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EGG CAPSULE FORMATION AND HATCHING IN THE MARINE SNAIL *LITTORINA SITKANA*

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[Plates 1–8]

CONTENTS

The process of egg capsule formation and hatching in *Littorina sitkana*, which includes the secretion of albumen layer, egg covering, egg capsule and jelly layer, has been studied with light and electron microscopy. Fertilization occurs in an expanded portion of the oviduct, or in the lumen of the albumen gland. There the zygotes are

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coated with a thick layer of albumen, followed by a thin egg covering in the covering gland. Specialized cilia with dense flexible tips manipulate the secretions into place. The characteristic shape of the egg capsule is moulded inside the capsule gland by opposing lips of the gland, which are under muscular control. The two halves of the capsule gland contain different types of gland cells, the secretions of which differ both in content and quantity. The resulting egg capsule is heterogeneous with a thick half composed solely of loose filamentous secretions and a thin half comprising a stratified mixture of loose and dense filamentous secretions. The dense filaments degrade more slowly. Thus during larval development the thick half of the egg capsule weakens first.

When the juvenile snail is close to hatching it uses both its shell and radula to wear down and chew through the egg covering. The pre-hatching juvenile crawls around the inside surface of the capsule and periodically exerts pressure on the capsule wall by rapidly expanding the foot and shell. Eventually the thick side of the capsule fractures and the juvenile uses the thin side as a platform for thrust and escape. Rapid dissolution of parts of the egg capsule during hatching indicates that a hatching enzyme may be released.

1. Introduction

Encapsulation of eggs has evolved with internal fertilization as a means of ensuring reproductive success. By making use of nutrient stores within the egg capsule embryos are able to pass some of the most vulnerable phases of their development in a relatively protected environment (D'Asaro 1986; Pechenik 1979, 1986), emerging at an advanced stage with well-developed nervous systems (Bickell & Chia 1979). This benefits their survival through an increased ability to avoid predators and other unfavourable situations, and to locate food sources (D'Asaro 1986). Although it is probably important that the egg capsule be impervious to bacteria and Protozoa, at least in the early stages (Lord 1986) and strong enough to provide some protection against predators (Pechenik 1986), there must also be a way out for the embryo when it is ready to hatch. In spite of a vast body of literature on reproductive and developmental biology of gastropod molluscs, dating back to the end of the last century (see Fretter & Graham 1962; Goodwin 1979, Fretter 1984), our current understanding of the process of egg capsule formation and its relation to hatching is restricted to only a few species of gastropods, (see Beeman 1970; Thompson 1966; Thompson & Bebbington 1969; Schmekel 1971; Coggeshall 1972; de Jong Brink 1969; Fretter 1941; Fretter & Graham 1962). Furthermore, there are no detailed studies in prosobranchs of the fine structure of the egg covering, the mechanisms involved in the production and release of secretions, and the moulding of the egg capsule, when it occurs within the capsule gland.

This paper presents for the first time fine structural details of the formation of the egg capsule and other egg envelopes in the marine snail *Littorina sitkana*. We include a comprehensive description of the techniques that have been developed to enable this kind of study in this and other animals. Furthermore, the nature of the programmed degeneration of the egg capsule, which is essential to hatching in this species, is examined in the light of existing knowledge of some hatching mechanisms in other gastropods.

2. Methods

Female *Littorina sitkana* were obtained from False Bay, San Juan Island, Washington, U.S.A., during the peak breeding season from March to April and were kept in aquaria with running

seawater at Friday Harbor Laboratories, University of Washington. Individuals in the process of egg laying were removed from their shells, decapitated and fixed immediately for either light microscopy (LM) or transmission electron microscopy (TEM), as follows.

(*a*) *Light microscopy*

Animals were fixed in seawater–Bouin's fluid overnight, followed by dehydration in an ethanol series. During dehydration picric acid was removed by adding a few drops of 3% (by mass) ammonium hydroxide to several changes of 70% (by volume) ethanol (this reduced brittleness in the block). To reduce brittleness further we found it necessary to use a double embedding technique (Humason 1967). In this a series of changes of a solution of 1% (10 g l^{-1}) celloidin in methyl benzoate followed absolute ethanol for up to 72 h. The tissue was then transferred through three changes of pure benzene, one change of 1:1 benzene:paraplast wax and two changes of pure paraplast wax, before the final embedding in paraplast wax (melting point 56 °C). Sections were cut at 7 μm with a steel knife mounted in a rotary microtome. The sections were stained by the Masson-Trichrome technique given in Humason (1967).

Alternatively, some animals (minus the rectum), were embedded in Historesin (LKB) after dehydration. These blocks were serially sectioned at 2 μm with 10 mm glass knives mounted in a JB4 ultramicrotome (Sorvall). The sections were stained either with Richardson's stain (Richardson *et al.* 1960) or by a modified Masson-Trichrome technique, which was specially developed for the staining of plastics (Sorvall P.N.45603-0).

The corrosion vinyl acetate technique was used to make plastic replicas of the genital ducts of female snails (Buckland-Nicks & Chia 1984), to increase our knowledge of the internal architecture of the pallial oviduct.

(*b*) *Electron microscopy*

For TEM shell-less females were fixed for 1 h in ice-cold (2.5% by volume) glutaraldehyde in millipore filtered seawater (pH 8.0). The pallial oviduct was then dissected away from the rest of the body tissues and cut transversely into six slabs, approximately 2 mm in thickness. No attempt was made to reduce the size of blocks further to obtain optimal fixation, for fear of disrupting egg capsules deeper in the block. Each slab was placed in a separate labelled vial and fixed again for 2 h in ice-cold 2.5% (by volume) glutaraldehyde buffered with 0.2 M Millonig's phosphate buffer (pH 7.6). The slabs were then washed in three changes of fresh 2.5% (by mass) sodium bicarbonate buffer (pH 7.2) and post-fixed with ice-cold 2% (by mass) osmium tetroxide in 1.25% (by mass) sodium bicarbonate buffer (pH 7.2) for 1 h. The slabs were dehydrated in an ethanol series, exchanged through propylene oxide, flat embedded in Medcast (Pelco) in the base of polypropylene 'Beem' capsules and baked in a 60 °C oven for 48 h.

The whole cross section of pallial oviduct was faced in each block and a series of 1 μm sections were cut and stained with Richardson's stain (Richardson *et al.* 1960) to identify an area of interest (figure 1*a–c*). A rectangle surrounding such an area was then marked on the face of the block with four shallow razor cuts (figure 1*d*). The block was mounted in a Sorvall MT2B ultramicrotome and a series of vertical passes was made with a glass knife on the right side of the rectangle to a depth of about 10 μm, just inside the first razor cut thus eliminating it (figure 1*e*). The block was then rotated 90° and the process repeated. This was done for each side of the rectangle until all four sides framing the area of interest had been cut to a depth of 10 μm (figure 1*f–i*). A diamond knife was mounted in the microtome and a few 0.5 μm sections were

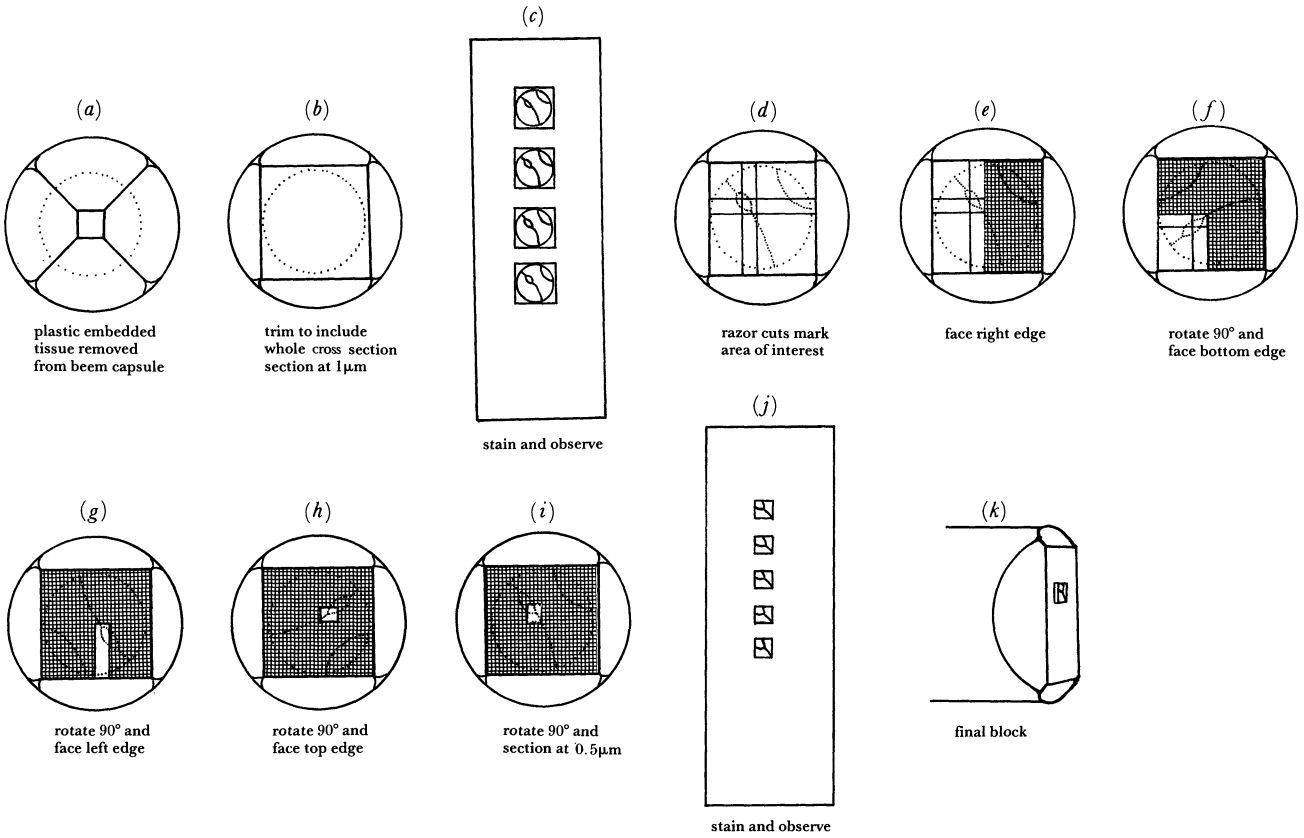


FIGURE 1. Schematic diagram of technique used to section several areas of interest in a single tissue block with minimal loss of tissue.

cut and stained as above to check the area of interest and to provide material for light microscopy (figure 1*j*–*k*). After this a series of silver sections were cut and picked up on naked 150 mesh copper grids. The sections were stained sequentially with alcoholic uranyl acetate (10 min) and aqueous lead citrate (Reynolds 1963) (8 min) and examined in a Philips 410 electron microscope operated at 80 kV. Subsequently, the rectangle was eliminated by refacing the block with a glass knife and then taking 1 μm sections until a second area of interest was encountered. The entire process was then repeated. In this way we were able to isolate each of the glands involved in manufacturing the egg investments and furthermore, since each female was in the process of egg laying, to observe directly several key events such as, the release of sperm, the site of fertilization, and how glandular secretions are released and contribute to the egg mass.

3. TERMINOLOGY

There has been a great deal of confusion in the literature over the naming of glands in the pallial oviducts of prosobranch molluscs. We have chosen the terms adopted by Fretter & Graham (1962), which in sequence are: albumen gland (secreting the albumen layer); covering gland (secreting the egg covering); capsule gland (secreting the egg capsule); jelly gland (secreting the jelly layer). We did not use the term 'membrane gland' and its secretion the 'egg membrane' (Fretter 1980, 1984) to describe the covering gland, for three reasons.

First, the egg covering is not a membrane but a complex stratified egg envelope comprising four different layers. Second, the term 'egg membrane' is frequently used in place of the terms 'oolemma' or 'oocyte membrane'. Third, these terms have been consistently used to describe egg envelopes in opisthobranchs (see review by Ghiselin 1965) and they may not be equivalent in prosobranchs, as the covering gland cell described here is very different from anything previously reported (Thompson 1966; Thompson & Bebbington 1969; Coggeshall 1972).

A previously undiscovered glandular area exists between the seminal receptacle and the oviduct, which we have termed here 'the accessory gland mass'. Preliminary experiments have indicated that the secretions of this gland may capacitate the sperm (Buckland-Nicks & Chia 1986). However, it would be premature to call it 'the capacitating gland' until these results are confirmed.

4. Results

(*a*) *Functional morphology of the pallial oviduct*

The pallial oviduct in *L. sitkana* comprises a series of four main glands: the albumen, covering, capsule and jelly glands (figure 2*a*), which become swollen during the peak breeding seasons in the spring and fall. In addition, there is a sperm receiving organ, the bursa copulatrix, next to the gonopore (figure 2*d*) and a sperm storage organ, the receptaculum seminis, adjacent to the albumen gland (figure 2*a*, *g*). The two are connected by the ventral channel of the oviduct (figure 2*d*), which is a tube formed by two flaps of tissue.

The ovary is a diffusely branching, orange-coloured mass embedded in the digestive gland (figure 2*a*). The ovarian tubules drain into the oviduct which passes close to the surface as a thin-walled tube just below the pericardial cavity (figure 2*a*). The oviduct is connected to this cavity by the gonopericardial duct. Anterior to the gonopericardial duct and adjacent to the albumen gland, the seminal receptacle duct connects the receptaculum seminis with the accessory gland mass (see terminology in §3). Apparently, within this gland the duct bifurcates, the distal (anterior) branch connects with the ventral channel of the oviduct (figure 2*e*) and the proximal (posterior) branch connects with the albumen gland (figure 2*f*) and oviduct (figure 2*g*). A sphincter muscle controls the flow of sperm into and out of the receptaculum seminis.

At the onset of egg-laying, oocytes are expelled in a continuous stream from the ovary and are moved in single file along the oviduct, largely by ciliary action. Fertilization occurs in a small expanded portion of the oviduct, here termed the 'fertilization chamber' adjacent to the entrance to the albumen gland into which the seminal receptacle duct delivers sperm from the receptaculum seminis via the accessory gland mass. However, sperm have also been found surrounding eggs in the albumen gland and it is likely that some oocytes are fertilized after they pass through the fertilization chamber.

The zygotes are moved into the albumen gland where they receive a copious layer of albumen up to 0.4 mm in thickness (figure 3*a*, *b*). Following this, the albumen is enclosed by a thin egg covering, measuring approximately 1.5 μm in thickness, which is secreted by the covering gland (figure 3*a*, *b*). This area of glandular tissue is difficult to distinguish from the albumen gland unless the zygotes are found within it. However, the physical appearance of this envelope, and its staining properties, are unique (figure 6, plate 1). The egg covering is not a simple membrane but a complex stratified egg envelope that is composed of four layers of variable thickness. Initially, a light granular layer is secreted, followed by a light and dark

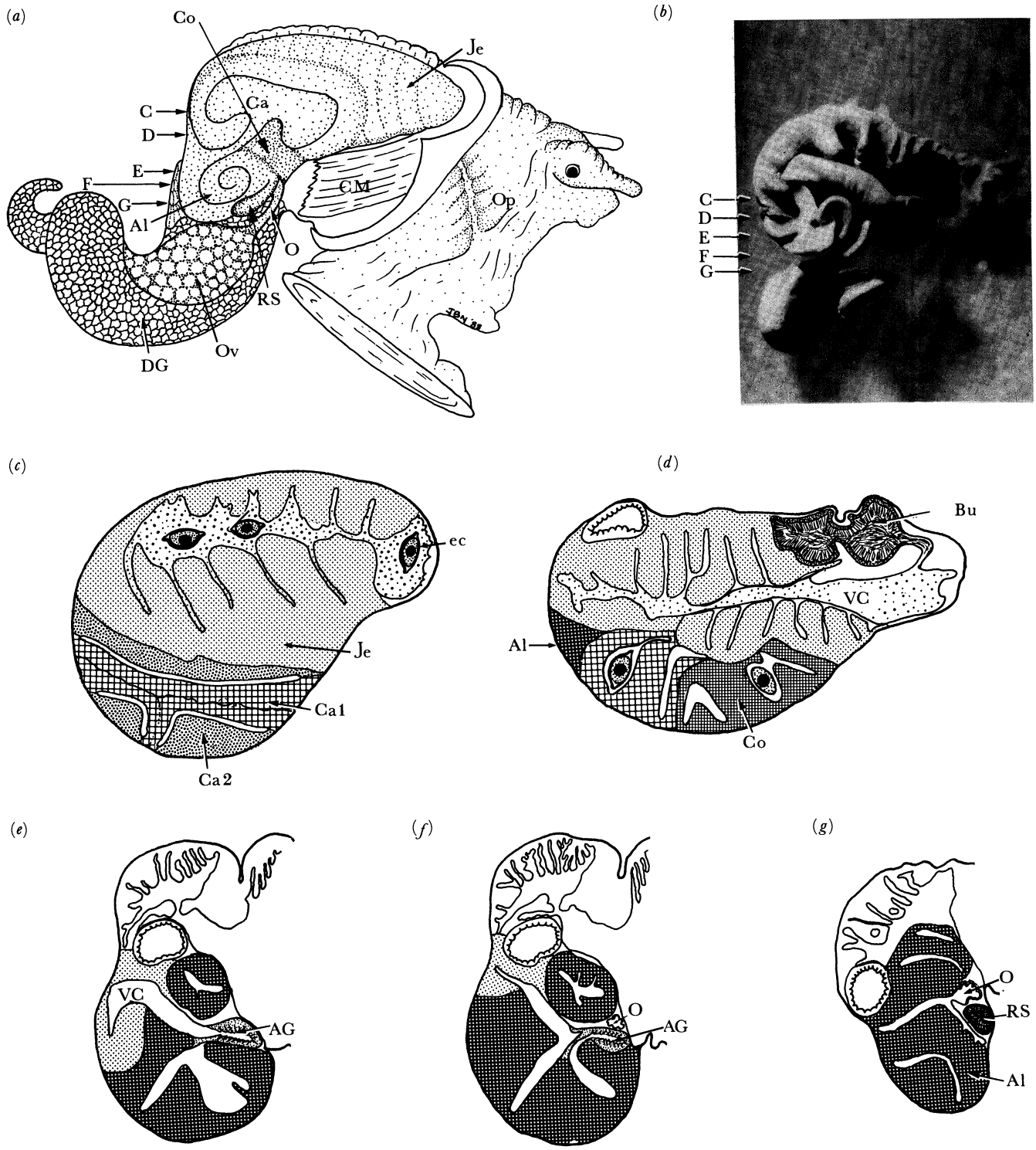


FIGURE 2. (*a*) Diagram of female *L. sitkana* removed from shell. (*b*) Photograph of a model of female genital ducts obtained by injecting vinyl acetate into gonopore of a female snail and dissolving away the tissues. (*c–g*) Longitudinal sections showing arrangement of glands in pallial oviduct, in relation to bursa copulatrix and receptaculum seminis. Sections were traced from photographs of stained wax sections and roughly correspond to levels indicated by arrows in Figure 2*a*, *b*. Ca: capsule gland; Je: jelly gland; CM: columellar muscle; Op: ovipositor; Al: albumen gland; RS: receptaculum seminis; O: oviduct; Ov: ovary; DG: digestive gland; Co: covering gland; Ca1: side of capsule gland containing homofilamentous granule cells; Ca2: side of capsule gland containing heterofilamentous granule cells; AG: accessory gland; Bu: bursa copulatrix; VC: ventral channel of oviduct.

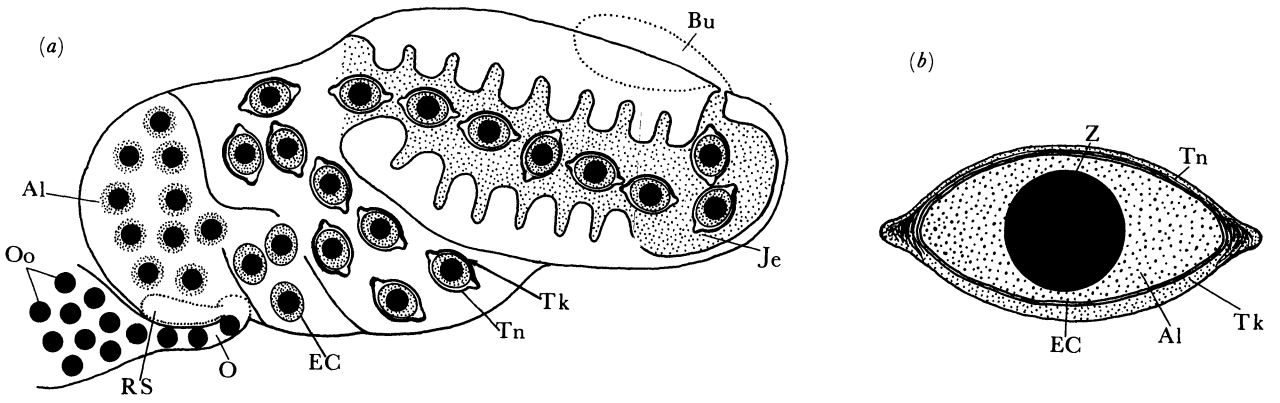


FIGURE 3. (*a*) Simplified diagram of main compartments of female genital system showing approximately where zygotes receive each egg envelope. (*b*) Diagram of section through encapsulated zygote of *L. sitkana*, omitting jelly layer. EC: egg covering; Tk: thick side of capsule; Tn: thin side of capsule; Al: albumen; Je: jelly layer; RS: receptaculum seminis; O: oviduct; Oo: oocytes; Bu: bursa copulatrix; Z: zygote.

granular layer, then a dense layer (only 0.005 μm in thickness) which forms from the coalescence of dense granules, and finally a fibrous layer (figure 6, plate 1). The egg covering presents the first major barrier to an emerging juvenile snail.

The zygotes pass on into the first portion of the capsule gland, where the characteristic shape of the egg capsule is moulded (figure 2*d*). Layers of smooth muscle fibres surrounding the gland cells enable precise control of the shape of the opposing lips of the gland. When a zygote is moved into the capsule gland, filamentous secretions are released from the gland cells by exocytosis and are whipped out onto their surfaces by specialised long, densely tipped cilia and there mixed into layers (figure 12, plate 3). The developing capsule is rotated in the plane of the opposed lips of the gland by the movements of the cilia, which draws out the secretions into long fibres (figure 14, plate 3), although some granules do not disperse. The strands appear to run in random directions and are not confined to a single plane. During egg laying several egg capsules are constructed simultaneously in the capsule gland.

Egg capsules of *L. sitkana* vary slightly in composition but the two halves are always different. In the first portion of the capsule gland the two halves secrete radically different products. In the one half virtually all the gland cells release copious amounts of a homogeneous loose filamentous material composed of 75 Å† filaments mixed with some granular secretions (figures 16 and 17, plate 4). In the opposing half the cells release a mixture of fine dense filamentous materials, composed of 40 Å filaments and smaller amounts of a loose filamentous secretion composed of 75 Å filaments (figures 16 and 18, plate 4). However, overall the amount of secretion released in the second half is less. Thus the two halves of the egg capsule differ in both thickness and composition: the homogeneous half is loose and thick (*ca.* 45 μm) and the heterogeneous half is stratified and thin (*ca.* 20 μm).

In the stratified half the proportions of the different types of secretion in the gland cells vary with the relative position in the gland. Initially this half releases pure dense filamentous secretions, composed of 40 Å filaments. In the middle of the gland the secretions begin to be mixed (i.e. a greater proportion of granules containing the 75 Å filaments). In the final part of the gland this half releases only the loose filamentous secretions, which are composed of

† 1 Å = 10^{-10} m = 10^{-1} nm.

Description of plate 1

Light and electron micrographs of the pallial oviduct of *L. sitkana*.

Figure 4. Nomarski light micrograph of a 1 μm section through part of zygote (Z) in lumen of albumen gland and adjacent receptaculum seminis, containing many sperm (Sp). Note also albumen secretions (S) and muscular wall (M) of receptaculum seminis. Magn × 1000.

Figure 5. Electron micrograph of a section of albumen gland similar to that shown in figure 4. Secretions (S) of dense granule cells are packaged in whorls of rough ER (ER). Adjacent ciliated epithelial supporting cells have long oval nuclei (N) and an apical aggregation of mitochondria (M). Magn × 8000.

