A STUDY OF THE OVIDUCAL GLANDS AND OVISACS OF *BALANUS BALANOIDES* (L.), TOGETHER WITH COMPARATIVE OBSERVATIONS ON THE OVISACS OF *BALANUS HAMERI* (ASCANIUS) AND THE REPRODUCTIVE BIOLOGY OF THE TWO SPECIES

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[Plates 1–6]

CONTENTS

A mainly microscopical study has been carried out on the oviducal glands and ovisacs of two hermaphroditic sessile barnacles, *Balanus balanoides* and *Balanus hameri*. In both species each gland secretes an ovisac, once a year, for a very restricted period before copulation. The morphology of the glands of *B. balanoides* has been worked out from serial sections and a Plasticine model. Three regions have been established

within the glands; two are ectodermal in origin, namely the main chamber and exit canal, while the third, the proximal chamber, is mesodermal. The exit canal is always lined with cuticle, but the main chamber for most of the year is not. However, in both species main chamber cells begin to secrete the ovisacs several weeks before copulation. Ovisacs are undoubtedly cuticular structures.

The cytology of *B. balanoides* main chamber cells during the sequence of events leading to the formation of ovisacs has been followed by means of transmission electron microscopy. The cells each develop a long apical cytoplasmic extension; from these extensions and the apical cell surfaces secretions pass out to form the ovisac wall. This wall has two zones, an outer electron-dense zone 14 μm thick and an inner flocculent zone 6 μm thick. When fully formed the ovisac is released from the main chamber cells to lie in the oviducal gland lumen, although the neck of the ovisac continues to be firmly attached to specialized anchor cells. The cytoplasmic extensions of the main chamber cells break away with the ovisac as it is released and eventually form the pores in the ovisac wall. Scanning electron microscopy was used to examine such unstretched sacs. In *B. balanoides* the main chamber cells then partially retrogress, shedding secretions and portions of cytoplasm into the gland lumen. It is proposed that these 'formed bodies', by their osmotic activity, draw water and low molecular mass solutes into the gland lumen from the haemolymph. The 'formed bodies' swell and burst, thereby accumulating fluid in the lumen of the gland, which becomes highly swollen. It is this fluid that has the activating factor(s), thought to be the ammonium ion, needed to activate inseminated sperm. In this condition *B. balanoides* becomes a receptive female. In *B. hameri*, although there is some retrogression of the main chamber cells with secretion of 'formed bodies', there is much less accumulation of fluid and so the oviducal glands do not become so highly swollen.

The copulatory act of *B. hameri* was observed closely and comparison was made with that of *B. balanoides*. In *B. hameri* a single male is involved and a single insemination is sufficient for egg laying to commence, while in *B. balanoides* more than one male may be involved and multiple inseminations are needed before egg laying begins.

Oocytes (eggs) pass from the ovaries down the oviducts and into the elastic ovisacs lying in the oviducal glands. As the ovisacs distend with eggs they first expel the oviducal gland fluid into the mantle cavity and then they themselves are forced out into the mantle cavity. As the stretching continues the ovisac wall becomes very thin and the pores enlarge. Although *B. balanoides* sperm, which are 0.5 μm in diameter, can easily pass into the 0.7 to 1.6 μm diameter pores of an unstretched ovisac, those of *B. hameri*, which are also 0.5 μm in diameter, need the ovisac to stretch and the pores to enlarge from their original diameter of 0.2 μm before sperm can pass through and fertilize the eggs.

Inseminated sperm, in both species, are deposited as gelled masses. In *B. balanoides* the expelled oviducal gland fluid activates such sperm, but inseminated sperm of *B. hameri* become active in seawater. At any one time, only those sperm on the outer surface of the masses are active, and so a staggered release takes place. This is essential when egg laying takes more than 30 min and the sperm of *B. balanoides* and *B. hameri* are active for only 5–6 min and 12–13 min respectively. The fully formed egg masses are finally freed from the anchor cells within the glands and moved to the bottom of the mantle cavity, where egg development takes place.

1. Introduction

In barnacles each oviduct opens to the exterior by way of an oviducal gland situated in the basal segment of the first cirrus. These glands secrete the paired ovisacs into which the eggs are delivered. Walley (1965) reviewed most of the early literature concerned with oviducal glands and ovisacs. Using the light microscope she described the development of the glands and the formation of the ovisacs in *Balanus balanoides*, while Klepal & Barnes (1977) have subsequently

described the fine structure of the ovisacs of *Pollicipes cornucopia* and *Balanus balanus*. The present study of the fine structure of the oviducal gland cells and ovisacs of *B. balanoides* extends a preliminary study with the scanning electron microscope (Walker 1977*a*); it also includes some comparative observations on copulation, sperm motility and the passage of sperm through the ovisac wall in *B. balanoides* and *B. hameri*.

2. Materials and methods

(*a*) *Observations on the copulation process*

(i) *B. balanoides*. Small rocks with adults attached were collected from the shore of the Menai Strait at different times throughout the breeding season in November and early December 1977, 1978 and 1979. The rocks were selected: those with some barnacles showing remnants of inseminated sperm masses on their opercular valves were particularly suitable, because there was a high probability that other barnacles on the rocks were ready to copulate. After a period of 8–10 h out of the water the rocks were immersed in small aquaria and the animals were observed at regular intervals for signs of sexual activity. The copulation process was observed for many pairs of animals.

(ii) *B. hameri*. Adults were collected by dredging off Langness Point, Isle of Man, in November 1978 and 1979; most were attached to the mussel, *Modiolus modiolus*. The *B. hameri* were maintained in the laboratory in running seawater tanks and a number of individuals were kept widely separated to prevent cross-fertilization. From mid-December, when the seawater temperature was 6–7 °C, the animals in groups were observed at regular intervals each day for signs of sexual activity. Each day also, the widely separated individuals were placed close together in an attempt to 'control' the onset of sexual activity. The complete copulation process was observed for only six pairs of animals. Most of the grouped barnacles were found to contain egg masses by 8 January.

(*b*) *Light microscope*

(i) The bodies, comprising the thorax and prosoma, of both *B. balanoides* and *B. hameri* were dissected out at regular intervals leading up to the respective copulation times and were fixed in seawater Bouin solution. The material was dehydrated and cleared in Cellosolve (2-ethoxy-ethanol) and placed in toluene before being embedded in Fibrowax. Transverse sections (7 μm thick) of the bodies were stained with Heidenhain azan.

(ii) Ovisacs of the two *Balanus* species were dissected out at the relevant times when they were lying free in the oviducal glands, and were examined in drops of seawater under coverslips.

(iii) *B. hameri* sperm were collected from the vesiculae seminales (vesicula sperm; see Barnes *et al.* 1971) and from the mantle cavity of recipients following insemination. They were placed in drops of filtered seawater on slides, covered with coverslips and examined, by means of dark field illumination, for signs of motility.

(*c*) *Transmission electron microscopy* (*t.e.m.*)

To allow the formation of the ovisacs to be followed, intact oviducal glands of *B. balanoides* were dissected out from animals collected on 19 October, 3 November and 25 November 1977. The glands were fixed in OsO_4 (20 g/l) in veronal buffer (pH 7.2) at 2 °C for 1½ h and then dehydrated in ethanol before embedding in Araldite. Sections 1 μm thick, stained in toluidine

blue (10 g/l) in aqueous borax solution (10 g/l), were examined with the light microscope to assess the quality of fixation before thin sectioning. Sections 600 to 700 Å thick were mounted on coated grids, stained, and then viewed in an A.E.I. Corinth 275 electron microscope.

(*d*) *Scanning electron microscope* (*s.e.m.*)

Oviducal glands of *B. balanoides*, with the secreted ovisacs still attached to the gland cells, were dissected out and the epithelial lining of each gland was slit and pulled away from the ovisac. This material was fixed in 2% (by volume) glutaraldehyde in seawater for 2 h at 2 °C, then dehydrated in ethanol and transferred to acetone; drying was by the critical-point method, by means of liquid carbon dioxide. Other material fixed and processed in the same way included free unstretched ovisacs and egg masses (early and late egg-laying stages) of both *Balanus* species.

After drying, the material was attached to specimen stubs by means of double-sided Sellotape, coated with gold and then viewed in a Cambridge Stereoscan Mark 2a scanning electron microscope, which was operated at 10 kV.

3. General description of oviducal glands

The oviducal glands are specialized regions forming the terminal part of the oviducts. Each of the pair of oviducal glands is situated within the basal segment of a first cirrus (first thoracic appendage). Klepal & Barnes (1977) partitioned the glands into two regions. The oviduct connects with the first region, which includes most of the gland and produces the ovisac; the second region takes the form of a short duct, which is lined with cuticle and leads to the exterior through a slit-like opening. By dissecting out the glands of *B. balanoides* and relating the observed morphology to serial sections, a model was constructed (figure 1, plate 1). Three regions were distinguished (figure 1*a*): two, herein designated main chamber (m.c.) and exit canal (e.c.), are as outlined by Klepal & Barnes; the third, the proximal chamber (p.c.), is situated at the junction of the gland and oviduct. The proximal chamber is lined by cells that at a specific time of the year are full of globular secretory material (see Walley 1965). The exit duct is always lined with cuticle, but in *B. balanoides* the main chamber for most of the year does not have such a lining. However, during late October and early November it forms the ovisac, which is a cuticular structure (see §11). In those barnacles that produce many broods, main chamber cells will be actively engaged in secreting a succession of ovisacs over a large part of the year. The main chamber and exit duct must, therefore, arise as an invagination of the ectoderm (Walley 1965), but the proximal chamber, which is a specialized part of the oviduct, is derived from mesoderm. Tighe-Ford (1967) has shown that oviducal gland development and ovisac formation in *B. balanoides* are inhibited when the animals are exposed to constant illumination; he implicates the neuroendocrine system in this control.

When an ovisac is fully formed it is released from the underlying cells of the main chamber to lie free in the lumen, although the oviducal end of the ovisac continues to be well anchored to specialized proximal chamber cells (figure 1*a*). Oocytes, hereafter referred to as eggs, pass from the ovaries along the oviducts and enter the ovisacs which, as they distend, pass out from the glands into the mantle cavity. When egg laying is completed the fully formed egg masses are released from their anchor points within the glands and manœuvred into the bottom of the mantle cavity on either side of the body, where development of the eggs takes place.

4. Development of the oviducal glands in *B. balanoides*

In an animal at least one year old the height of the cells in the main chamber of an oviducal gland increases from 70 μm in June to 130 μm in October (Walley 1965). At the beginning of October the gland increases in size and mitotic figures occur in the cells; secretion of the ovisac by main chamber cells starts about four weeks before copulation (Walley 1965). In the present study Araldite sections (1 μm thick) of glands from animals over one year old collected on 19 October showed projections from cells of the main chamber extending into the gland lumen (figure 3, plate 2). Walley (1965) considered these projections to be extensions of the cytoplasm. At the fine structure level (t.e.m.) this was confirmed (figure 4, plate 2) and, furthermore, in animals collected on 19 October, secretion of the ovisacs had begun. Cells varied from 35 to 110 μm in height, with usually a single cytoplasmic extension 20 μm long. The nucleus, with single nucleolus, is situated basally in the cell. There are several Golgi bodies within the apical half of a cell and associated with these are secretory vesicles of various sizes, all with similar slightly electron-dense contents. A notable feature within the cytoplasm is the large number of microtubules, 250 Å in external diameter, which extend throughout the cell and are usually aligned along the longitudinal axis. Apically, many of the microtubules aggregate together and pass into the cytoplasmic extension (figure 5, plate 2). Much swollen rough endoplasmic reticulum and numerous mitochondria occur basally in the cell. The basal plasma membrane makes firm contact with the basal lamina at hemidesmosomes (figure 6, plate 2), while adjacent lateral plasma membranes are connected by desmosomes apically and basally (figure 2; figure 7, plate 2). A further distinctive feature basally is the direct contact between certain oviducal gland cells and underlying connective tissue or parenchyma cells (figure 7). Such contact, however, is not unusual in barnacles (Koulish 1971, 1976; Rainbow & Walker 1977). Many of these underlying cells contain small crystals (figure 8, plate 3), the chemical identification of which is shown, in a separate study, to be tyrosine (Walker 1980).

By 3 November the main chamber cells were slightly taller, ranging from 35 to 130 μm, and an additional type of secretory vesicle was evident, which had very electron-dense contents (figure 9, plate 3). This particular secretory material is released only across the plasma membrane of the cytoplasmic extensions at this time (figure 10, plate 3). Multivesicular bodies were then also present in the apical cytoplasm.

The single secretory product within the gland cells of animals at 19 October gave rise to flocculent, slightly electron-dense material outside the cells, which eventually formed the inner zone (*ca.* 6 μm thick) of the ovisac wall (figure 2), while in animals at 3 November the additional electron-dense secretion was released from and remained around the cytoplasmic extensions. This electron-dense secretion, which is also later released from the apical cell surface, eventually forms the outer zone (*ca.* 14 μm thick) of the ovisac wall. Between 3 and 25 November the ovisacs became fully formed and separated from the main chamber cells. Figure 2 illustrates the build-up and final form of the ovisac wall. As the ovisac is released into the gland lumen the cytoplasmic extensions break away; those portions of the extensions within the walls of the ovisac then degenerate, leaving small perforations.

In animals on 25 November the height of the main chamber cells was reduced to between 17 and 35 μm as secretion vesicles and portions of cytoplasm were released apically (figure 2; figure 11, plate 3). This process of general cytoplasmic breakdown continued until the cells were only 7–25 μm in height, and at this stage many lysosomes were present (figure 12, plate 3),

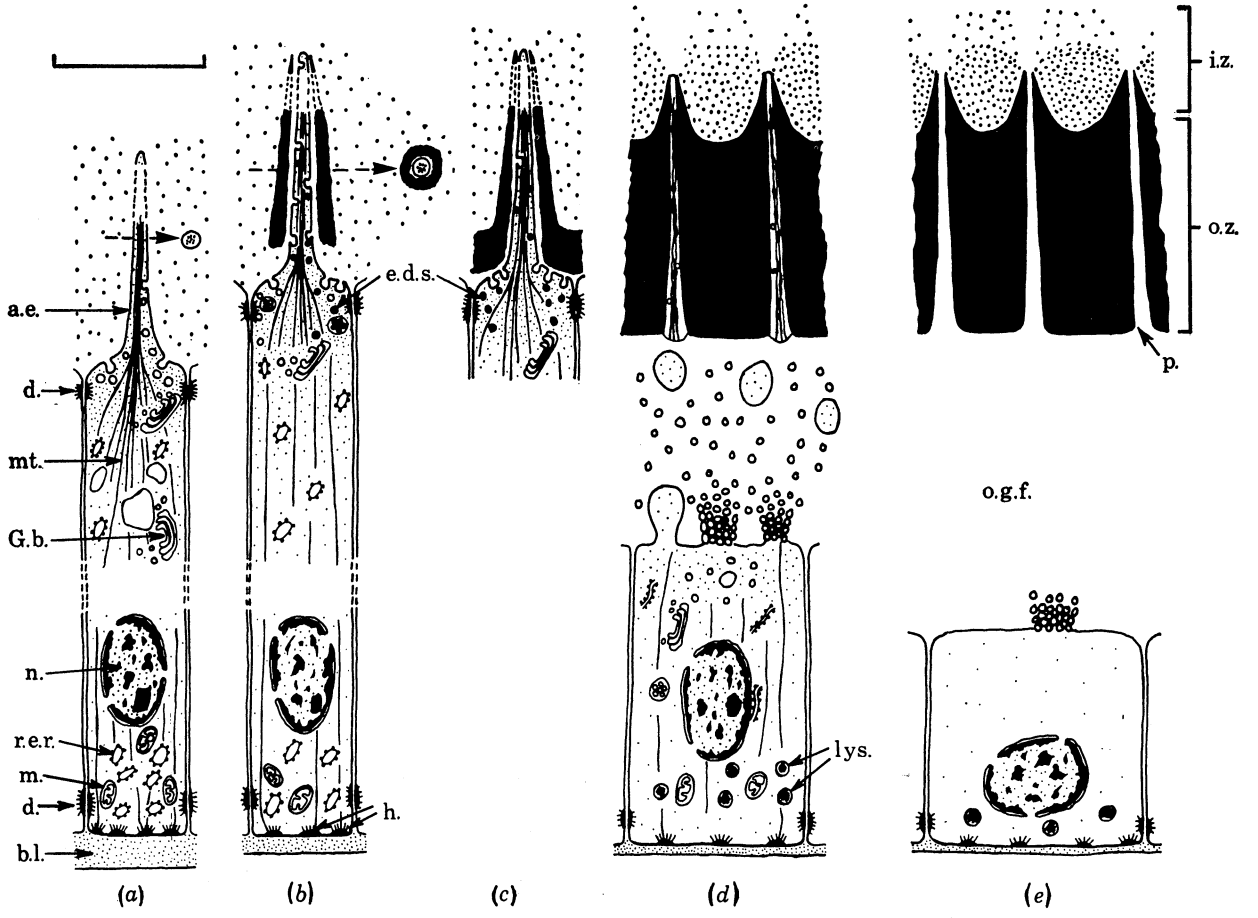


FIGURE 2. *B. balanoides*. Schematic diagrams illustrating a main chamber cell through its secretory cycle leading to the formation of an ovisac ((*a*)–(*c*)) and following ovisac release ((*d*), (*e*)). Scale bar, 10 μm.

a further indication of rigorous autophagy. The secreted membrane-bound packages of various sizes constitute the initial stage in the formation of the oviducal gland fluid (Walley *et al.* 1971, see §11).

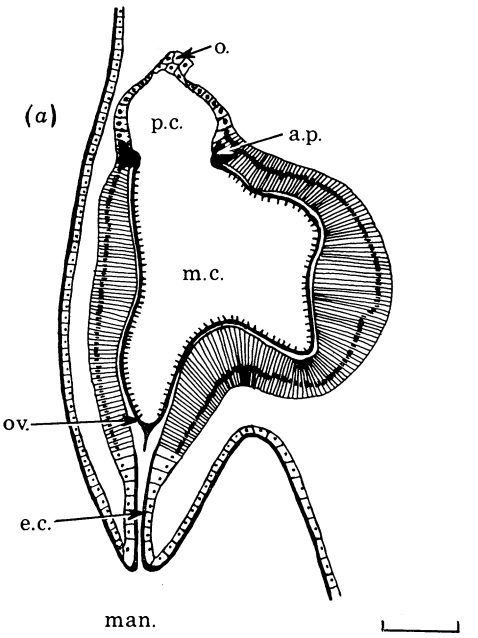
5. OBSERVATIONS ON FULLY FORMED OVISACS

B. balanoides

When glands, with secreted ovisacs attached to main chamber cells, are torn apart and prepared for s.e.m. the cytoplasmic extensions connecting the cells with an ovisac are intact in some areas (figure 13, plate 4), while in others the extensions have been pulled out, leaving distinct pores in the ovisac wall (figure 14, plate 4).

