969) or from narrow channels projecting into the axons (Bunt and Ashby, 1967). However, the most adequate hypotheses seem to be that they may originate either by a process of budding from the elementary granules (Scharrer, 1968) or from the membranes of these granules after their contents have been extruded into the extracellular space via exocytosis (e.g., Weitzmann, 1966; Normann, 1969). In *L. stagnalis* indications that these vesicles are budded off from the elementary granules were scarce. On the other hand, indentations of the axonal membrane indicating exocytosis were regularly found. Presumably the clear vesicles arise from the membranes of the elementary granules after exocytosis. Apparently in *L. stagnalis* the elementary granules change in size and appearance prior to exocytosis since in axon terminals containing many clear vesicles elementary granules of relatively small size and varying electron density occur. These almost certainly originate from normal-sized granules: in a number of cases a series of transitional size-stages was found in a gradient towards the axonal membrane. In such axons structural changes of the granules were indicated by the occurrence of fixation artifacts (rupture of the membranes of the elementary granules) after inadequate fixation. Comparable phenomena are known to occur in other animals during release of neurohormones (Fridberg *et al.*, 1966; Shivers, 1969; Weitzmann, 1969). A decrease of the diameter of the granules in the axon terminals has been found in the crayfish



*Orconectes nais* (Shivers, 1969) and in the intercerebral commissure of the pulmonate snail *Helisoma tenue* (Simpson, 1970).

The release of the neurosecretory substances from the neurohaemal regions of the cerebral ganglia of *L. stagnalis* is a rhythmical (daily) process. This observation offers the opportunity to study release phenomena in detail without previous artificial stimulation of the neurosecretory system as carried out in other investigations (e.g., Scharrer and Kater, 1969; Normann, 1969). The results will be dealt with in a future publication.

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S. E. Wendelaar Bonga  
 Department of Zoology  
 Free University  
 Amsterdam/The Netherlands