Figure 6. Section of a zygote in final portion of covering gland to show, in particular, the four layers of the egg covering (EC): light granular (LG), light and dark granular (LDG), dense (D), and fibrous (F). Note also zygote (Z) and albumen (Al). Magn × 3500.

Description of plate 2

Electron micrographs of gland cells of the pallial oviduct of *L. sitkana*.

Figure 7. Section showing general appearance of microgranule cells in the albumen gland. Note small size of secretion granules (S), nucleus with one or more nucleoli (N), Golgi bodies (G) and rough ER (ER). Magn × 4800.

Figure 8. Section showing general appearance of a covering gland cell. Note large apical secretion mass (SM) that contains several dense granules with intact membranes (arrowheads), as well as pale secretions that have fused. Ciliated epithelial supporting cells (Ci) separate the gland cells. Magn × 9000.

Figure 9. Section through base of a macrogranule cell in the albumen gland showing formation of secretions (S) by rough ER (ER) adjacent to nucleus (N). Note Golgi body (G) in adjacent area of cell. Magn × 4300.

Figure 10. Section through base of a covering gland cell, showing formation of secretions (S) of radically different densities in vicinity of Golgi bodies (G). Nucleus (N). Magn × 13300.

Description of plate 3

Electron micrographs of secretions in the pallial oviduct of *L. sitkana*.

Figure 12. Section through specialized densely-tipped cilium (arrowhead) in the process of moving filamentous capsule secretions (FS) into the lumen. For size comparison note sections through full width of cilium to right. Magn × 65000.

Figure 13. Section through short-pointed cilia (arrowheads) from jelly gland, fixed during act of manipulating jelly layer (JM). Magn × 25000.

Figure 14. Scanning electron micrograph of capsule wall, showing how secretions are drawn into fibres. Note some granules do not disperse. Magn × 40000.

Figure 15. Cross section of specialized densely-tipped (DT) cilia fixed in act of moving filamentous secretions (FS) into lumen from among microvilli (Mi) of a heterofilamentous granule cell in capsule gland. Magn × 45000.

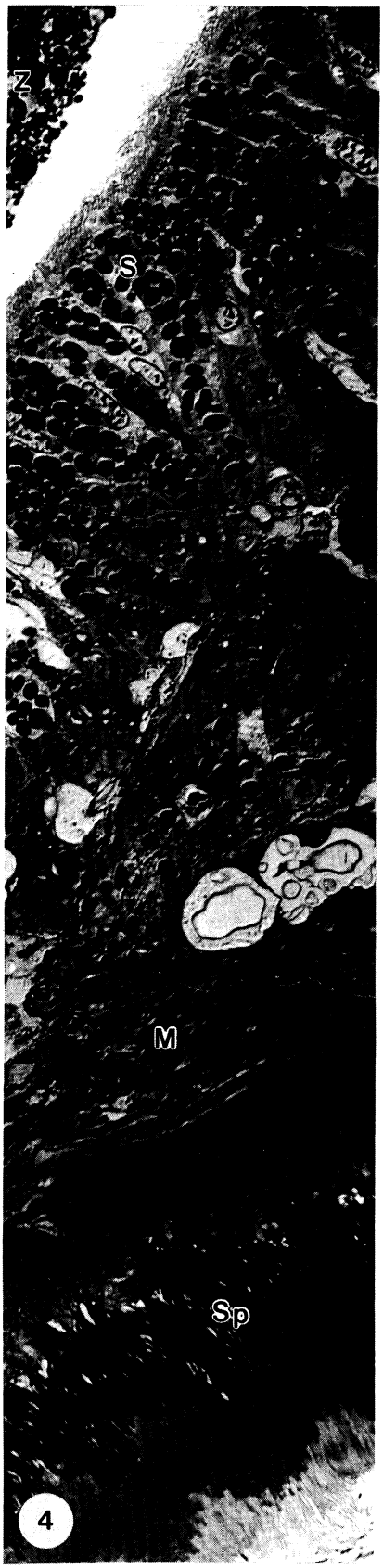
Description of plate 4

Electron micrographs of capsule gland of *L. sitkana*.

Figure 16. Section through first portion of capsule gland showing the radically different secretions produced by the two halves. Left half is homofilamentous granule cells; right half is heterofilamentous granule cells. Magn × 4250.

Figure 17. Section through homofilamentous granule cell. Loose filamentous secretions (LFS) composed of 75 Å filaments are released at apex of cell by exocytosis and are moved into place by specialized cilia to form one half of egg capsule (EC). Magn × 10000.

Figure 18. Section through heterofilamentous granule cell. Dense filamentous secretions (DS) composed mainly of 40 Å filaments are released by exocytosis and brushed into position by specialized cilia to form one half of egg capsule (EC). Some loose filamentous secretions composed of 75 Å filaments (arrowheads) may also be produced. Magn × 8000.



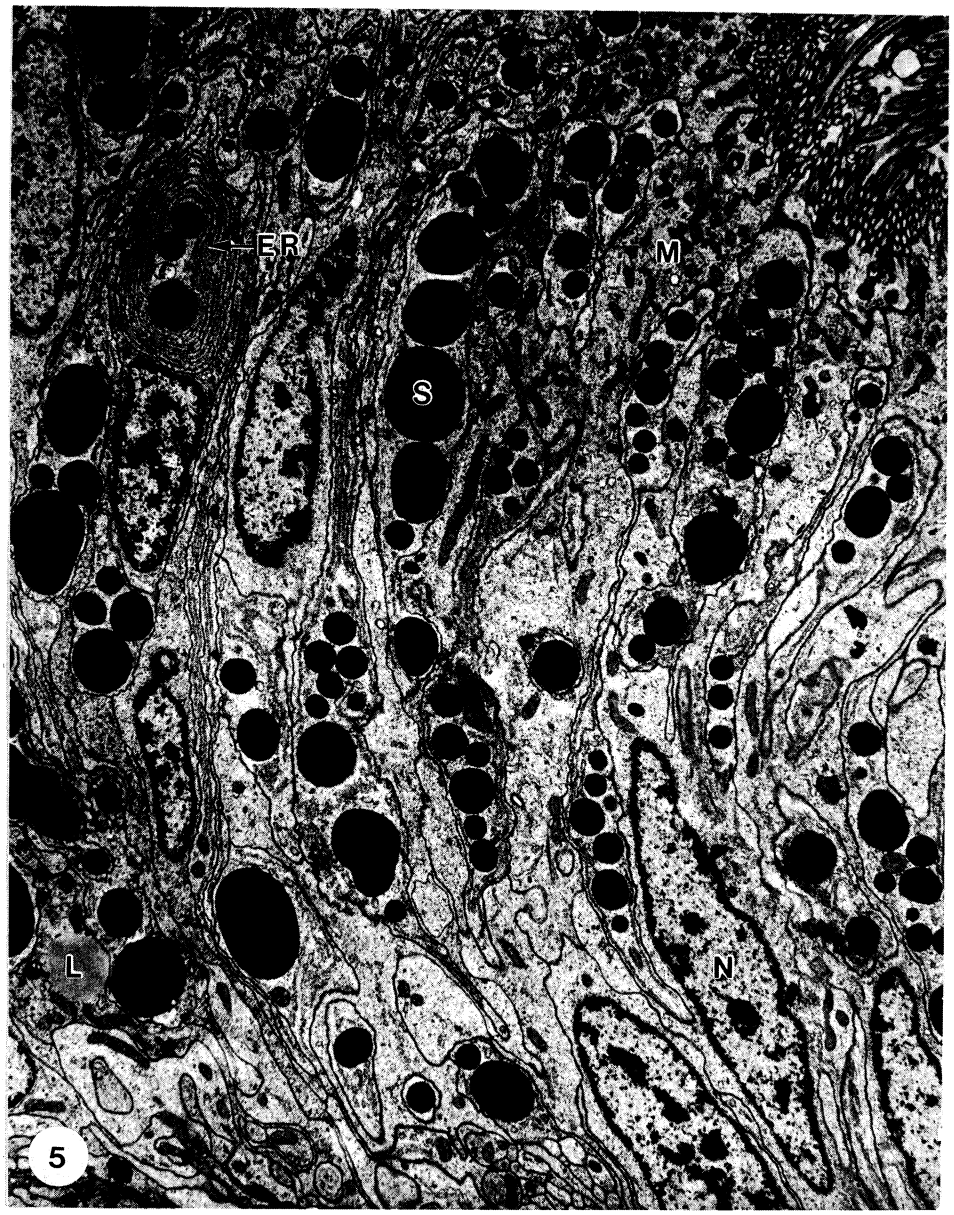


FIGURE 4–6. For description see opposite.

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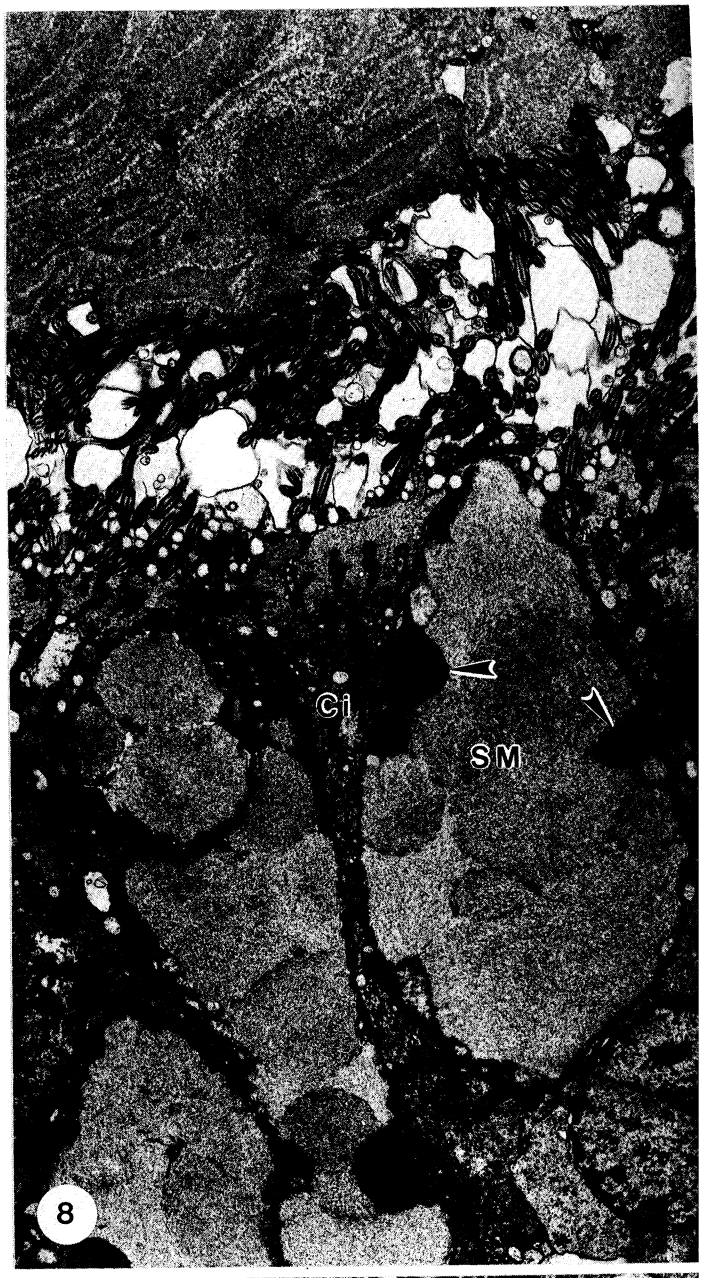
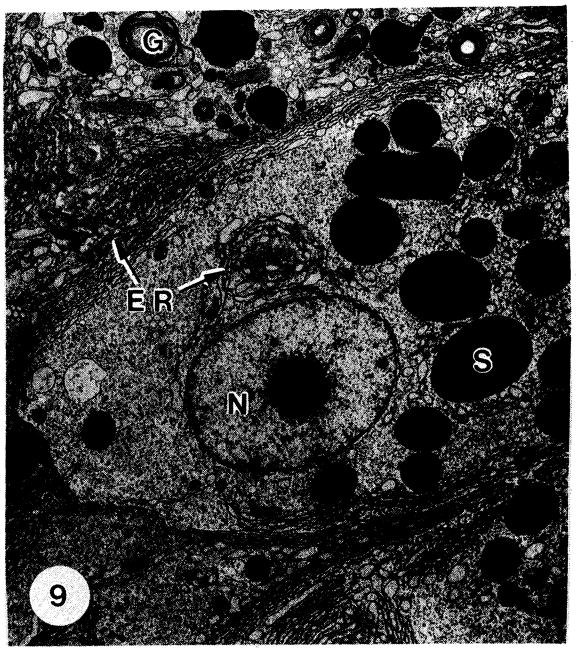
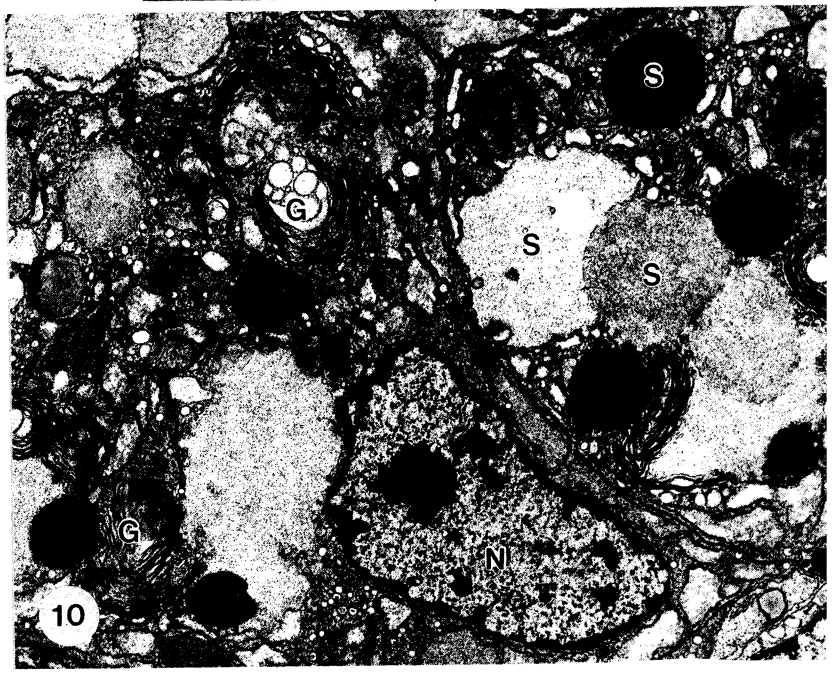
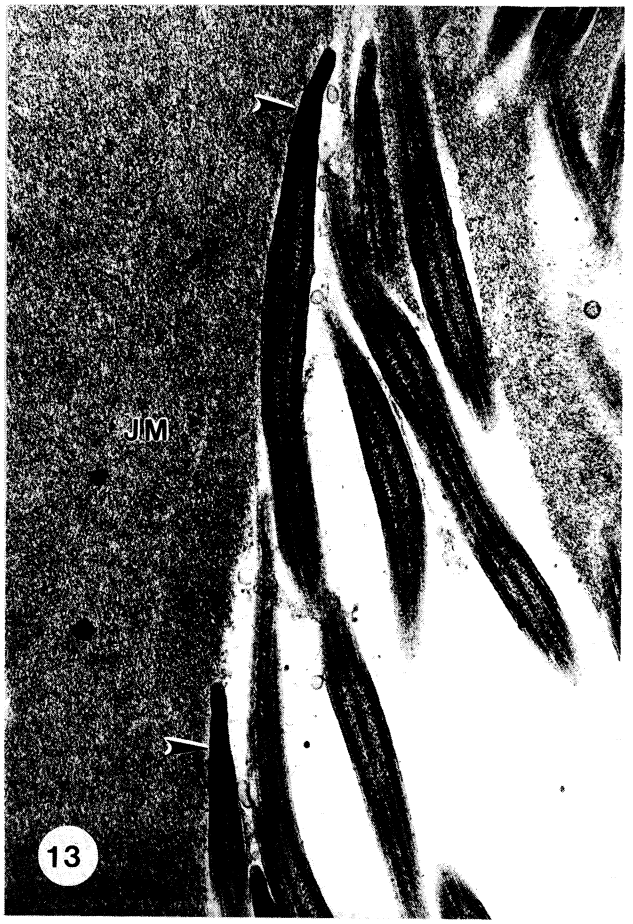
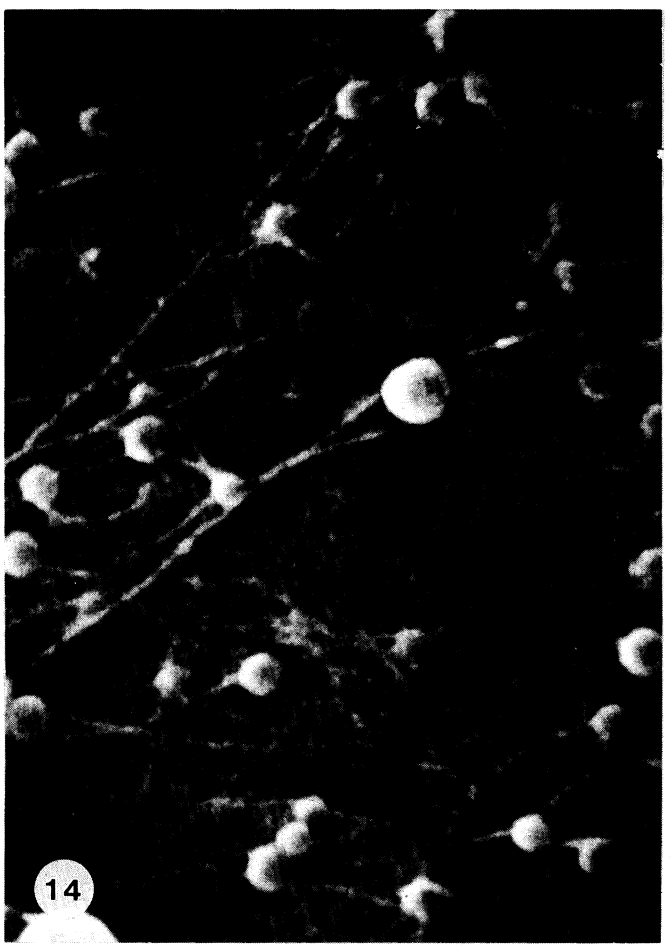




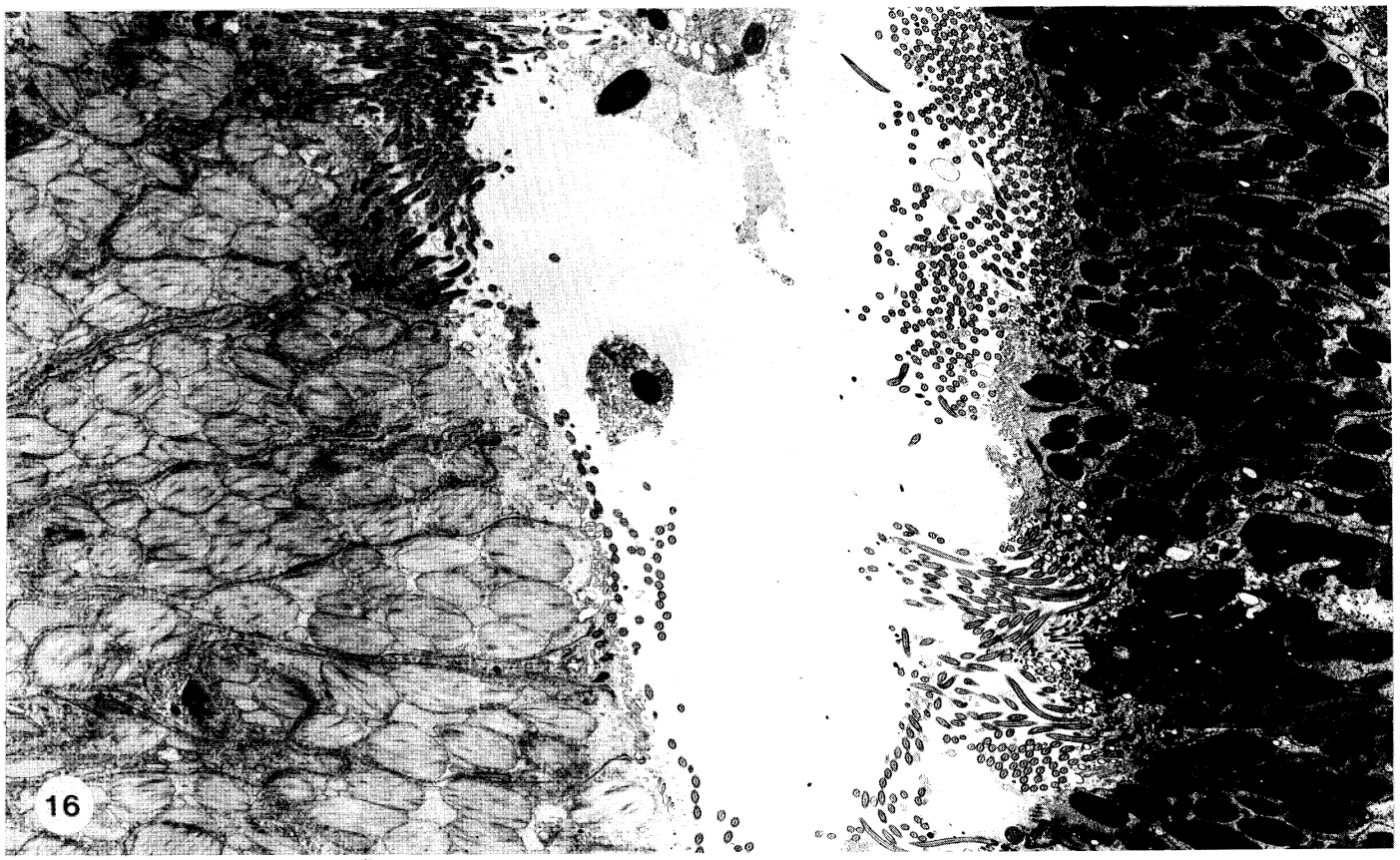


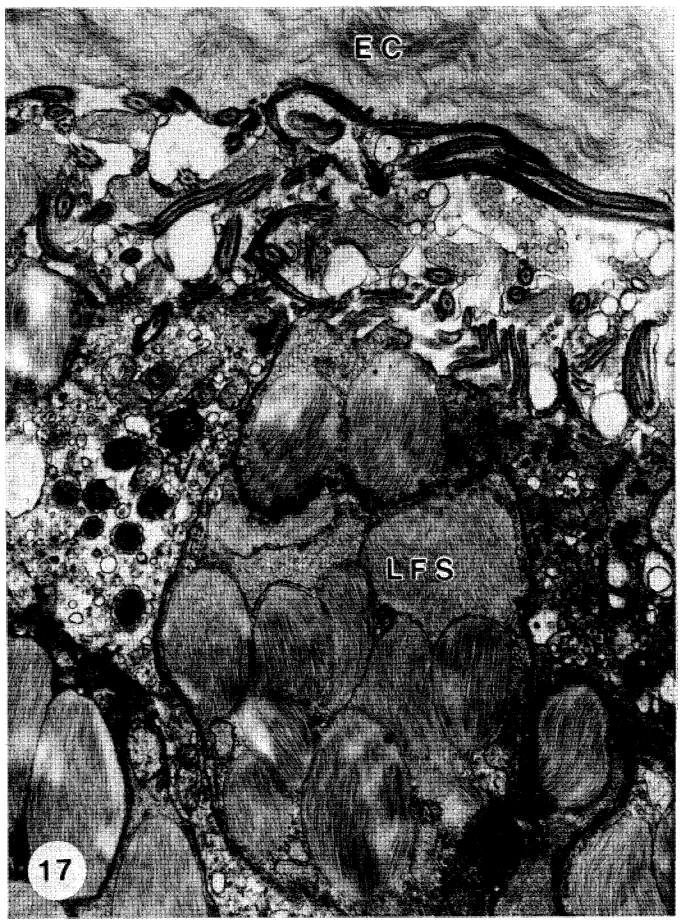
FIGURES 7–10. For description see p. 166.