When dissected out from the glands the free, fully formed ovisacs are contorted and contracted (figure 15, plate 4). At higher magnification, the outer ovisac surface clearly shows imprints of the apical cell surfaces of the main chamber cells that produced it (figure 16, plate 4). Each polygonal cell imprint has a boundary 4–6 μm in diameter and a single central feature resembling a pore. At this stage, before egg laying, the ovisacs have elastic properties.

S.e.m. observations of such ovisacs confirm the light microscope findings. The imprints of cell boundaries on the outer sac surface are readily apparent (figure 17, plate 4) and within each



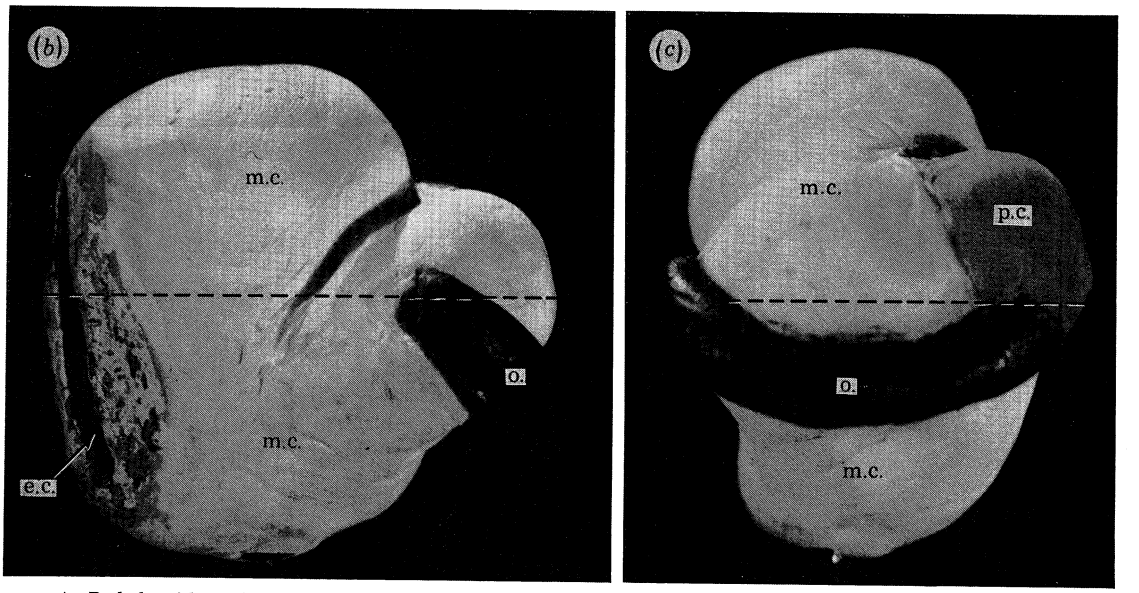
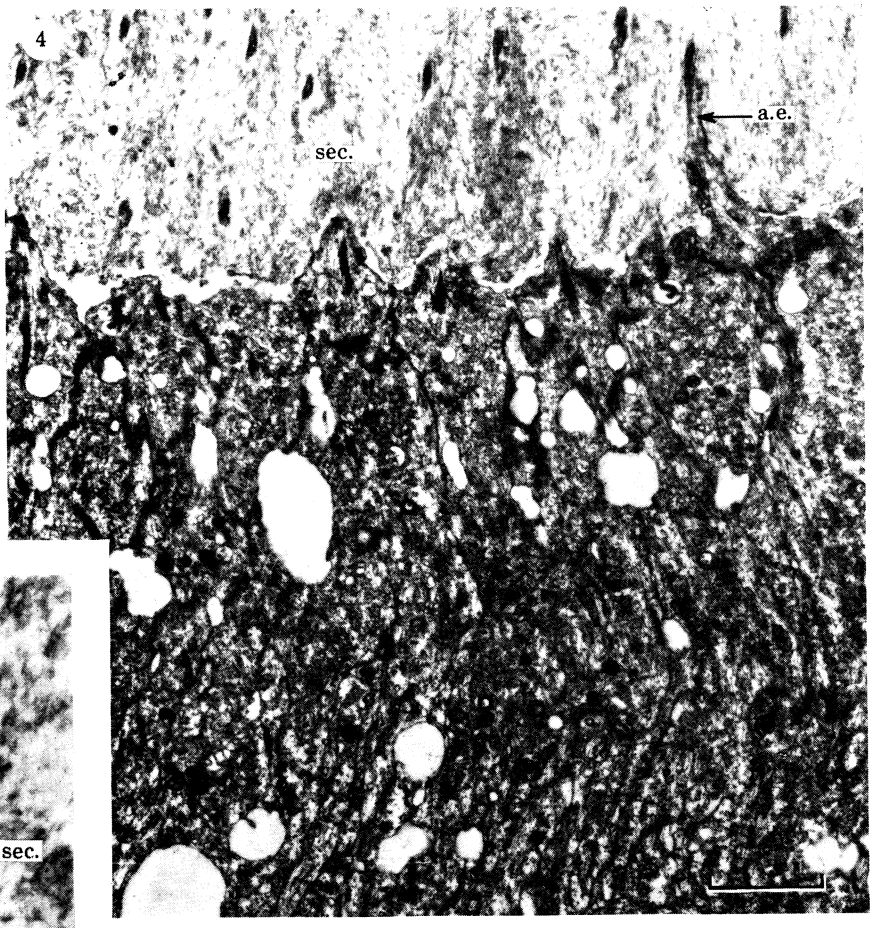
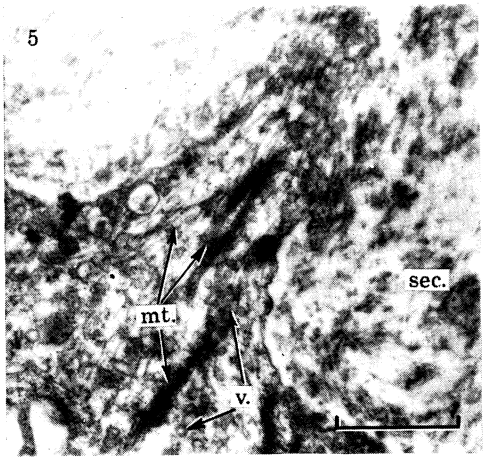
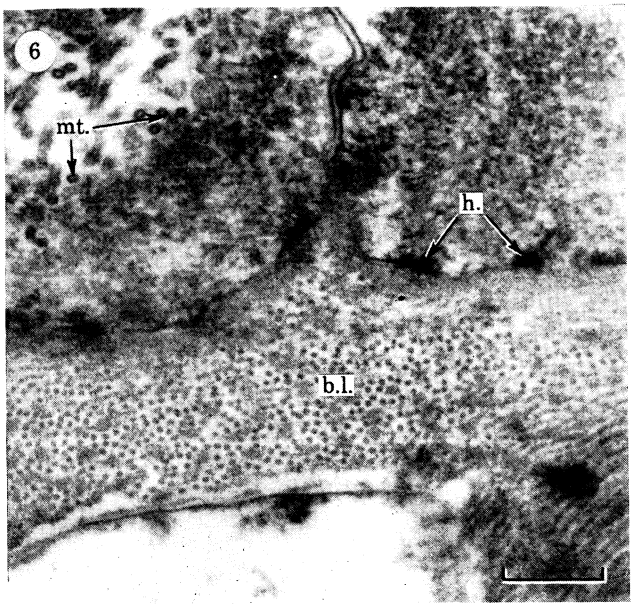


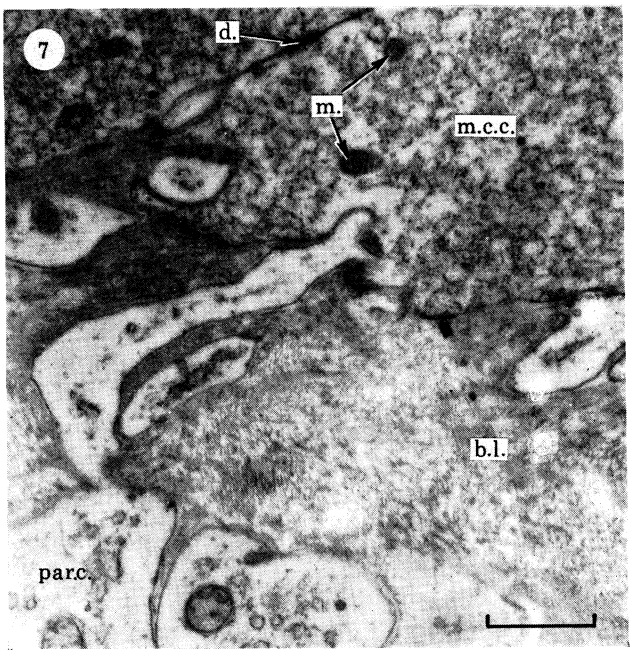
FIGURE 1. *B. balanoides.* (*a*) Diagrammatic section through the oviducal gland illustrating the three main regions. Scale bar, 120 μm. (*b*), (*c*) Views of the Plasticine model constructed from serial sections; the broken lines represent the position of the section drawn in (*a*).

(*Facing p.* 152)

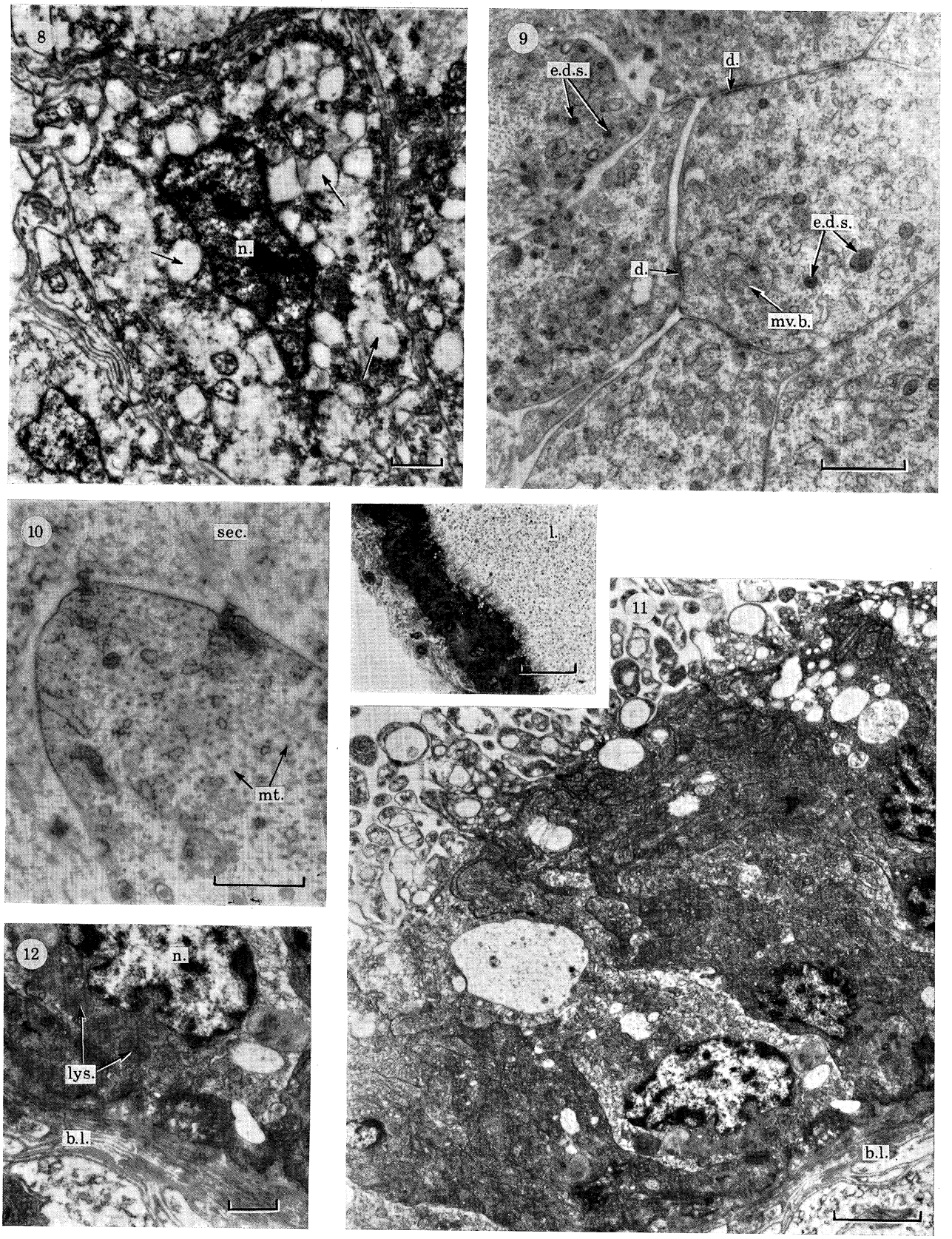




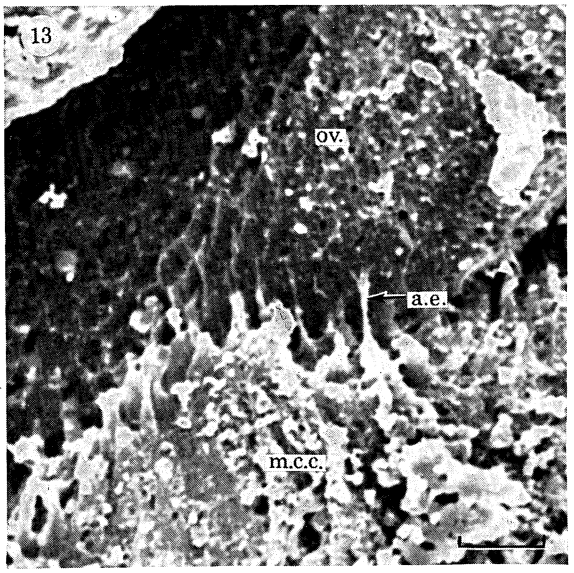


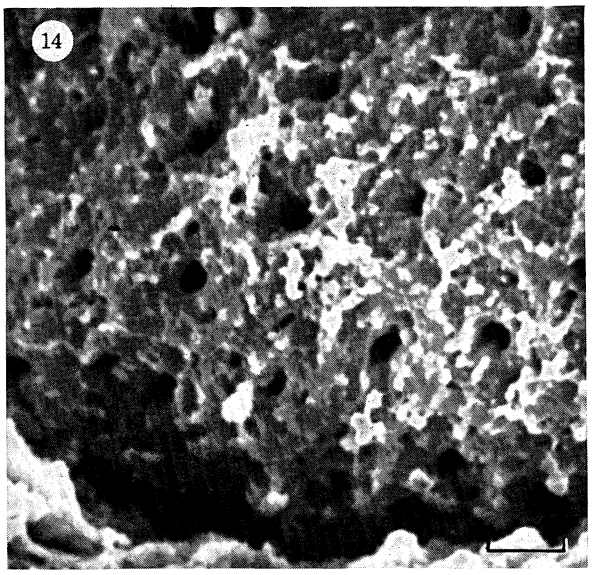


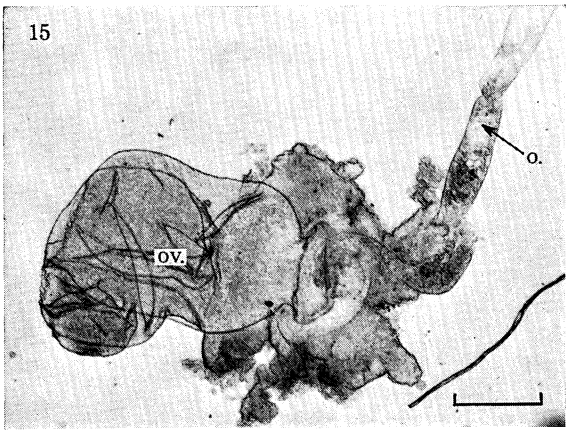
FIGURES 3–7. For description see p. 153.

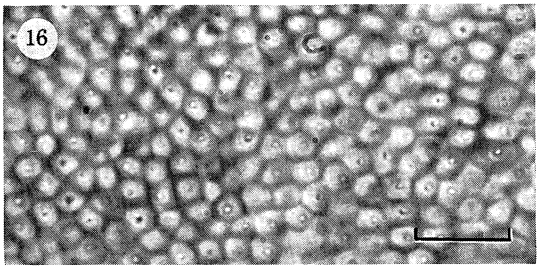


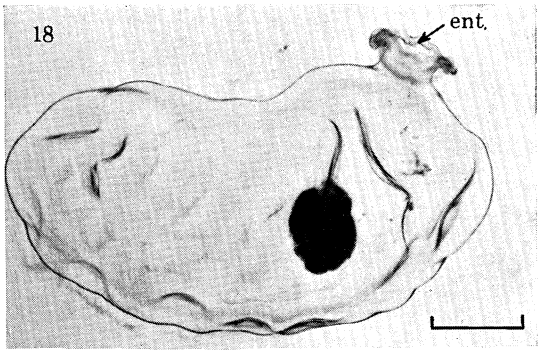
FIGURES 8–12. For description see p. 153.