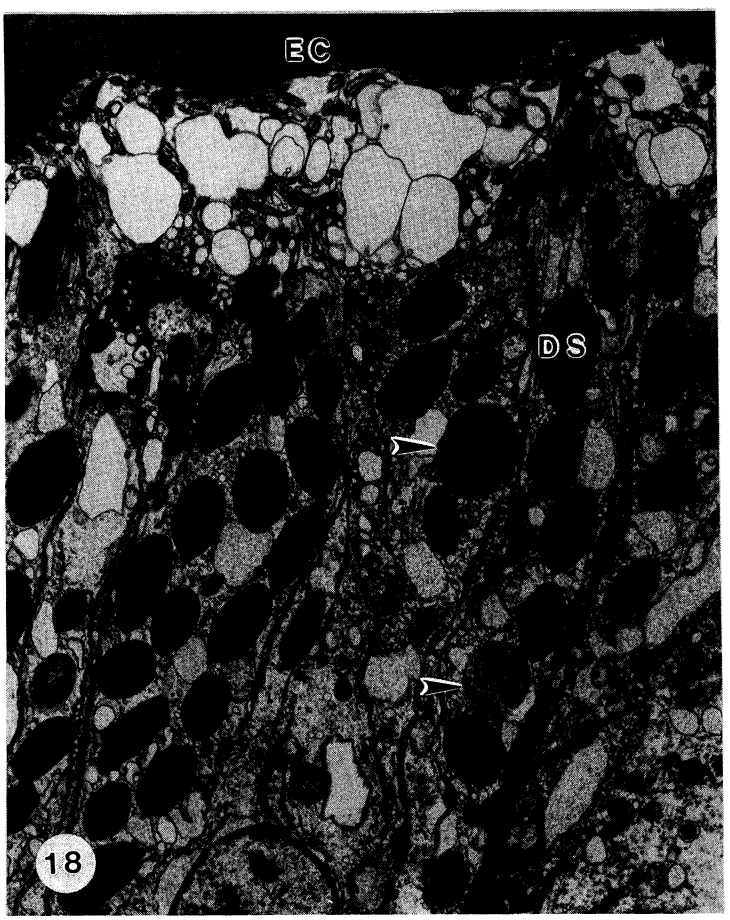




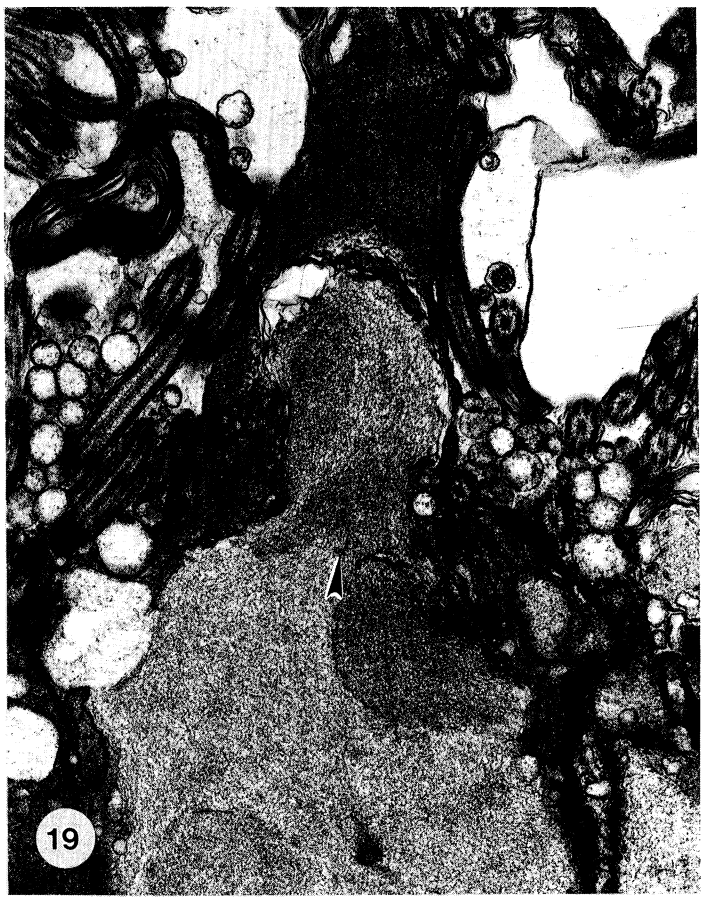
FIGURES 12–15. For description see p. 166.

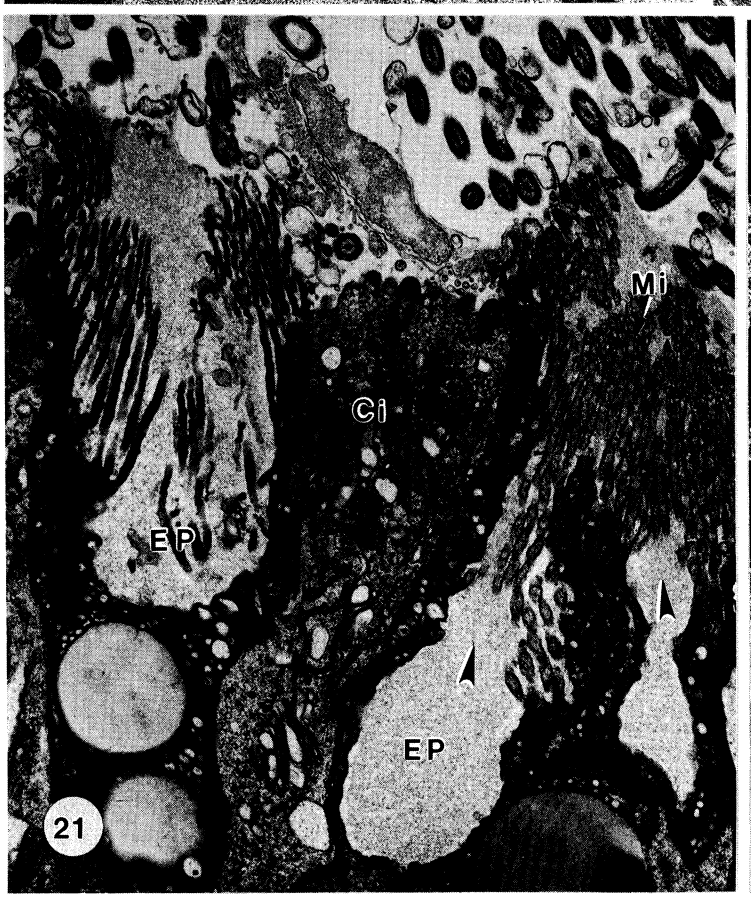


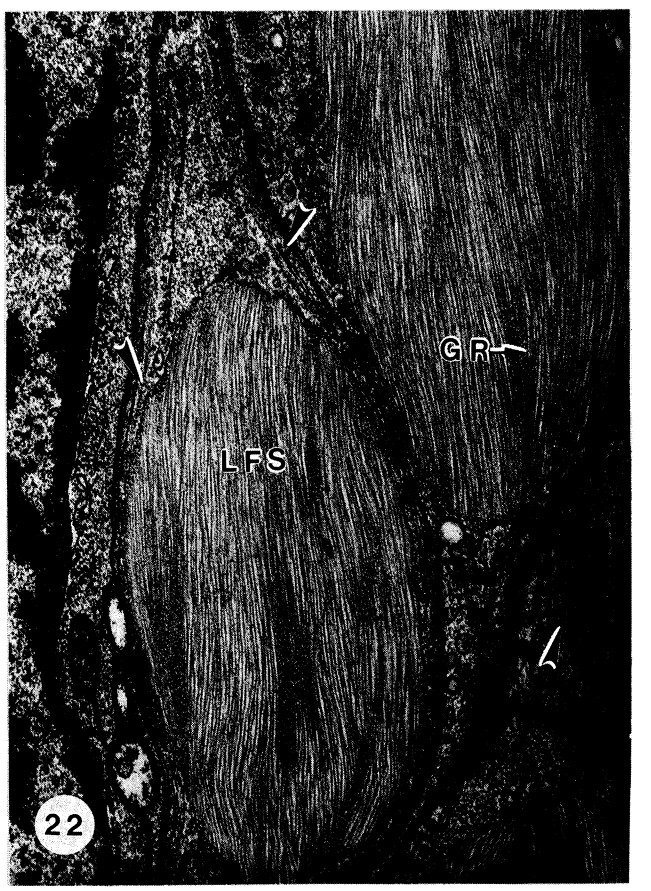




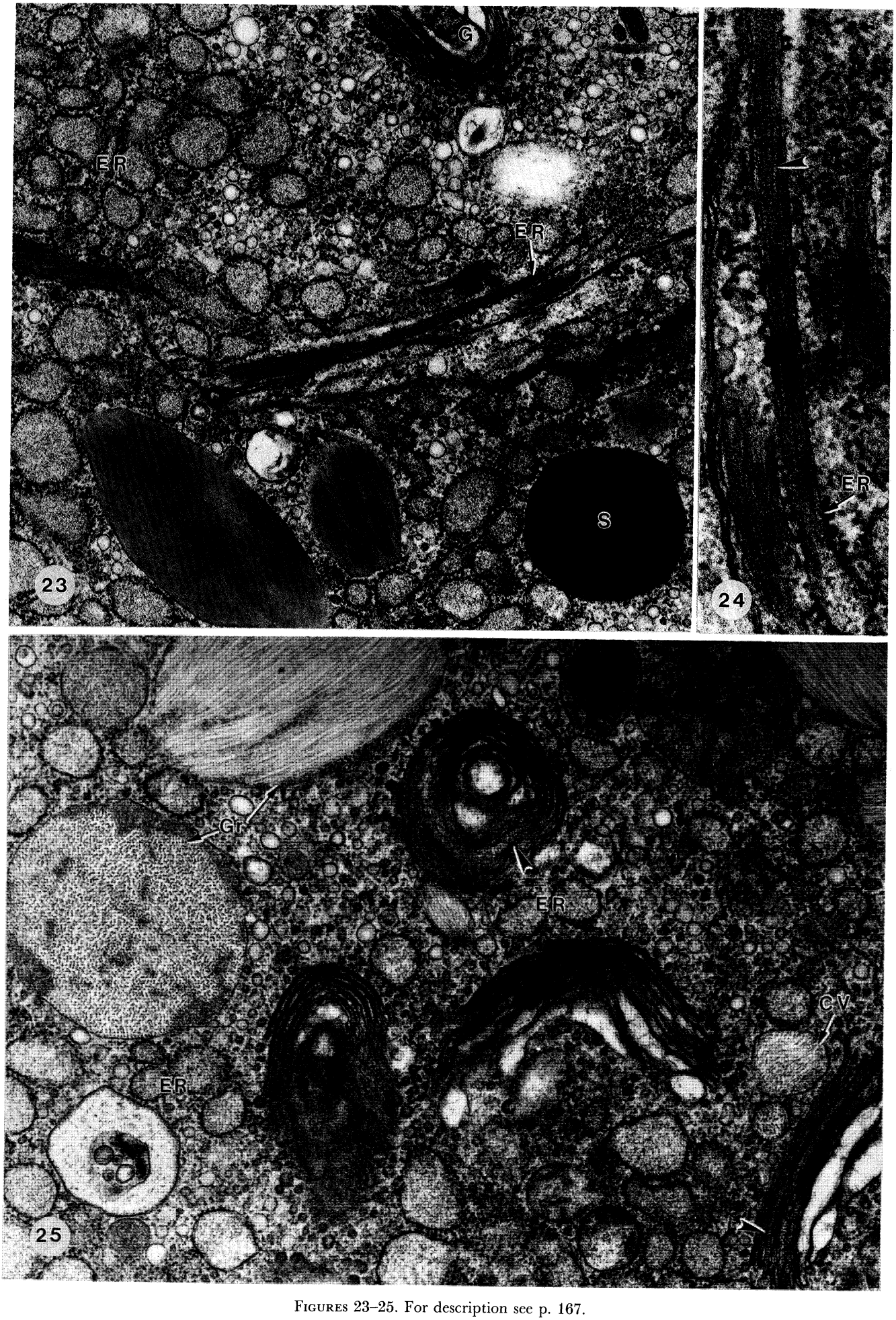
FIGURES 16–18. For description see p. 166.



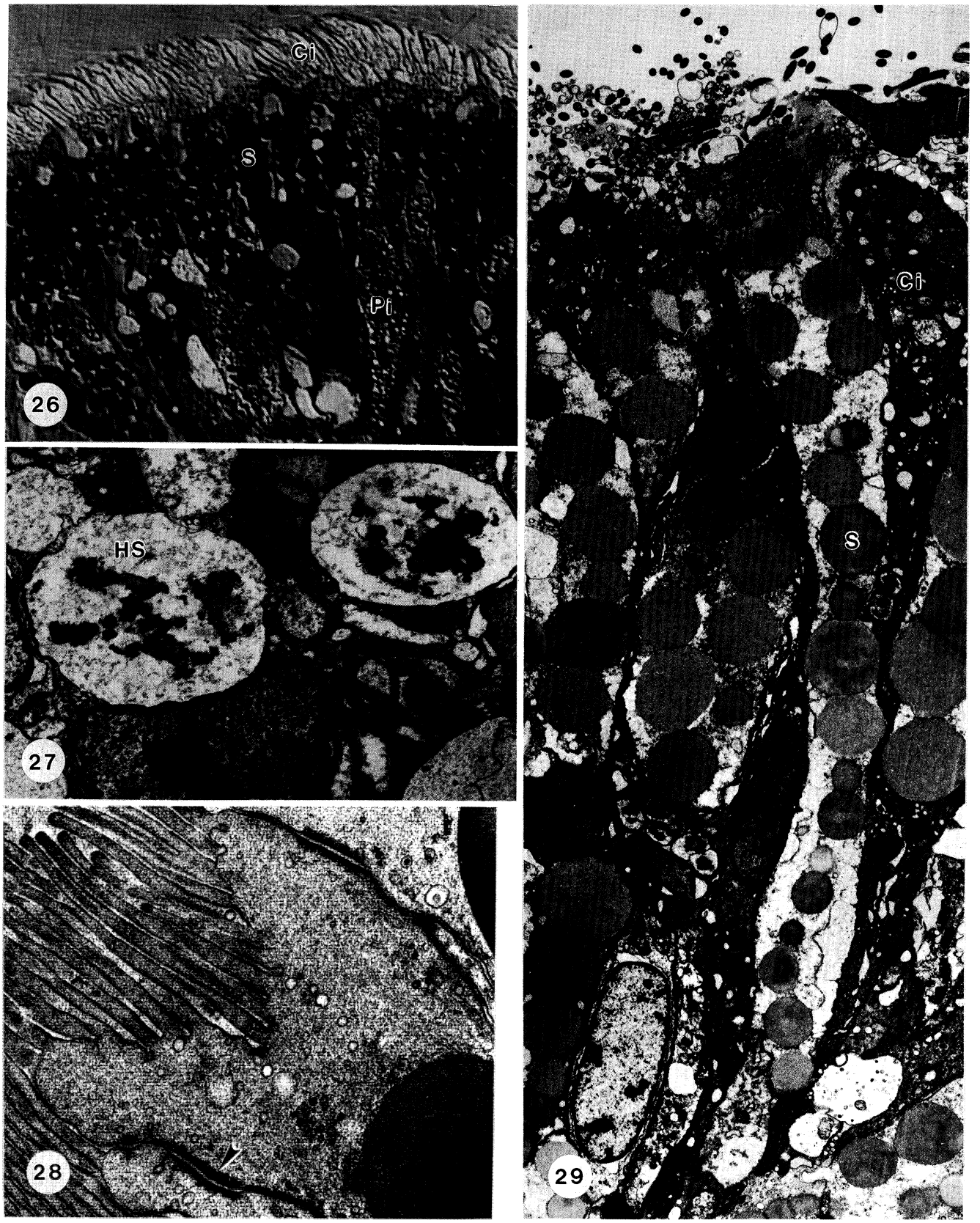




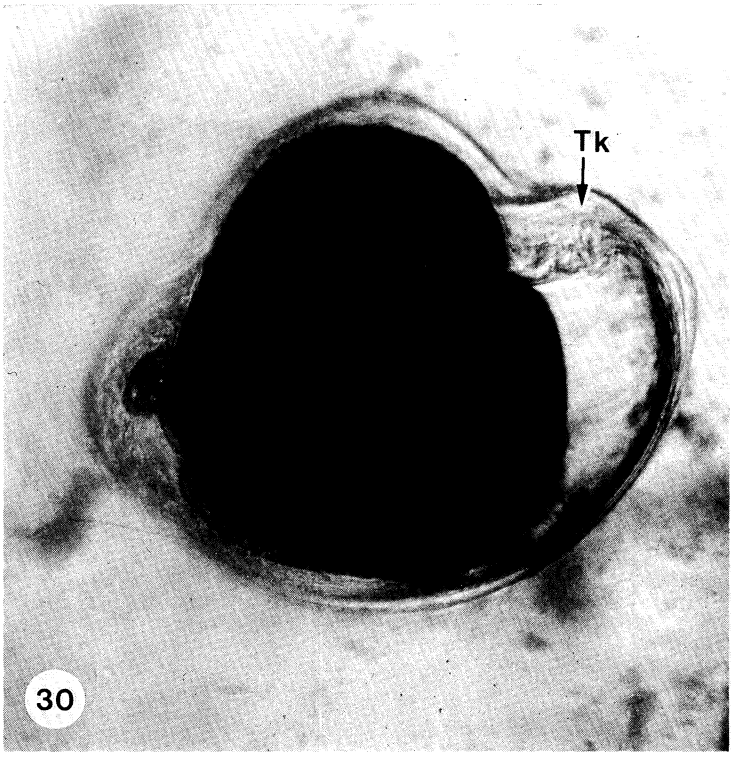
FIGURES 19–22. For description see p. 167.

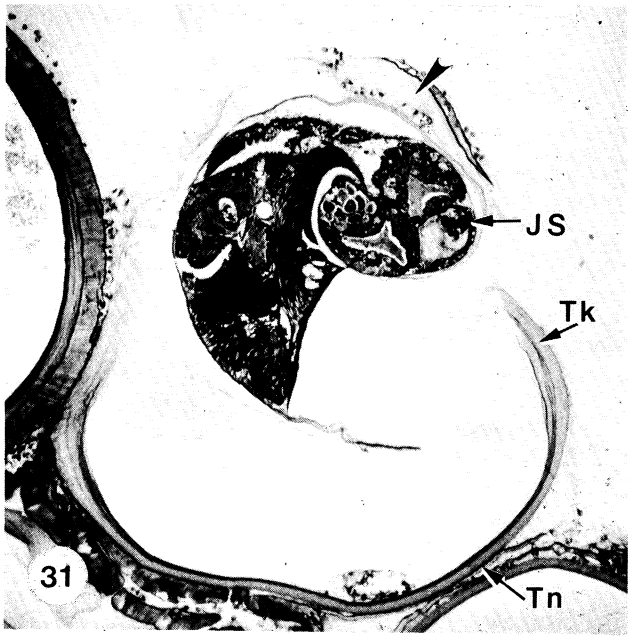


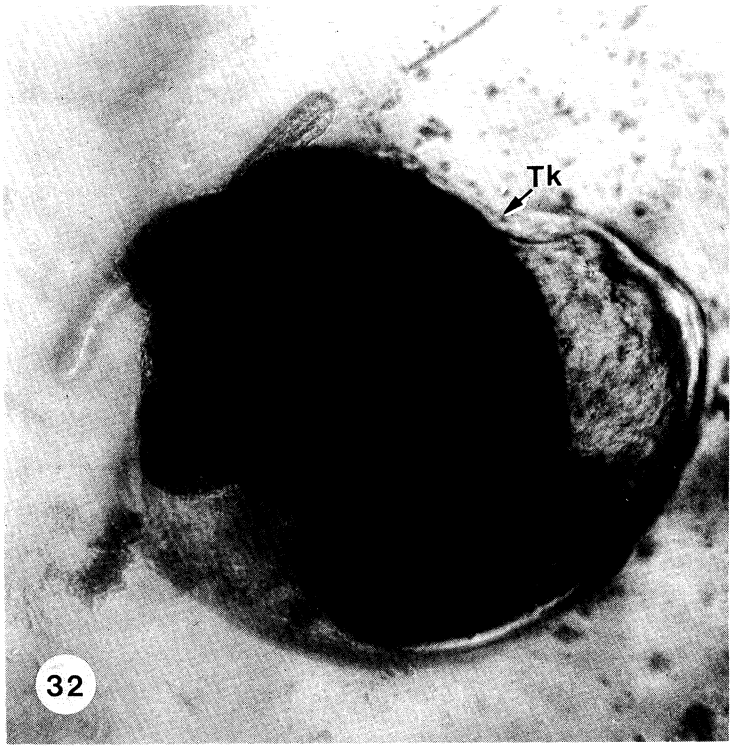
FIGURES 23–25. For description see p. 167.

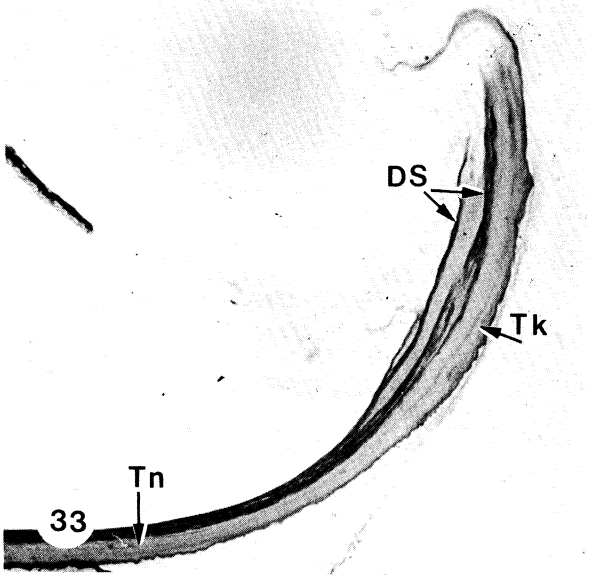


FIGURES 26–29. For description see p. 167.









FIGURES 30–33. For description see opposite.

Description of plate 5

Electron micrographs of secretions and secretion release in the pallial oviduct of *L. sitkana*.

Figure 19. Section through apex of covering gland cell, showing exocytosis of fibrogranular secretions (arrowhead). Magn × 23000.

Figure 20. Section through apex of a homofilamentous granule cell in the capsule gland, showing exocytosis of loose filamentous secretions (LFS), composed of 75 Å filaments and dense tips (DT) of specialized cilia that brush them into position. Notice how secretions apparently adhere to cilia (arrowhead). Magn × 22000.

Figure 21. Section through apex of jelly gland cell, showing release of fine granular secretions (arrowheads) through the end-pieces(EP). Note also numerous microvilli (Mi) and ciliated epithelial supporting cells (Ci) separating gland cells. Magn × 14000.

Figure 22. Section of homofilamentous granule cell in capsule gland showing microtubular tracts (arrowheads) adjacent to loose filamentous secretion granules (LFS). Note also fine granular secretions (Gr). Magn × 31000.

Description of plate 6

Electron micrographs of secretion formation in capsule gland of *L. sitkana*.

Figure 23. Section through base of heterofilamentous granule cell in capsule gland, showing formation of dense filamentous secretions. Note long cisterna of rough ER containing 40 Å filaments (ER). Mature secretion granule (S), Golgi body (G) and swollen vesicles of rough ER (ER) are visible. These cells are also capable of forming loose filamentous secretions, comprising 75 Å filaments. Magn × 33000.

Figure 24. Portion of figure 23 enlarged to show 40 Å filaments (arrowhead) inside rough ER (ER). Magn × 100000.

Figure 25. Section through base of homofilamentous granule cell in capsule gland, showing formation of secretion granules containing 75 Å filaments. Swollen vesicles of rought ER (ER) contribute to *cis* faces of Golgi bodies. Inside Golgi cisternae one can see filamentous secretions under production (arrowheads). Condensing vacuoles (CV) containing 75 Å filaments are released from *trans* faces of Golgi bodies. Considerable growth in vacuoles occurs after their release, before reaching maturity. Note also granular secretions (Gr) in mature granules. Magn × 40000.