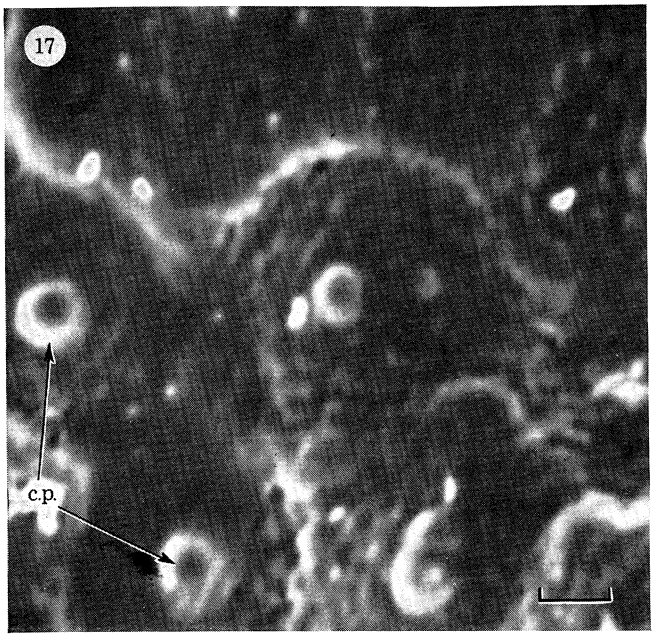


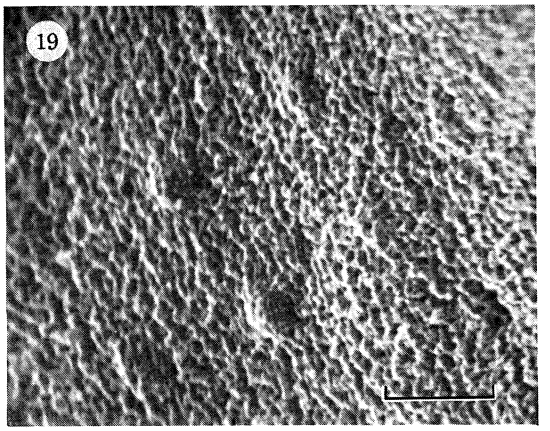




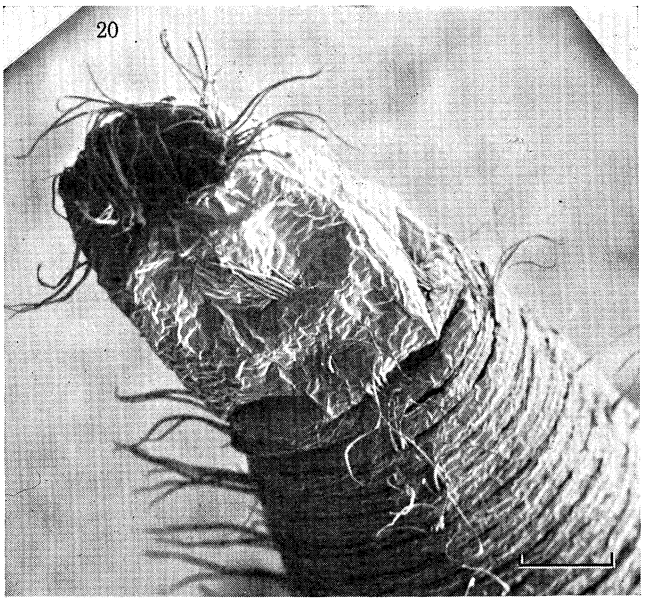


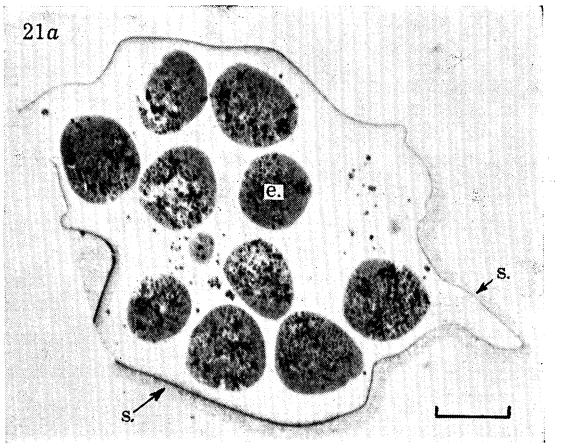


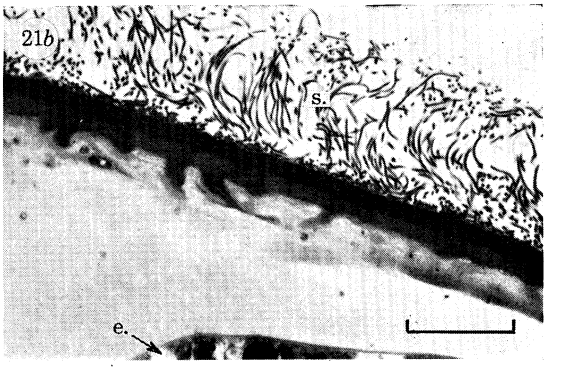


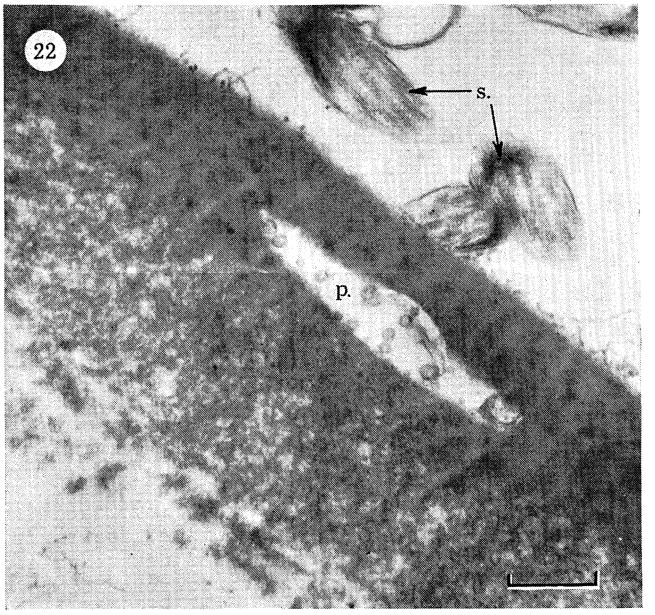


FIGURES 13–19. For description see p. 153.









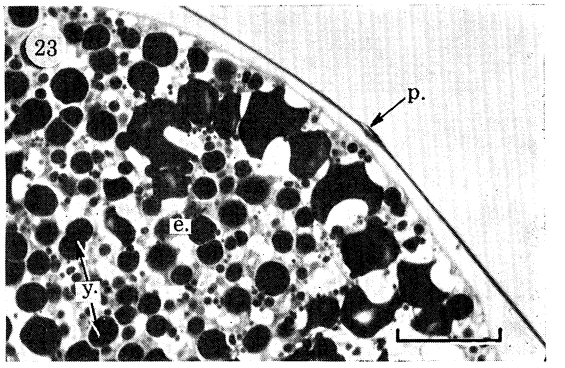


FIGURE 20. *B. hameri*: scanning electron micrograph showing the tip of a penis with its array of setae. Three of the four rows of setae that extend down the penis can also be seen. Scale bar, 50 μm.

FIGURE 21. *B. balanoides*: (*a*) 1 μm Araldite section through an early egg mass, showing the mass of surrounding sperm. Scale bar, 100 μm. (*b*) Higher magnification, showing the ovisac wall and sperm. Scale bar, 20 μm.

FIGURE 22. *B. balanoides*: electron micrograph of a section across a partially stretched ovisac wall, showing cytoplasmic debris within a pore. Scale bar, 0.3 μm.

FIGURE 23. *B. balanoides*: 1 μm Araldite section through a late egg mass, showing the thinned ovisac wall with a pore and underlying egg. Scale bar, 50 μm.

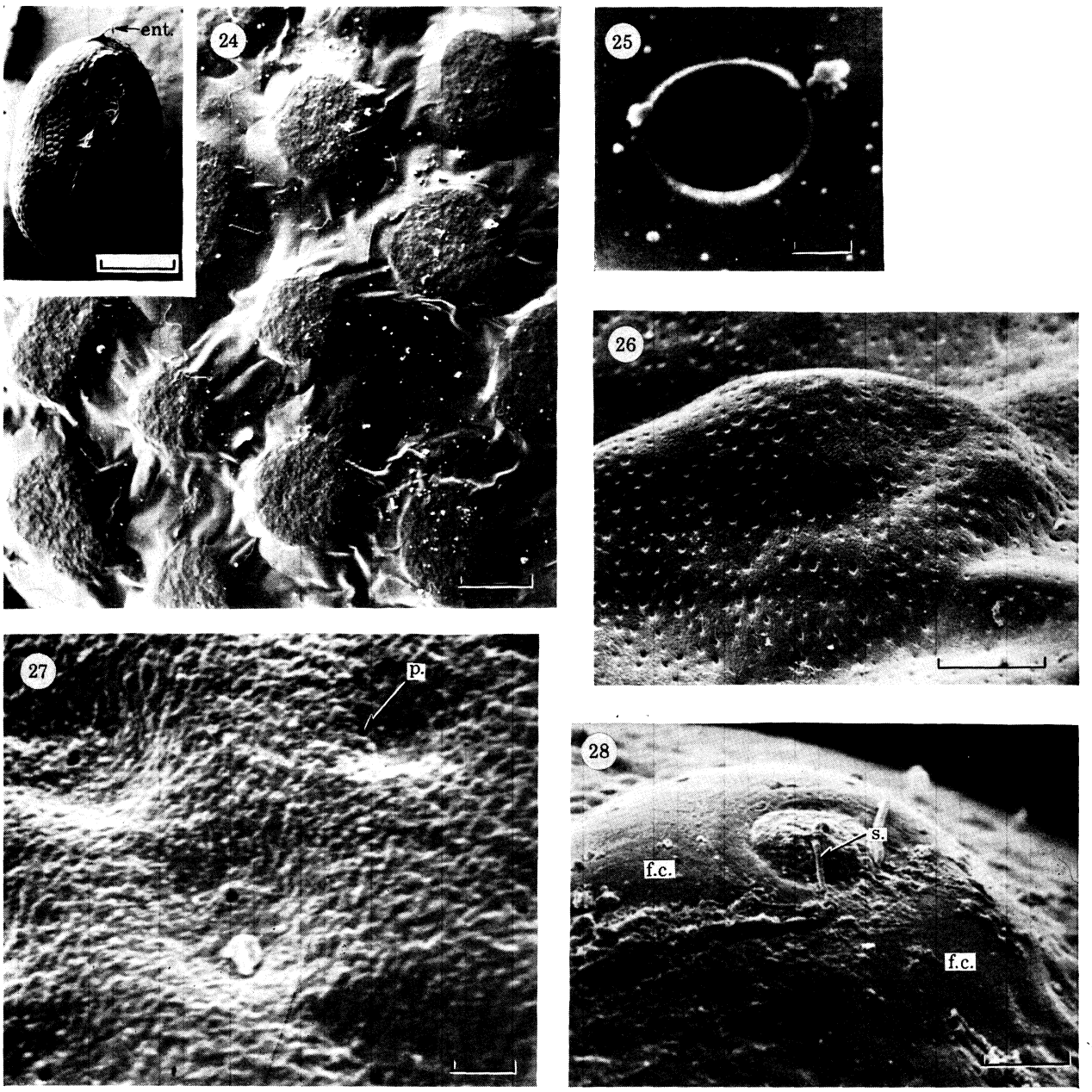


FIGURE 24. *B. balanoides*: scanning electron micrograph showing the areas of contact between eggs and ovisac wall in a fully formed egg mass. Scale bar, 50 μm. Inset: the same whole egg mass (scale bar, 1 mm). The slight damage occurred during preparation.

FIGURE 25. *B. balanoides*: scanning electron micrograph showing a pore in the ovisac wall surrounding a fully formed egg mass. Scale bar, 1 μm.

FIGURE 26. *B. hameri*: scanning electron micrograph showing the pitted appearance of the outer surface of an ovisac fixed during early egg laying. Scale bar, 50 μm.

FIGURE 27. *B. hameri*: higher magnification micrograph of an ovisac fixed during early egg laying, showing the outer ovisac surface with pores within pits. Scale bar, 2 μm.

FIGURE 28. *B. hameri*: scanning electron micrograph showing the fertilization cone thrown up by a fertilized egg. Scale bar, 5 μm.

boundary is a single central protrusion (0.6–0.8 μm in diameter), which has no discernible ultrastructure. These protrusions are the bases of the cytoplasmic extensions that have broken away from the main chamber cell apices as the ovisac is released into the gland lumen.

B. hameri

Unlike the ovisacs of *B. balanoides*, which become contorted when released from the gland cells, those of *B. hameri* tend to retain the gland shape although they too are contracted (figure

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Description of plate 2

Figure 3. *B. balanoides*, 19 October: 1 μm Araldite section showing main chamber cells with apical cytoplasmic extensions. Scale bar, 20 μm.

Figure 4. *B. balanoides*, 19 October: transmission electron micrograph showing the apical cytoplasm of main chamber cells. Scale bar, 3 μm.

Figure 5. *B. balanoides*, 19 October: electron micrograph showing the base of a cytoplasmic extension of a main chamber cell. Scale bar, 1 μm.

Figure 6. *B. balanoides*, 19 October: electron micrograph showing various basal cytoplasmic features of main chamber cells. Scale bar, 0.2 μm.

Figure 7. *B. balanoides*, 19 October: electron micrograph showing direct cell-to-cell contact between a main chamber cell and underlying parenchyma cells. Scale bar, 1 μm.

Description of plate 3

Figure 8. *B. balanoides*, an electron micrograph of a section through a parenchyma cell containing crystals (arrows). The crystals have in fact been leached away during preparation. Scale bar, 1 μm.

Figure 9. *B. balanoides*, 3 November: an electron micrograph of a section across the apical cytoplasm of main chamber cells. Scale bar, 1 μm.

Figure 10. *B. balanoides*, 3 November: electron micrograph of a section across the base of a cytoplasmic extension, showing the electron-dense secretion passing out to form part of the ovisac wall. Scale bar, 0.5 μm.

Figure 11. *B. balanoides*, 25 November: electron micrograph of a section through main chamber cells. The ovisac has been shed and the cells are actively shedding secretion and portions of cytoplasm apically. Inset: 1 μm section (light microscope), showing the extent of such apical secretion within the lumen of the oviducal gland. Scale bar, 3 μm.

Figure 12. *B. balanoides*, 25 November: electron micrograph showing the presence of a large number of lysosomes in the basal cytoplasm of a main chamber cell. Scale bar, 1 μm.

Description of plate 4

Figure 13. *B. balanoides*: scanning electron micrograph showing apical cytoplasmic extensions connecting main chamber cells to the newly formed ovisac. Scale bar, 5 μm.

Figure 14. *B. balanoides*: scanning electron micrograph of the outer ovisac surface showing the pores left when the cytoplasmic extensions have been pulled out. Scale bar, 2 μm.

Figure 15. *B. balanoides*: an ovisac as seen with the light microscope. The rim around the entrance to the ovisac has remained firmly attached to anchor cells. Scale bar, 20 μm.

Figure 16. *B. balanoides*: a light microscope of an ovisac wall, showing the imprints of the apical surfaces of main chamber cells. Each imprint has a pore at its centre. Scale bar, 2 μm.

Figure 17. *B. balanoides*: scanning electron micrograph of the outer surface of an unstretched ovisac, showing imprints of the apical surface of main chamber cells. The central protrusions are the remnants of the cytoplasmic extensions. Scale bar, 1 μm.

Figure 18. *B. hameri*: an ovisac as seen with the light microscope. Note the relatively narrow entrance and the large secretion mass within the ovisac. Scale bar, 30 μm.

Figure 19. *B. hameri*: scanning electron micrograph showing the regular protrusions from the outer surface of an unstretched ovisac. Scale bar, 5 μm.

18, plate 4). With the s.e.m. the imprints of cell boundaries cannot be seen on the outer ovisac surface, only regularly spaced protrusions (*ca.* 1.25 μm diameter) being apparent (figure 19, plate 4). The ovisacs of *B. hameri* also have elastic properties at this stage.

6. Description of the copulation process and egg laying of *B. hameri*

B. hameri is a cross-fertilizing hermaphrodite. Initially the 'acting female' and 'acting male' (hereafter referred to as female and male respectively) extend and retract their cirri rhythmically and at times the penes of both are extended. The female then becomes distinguished by the fact that the cirri no longer extend fully and the penis is permanently retracted. The male continues rhythmically to extend and retract the cirri fully, and periodically the penis is extended. When the penis is extended, for some time it is merely waved around aimlessly in the water, but eventually contact is made with neighbouring individuals, apparently by chance. The tip is brought down and searching behaviour is initiated. This behaviour is essentially random, because if a female is not found the male will eventually retract the penis. However, if a female is located she appears to be immediately attractive to the male. Such attraction is likely to be chemical, with the secretions perhaps of the cirral glands (Walley 1967) being sensed by some of the array of setae protruding from the penis (figure 20, plate 5).

Once a female is located the penis of the male is inserted between the bases of the cirri of the female and its tip explores various areas within the mantle cavity. After 3–4 min, during which time both partners are presumably mutually stimulated, the female retracts the cirri fully and the penis of the male is trapped at the tergo-scutal junction of the female's opercular valves as they close. The female rocks the opercular valves several times over the next 3–4 min, after which the male begins to retract and expand the cirri intermittently; even when the cirri are fully retracted the opercular valves of the male remain agape. The female continues intermittently to rock the opercular valves up and down, until, approximately 12 min after trapping the penis, she shuts the valves tight. At the same time the male retracts the cirri fully and closes the opercular valves, leaving only a small gap for the penis. The penis is then released by the female and shortly afterwards she expels some of the inseminated sperm from the mantle cavity into the surrounding seawater, indicating that insemination has taken place.

To study semen transfer more closely a copulating pair were removed from the tank and placed in a crystallizing dish, filled with seawater, under a stereomicroscope. They remained joined by the penis and were, as far as could be determined, unaffected by the transfer. Under the microscope it was observed that semen transfer is not a continuous process, but happens intermittently. Initially, as the female rocks the valves they will open slightly and then close, allowing semen to pass only when open. At the same time the male may be presumed to generate a high haemolymph pressure which, together with contractions of the muscles around the vesiculae seminales, will ensure semen transfer, when allowed, along the ductus of the penis. High haemolymph pressure is indicated by the permanent extension of the cirri at this time (Crisp & Southward 1961). After 3–4 min, when a certain volume of semen has been transferred, the male must begin to transport more semen along the vesiculae seminales towards the penis. Haemolymph pressure varies during this process, as indicated by cirral movement and the changing turgidity of the penis itself. When semen is being inseminated, however, the cirri of the male are always fully extended, indicating high haemolymph pressure at this time.