Description of plate 7

Light and electron micrographs of jelly gland of *L. sitkana*.

Figure 26. Nomarski light micrograph of jelly gland cells. Note also: ciliary border (Ci); secretion granules (S); and pigment cells (Pi) that are found throughout the pallial oviduct. Magn × 1600.

Figure 27. Section through base of jelly gland cell showing formation of heterogeneous secretion granules (HS). Magn × 12500.

Figure 28. Section through apex of jelly gland cell showing characteristic depression of end-piece with dense microvilli. Note junctions between cells composed of desmosomes and septate junctions (arrowhead). Magn × 40000.

Figure 29. Section through jelly gland cells separated by ciliated epithelial supporting cells (Ci). Some jelly gland cells are extremely narrow and long (up to 1 mm) and secretions (S) pass along in single file to the apex where they are released by exocytosis. Magn × 7000.

Description of plate 8

Light micrographs of hatching in *L. sitkana*.

Figure 30. Juvenile snail about to hatch from thick side (Tk) of egg capsule. Magn × 80.

Figure 31. Light micrograph of section of juvenile snail (JS) killed at moment of hatching from thick side (Tk) of egg capsule. Thin side (Tn) remains strong and acts as a platform for thrust. Note part of thick side of egg capsule above shell (arrowhead). Magn × 70.

Figure 32. Juvenile snail emerging from thick side (Tk) of egg capsule. Magn × 80.

Figure 33. Portion of Figure 31 enlarged to show dense secretions (DS) extending from thin side (Tn) into part of thick side (Tk) of egg capsule. Unsupported thick side has fractured. 40 Å filaments of thin side do not deteriorate as quickly as 75 Å filaments of thick side. Magn × 170.

75 Å filaments. Thus in the thin half of the egg capsule, the dense filamentous secretions are enclosed by loose filamentous secretions (figure 33, plate 8).

The fully formed egg capsules pass through a wide duct into the jelly gland, where they are embedded *en masse* in a viscous jelly layer (figure 2*c*). The cilia in this gland have shorter tapered tips (figure 13, plate 3), like those of typical cilia. The egg mass accumulates in the vestibule and slowly passes out of the gonopore on the right side of the head along a small groove that leads to the foot (figure 2*a*). This is a glandular region, usually called the 'ovipositor' that coats the egg mass with a sticky secretion, which helps glue it to a suitable substratum, such as a rock or a piece of driftwood. The foot may aid in placing the egg mass in position on the substratum but does not have any influence on the shape of the egg capsules which are fully formed in the capsule gland.

The exact path that zygotes take through the pallial oviduct is illustrated in Figures 2*b* and 3*a*, together with a series of longitudinal sections (figure 2*c–g*) that show which glands are located at specific levels in the genital system. The levels at which these longitudinal sections were taken are indicated by arrows in figure 2*a*, *b*. A diagrammatic summary of the various compartments of the pallial oviduct, their contributions to the egg mass and the structure of the egg capsule itself are illustrated in figure 3*a*, *b*.

(*b*) *Fine structure of the albumen gland*

The albumen layer is made of nutrient-rich materials that the juvenile can ingest before hatching. The gland cells are interspersed with epithelial supporting cells and the latter have the specialized long densely tipped cilia. The bulk of the albumen layer is secreted from three types of unciliated gland cells; the dense granule cell, the microgranule cell and the macrogranule cell:

Dense granule cell

This gland cell type produces mainly a dense granular secretion that is packaged within the endoplasmic reticulum (ER). Groups of dense secretion granules aggregate within whorls of rough ER (figure 5, plate 1). The secretions are enclosed in membrane to form granules that move to the apex of the cell and pass out by exocytosis. Mature granules vary in size but on average measure 2.3 μm in length. On several occasions lipid droplets have been observed in these cells (figure 5, plate 1). General features of this cell type are summarized in figure 11*b*.

Microgranule cell

This gland cell type produces two kinds of granule. One kind of granule is heterogeneous with a light periphery and a dense core, whereas the other kind are homogeneous. The secretions are manufactured in the basal part of the cell within elaborate whorls of rough ER (figure 7, plate 2) and are then packaged into discrete membrane bound granules by the Golgi bodies. Mature granules average 0.7 μm in length. The secretions collect at the apex of the cell prior to release by exocytosis. The general features of this cell type are illustrated in figure 11*a*.

Macrogranule cell

The macrogranule cell is similar to the microgranule cell except that mature granules are fewer in number and are much larger (averaging 5 μm in length). Both rough ER and Golgi bodies are prominent in the cell during the formation and release of granules (figure 9, plate 2).

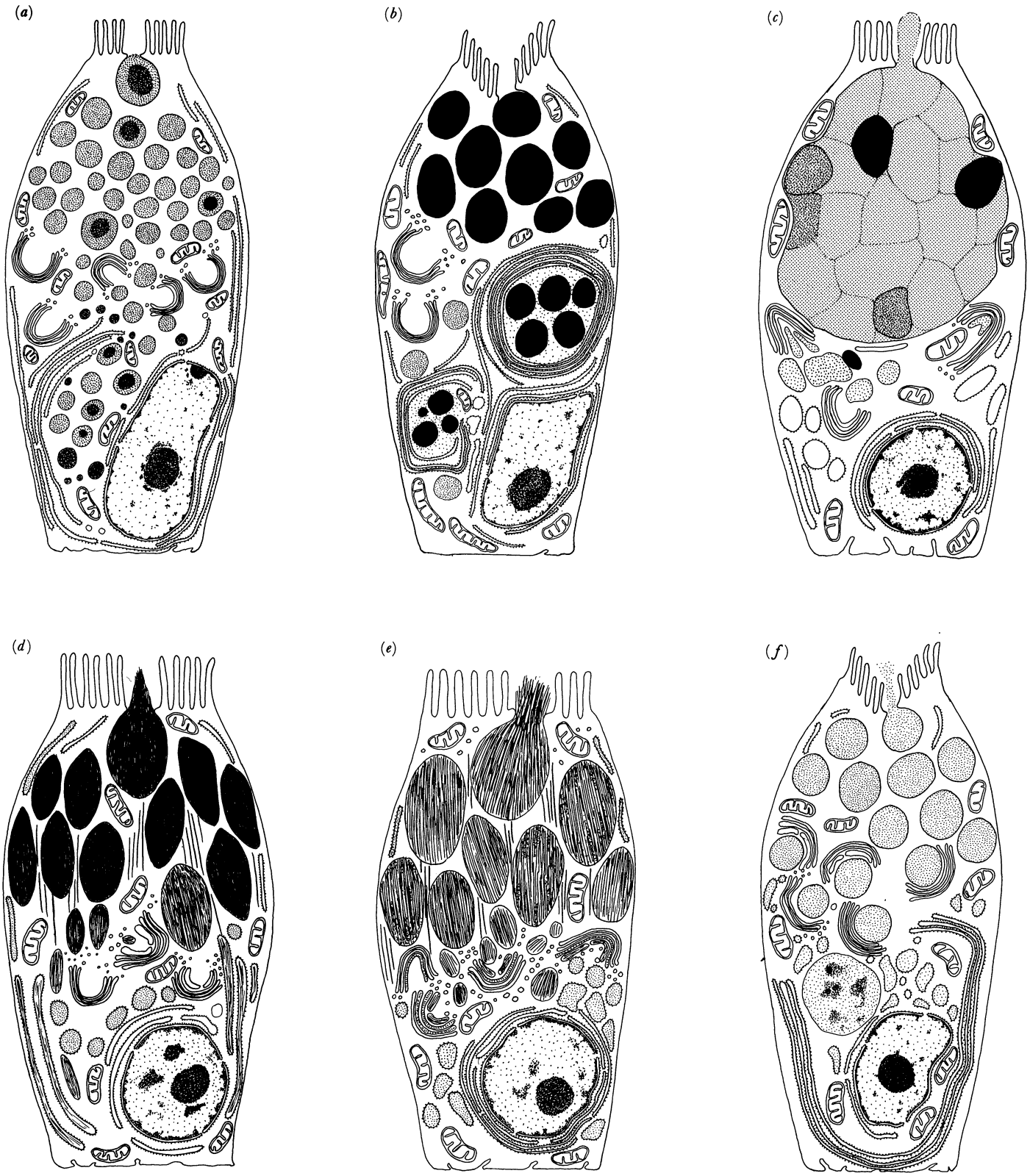


FIGURE 11. Schematic diagram summarizing fine structure of gland cells found in pallial oviduct. (*a*) Microgranule cell of albumen gland; (*b*) Dense granule cell of albumen gland; (*c*) Covering gland cell; (*d*) Heterofilamentous granule cell of capsule gland; (*e*) Homofilamentous granule cell of capsule gland; (*f*) Jelly gland cell. The shape of each cell varies greatly depending on factors such as; position in the gland, state of nutrition and seasonal cycle. To avoid bias, all cells have been represented with a similar shape.

(*c*) *Fine structure of the covering gland*

The covering gland is located between the albumen and capsule glands. The egg covering it produces is a thin stratified layer enclosing the albumen and zygote (figure 6, plate 1). One type of gland cell predominates and it secretes several kinds of granule, which range from small and very dense to large and pale. At the apex of the cell the less dense granules tend to coalesce, forming a large secretion mass (figure 8, plate 2). The denser granules retain their outer membranes but may become incorporated into the large secretion mass (figure 8, plate 2). When the outer membrane of the mass fuses with the plasma membrane, the entire contents are released at once. However, some of the dense granules do not discharge their contents before release and are visible in the egg covering as dense dots (figure 6, plate 1). The proportion of fibrous, pale and dense granules in the covering gland cells varies in a predictable way throughout the covering gland as described previously. Both rough ER and Golgi bodies appear to be involved in manufacturing the secretions (figure 10, plate 2). Once again gland cells roughly alternate with ciliated epithelial supporting cells and the specialized densely tipped cilia are used to move secretions into the lumen of the covering gland as they are exocytosed (figure 8, plate 2; figure 19, plate 5). It should be mentioned that variations in the thickness of the egg covering around an embryo have occasionally been observed in sections. The main features of the covering gland cell are illustrated in figure 11*c*.

(*d*) *Fine structure of the capsule gland*

The egg capsule is secreted by two types of gland cell that roughly alternate with ciliated epithelial supporting cells. In the first part of the gland the duct is surrounded on one side by heterofilamentous granule cells that secrete two types of filamentous granule (figures 16 and 18, plate 4), and on the other side by homofilamentous granule cells that secrete a single type of granule (figures 16 and 17, plate 4).

Heterofilamentous granule cell

The dense filamentous granules that characterize this type of gland cell are formed as 40 Å filaments within saccules of the rough ER and later released as ribosome-free condensing vacuoles (figures 23 and 24, plate 6). The vacuoles continue growing after their release and reach an average size of 1.6 μm as mature secretion granules (figure 23, plate 6). The 40 Å dense filaments are not observed in the Golgi cisternae. However, secretion granules containing loose 75 Å filaments are also produced in certain parts of the gland. These granules are predominantly formed in the Golgi cisternae, not the rough ER. The main features of the heterofilamentous granule cell are illustrated in figure 11*d*.

Homofilamentous granule cell

In the bases of homofilamentous granule cells swollen endoplasmic reticulum is seen budding off vesicles towards the forming face of the Golgi bodies (figure 25, plate 6). 75 Å filaments are formed within the Golgi cisternae and are released initially in small vesicles from the *trans* face (figure 25, plate 6). These small vesicles fuse into one or more condensing vacuoles, within which one can see the individual filaments (figure 25, plate 6). The vacuoles grow and develop into mature granules and are transported to the apex of the cell prior to release by exocytosis. In this and other gland cells, tracts of microtubules are often visible running

between the base and apex of the cell (figure 22, plate 5). Mature granules reach an average length of 2.3 μm, when they contain numerous 75 Å filaments aligned roughly parallel to each other (figure 22, plate 5; figure 25, plate 6). These filaments appear to have a helical structure. The main features of the homofilamentous granule cells are illustrated in figure 11*e*.

(*e*) *Fine structure of ciliated epithelial supporting cells*

The ciliated epithelial supporting cell has a typical triangular appearance with a narrow base and stem and a broad apex (figure 8, plate 2). The large oval-shaped nucleus is located either medially or apically. There is an apical aggregation of mitochondria, which may interdigitate with ciliary rootlets (figure 8, plate 2). The apical surface of these epithelial cells in the albumen, covering, capsule and accessory glands, comprises a few microvilli and numerous long densely tipped cilia, which have become specialized for manipulating the secretions (figures 12 and 15, plate 3). The nine outer doublet and two central microtubules of the cilium, gradually reduce in number towards the small bulbous tip, which is filled with a dense material (figures 12 and 15, plate 3). In the jelly gland, however, the cilia of epithelial cells have a more typical appearance with a shorter tapered tip (figure 13, plate 3).

(*f*) *Fine structure of the jelly gland*

The internal architecture of the jelly gland is formed into a series of deep ridges that effectively increase the surface area for the release of its viscous secretion. The bulk of the secretion is produced by jelly gland cells, although a small contribution is made by mucous and pigment cells, which are also found elsewhere in the pallial oviduct.

The jelly gland cells are characterized by fine secretion granules that form initially within whorls of rough endoplasmic reticulum, and collect in the cytoplasm near the base of the cell. Sometimes these granules become heterogeneous with the addition of secretions of different densities (figure 27, plate 7). Jelly gland cells are often extremely elongate (up to 1 mm in length) and the secretion granules become arranged in single file all the way to the apex of the cell, where they collect in clusters before release. Exocytosis occurs through a small depression in the apex of the cell that is often referred to as the 'end-piece', and contains numerous microvilli (figure 21, plate 5; figure 28, plate 7). Secretion granules fuse with the plasma membrane in quick succession and are expelled through this depression in a stream that is whipped out onto the surface of the cell by long cilia of adjacent epithelial supporting cells (figures 26 and 29, plate 7). Lateral bands of microfilaments, that have been observed near junctions between adjacent cells, may be responsible for causing contractions of the cell, which expel the secretions as they are exocytosed. The ciliated epithelial supporting cells of the jelly gland are different from those of the other three glands, as the apical end of the cilium does not extend into a long dense process but has much more the typical appearance of a cilium (figure 13, plate 3). In other respects the cells appear to be similar. The main features of the jelly gland cell are illustrated in figure 11*f*.

(*g*) *The hatching process*

In *L. sitkana* the egg capsule is uniformly strong when first deposited but the thick side degenerates faster and after about two weeks exposure it is noticeably weaker. One can easily detect this by testing the resistance of each side to puncturing with a needle. By the time the juveniles are ready to hatch the thick side is quite fragile and is occasionally invaded by protozoans. The thin side of the egg capsule remains resilient, although not as strong as when

first released. Hatching requires the exertion of physical force by the juvenile both by the repeated expansion of the foot and shell against the egg capsule (figure 30, plate 8), and by the rapid contraction of the columellar muscle which causes the shell tubercles to grate against the inside surface of the egg capsule (Buckland-Nicks *et al.* 1973). These movements gradually stretch the egg covering that encloses the juvenile until it tears. An action that was not observed previously, but became evident after detailed examination of ciné films and video tapes of hatching, is that the radula is also used to chew through the egg covering. The pre-hatching juvenile crawls around the inside surface of the capsule, periodically stopping and rapidly expanding the foot and shell against the capsule wall. This creates pressure on the capsule wall which eventually fractures on the thick side (figures 31 and 33, plate 8) and the juvenile uses the thin side as a platform for thrust and escape (figures 31 and 32, plate 8). In addition to these physical mechanisms it appears that if hatching is prolonged, which sometimes happens, the capsule becomes extremely soft and pliable (figure 30, plate 8). When the juvenile emerges the capsule wall appears to dissolve at the point of emergence but not elsewhere.

5. Discussion

(*a*) *Site of fertilization*

The points of entry of sperm and oocytes into the albumen gland vary with different species. According to Linke (1933), the oviduct joins the albumen gland in the vicinity of the receptaculum seminis aperture in *L. obtusata*. In *L. sitkana* the situation is similar to that described by Linke (1933) but the seminal receptacle duct opens into a slight expansion of the oviduct just as it enters the albumen gland. It is in this area of the oviduct that the eggs are fertilized and we refer to it as the 'fertilization chamber'. Oocytes have also been found surrounded by sperm in the albumen gland and it is likely that fertilization of some oocytes occurs there. In fact this has generally been assumed to be the site of fertilization in prosobranchs in the past (Fretter 1941, 1984; Fretter & Graham 1962).

In *L. sitkana* the ventral channel of the oviduct delivers sperm from the bursa copulatrix to the distal side (anterior) of the bifurcation of the seminal receptacle duct, which leads into the receptaculum seminis. During egg laying sperm are forced out by muscular contractions of the wall of the receptaculum seminis and pass through the proximal side of the seminal receptacle duct through the accessory gland mass and into the albumen gland. Previously it was thought that there was a single channel into the receptaculum seminis (Linke 1933; Johansson 1957) and these authors disagreed on the direction of ciliary beating in this channel. Johansson (1957) stated that the sperm must swim against a ciliary excurrent; whereas Linke (1933) suggested that the cilia beat towards the receptaculum seminis, aiding the movements of sperm. Our observations suggest that the ciliary beat tends to aid the locomotion of sperm in each channel, that is, towards the receptaculum seminis in the distal side and away from it in the proximal side. However, the muscular contraction of the receptaculum seminis probably plays the key role in delivering sperm to the oviduct, rather than the action of cilia.

(*b*) *Egg capsule design and hatching*

In *L. sitkana* the shape of the egg capsule is fully formed in the capsule gland by opposing lips of the gland, which are under muscular control. Several egg capsules are formed simultaneously in different parts of the gland. Although some hardening probably occurs after

they leave the capsule gland, the final shape is completed there. Preliminary evidence suggests that this also occurs in *L. scutulata*, which has a more complex capsule shape, but at least part of the moulding process occurs in the vestibule in this species (J. Buckland-Nicks & F.-S. Chia, unpublished observations). These results differ from Linke's (1933) assertion that the foot is involved with moulding the egg capsule in *L. littorea*.

Kawaguti & Yamasu (1961) demonstrated that egg envelopes are secreted in layers in saccoglossans. This was supported in studies of opisthobranchs (see Thompson 1961; Ghiselin 1965) and prosobranchs (see Sullivan & Maugel 1984); although others have suggested that eggs were forced into a pre-existing mass of mucus material by ciliary action (Fretter 1941; Fretter & Graham 1954). Our observations agree with Kawaguti & Yamasu (1961) that the egg envelopes are added in successive layers around the zygotes.

The formation of more than one secretion by one gland-cell type has been reported previously in prosobranchs by Fretter (1941) and Fretter & Graham (1962), but how these secretions are formed or the unique way in which they produce a heterogeneous egg capsule in *L. sitkana* was not previously understood. In the capsule gland of *L. sitkana* this has evolved to give rise to two completely different halves of the egg capsule, one of which degenerates more rapidly than the other. This is the first report of this type of heterogeneity in an egg capsule. Preliminary evidence in this laboratory suggests that there may be considerable variation within the Littorinidae. Heterogeneity is found in egg capsules of advanced mesogastropods, such as *Ilyanassa obsoleta* (Sullivan & Maugel 1984) and neogastropods such as *Nucella lapillus* (Fretter 1941), which make capsules that house many embryos. In these species the egg capsule is fully formed in the pallial oviduct, but is not shaped there. Final hardening and moulding of the capsule wall is done by the ventral pedal gland (a structure that is absent in *L. sitkana*), but the lid remains unpolymerized (Sullivan & Maugel 1984). At an appropriate moment in development the veligers release a hatching enzyme, which dissolves (Pechenik 1975), or loosens the lid. This provides a perfect escape route for the encapsulated veligers.

Hatching enzymes occur widely among gastropods and are usually released by the embryo at a specific stage in the hatching process (Ankel 1937; Bondesen 1950; Hancock 1956; Davis 1967; Pechenik 1975). However, mechanical means of rupturing the egg covering and capsule can also be effective hatching mechanisms in prosobranchs (see Hertling 1928; Davis 1967) and other gastropods (see Noland & Carriker 1946; Vaughn 1953; Bondesen 1950). Our more recent observations of ciné film and video tapes of the hatching process in *L. sitkana*, together with those of our previous study (Buckland-Nicks *et al.* 1973) suggest that although the mechanical means of breaking through the egg covering and capsule are usually effective, a hatching enzyme may be released in later stages to ensure emergence of the juvenile.

(*c*) *The covering gland*

The covering gland (also called the 'shell gland' (Linke 1933) and sometimes the 'membrane gland' (Fretter 1980)) has been very much misunderstood, although accurately described by Fretter & Graham (1962) and Fretter (1980) for *L. littorea*, as secreting a thin shell around the albumen, which is subsequently enclosed by an egg capsule secreted in the capsule gland. This description agrees closely with that of Linke (1933) for *L. obtusata* although in this species, as in *L. sitkana*, there is an additional layer, the jelly layer, secreted by the jelly gland. Our description of *L. sitkana* is in close agreement with these authors and we have adopted the terminology of Fretter & Graham (1962). However, since then the literature has become very

confusing, particularly for the novice biologist. For example, in an article on the egg masses of *L. obtusata* and *Lacuna pallidula*, Goodwin (1979) refers to the albumen as the 'egg jelly layer' and he 'presumes that the egg covering is a hardening or concentration of this layer'. In other studies of *Littorina* the egg covering is assumed to be produced by the albumen gland (Hannaford-Ellis 1979).

Fretter (1980) summarizes the situation as follows: 'the precise function of this membrane gland [= covering gland] is unknown. It is presumed that it is concerned with the production of the external bounding layer of the albumen'. Indeed this is true, as we have shown. However, in a more recent review Fretter (1984) refers only to albumen, membrane and jelly glands in diagrams (figure 1*b*) and text (p. 7) in four species of *Littorina* (*L. arcana*, *L. obtusata*, *L. nigrolineata* and *L. mariae*), all of which have the same egg mass type as *L. sitkana*. Where then is the capsule gland? Hannaford-Ellis (1979), with whom we agree in this instance, labels the capsule gland of *L. arcana* in the position that Fretter (1984) located the membrane gland of this species. Furthermore, Hannaford-Ellis labels a 'translucent section of the albumen gland', which may be the covering gland in *L. arcana*. It should also be noted that in Webber's (1977) review article, the diagram of the female genital system of *Littorina* has been accidentally mislabelled as figure 3*d*; it should be figure 3*c*.

Clearly, the egg covering is a unique envelope that is produced by a separate area of glandular tissue, here and elsewhere termed the covering gland (Fretter & Graham 1962). This layer may be equivalent to the 'egg membrane' of opisthobranchs (see Ghiselin & Wilson 1966; Schmekel 1971) as suggested by Ghiselin (1965). However, in *L. sitkana*, and perhaps in other gastropods too, this layer is not really a membrane as it has been termed previously (Buckland-Nicks *et al.* 1973; Fretter 1980; 1984), but a complex stratified egg envelope comprising four distinct layers. The gland cells which secrete the egg covering differ from all others in the pallial oviduct by having the ability to produce up to four types of secretion granule simultaneously. The morphology of such a cell type has not been reported in any previous study of gastropod development.

(*d*) *Ciliary specialization*

Cilia have become specialized to perform a wide variety of functions in various organs of the body (for review see Satir 1961). In certain glands of the pallial oviduct of *L. sitkana* the cilia coordinate like a paint brush to manipulate the secretions into place. The elongated dense tips of the cilia enable this specific adaptation. However, in the jelly gland, where the secretions are much more viscous, the cilia are shorter and more robust without the thin dense tips. Specialization of cilia has not been reported before in this context and it will be interesting to see how widespread this phenomenon is among gastropods.