The end of copulation is marked when the male retracts the penis fully and closes the opercular aperture. At this time the opercular valves of the female gape, and intermittently some of the inseminated sperm are expelled from the mantle cavity. This activity lasts for about 3 min. Towards the end of this period the male begins again to beat the cirri rhythmically, but the penis remains retracted. The female then rocks the opercular valves violently upwards from side to side, then shuts tight. She remains tightly shut for about 30 min, after which she again rocks the opercular valves violently, then leaves a slight opercular gape through which further sperm are expelled. Since females dissected at this stage had completed egg laying it may be presumed that the $\frac{1}{2}$ h during which the opercular valves remain closed represents oviposition. During this process the male partner continues to extend and retract the cirri, but the penis is never extended.

7. A comparison of the copulation process in *B. hameri* and *B. balanoides*

Observations, over several seasons, of the copulation process in *B. balanoides*, also a cross-fertilizing hermaphrodite, taken together with observations by Clegg (1955) and by Barnes *et al.* (1977) on the same species can best be compared with those of the process in *B. hameri* by reference to table 1. The sequence of events in *B. balanoides* is agreed upon by all workers, but there is considerable variation in the timing.

8. The ovisac during egg laying

B. balanoides

Walley (1965) showed that the ovisacs of *B. balanoides* gradually distend as eggs are laid into them, first expelling the oviducal gland fluid (Walley *et al.* 1971), then themselves being forced out from the oviducal glands into the mantle cavity. Such early egg masses become surrounded by a mass of motile sperm (figure 21*a*, plate 5); at this time the sperm have not yet passed across the ovisac wall in large numbers (figure 21*b*, plate 5). The ovisacs remain attached within the glands until the egg masses are completely formed. S.e.m. observations of ovisacs fixed during the early stages of egg laying show that the small protrusions (broken off cytoplasmic extensions) have now become regularly spaced holes or pores (Walker 1977*a*), 0.7–1.6 μm in diameter and 5–6 μm apart. In section these pores are seen to contain the degenerating remnants of the cytoplasmic extensions: microtubules and small vesicles (figure 22, plate 5).

Walker (1977*a*) and Klepal *et al.* (1977) have already shown that sperm use these pores to pass through the ovisac wall before fertilizing the enclosed eggs.

As the eggs pass into an ovisac its wall will stretch in all directions, so enlarging the diameter of the pores. Stretching progressively reduces the wall thickness from the initial 20 μm down to about 1 μm when all the eggs have been laid (figure 23, plate 5). Parts of the ovisac wall surrounding a fully formed egg mass make contact with the underlying eggs (figure 24, plate 6), the intervening areas being contact-free. Klepal *et al.* (1977) assumed that the pores close as the ovisacs expand, partly because they were unable to find pores in the ovisac wall surrounding fully formed egg masses. This is certainly *not* the case, for enlarged pores (up to 6 μm in diameter) are readily found in the ovisac wall surrounding fully formed egg masses (figure 25,

TABLE 1. COMPARATIVE OBSERVATIONS OF THE COPULATORY PROCESS AND EGG LAYING OF *B. BALANOIDES* AND *B. HAMERI*

stage	*B. balanoides*	*B. hameri*
1. exploration	The penis of the male is extended and curled downwards so that the tip repeatedly touches the substratum and more particularly other individuals. The penis is extended for short periods only if a female cannot be located. Once retracted a period of regular cirral beating intervenes before the penis is extended again.	The penis of the male is extended and waved in the water. Eventually the penis is curled downwards, the tip exploring the substratum and other individuals. The penis is extended for long periods and can be extended again immediately after retraction. A male unable to locate a female will release semen into the water.
2. location	The male locates a female by random searching with the penis, but once contact is made the female appears to be chemoattractive.	
3. copulation	Semen is transferred following acceptance of a penis by the female; more than one male may be involved. There are *multiple inseminations*. The mean time of each insemination is 38 s (44 s (Clegg 1955); 40 s (Barnes *et al.* 1977)). The male(s) locates the female very easily following a first insemination. The penis is immediately withdrawn if disturbed, even by shading. Sperm are deposited as gelled masses in the mantle cavity of the female.	A single penis is accepted, then trapped by the opercular valves of the female as they close. Semen transfer is intermittent, taking place only when the female opercular valves gape slightly. The penis is not withdrawn even if disturbed. There is thus a *single insemination*, which lasts about 12 min. Sperm are deposited as gelled masses among the bases of the cirri.
4. before egg laying	Following the 4–7 inseminations (2–14 (Clegg 1955); 6–8 (Barnes *et al.* 1977)), which occur over a 30–90 min period, the female raises the opercular valves, leaving a large gape. After 3 min the opercular valves shut tight and 16 min later the valves are rocked violently and some motile sperm are expelled from the mantle cavity. (Barnes *et al.* (1977) observed this period to vary from 10 to 30 min.)	The penis is released by the female. The opercular valves of the female gape after 3 min and motile sperms are expelled from the mantle cavity. The valves are then rocked violently before shutting tight.
	Inseminated sperm require oviducal gland fluid for activation and once activated are motile for only 5–6 min (Walker 1977*b*).	Inseminated sperm become active in seawater, motility lasting for 12–13 min.
5. egg laying	Eggs are laid over a period that may extend to 45 min (Barnes *et al.* (1977) give a 60–90 min period).	Eggs are laid over a 30 min period.
6. after egg laying	Egg laying is completed when the female rocks the opercular valves violently and passes out the remaining gelled sperm masses from the mantle cavity. These tend to lie on or around the opercular valves.	As for *B. balanoides*, except that the sperm remaining in the mantle cavity are not in the form of gelled masses, being expelled freely suspended in mantle fluid.

plate 6), although pores situated in those parts of the wall making contact with the eggs will be blocked by the surface of the eggs.

Calculations based on observed dimensions of ovisacs, pores, etc. yield some interesting information. If one assumes an ovisac to be a sphere, then the surface area of an unstretched ovisac of 0.75 mm diameter is 1.77×10^6 μm². Most of this surface area will have pores, only a small part close to the ovisac entrance being without (Klepal *et al.* 1977). The part without pores was estimated by Klepal *et al.* to be 10% of the total surface area, which gives a surface area with pores of about 1.6×10^6 μm².

From sections, the diameter of the main chamber cells producing the sac is of the order of 5 μm, giving an apical cell surface area of approximately 20 μm². Alternatively, by observing unstretched ovisac walls with the light microscope the number of cell imprints in a given area can be counted directly and the apical cell surface area is calculated as 22.7 μm², in close agreement with the earlier figure. As a cell produces a single cytoplasmic extension (pore), there are therefore approximately $(1.6 \times 10^6)/20 = 0.8 \times 10^5$ pores per ovisac.

B. hameri

The fine structure of the ovisacs of *B. hameri* reveals features that are similar to those of *B. balanoides*. At the early egg-laying stage the outer sac wall appears pitted (figure 26, plate 6) and within each pit (about 2 μm in diameter) is a pore (figure 27, plate 6), which is about 0.2 μm in diameter. The pores are 5–6 μm apart. As for *B. balanoides*, enlarged pores (up to 0.5 μm in diameter) are readily found in the sac wall surrounding fully formed egg masses.

Calculations similar to those carried out for *B. balanoides* give a figure of 2.4×10^5 pores per unstretched ovisac of 1.5 mm diameter.

9. Sperm activation

It is well documented that inseminated sperm of *B. balanoides* are inactive (Barnes & Crisp 1956; Walley *et al.* 1971). Clearly the time at which motility is initiated is critical in this species because the sperm are motile for only 5–6 min (Walker 1977*b*). Walley *et al.* (1971) demonstrated that oviducal gland fluid that accumulates in the glands of the female before copulation contains a factor or factors responsible for initiating such motility. The fluid is expelled into the mantle cavity as the ovisacs distend with eggs, so that egg laying and initiation of sperm motility are closely linked. Sperm taken from the vesiculae seminales, however, are not activated by oviducal gland fluid (Walley *et al.* 1971).

Inseminated sperm of *B. hameri* are always motile when collected. A large proportion of vesicula sperm also become motile when placed in seawater. Oviducal glands of *B. hameri* do not become highly swollen with oviducal gland fluid in the female before copulation.

10. Fertilization

Oocytes enter the ovisacs at egg laying, in the metaphase stage of the first meiotic division, and it is in this condition that they are fertilized (Walley *et al.* 1971). Fertilization is marked by the eruption of a fertilization cone at the point where the sperm enters (figure 28, plate 6).

Sperm entry has two effects. First, the fertilization membrane (Groom 1894; Austin *et al.* 1958; Walley 1965; Klepal *et al.* 1979) lifts from the egg surface and presumably acts as a barrier helping to prevent polyspermy, and secondly the maturation divisions are completed.

11. Discussion

Most crustaceans brood their eggs for varying periods. Eggs may be directly attached to certain appendages (Nebaliacea, Decapoda), contained within a brood pouch or chamber (Cladocera, Amphipoda, Isopoda, Tanaidacea, Mysidacea, Cumacea), or retained within an ovisac (Copepoda, Cirripedia). Barnacles have ovisacs that remain attached to the body as the eggs are laid into them, but when egg laying is completed the fully formed egg masses are released to lie in the mantle cavity. In some pedunculate barnacles the released egg masses become attached to the mantle lining at the ovigerous frena (Darwin 1851). Some crustaceans have internal fertilization, but for most, including barnacles, since the mantle cavity is continuous with the external medium, it is strictly external. Barnacle sperm must pass thence through the ovisac wall before fertilization can take place. There is a restricted period of 2–3 weeks during which copulation takes place in both species of *Balanus* considered here, and for *B. balanoides* the timing of the event at any one locality depends to a large extent on the position on the shore (Crisp 1959).

The copulation process of sublittoral *B. hameri* described in the present study is different in several important respects from that of littoral *B. balanoides*. In *B. hameri* only a single insemination is needed for egg laying to commence, the penis being held tightly between the closed opercular valves of the female. In *B. balanoides* more than one male may be involved and the female leaves the opercular valves gaping, so permitting several inseminations at the same time; even if only a single male is involved, multi-inseminations seem to be essential for egg laying to commence. Specimens of *B. hameri* collected for the present study were dredged up from a *Modiolus modiolus* bed; at this site barnacle settlement takes place directly on to the mussel shells or the shell plates of already attached *B. hameri*. Thus, although *B. hameri* is only found on a minority of mussels in this population ($< 3\%$, S. J. Hawkins, personal communication), there are usually several barnacles present on a mussel, but there is not space for large numbers. *B. hameri* therefore has a limited chance of finding a partner. Interestingly, some of the small young individuals in their first season produce no ovaries, and so they act only as males; such protandry must increase the opportunity for cross fertilization. *B. balanoides*, on the other hand, is normally more crowded; so at any one time the female can behave with the expectation that more than one male will make contact. If several males successfully inseminate a female, gene flow is enhanced.

Another difference is the inactivation in seawater of inseminated sperms of *B. balanoides* compared with the activity induced in those of *B. hameri* by seawater. This difference stems from the number of inseminations, the time over which insemination(s) occurs and the time taken to lay the eggs. In *B. balanoides* multi-inseminations take place over a prolonged period (> 30 min), so that deposition of inactive sperm must be a prerequisite, because, once activated, they remain motile for only 5–6 min. The single insemination by *B. hameri* lasts about 12 min and the sperm are motile for 12–13 min. Inseminated sperm of both species are deposited as gelled masses. Sections show that secretory material released from the epithelial cells lining the vesiculae seminales is likely to form the matrix of such masses. The gelled masses ensure the

liberation of motile sperm over a protracted period, by allowing only those sperm on the immediate outside of a mass to be released. As egg laying in both species takes at least 30 min, sperm release must be staggered over this period if all the eggs are to be fertilized. It has still to be shown conclusively whether the gelled masses break down progressively by a biochemical process or whether they do so by the purely mechanical process of the sperm actively swimming away. The latter process seems more likely because inseminated sperm masses taken for *B. balanoides* immediately after deposition remain intact when placed in seawater.

In summary, sublittoral *B. hameri* requires only a single insemination and no activating factor needs to be produced by the animal for initiating sperm motility. Littoral *B. balanoides*, on the other hand, requires more than one insemination for egg laying to commence, which, together with the delay between the final insemination and the start of egg laying, makes it essential that some specialized control for initiating sperm motility operates.

The timing of the initiation of sperm motility in *B. balanoides* is all important. Walley *et al.* (1971) clearly showed that the fluid accumulated in the oviducal glands contains the essential factor(s) required to initiate motility of inseminated sperm. Barnes *et al.* (1971) believed, though without direct evidence, that the passage of sperm through the penis at insemination was a necessary and sufficient condition for the initiation of motility. Later Klepal *et al.* (1972) modified their earlier conclusion in the light of the findings of Walley *et al.* and predicted that passage through the penis plus the effect of oviducal gland fluid were necessary for full sperm motility.

Walker (1977*b*) has shown recently that motility of *B. balanoides* sperm can be induced artificially with ammonium ions. A further interesting discovery by Walker was the sperm-activating property of *B. balanoides* haemolymph. White (personal communication) has shown that adult *B. balanoides* excrete elevated levels of ammonia nitrogen over the period leading up to copulation, and so it is likely that the haemolymph ammonia nitrogen level is also elevated at this time. The most feasible explanation for the accumulation of the fluid in the oviducal glands would seem to be the movement of water and low molecular mass solutes (including ammonia nitrogen) from the haemolymph, across the oviducal gland epithelium. The mechanism for such movement, however, is not obvious. Riegel (1970) propounded the 'formed body' theory for transepithelial fluid movement, which has certain attractions in explaining the accumulation of oviducal gland fluid. In the theory, 'formed bodies', which are membrane-bound bodies with contents having high hydrolytic activity, are released apically from epithelial cells and become osmotically active. An osmotic gradient is set up which results in a net flow of water into the bodies. Osmotic pressure increases in the spaces between the bodies, so that water, accompanied by solutes, moves across the epithelium into the spaces. In *B. balanoides*, after the ovisacs are formed and released, the gland cells rapidly retrogress, discharging secretion vesicles and portions of cytoplasm apically. Such 'formed bodies' probably swell up, then burst, resulting in the clear oviducal gland fluid.

As the epithelial cells of the main chamber of an oviducal gland develop, they more closely resemble cytologically the epidermal (hypodermal) cells of arthropods (see Neville 1975). Apical cytoplasmic extensions are known to form pore canals within cuticle (Wigglesworth 1948). In the barnacle, extensions of the main chamber cells form such pore canals, which eventually become the perforations of the ovisac wall. Bubel (1975) did not find pore canals in the mantle cuticle of adult *Elminius modestus*, while Walker & Lee (1976) found pore canals only in the exocuticle of the cypris larva of *B. balanoides*. The oviducal gland therefore produces

a rather specialized cuticular structure, the ovisac. The specialization of ovisacs is further borne out by their biochemical composition (Barnes & Blackstock 1977); analyses show that the ovisac wall contains protein but no chitin.

When fully formed, an ovisac is released from the main chamber cells to lie in the gland lumen. Such a release is likely to coincide with a moult, but this has not been confirmed. A female moults before copulating (Patel & Crisp 1961) and then moulting is inhibited for some time after oviposition; so the ovisacs are probably released at the moult preceding copulation. In insects an exuvial space forms as the old cuticle separates from the hypodermis at a moult and enlarges by dissolution of the endocuticular lamellae and fluid pressure (Zacharuk 1976); the fluid in this space is the moulting fluid. Barnacle oviducal gland fluid should perhaps be regarded as a modified moulting fluid.

Before egg laying, the ovisacs of both *Balanus* species are usually filled with secretion, which originates from the proximal chamber cells (see Walley 1965). Walley also surmises that this secretory material, released into the lumen of the oviducal glands before the ovisacs begin to form, may in some way provide the right 'chemical environment' for the subsequent formation of the ovisacs. Its further involvement may be to act as a chemoattractant for sperm. At egg laying, as each ovisac distends with eggs, the secretion is likely to be forced out through the pores in the ovisac wall, thereby attracting motile sperm, maintaining them close to the enlarging egg mass as well as possibly aiding pore location.

In the earlier s.e.m. study, Walker (1977*a*) showed that the filiform sperm (0.5 μm in diameter) of *B. balanoides* can easily pass through the pores (0.7 to 1.6 μm in diameter) of the ovisac wall surrounding an early egg mass, i.e. one containing relatively few eggs. However, though the filiform sperm of *B. hameri* are of the same diameter, the pores in the ovisac wall surrounding an early egg mass are only about 0.2 μm in diameter. Sperm are therefore unlikely to pass through these pores until the latter have enlarged; this happens in due course since the mean diameter of a pore in the ovisac wall surrounding a fully formed egg mass of *B. hameri* reaches 0.5 μm. These observations for *B. hameri* refute the suggestion of Klepal *et al.* (1977) that sperm enter the ovisacs before the arrival of the eggs, before the pressure of the incoming eggs and secretions stretch the ovisac wall and supposedly close the pores. The stretching of the ovisac wall with consequent enlargement of the pores (see Gruvel 1893) is essential for sperm passage in *B. hameri*, while for *B. balanoides* the conclusion reached by Walley *et al.* (1971), that as the ovisacs emerge from the glands distended with eggs they enter a bath of vigorously active sperm which then pass into the ovisac, is vindicated.

Before egg laying the ovisac wall is highly elastic; because it is composed only of protein (Barnes & Blackstock 1977), similarities with resilin, the elastic protein of insect cuticle (Andersen 1971), were looked for. With data from both studies the amino acid compositions were compared by means of the method of Marchalonis & Weltman (1971), and an $S\Delta Q$ value of 97 (leaving out glycine), which indicates relatedness, was obtained. As egg laying proceeds the sacs become progressively less elastic and more plastic, i.e. capable of being moulded.

After fertilization each egg throws off a fertilization membrane, which acquires stickiness so that eggs adhere to one another, forming cohesive masses. The main function of the ovisac, that of holding the eggs together and so preventing their extrusion from the mantle cavity in the respiratory water current, is therefore superseded. As incubation of the eggs proceeds the ovisacs slowly disintegrate. Barnes & Barnes (1977) believe that such disintegration takes place

enzymatically, the enzyme, ovisacase, being produced by the embryos at a certain stage in their development.

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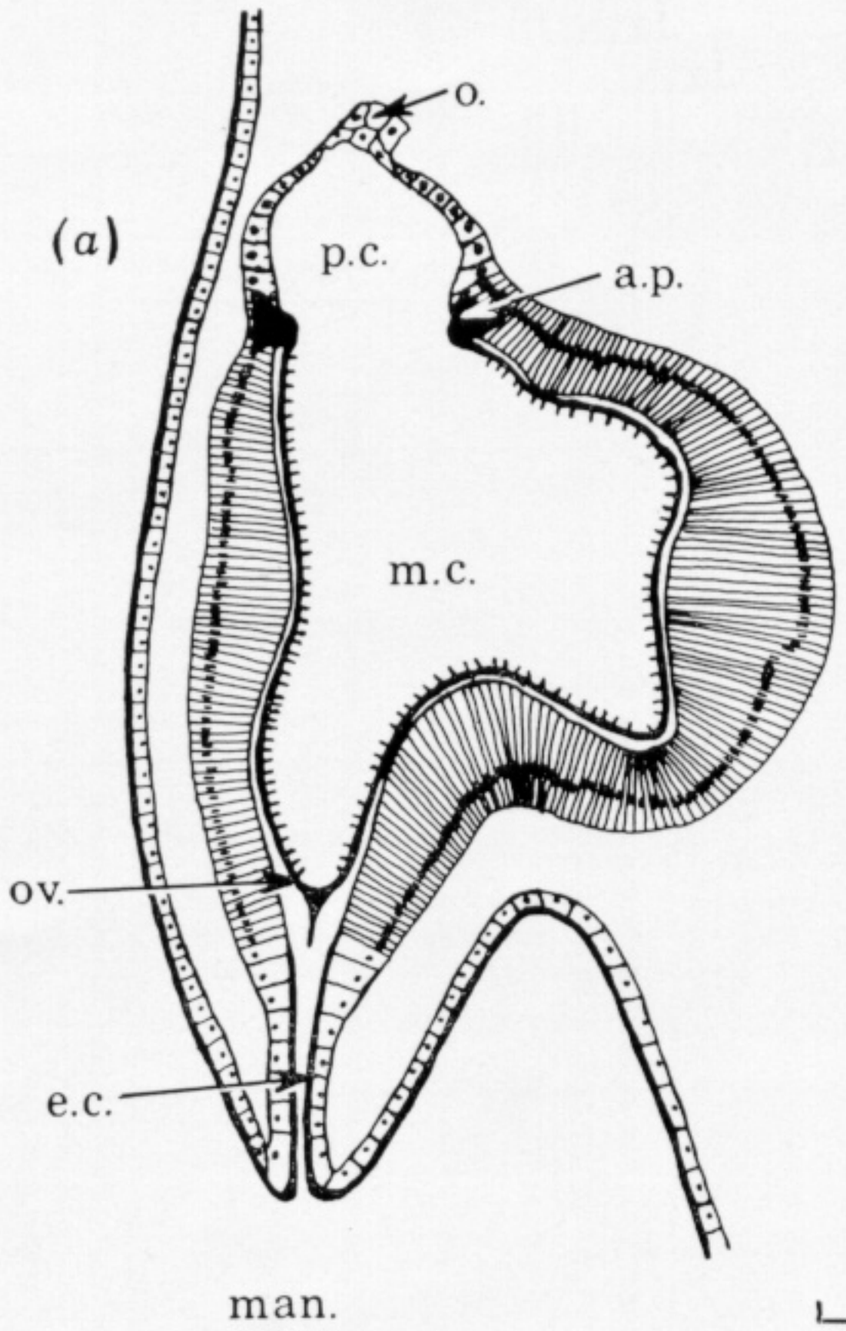
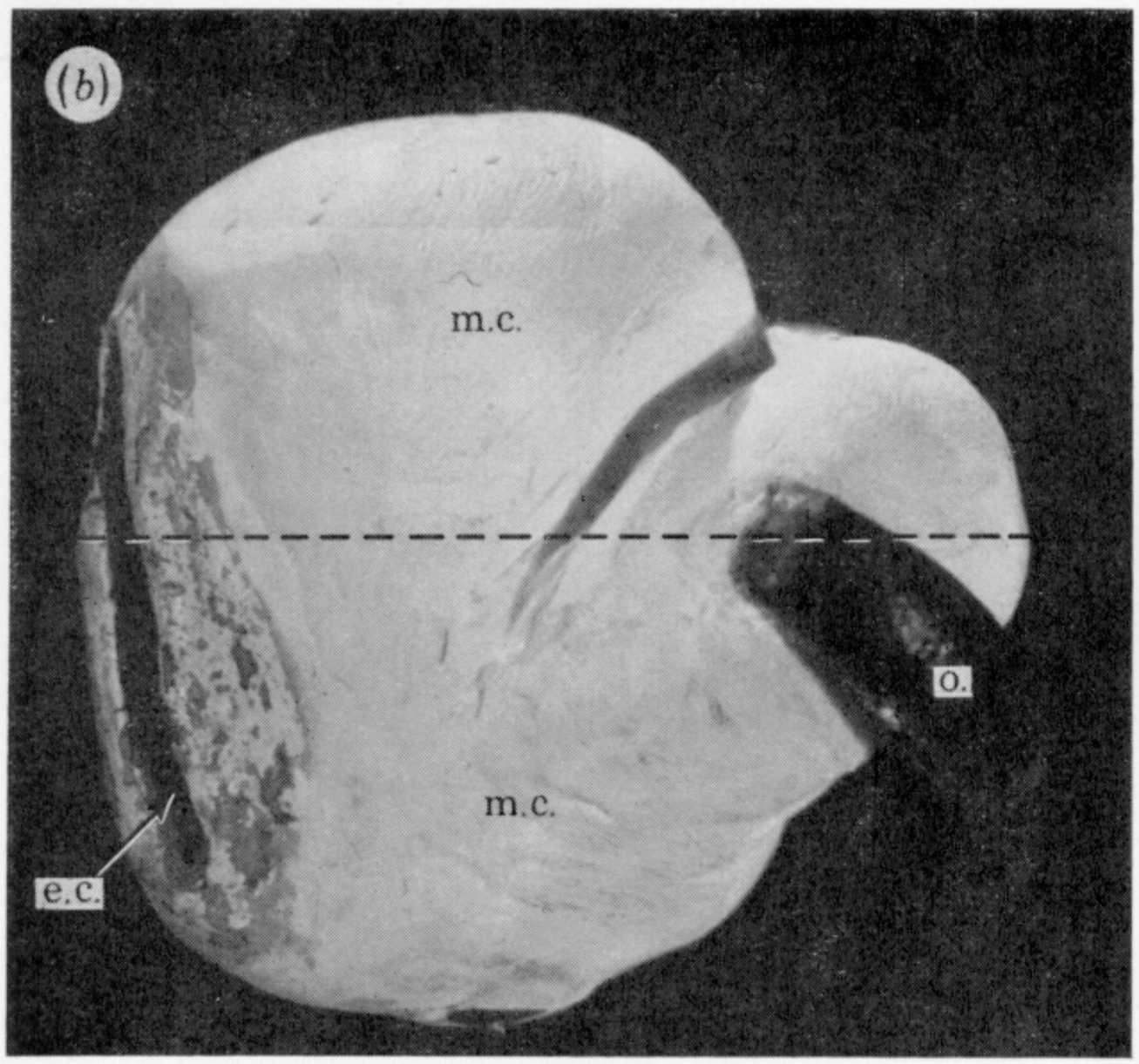
Key to abbreviations used in the figures

a.e.	apical cytoplasmic extension
a.p.	anchor point
b.l.	basal lamina
c.p.	central protrusion
d.	desmosome
e.	egg
e.c.	exit canal
e.d.s.	electron-dense secretion
ent.	ovisac entrance
f.c.	fertilization cone
G.b.	Golgi body
h.	hemidesmosome
i.z.	inner zone of ovisac wall
l.	lumen of oviducal gland
lys.	lysosome
m.	mitochondrion
man.	mantle cavity
m.c.	main chamber
m.c.c.	main chamber cell
mt.	microtubule
mv.b.	multivesicular body
n.	nucleus
o.	oviduct
o.g.f.	oviducal gland fluid
ov.	ovisac
o.z.	outer zone of ovisac wall
p.	pore
par.c.	parenchyma cell
p.c.	proximal chamber
r.e.r.	rough endoplasmic reticulum
s.	sperm
sec.	secreted material forming the ovisac wall
v.	vesicle
y.	yolk granule





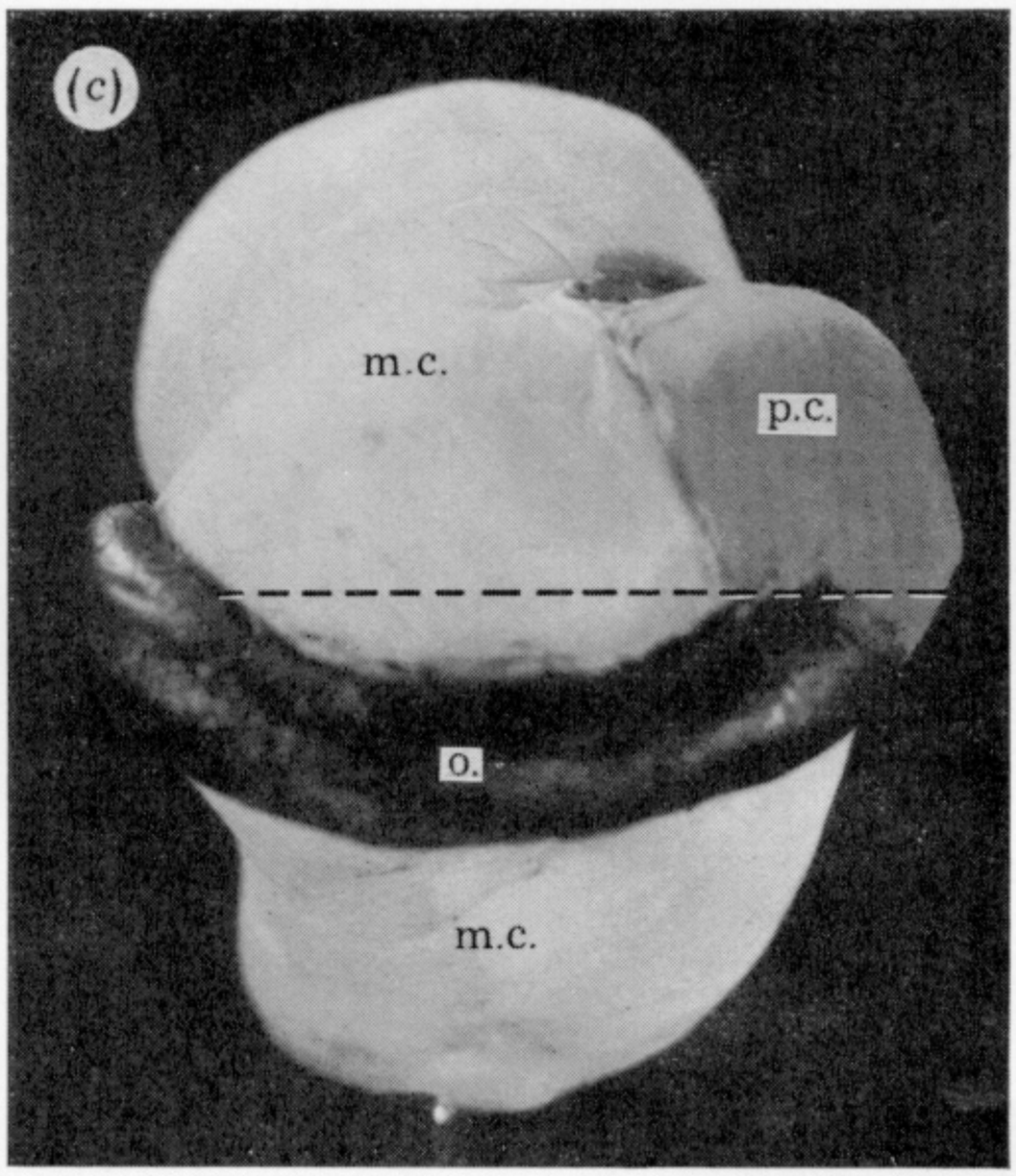
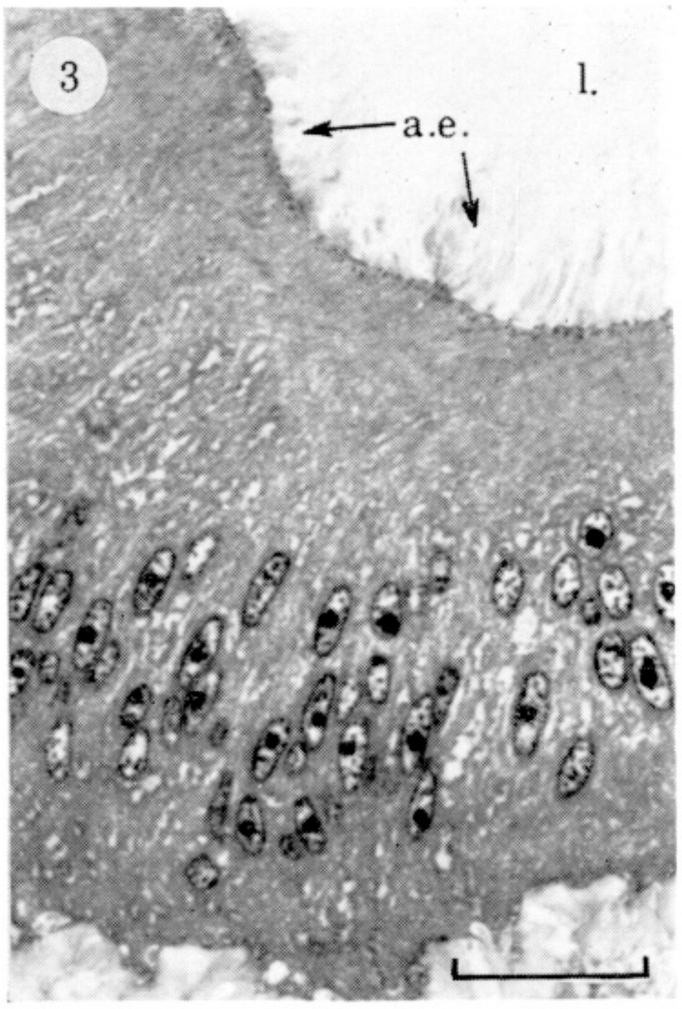
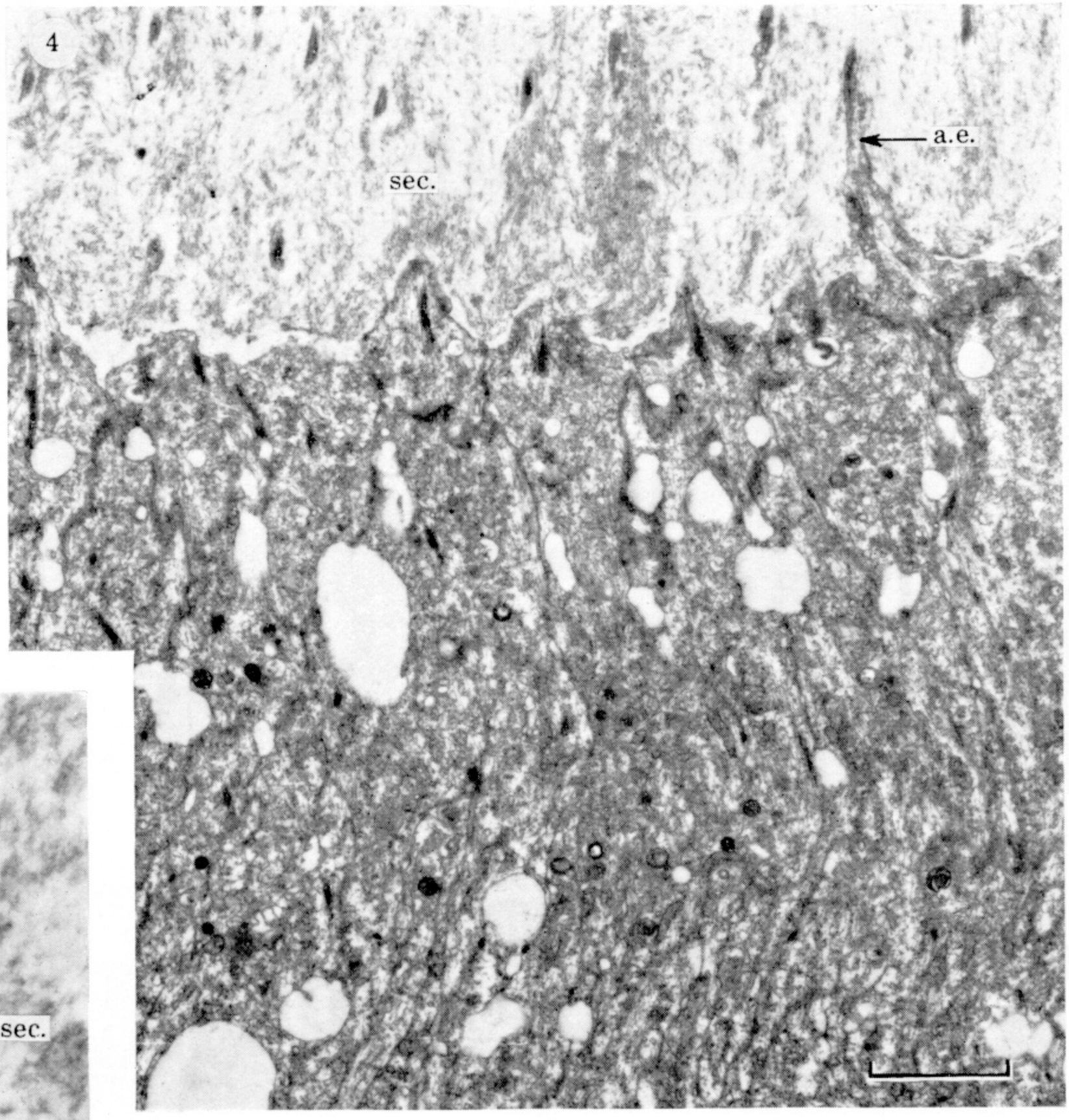
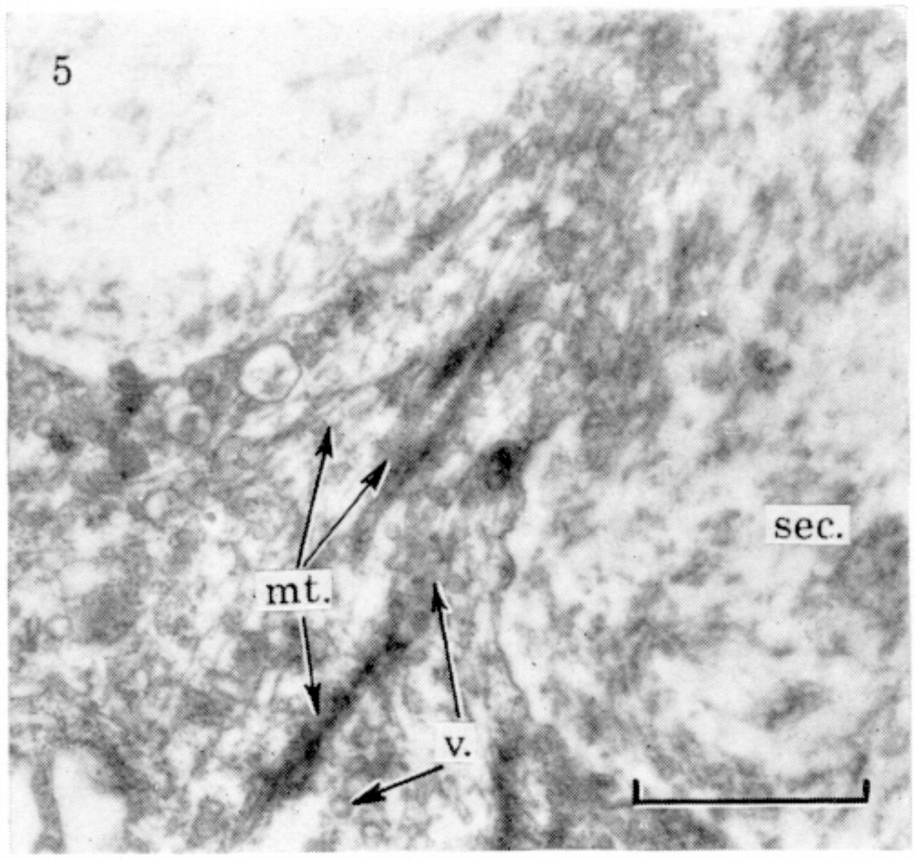
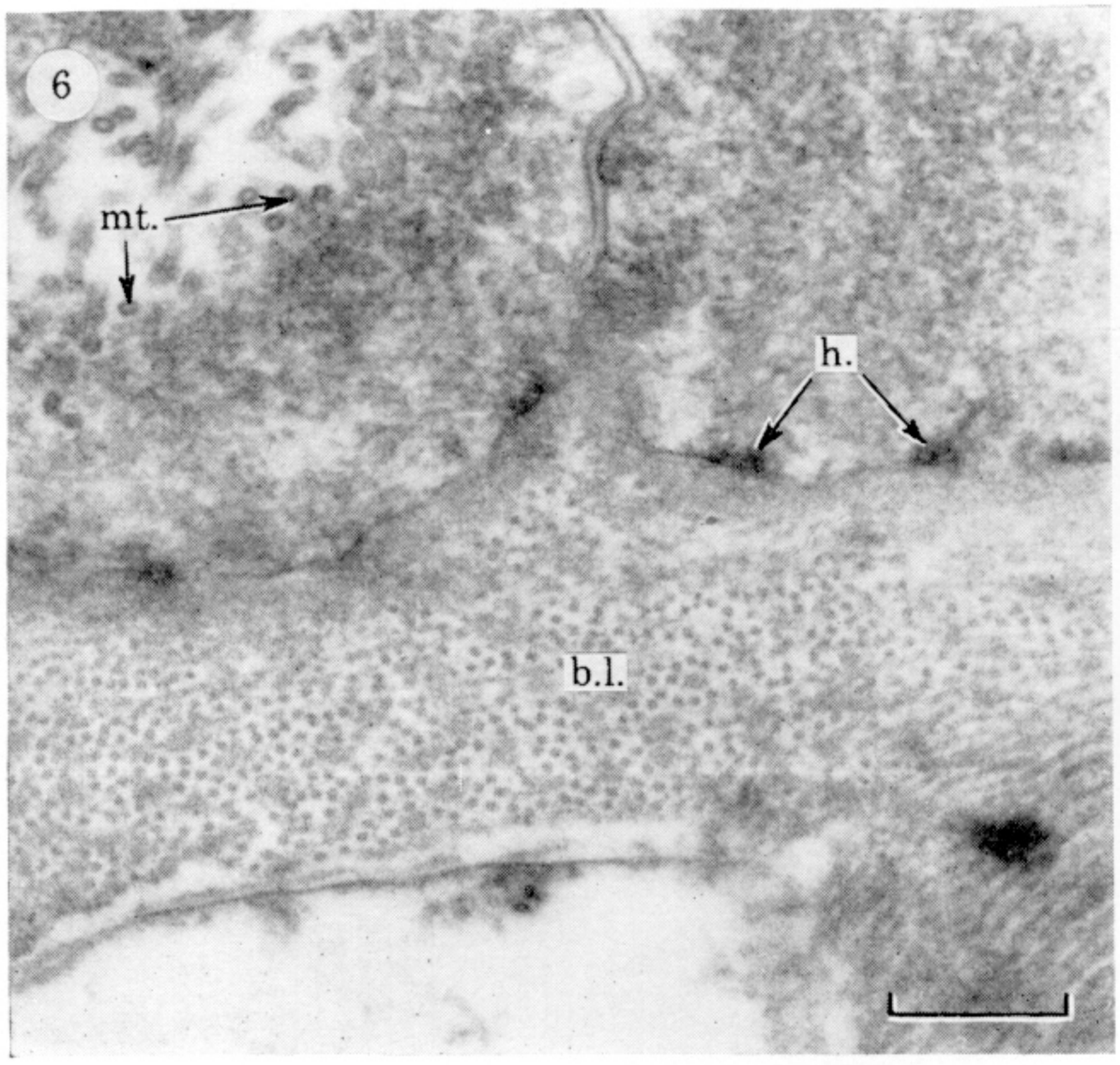