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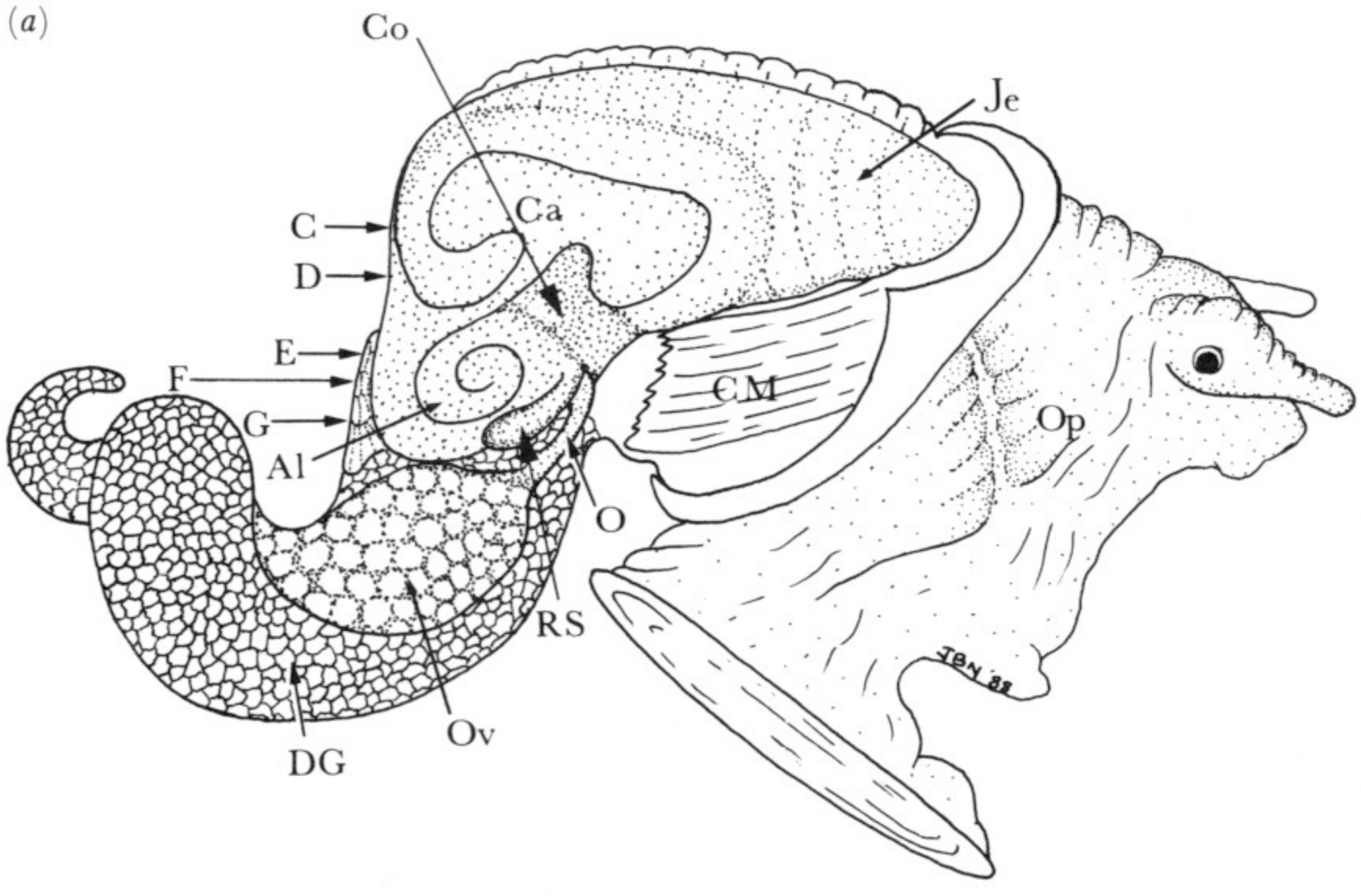
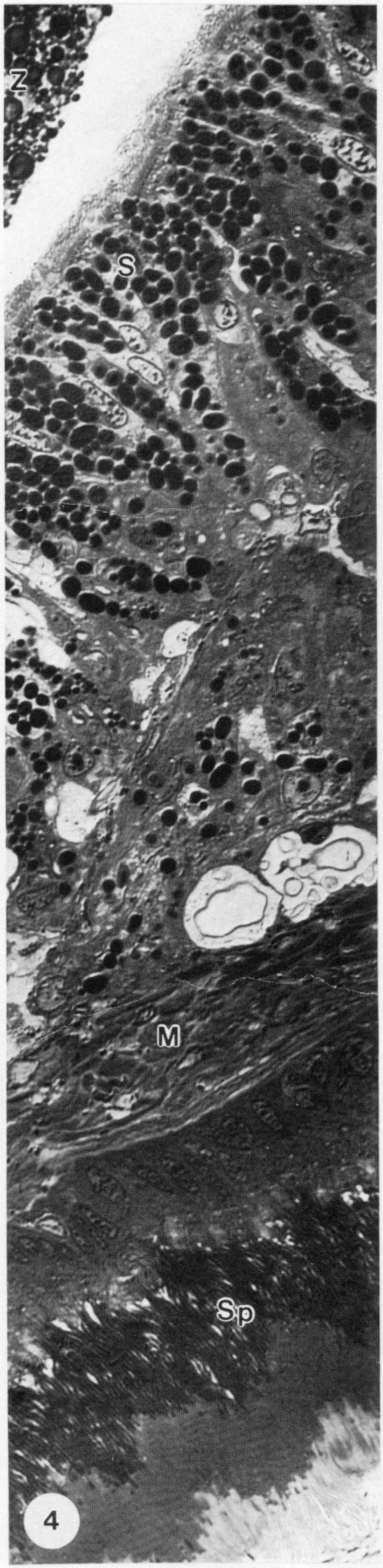
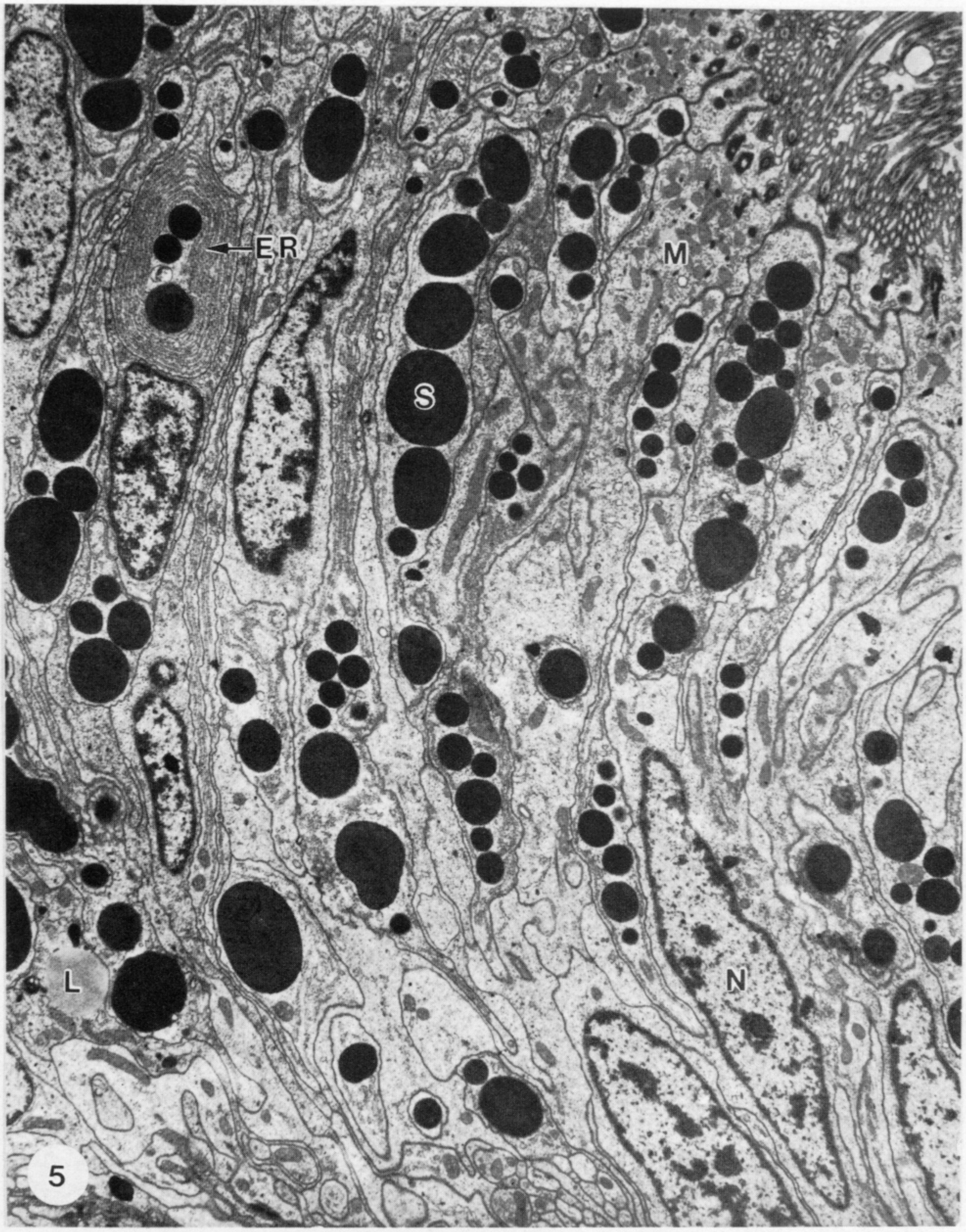


FIGURE 2. (*a*) Diagram of female *L. sitkana* removed from shell. (*b*) Photograph of a model of female genital ducts obtained by injecting vinyl acetate into gonopore of a female snail and dissolving away the tissues. (*c–g*) Longitudinal sections showing arrangement of glands in pallial oviduct, in relation to bursa copulatrix and receptaculum seminis. Sections were traced from photographs of stained wax sections and roughly correspond to levels indicated by arrows in Figure 2*a*, *b*. Ca: capsule gland; Je: jelly gland; CM: columellar muscle; Op: ovipositor; Al: albumen gland; RS: receptaculum seminis; O: oviduct; Ov: ovary; DG: digestive gland; Co: covering gland; Ca1: side of capsule gland containing homofilamentous granule cells; Ca2: side of capsule gland containing heterofilamentous granule cells; AG: accessory gland; Bu: bursa copulatrix; VC: ventral channel of oviduct.





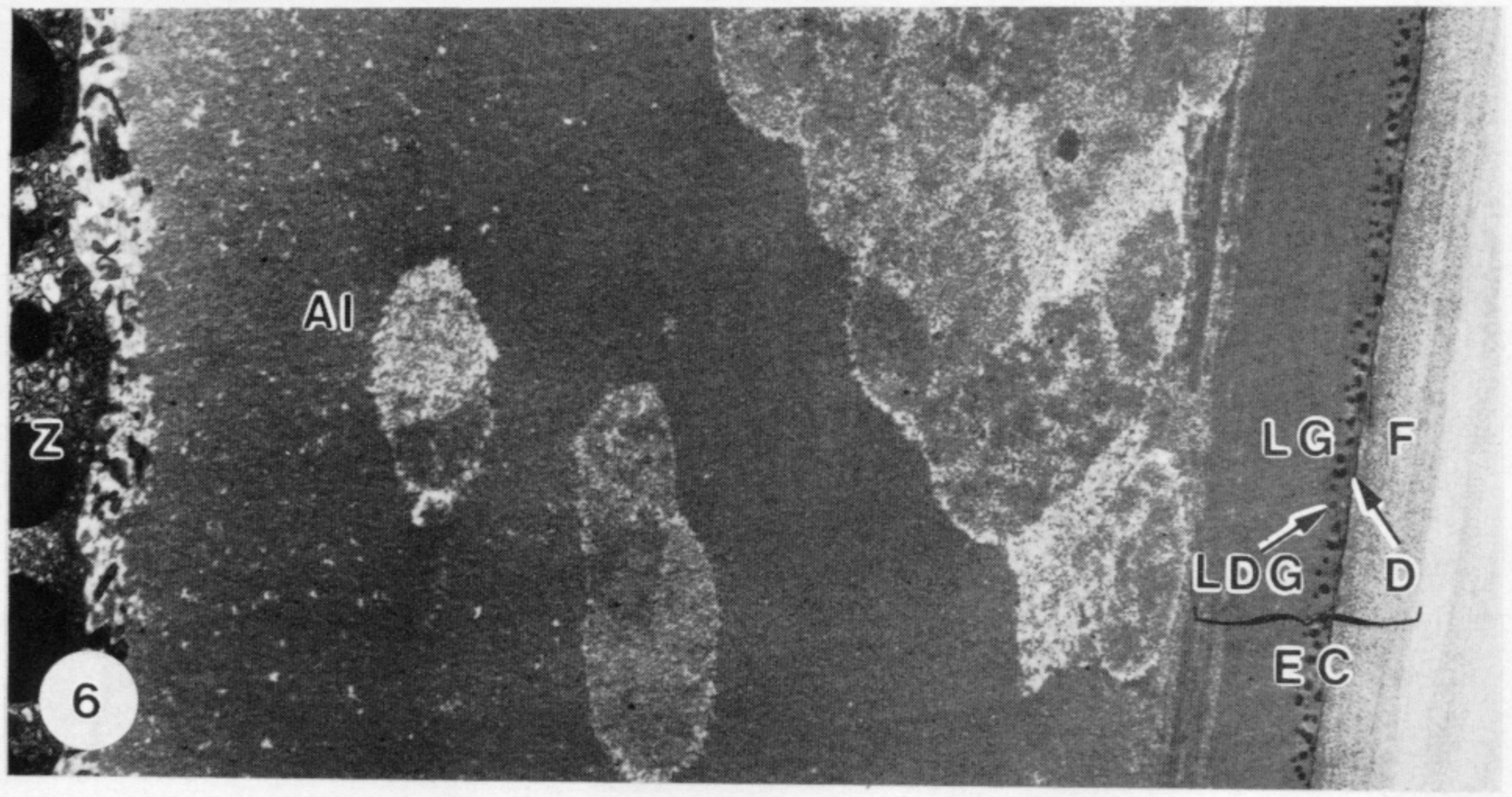
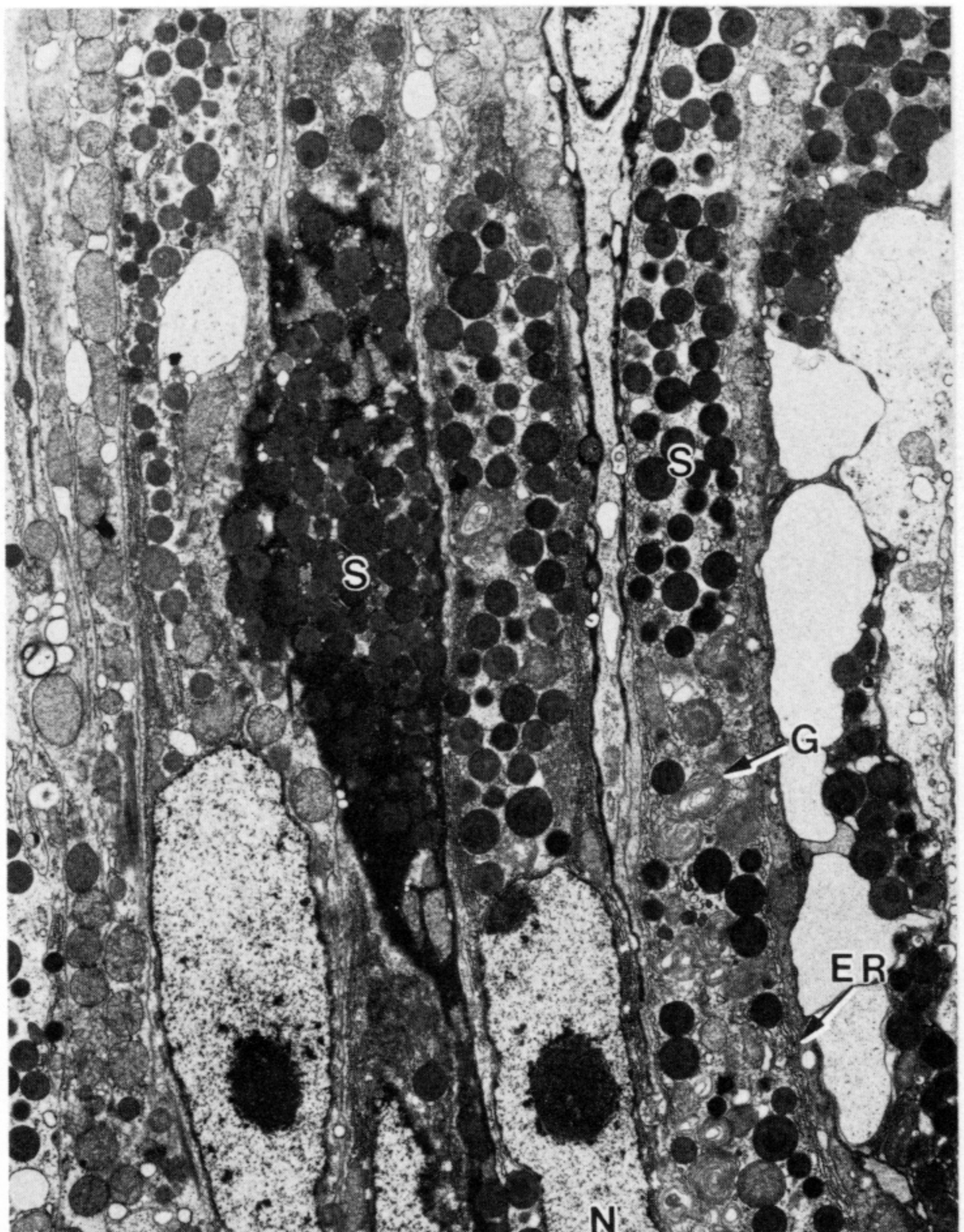


FIGURE 4–6. For description see opposite.



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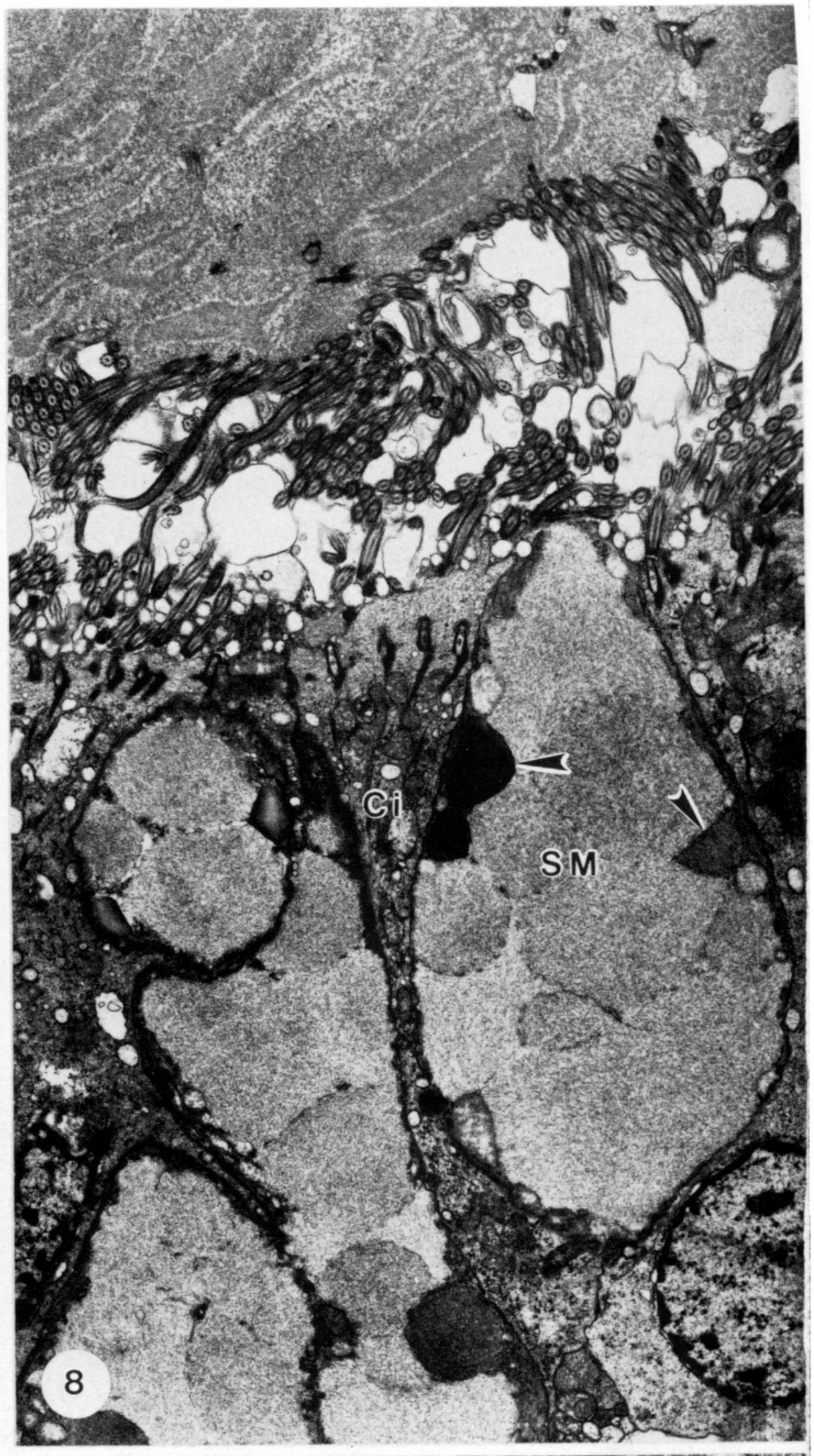

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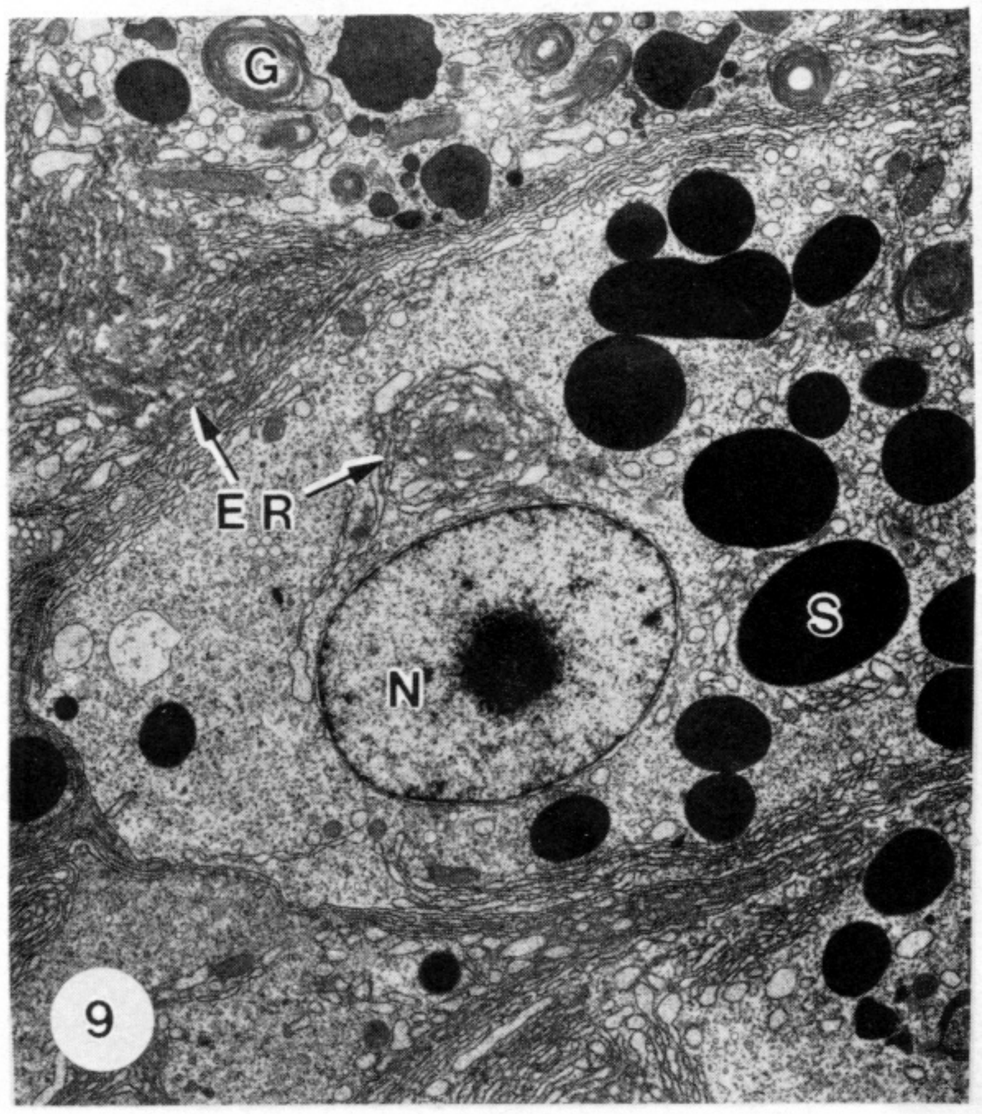