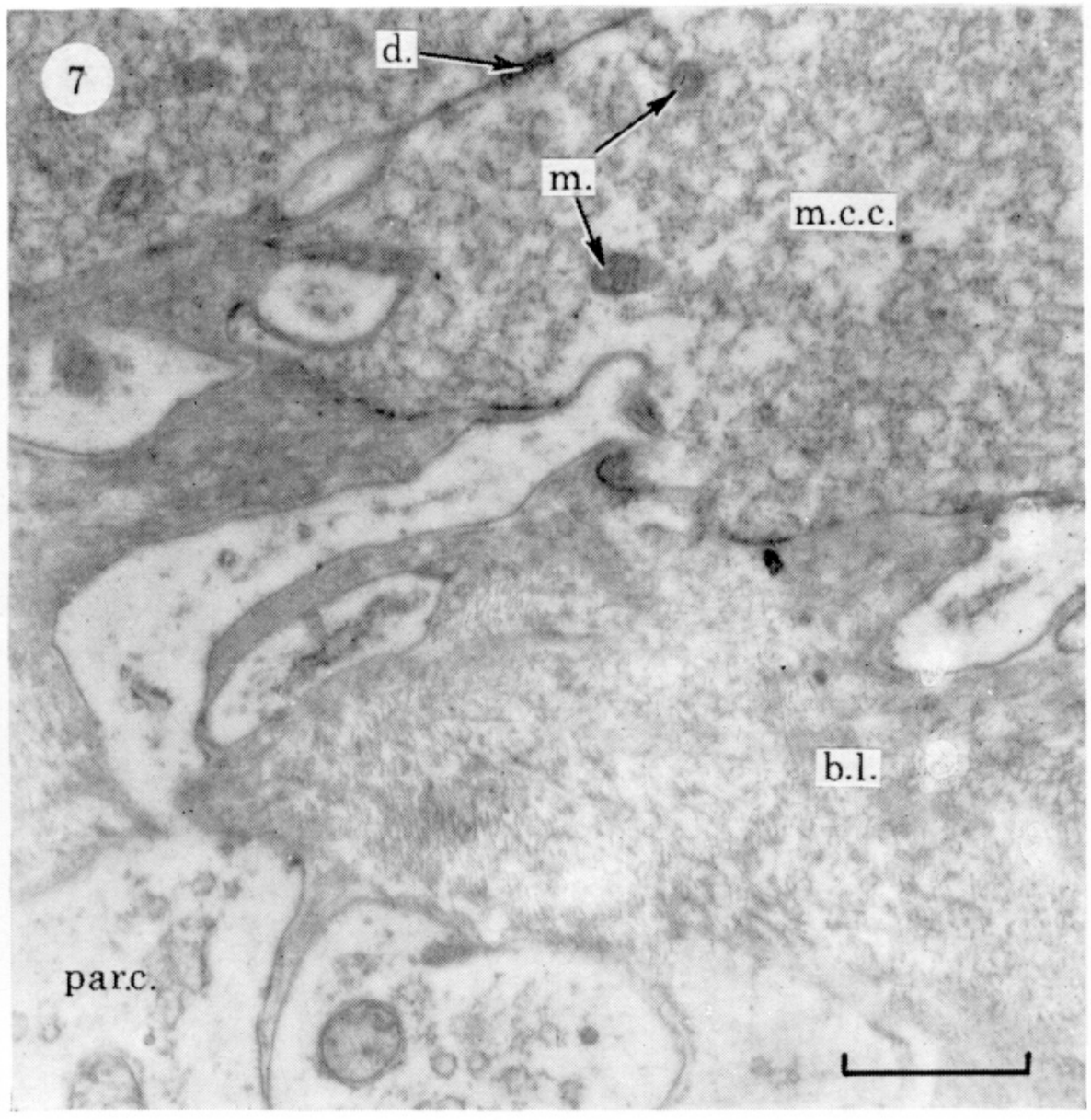
FIGURE 1. *B. balanoides*. (*a*) Diagrammatic section through the oviducal gland illustrating the three main regions. Scale bar, 120 μm. (*b*), (*c*) Views of the Plasticine model constructed from serial sections; the broken lines represent the position of the section drawn in (*a*).