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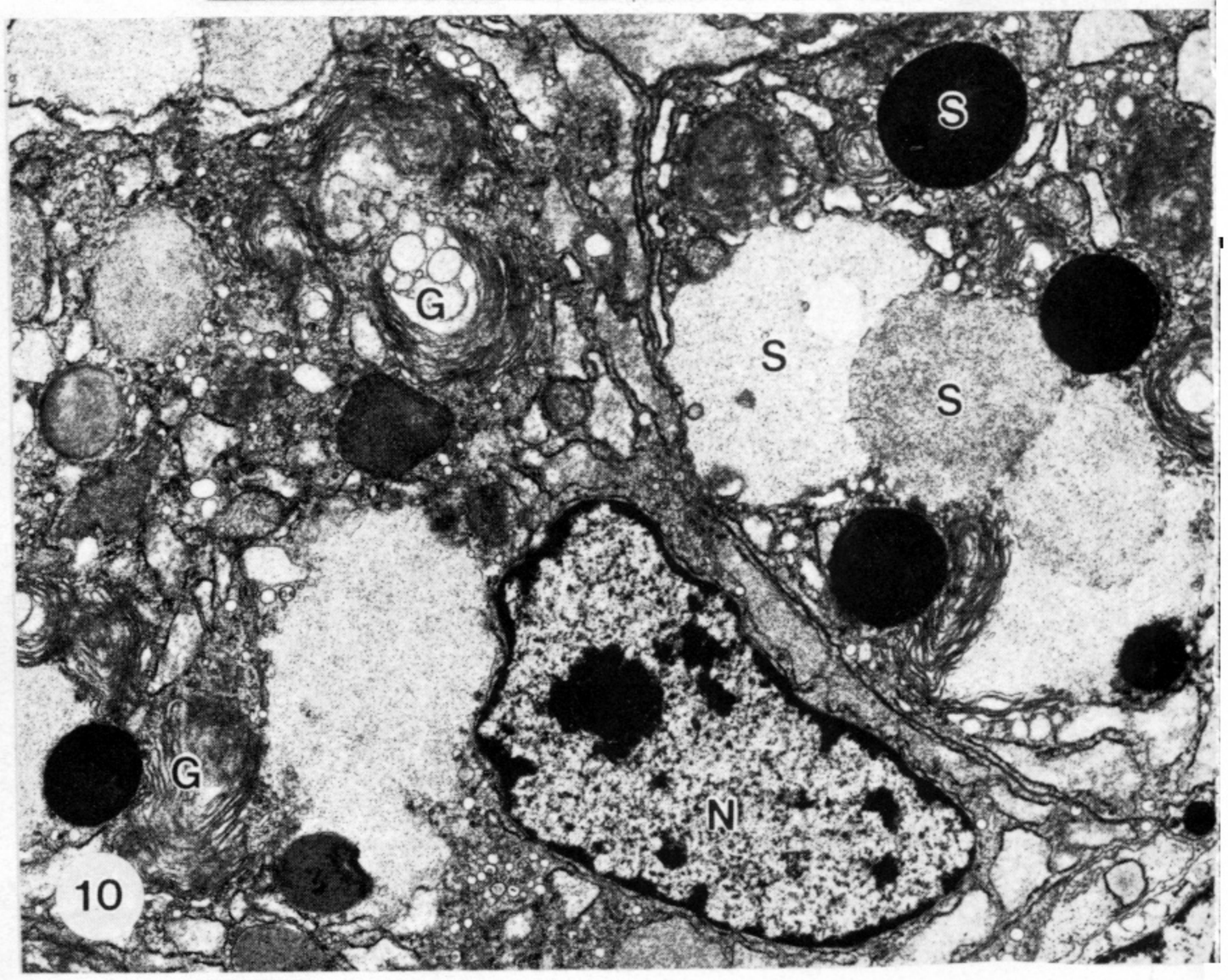

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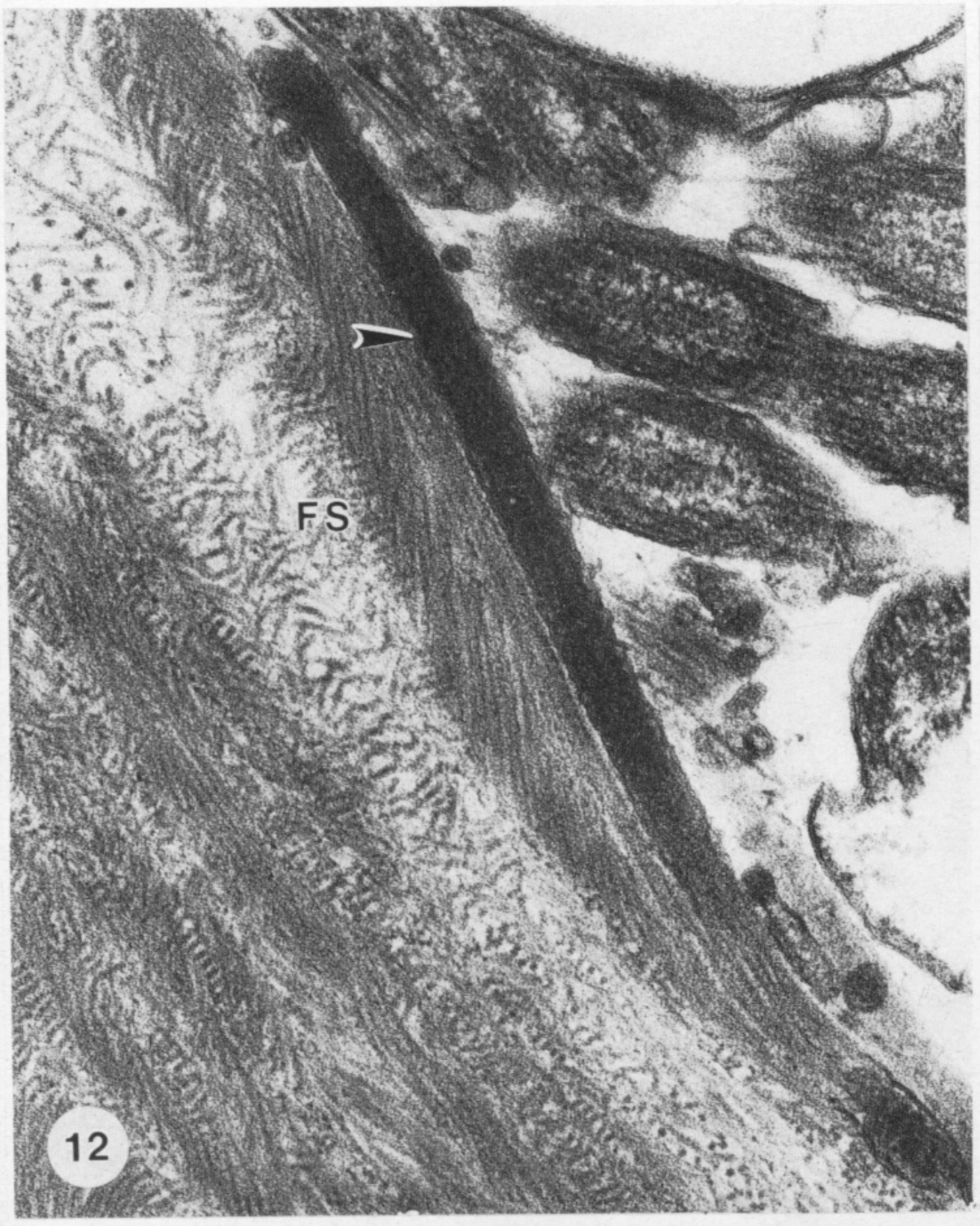
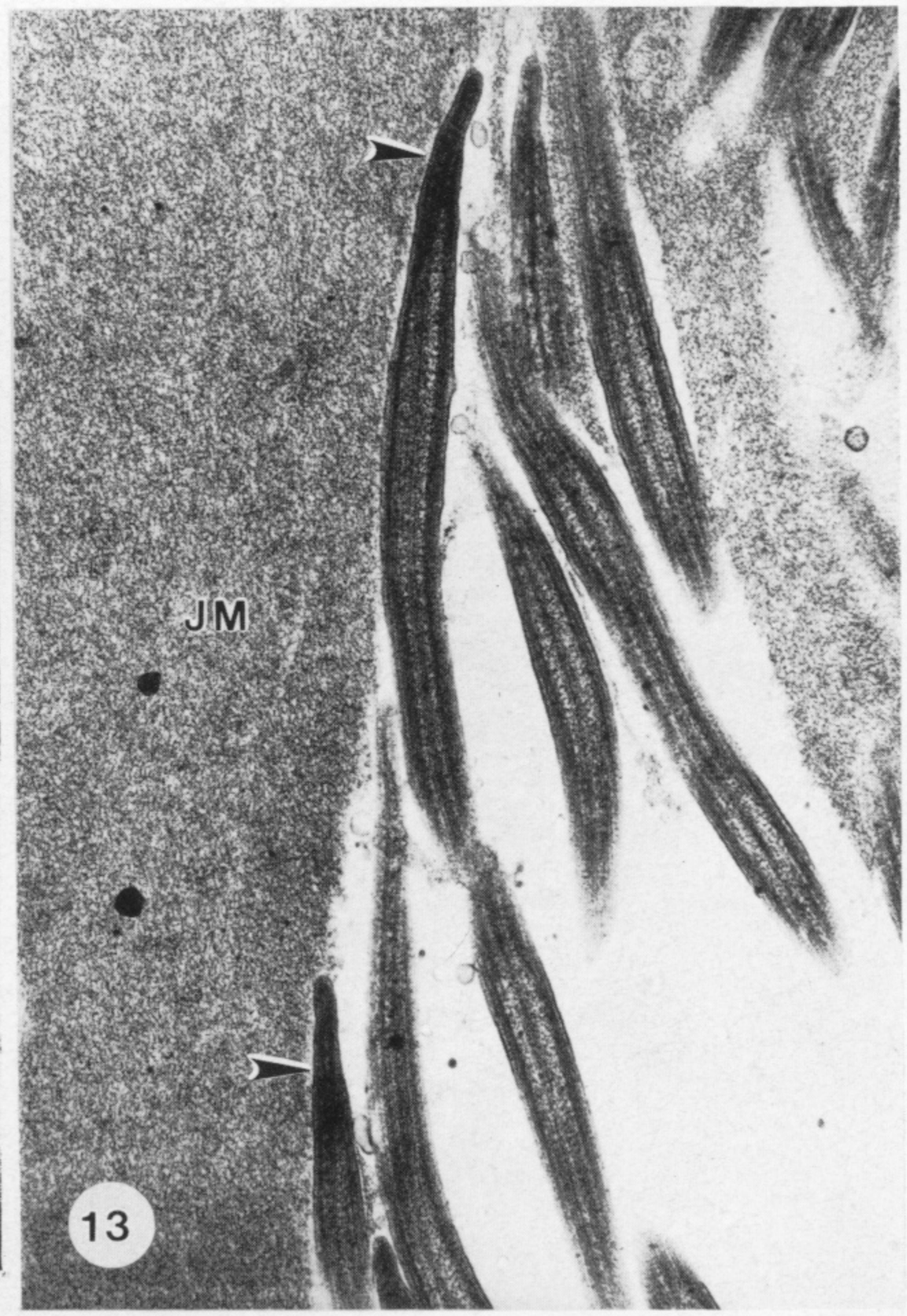
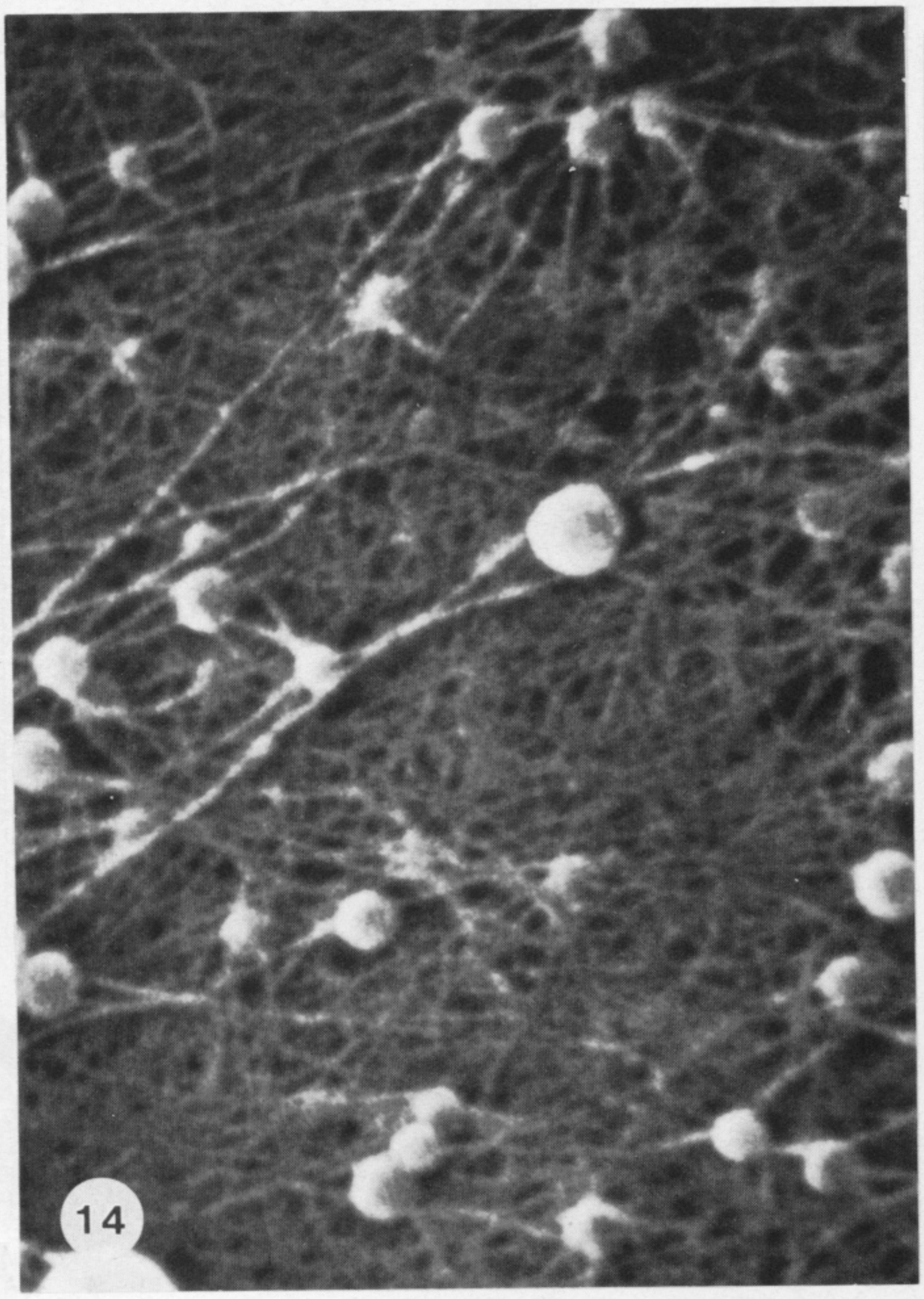
FIGURES 7–10. For description see p. 166.







FIGURES 12–15. For description see p. 166.

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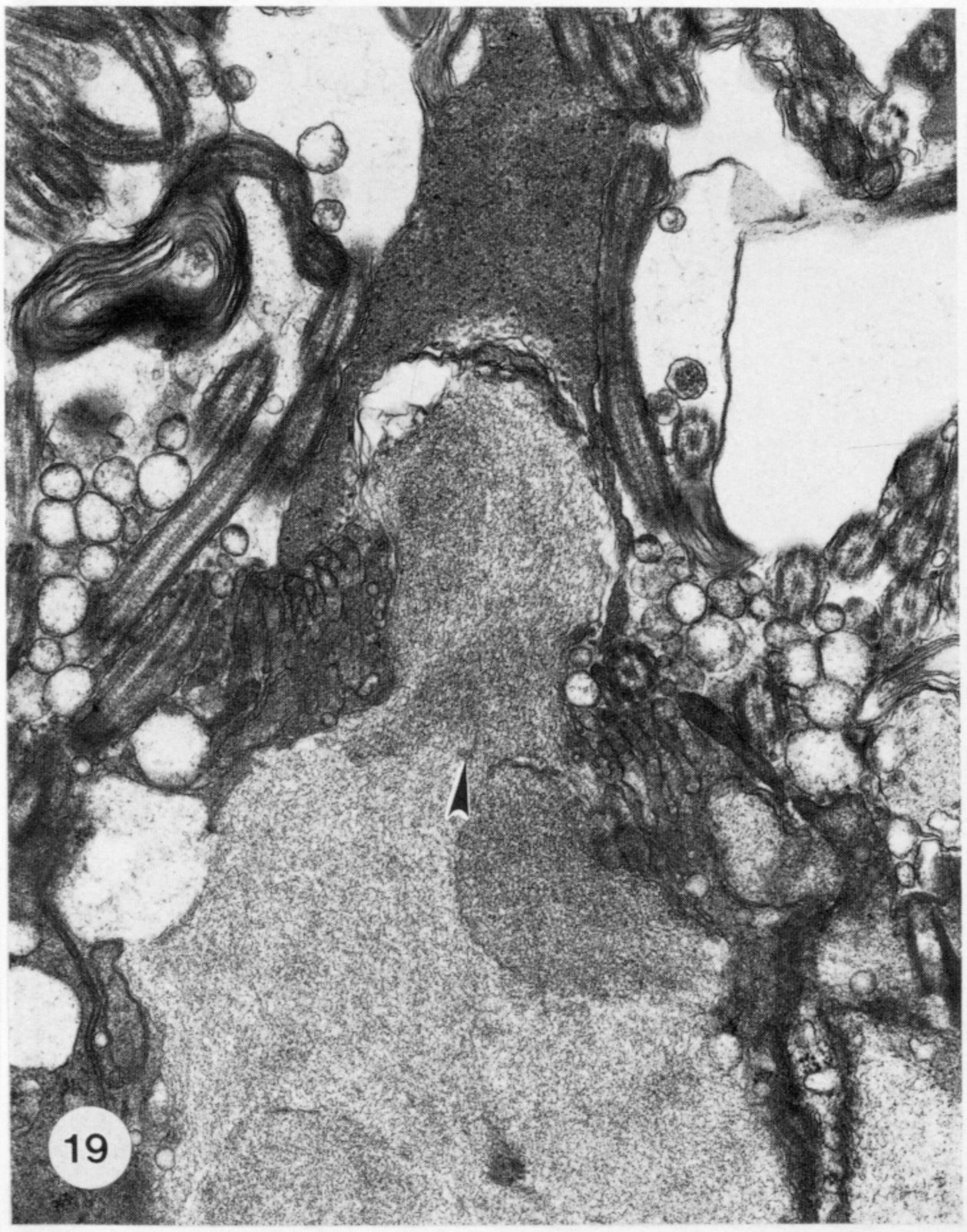
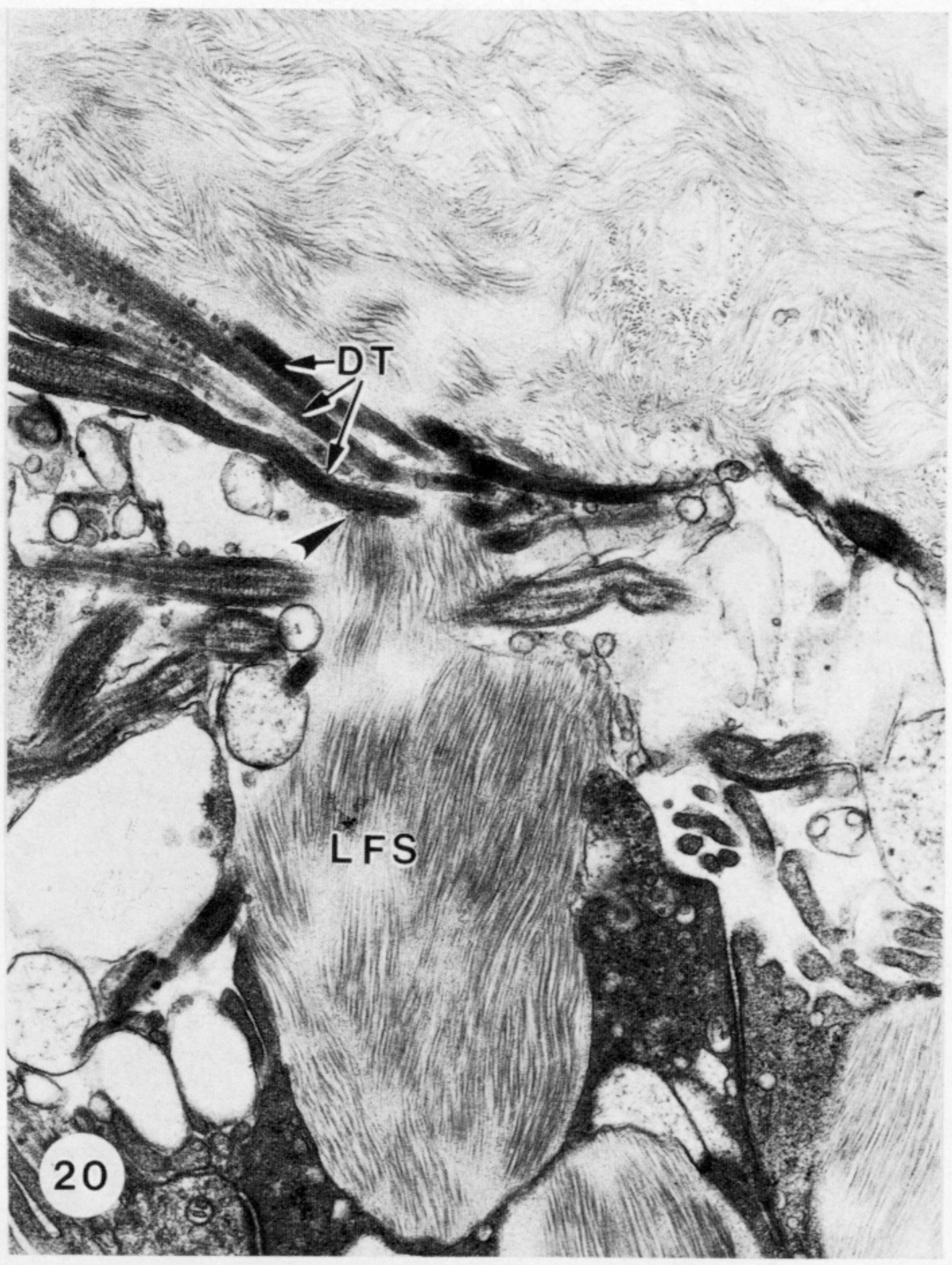
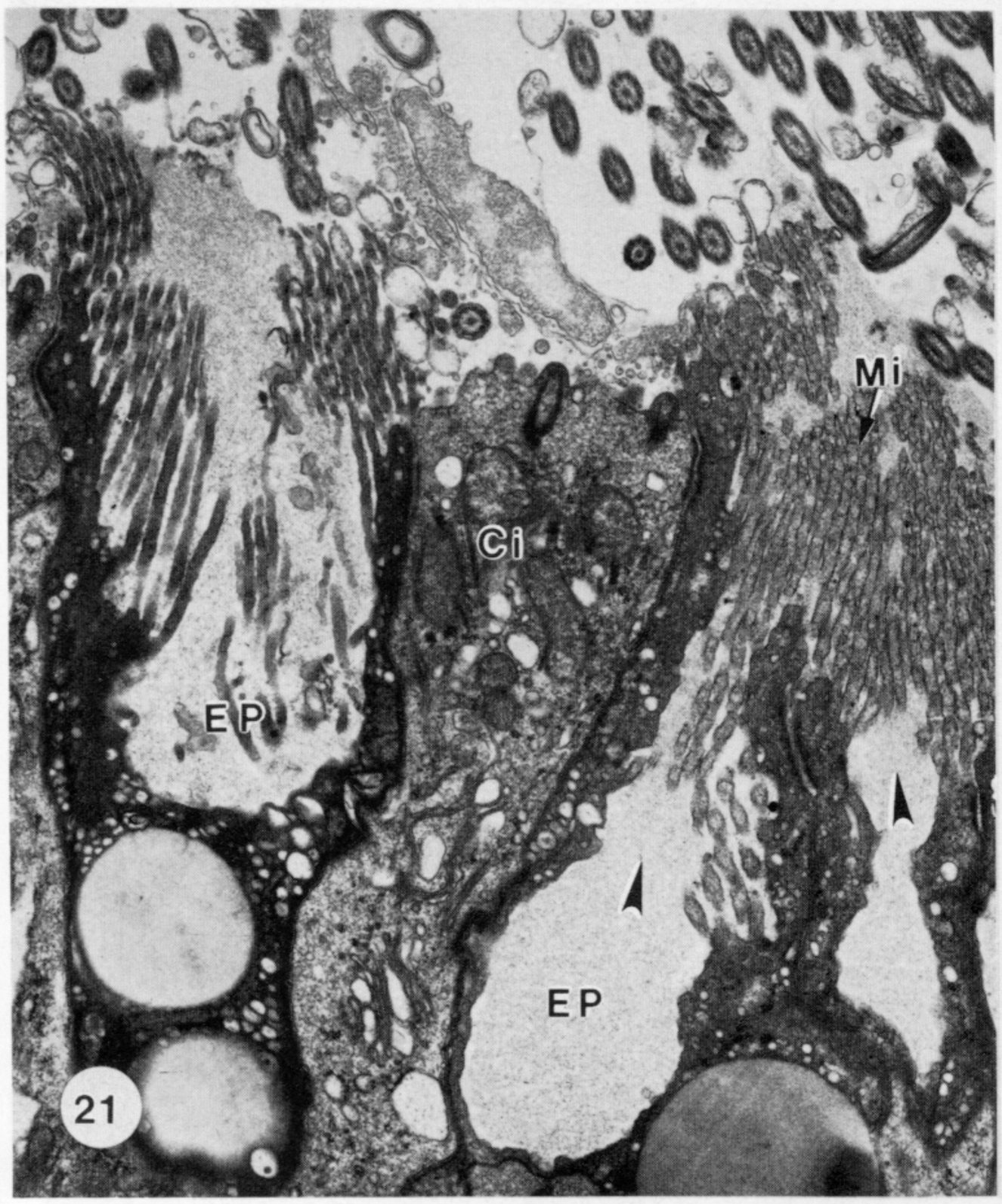
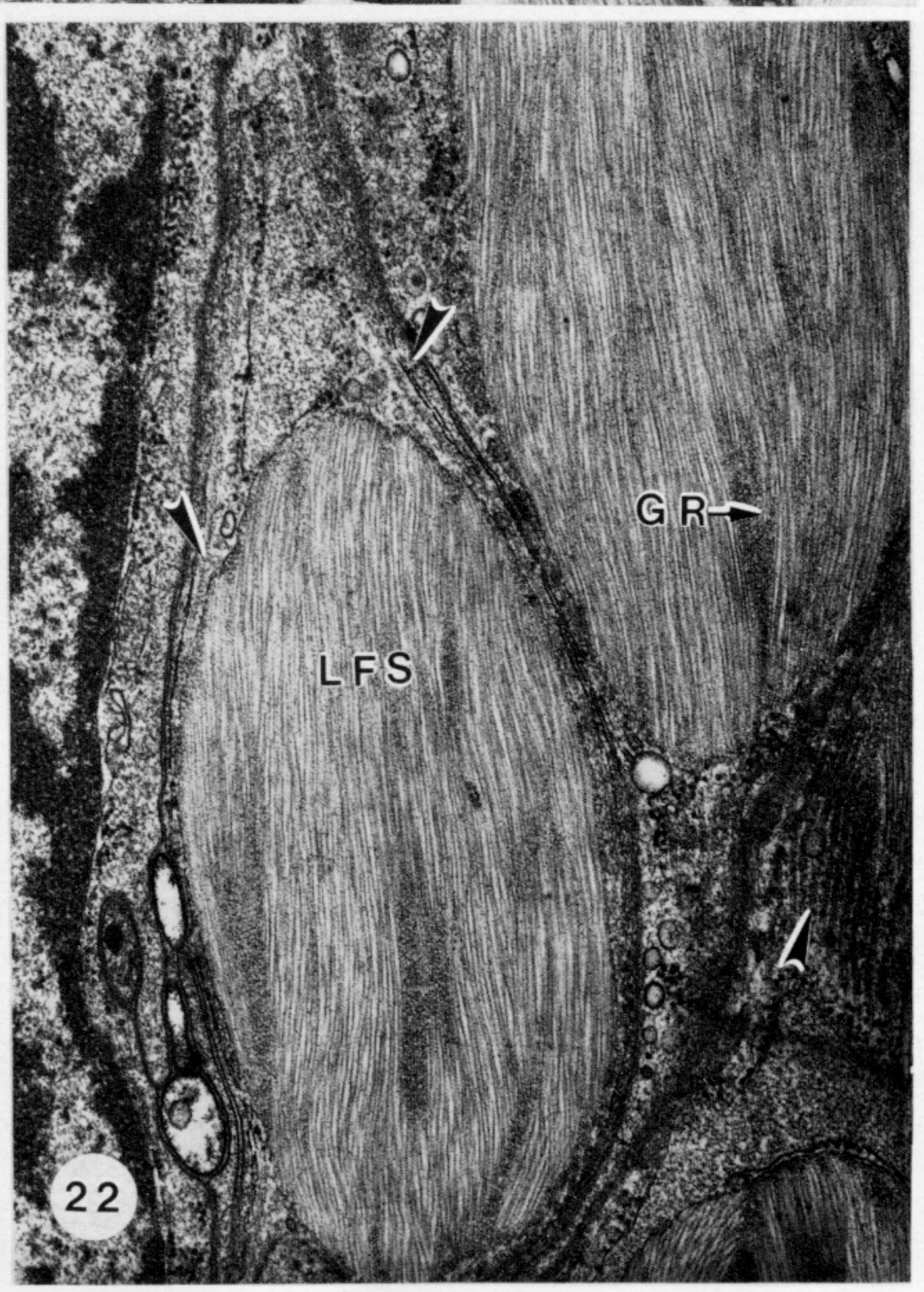
FIGURES 16–18. For description see p. 166.

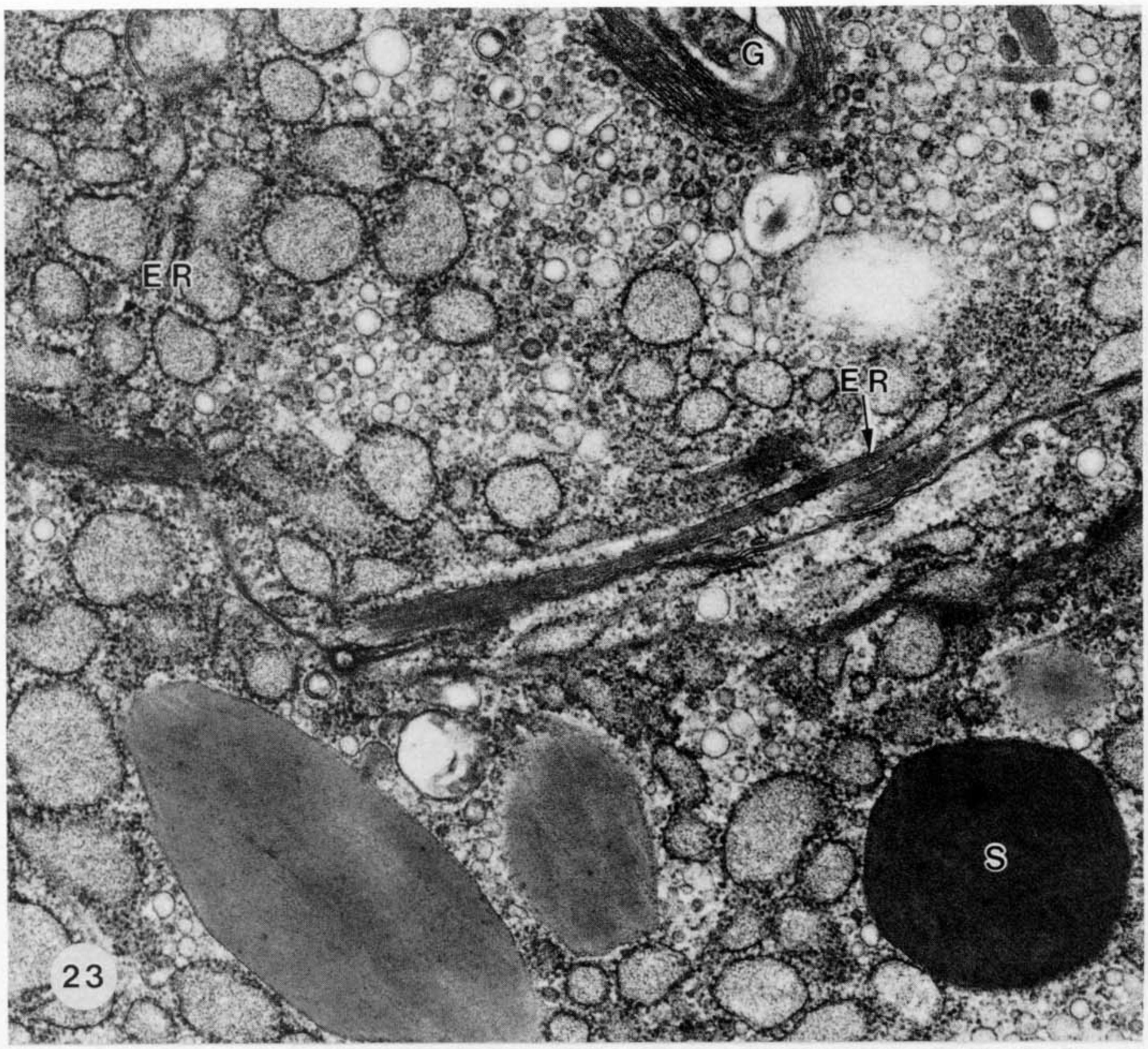


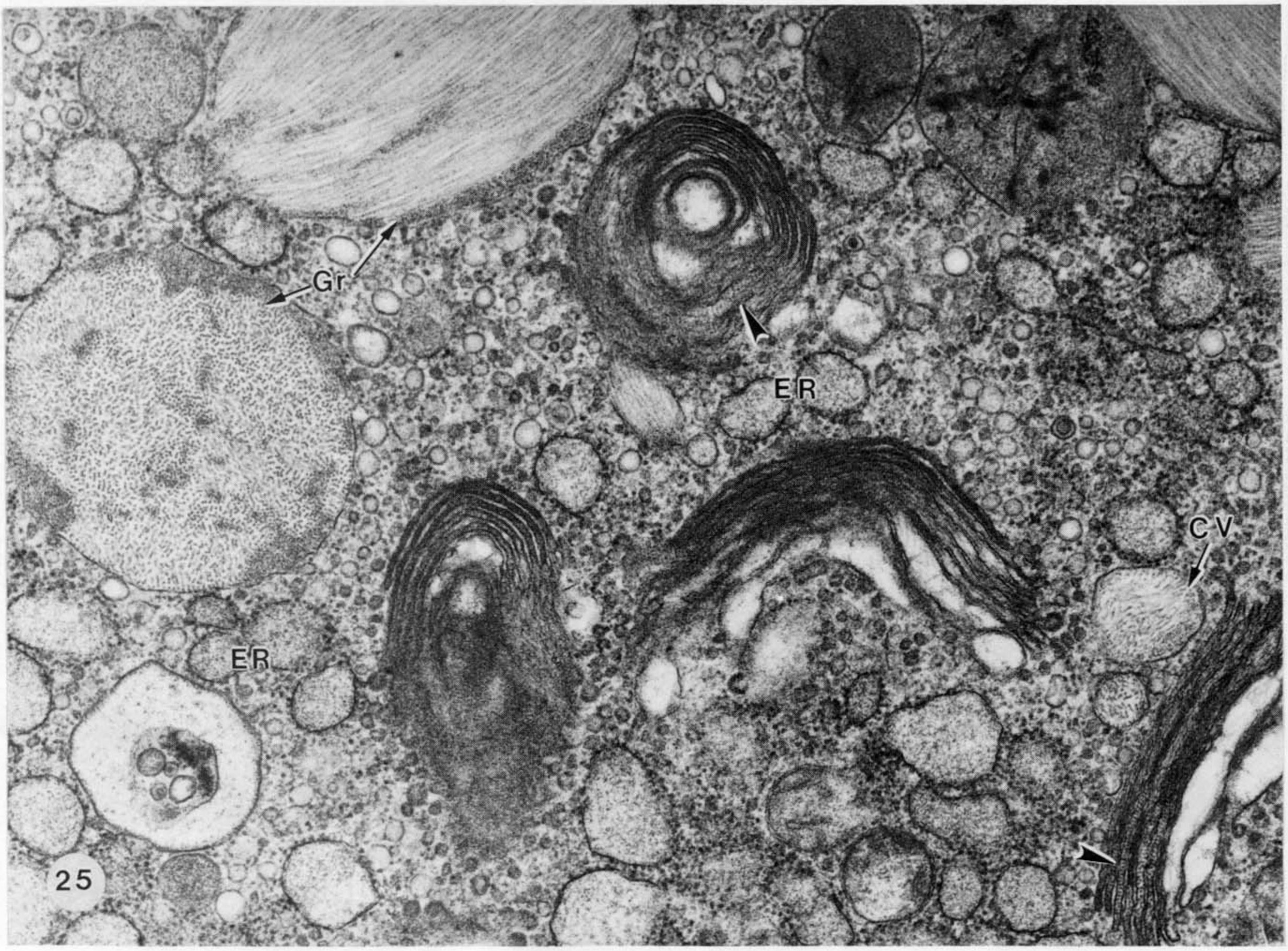




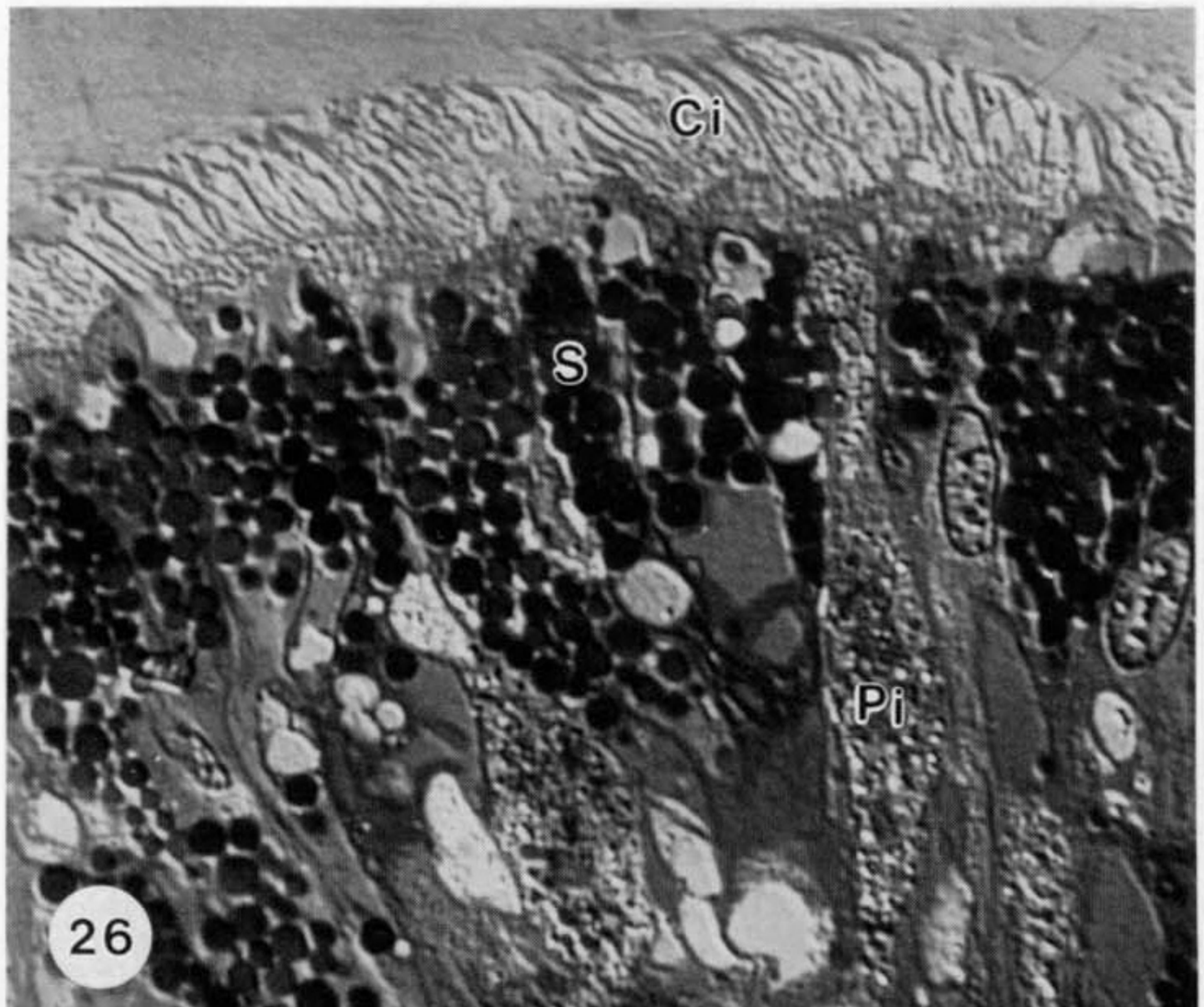


FIGURES 19–22. For description see p. 167.





FIGURES 23–25. For description see p. 167.



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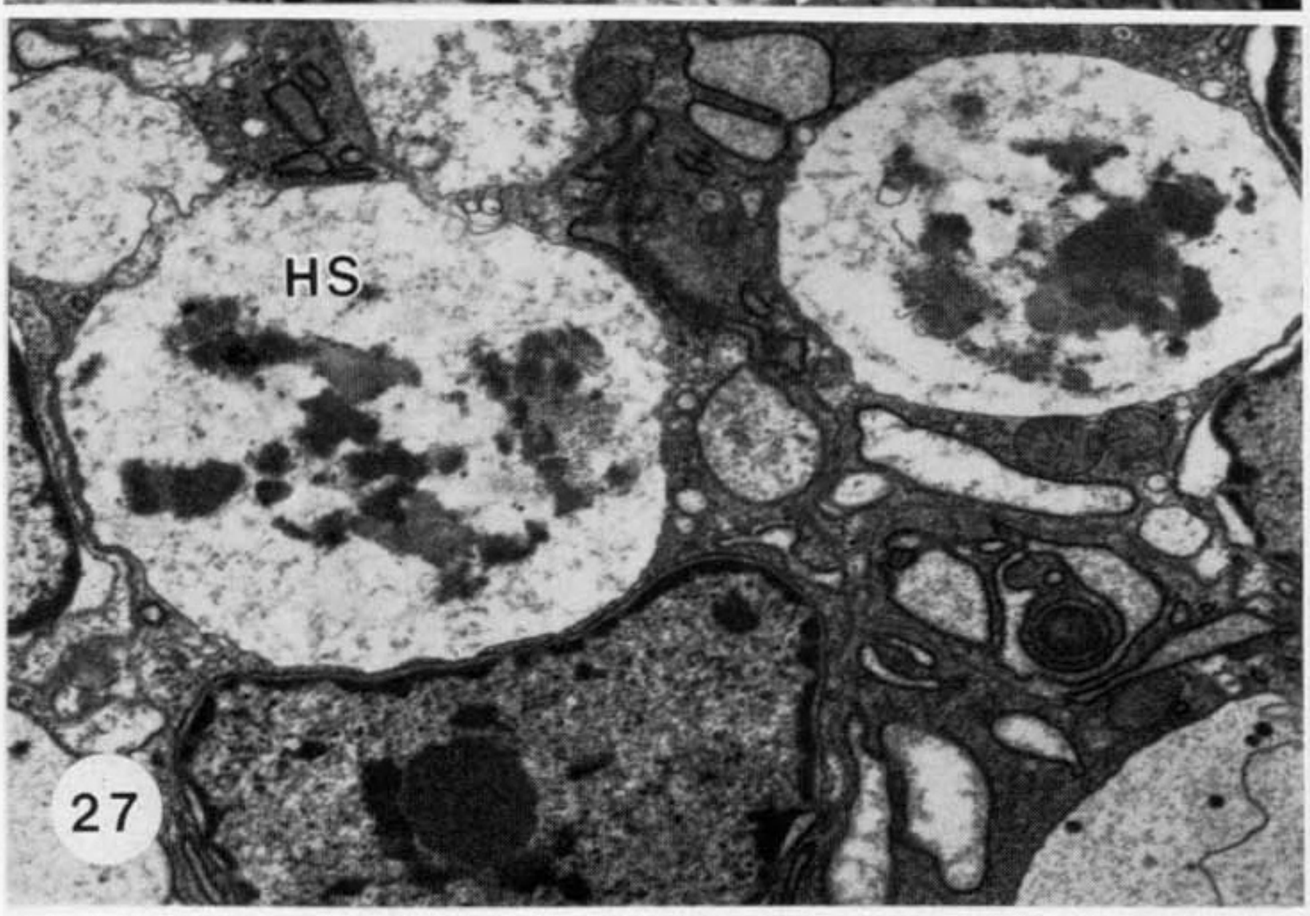

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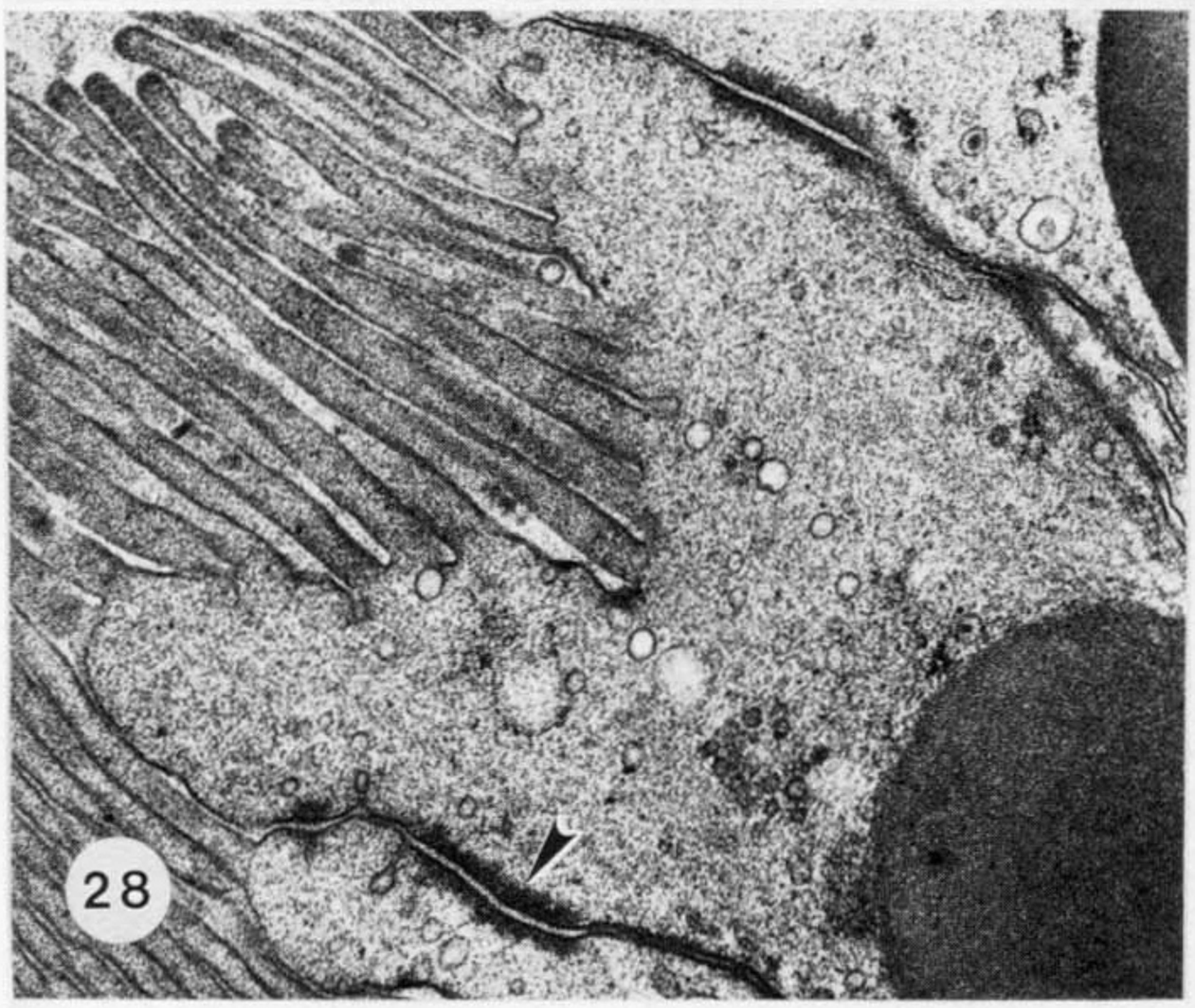