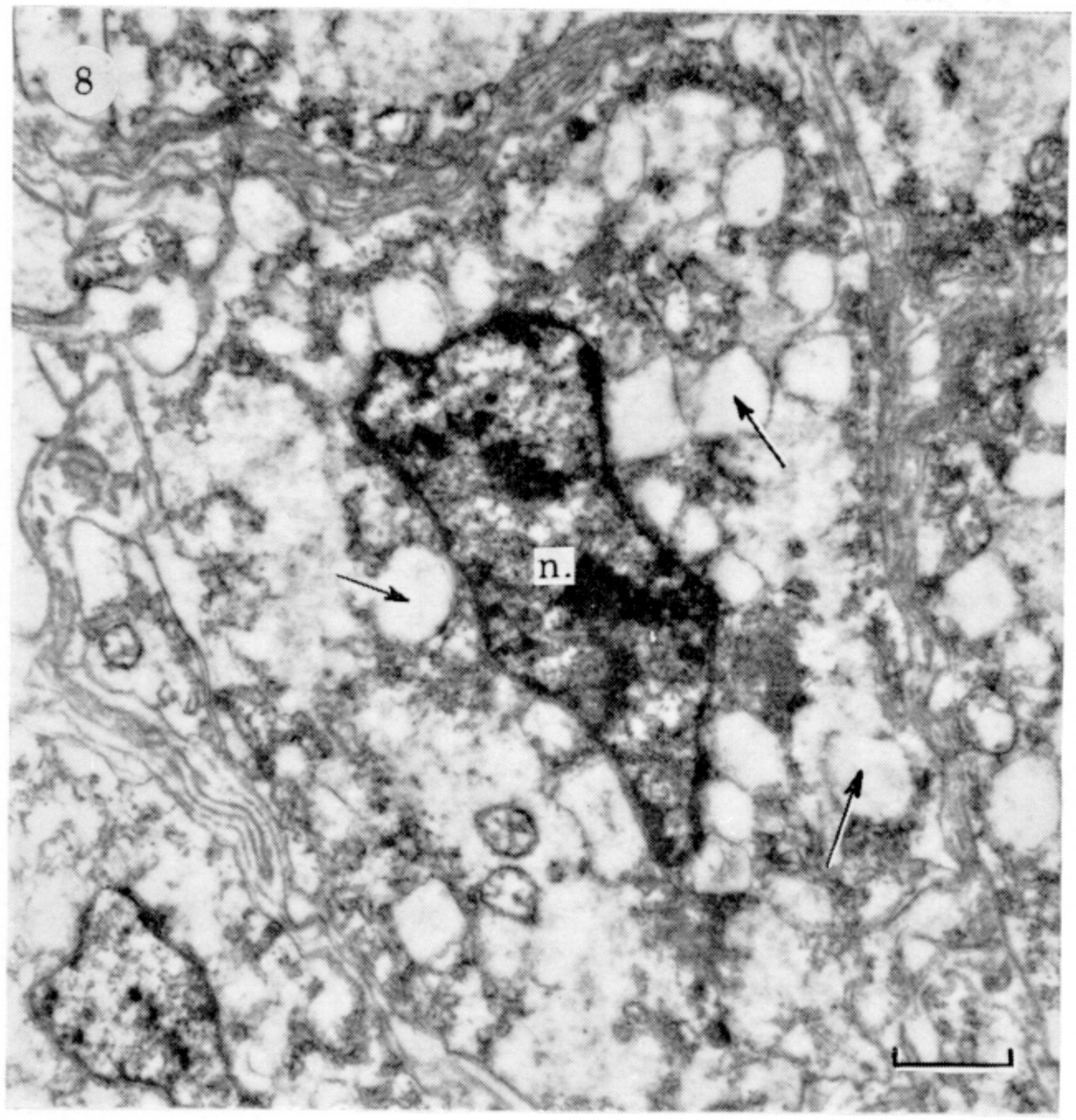


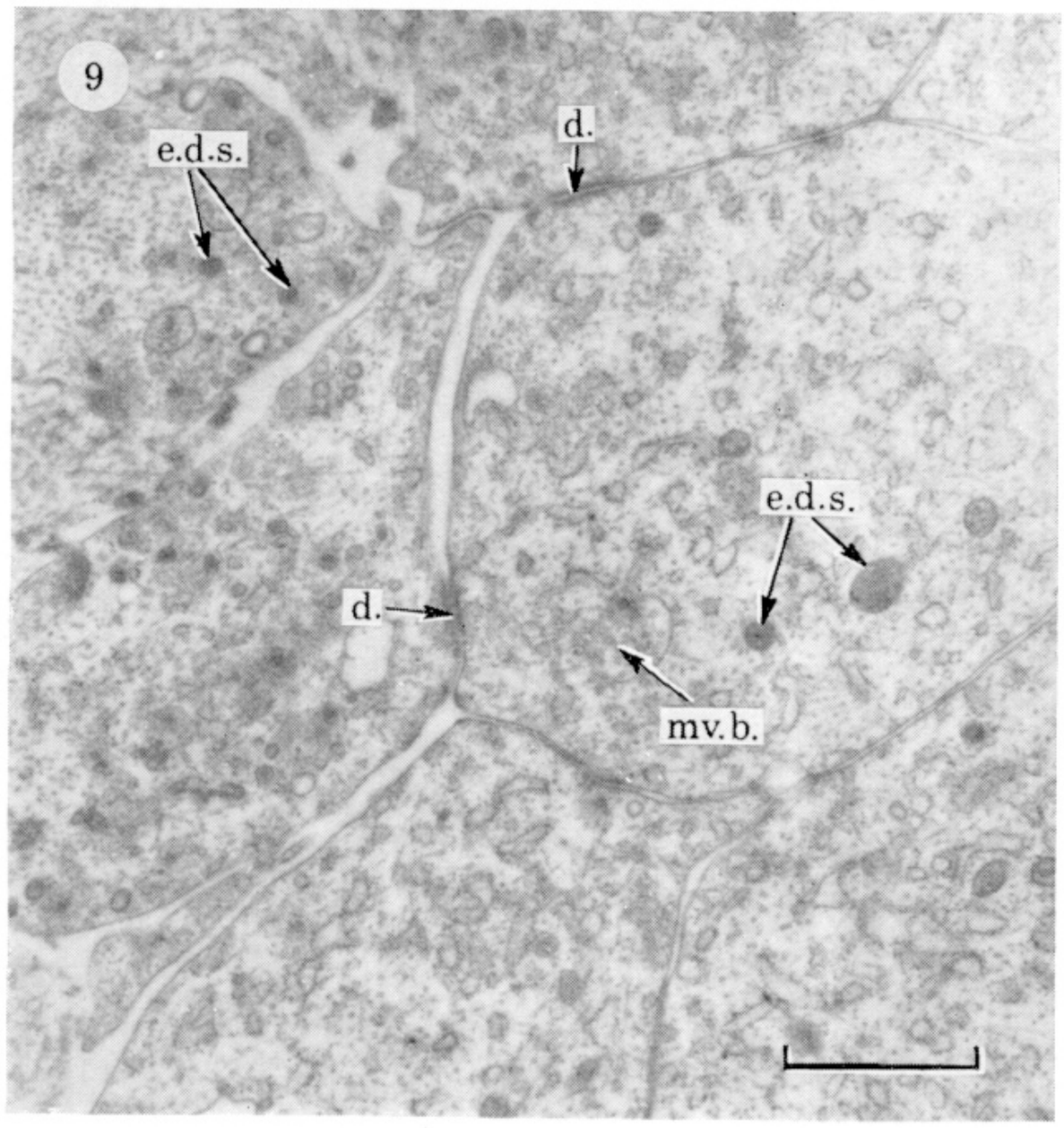


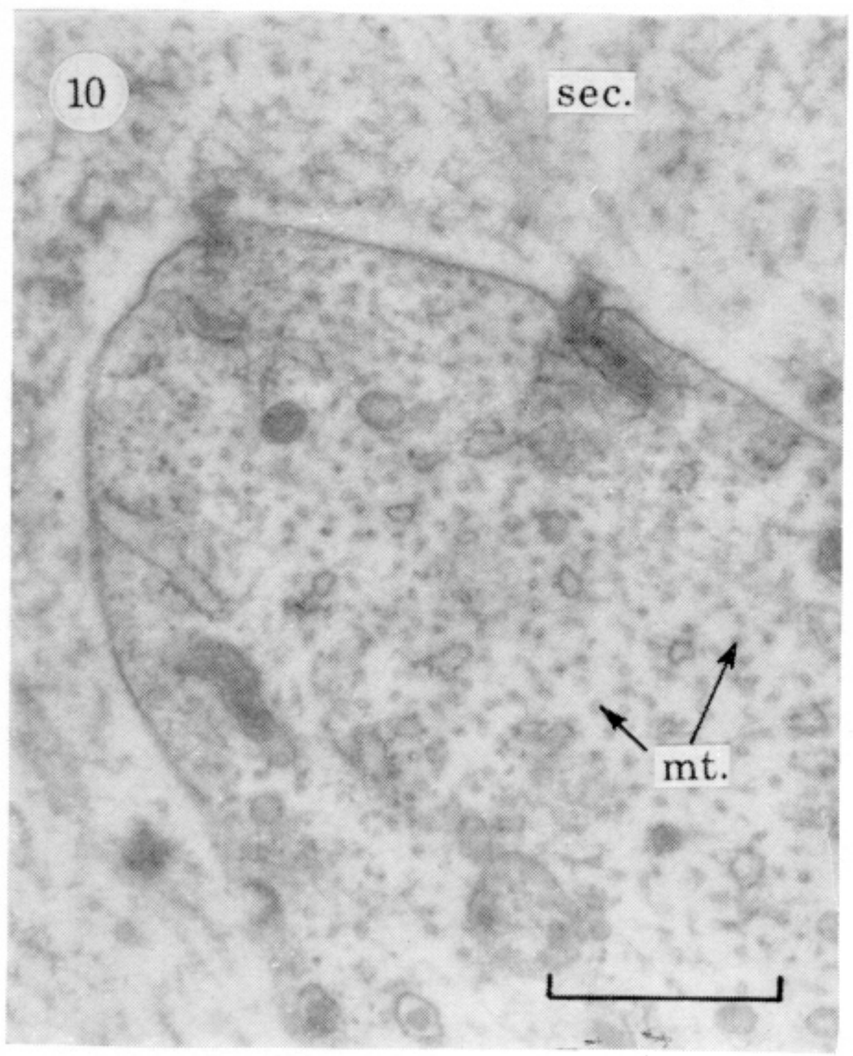


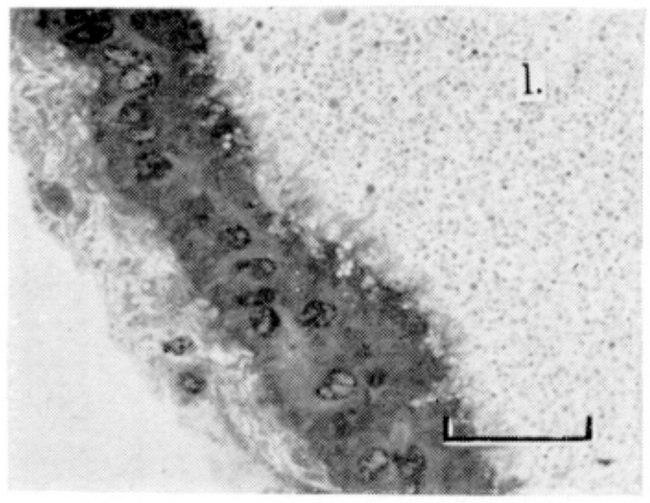


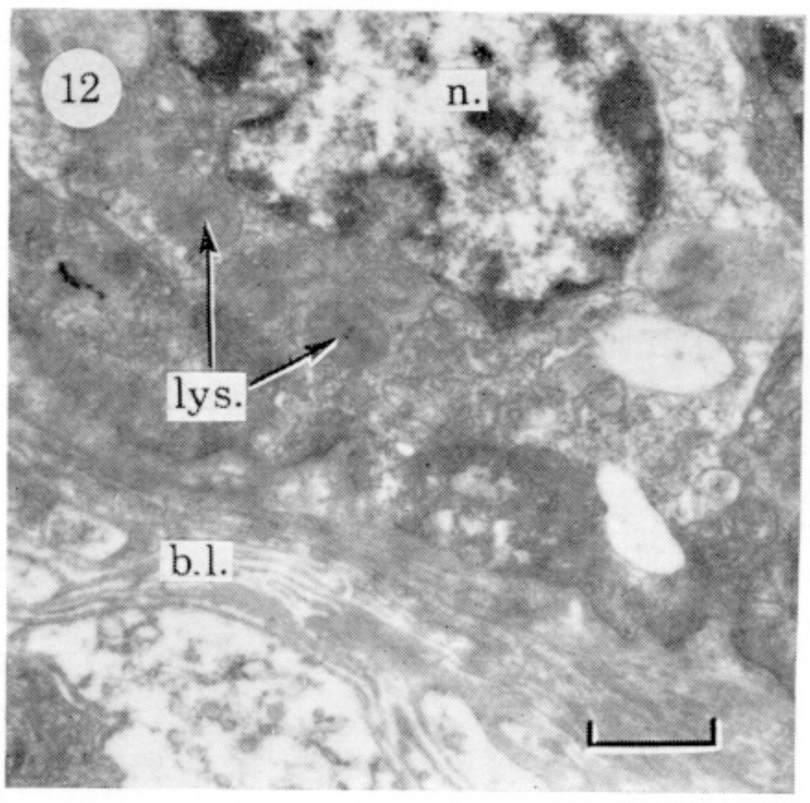
FIGURES 3–7. For description see p. 153.



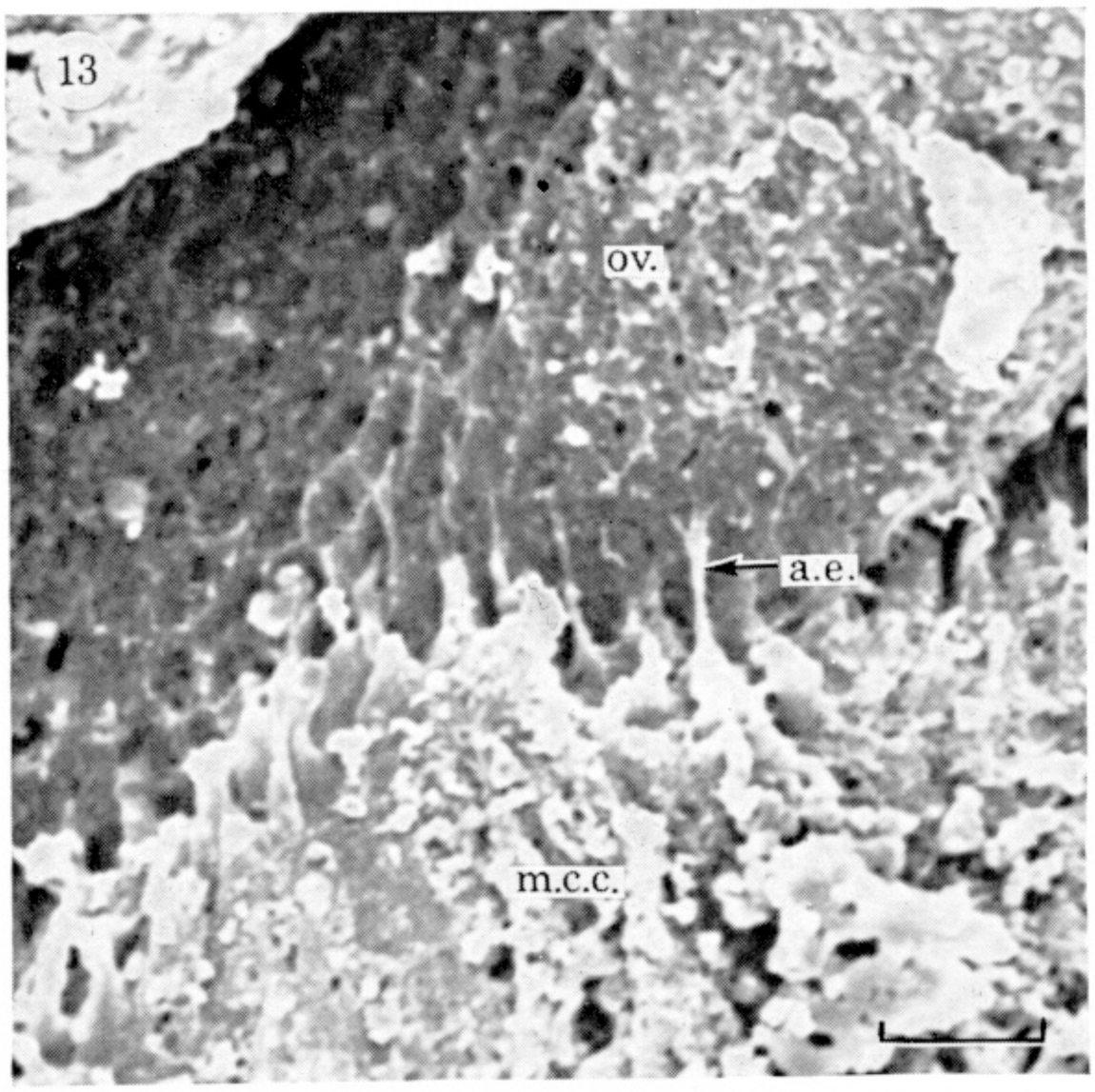


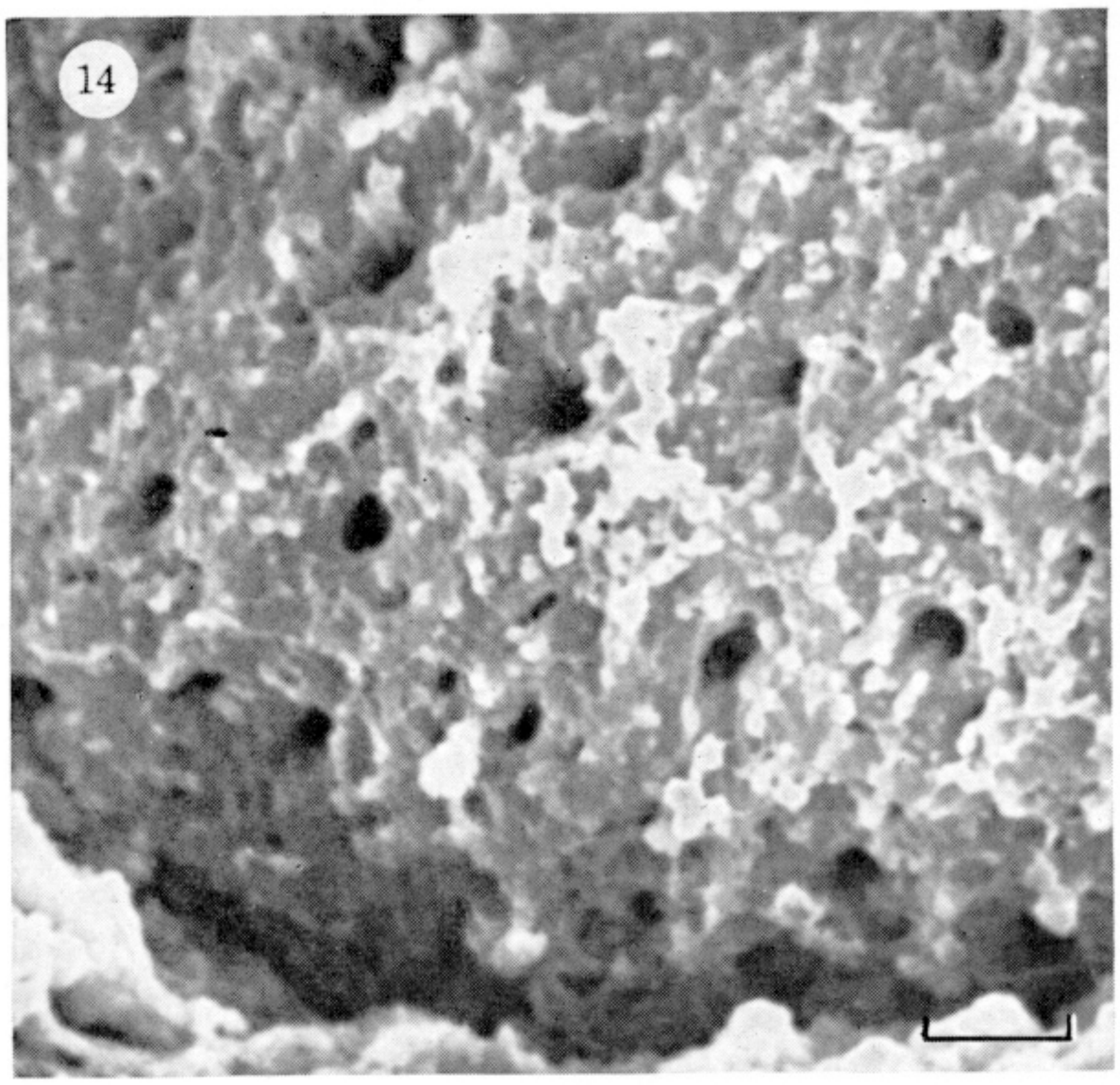


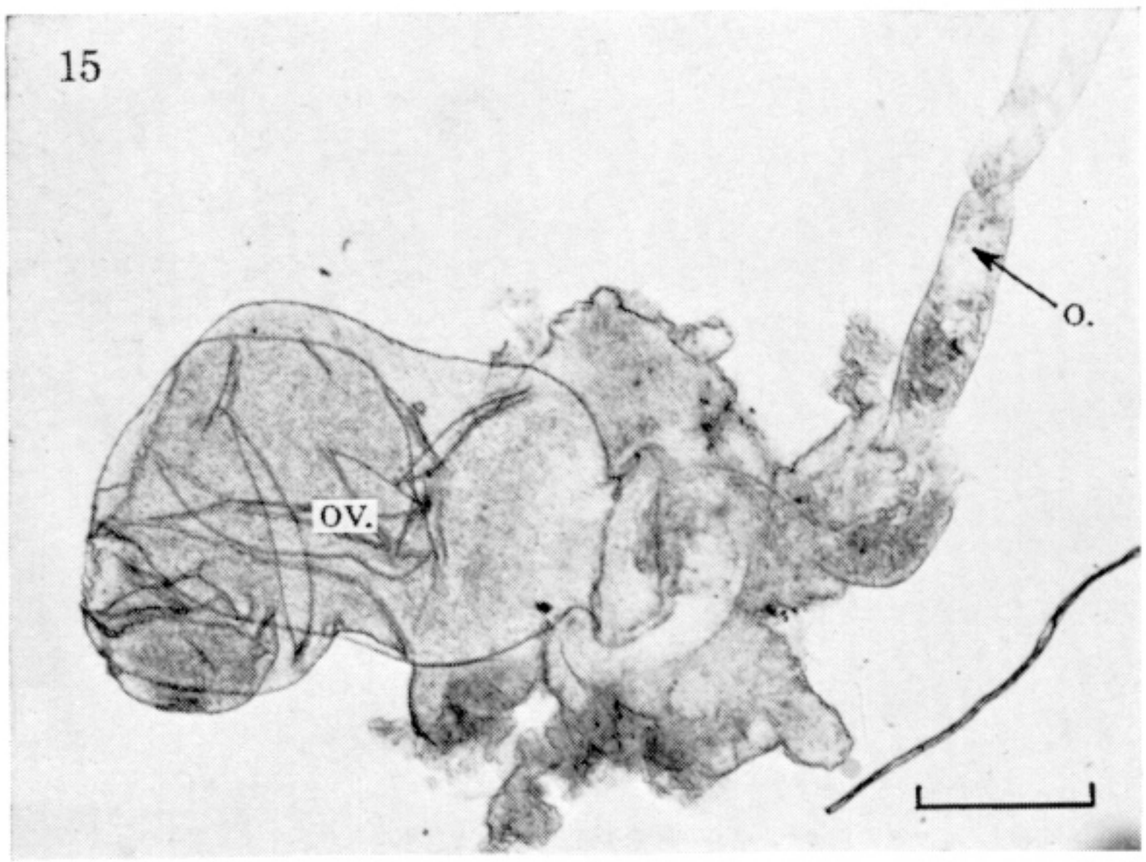


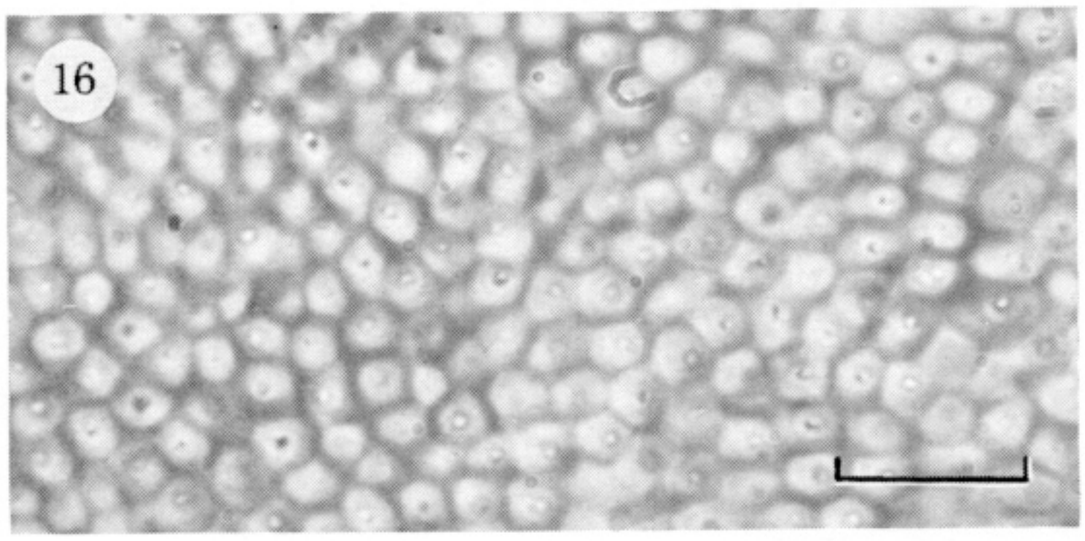


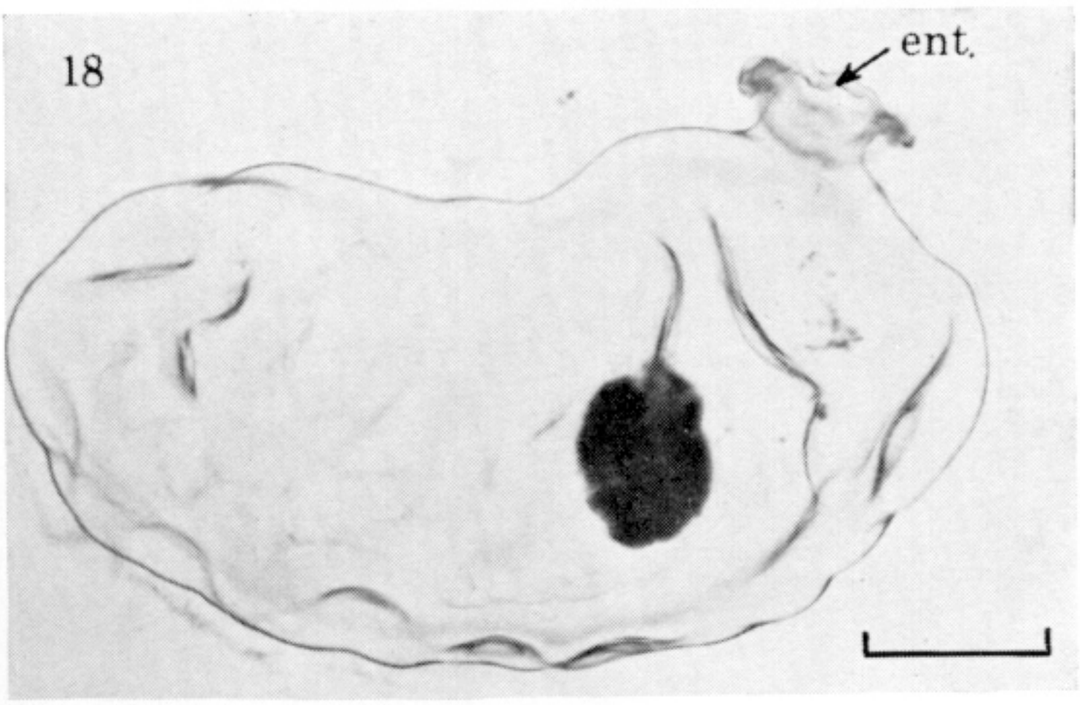
FIGURES 8–12. For description see p. 153.