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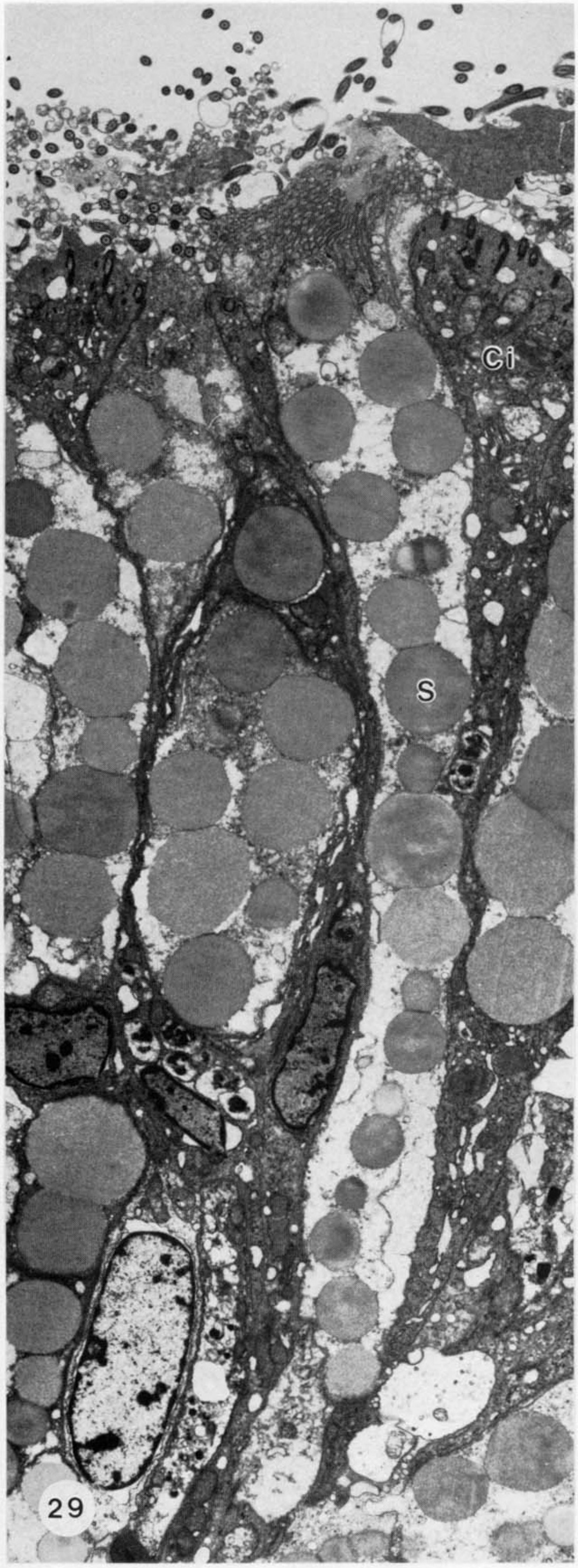

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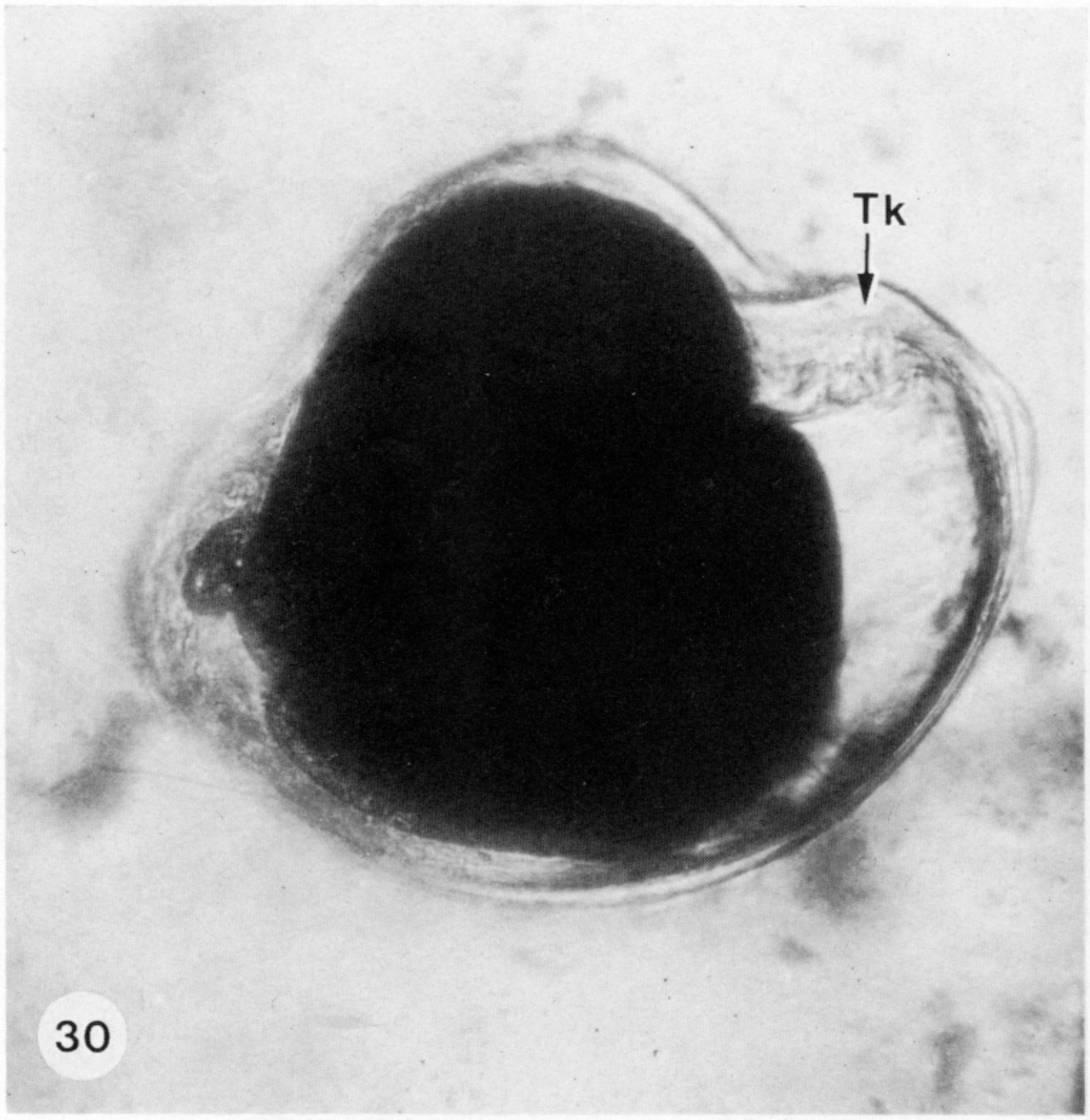
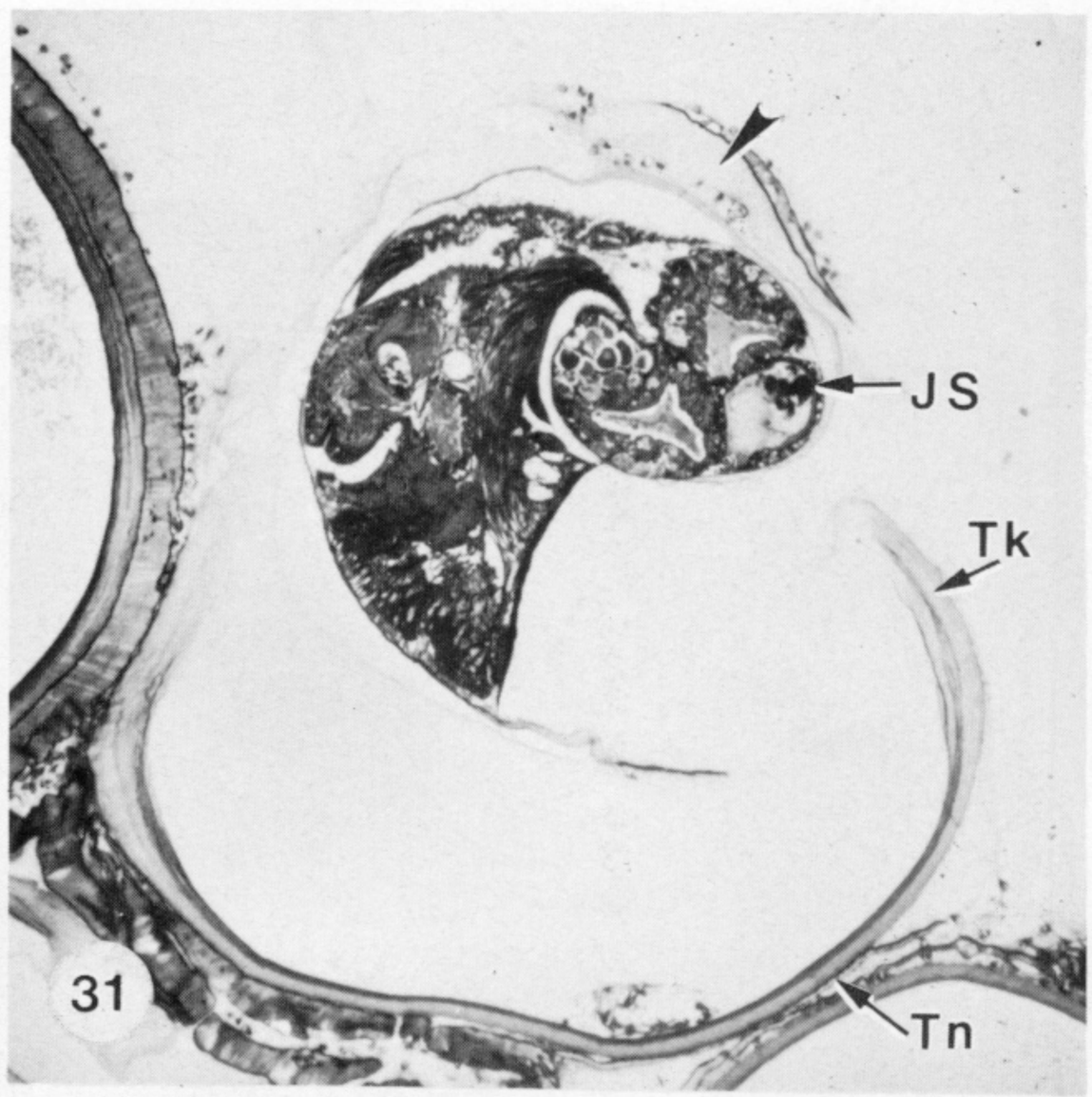
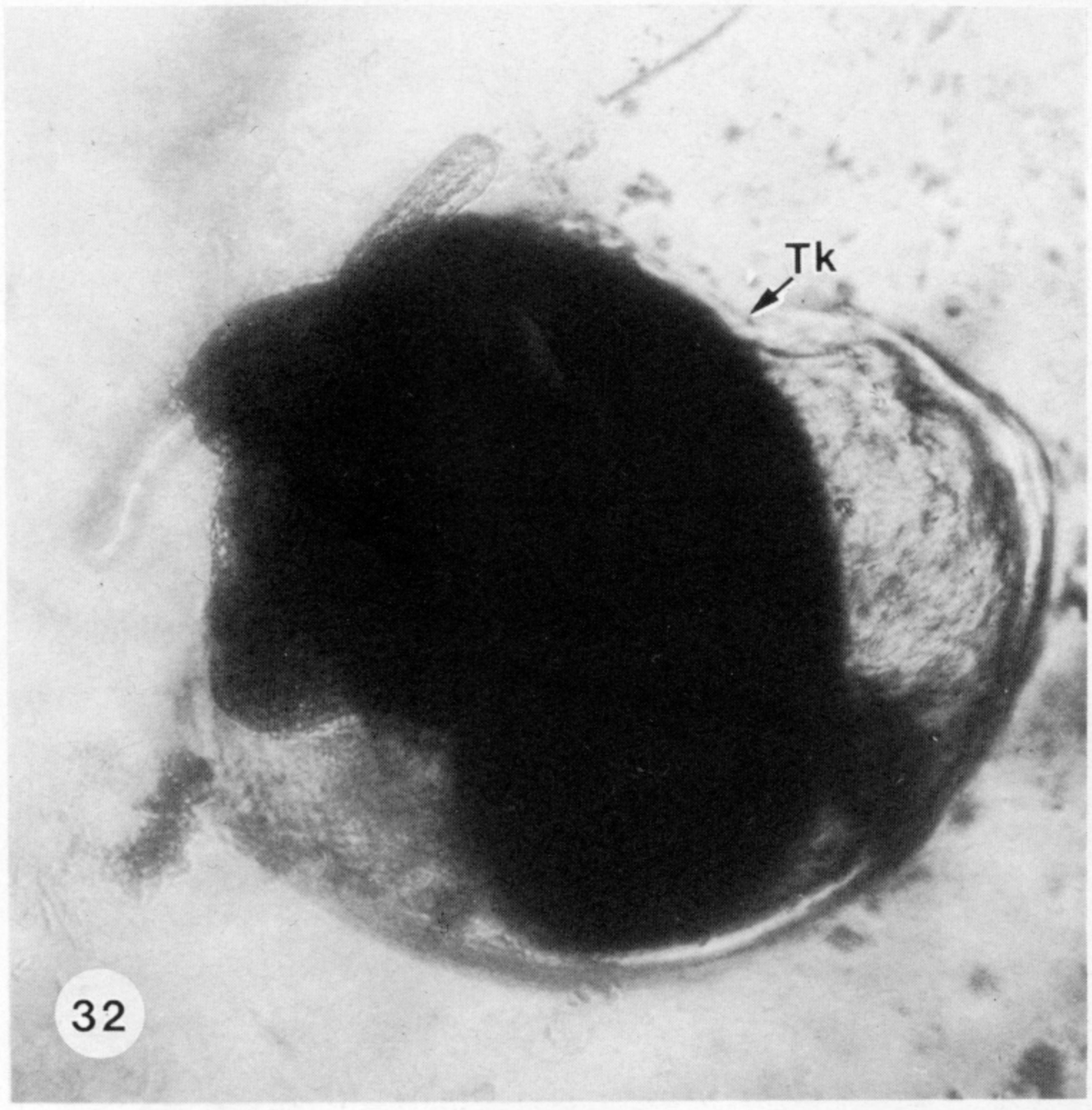
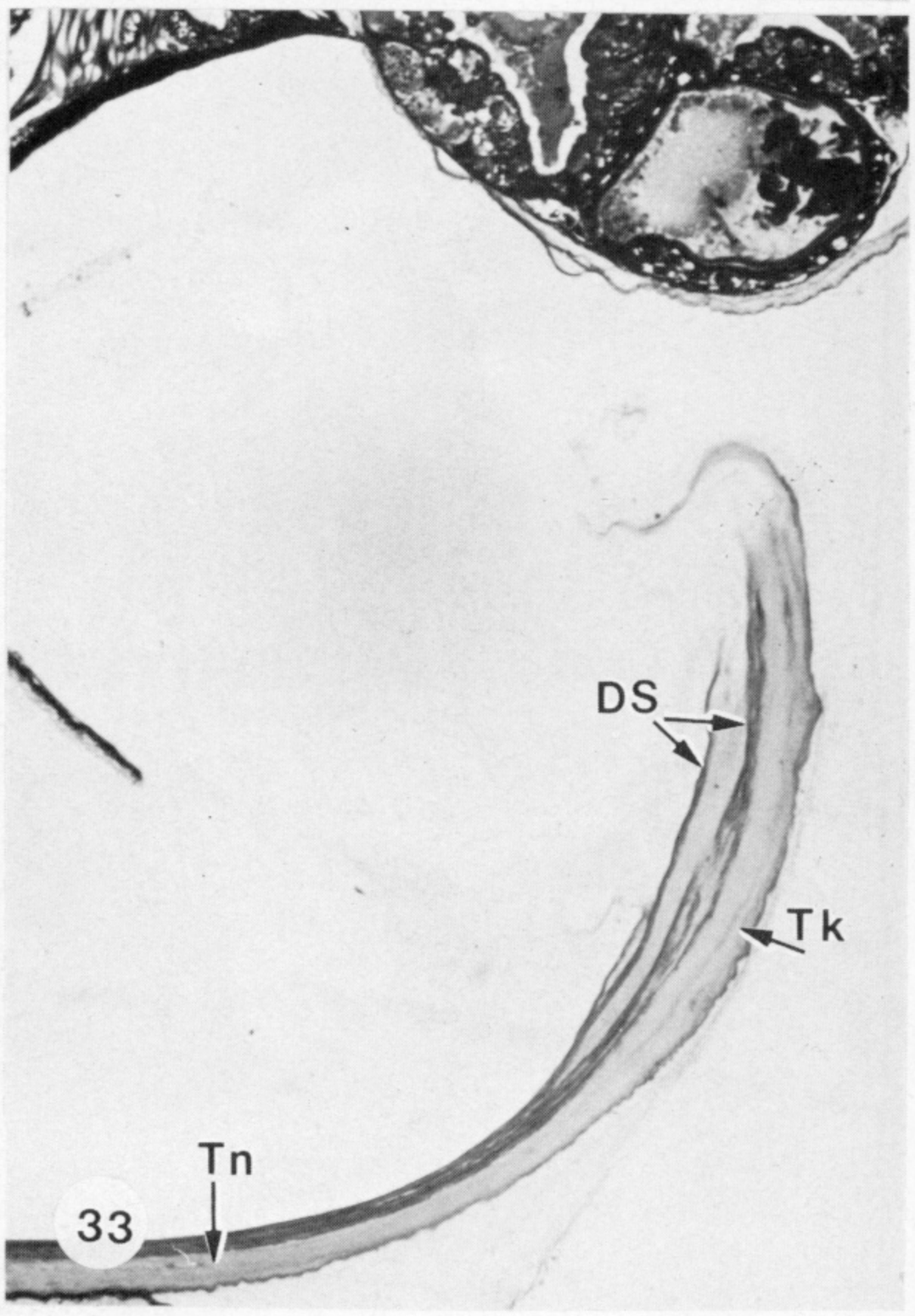
FIGURES 26–29. For description see p. 167.









FIGURES 30–33. For description see opposite.

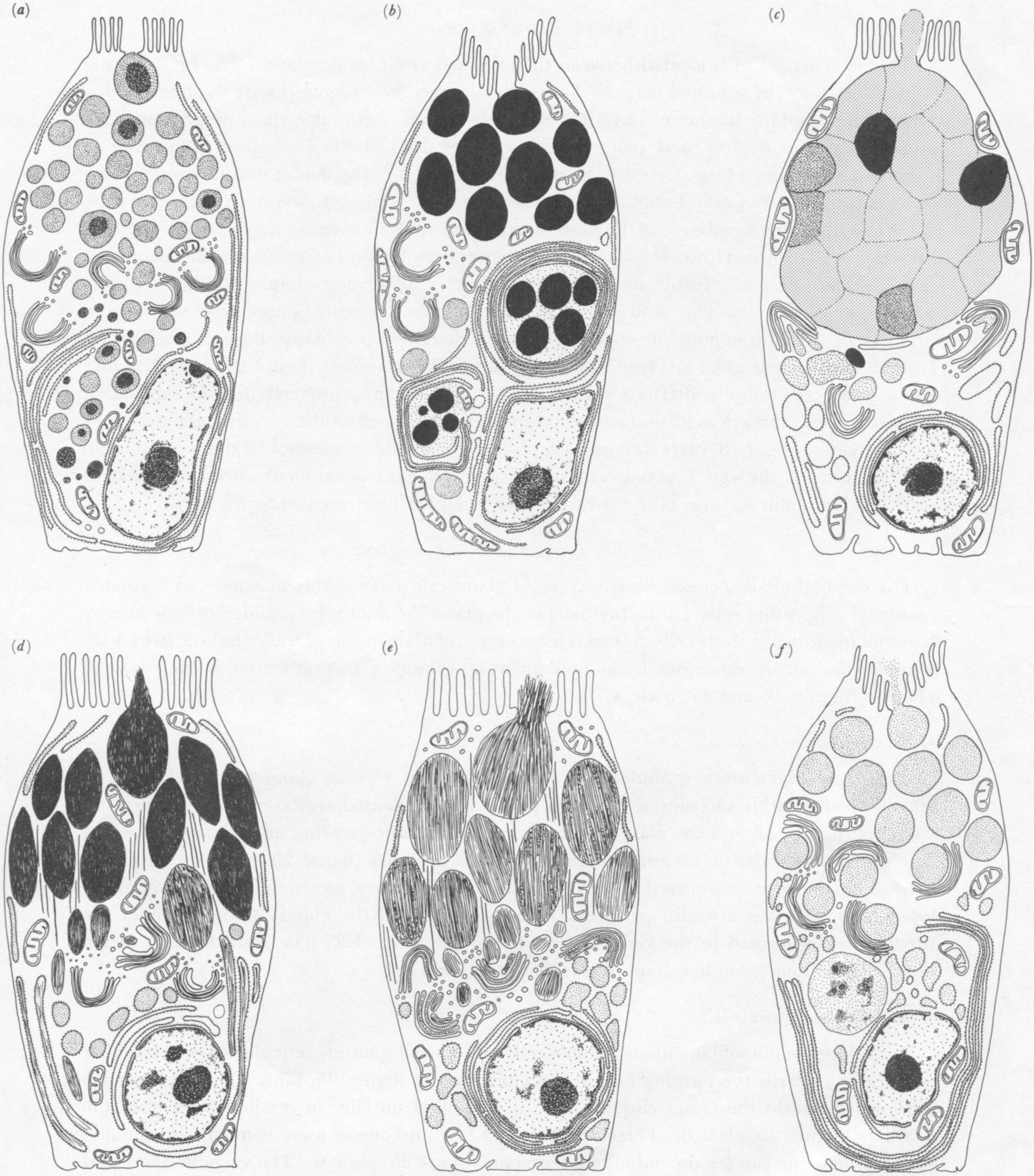


FIGURE 11. Schematic diagram summarizing fine structure of gland cells found in pallial oviduct. (*a*) Microgranule cell of albumen gland; (*b*) Dense granule cell of albumen gland; (*c*) Covering gland cell; (*d*) Heterofilamentous granule cell of capsule gland; (*e*) Homofilamentous granule cell of capsule gland; (*f*) Jelly gland cell. The shape of each cell varies greatly depending on factors such as; position in the gland, state of nutrition and seasonal cycle. To avoid bias, all cells have been represented with a similar shape.