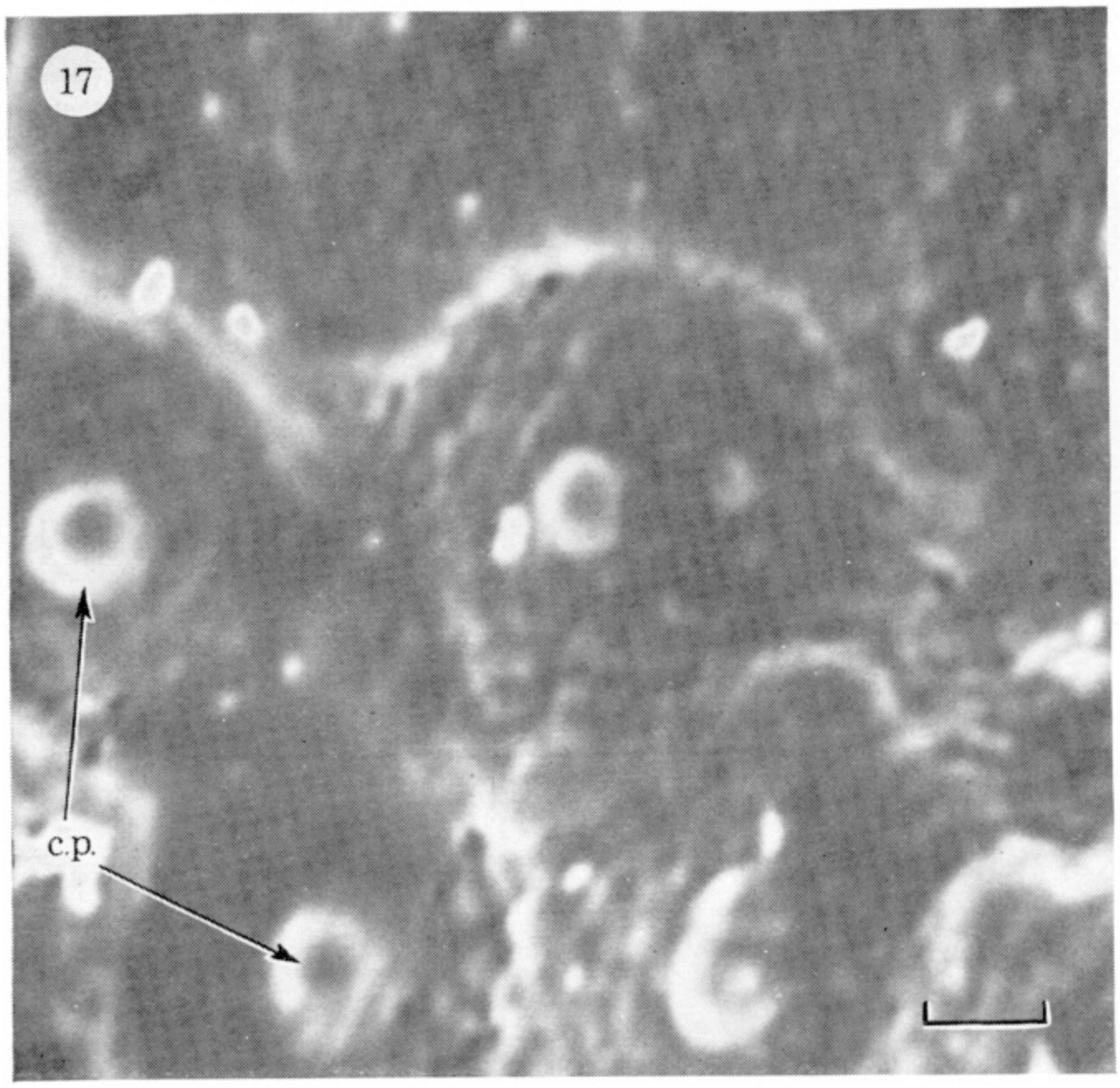


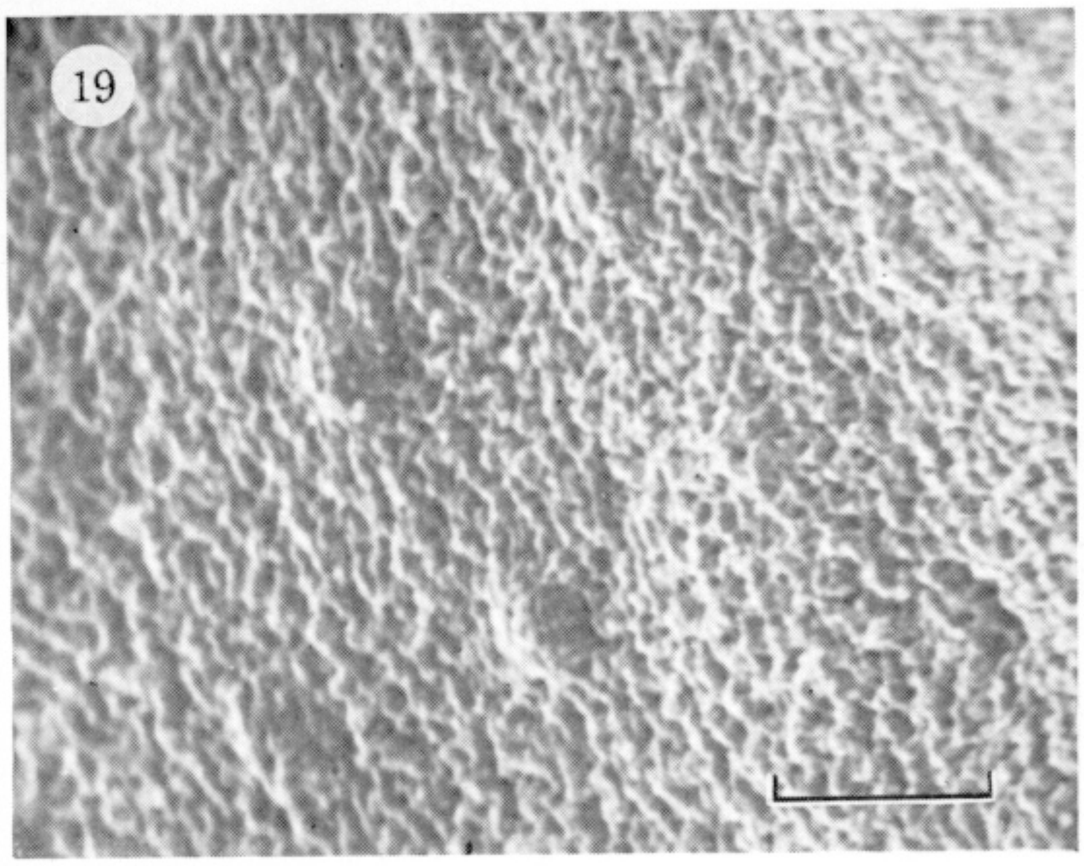




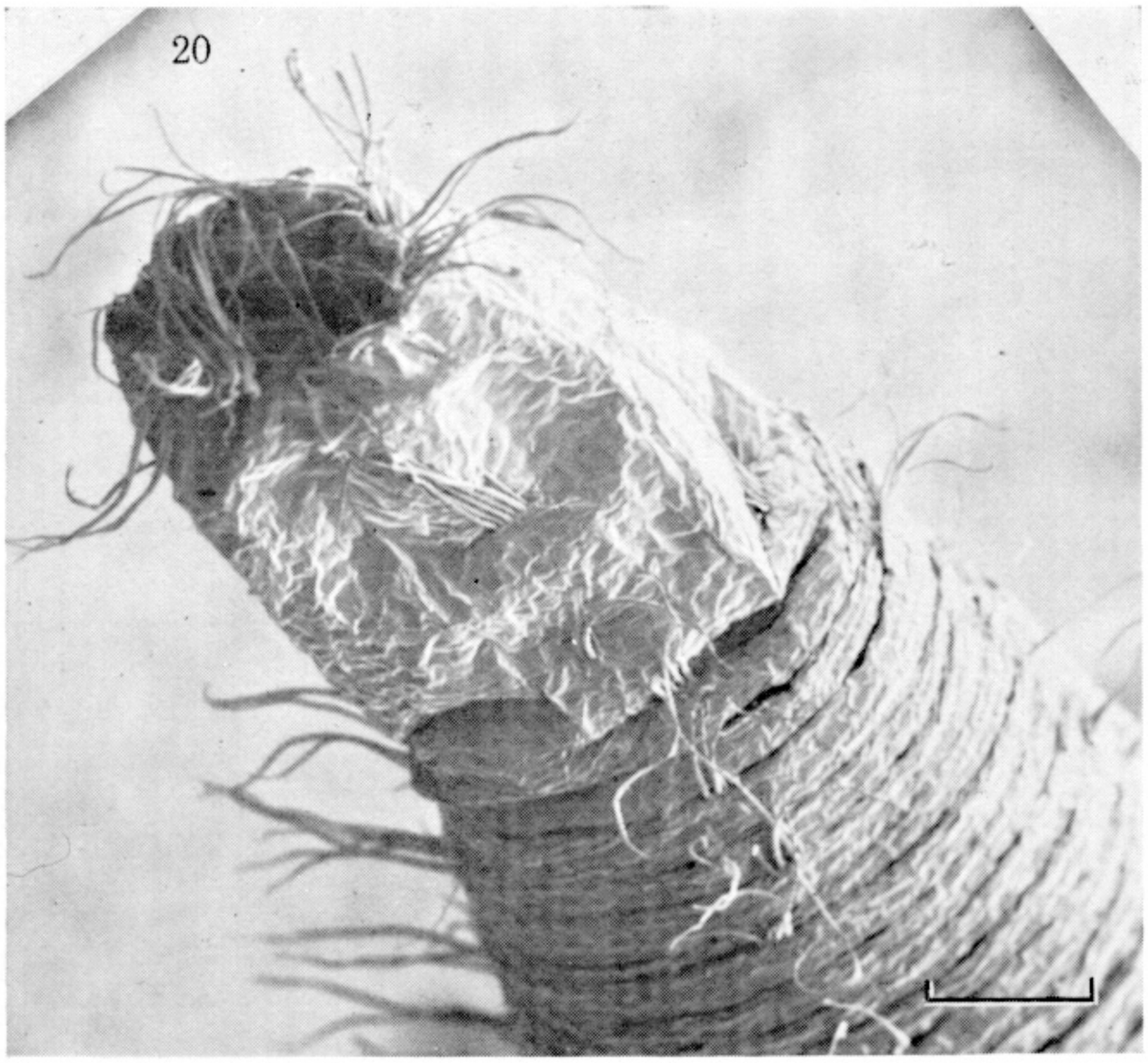


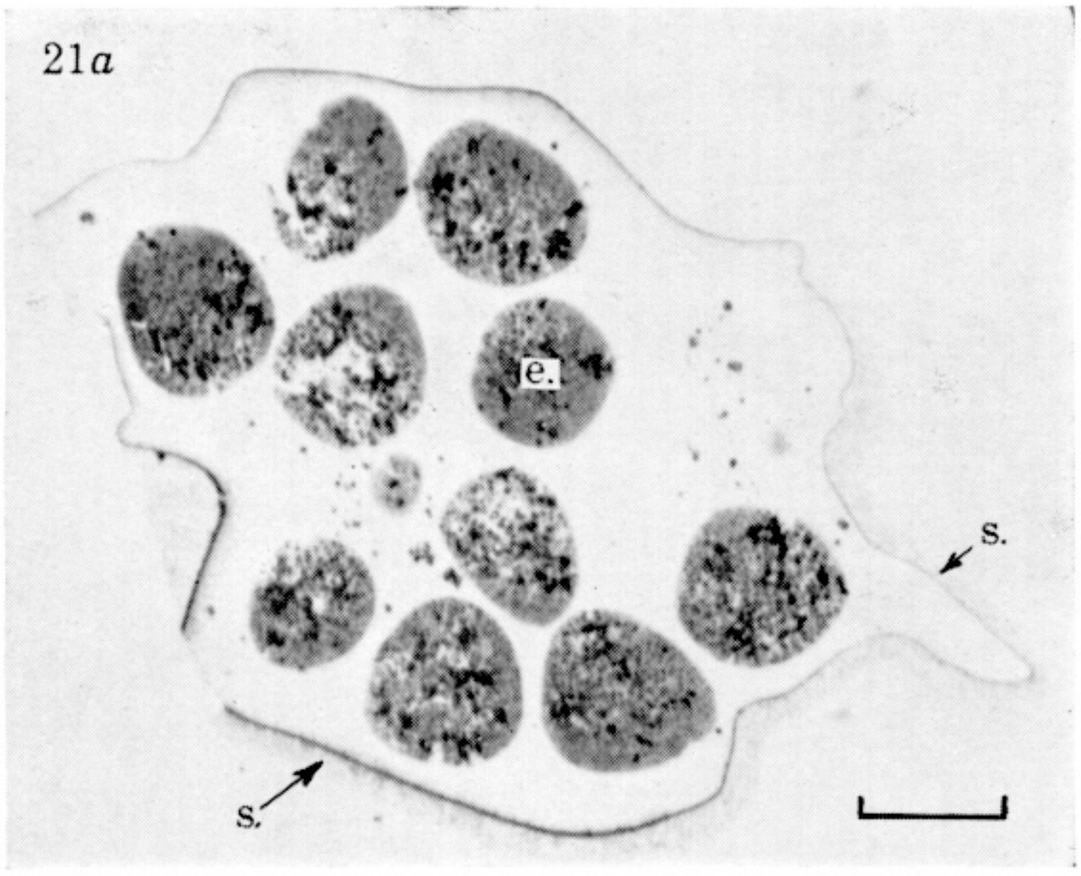


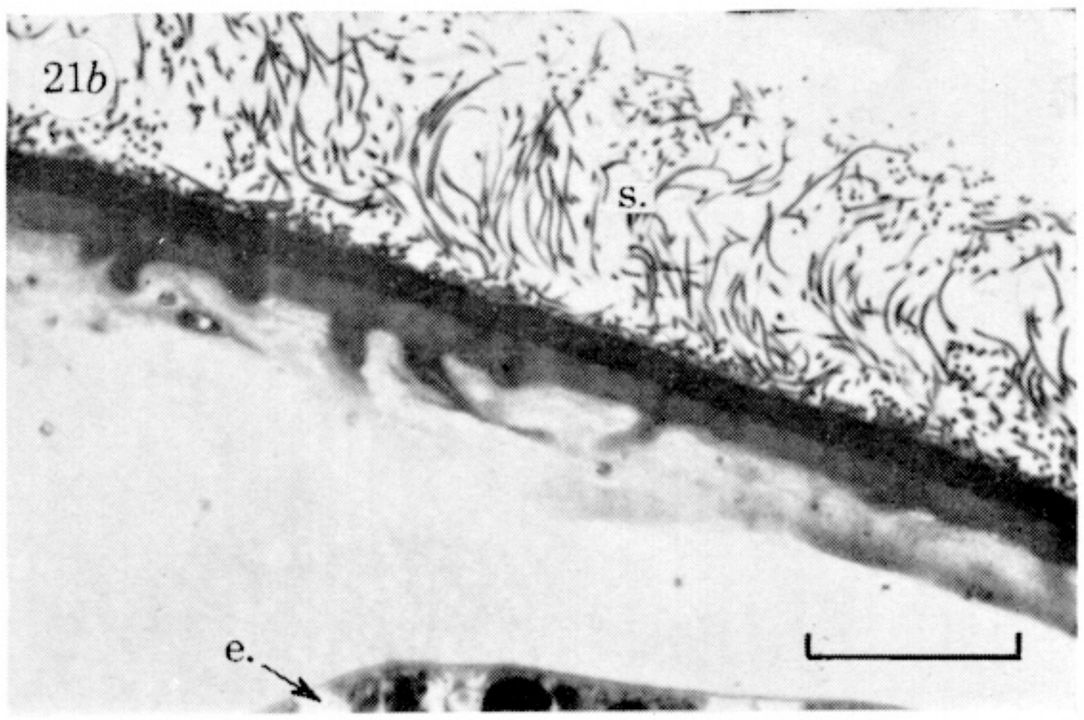


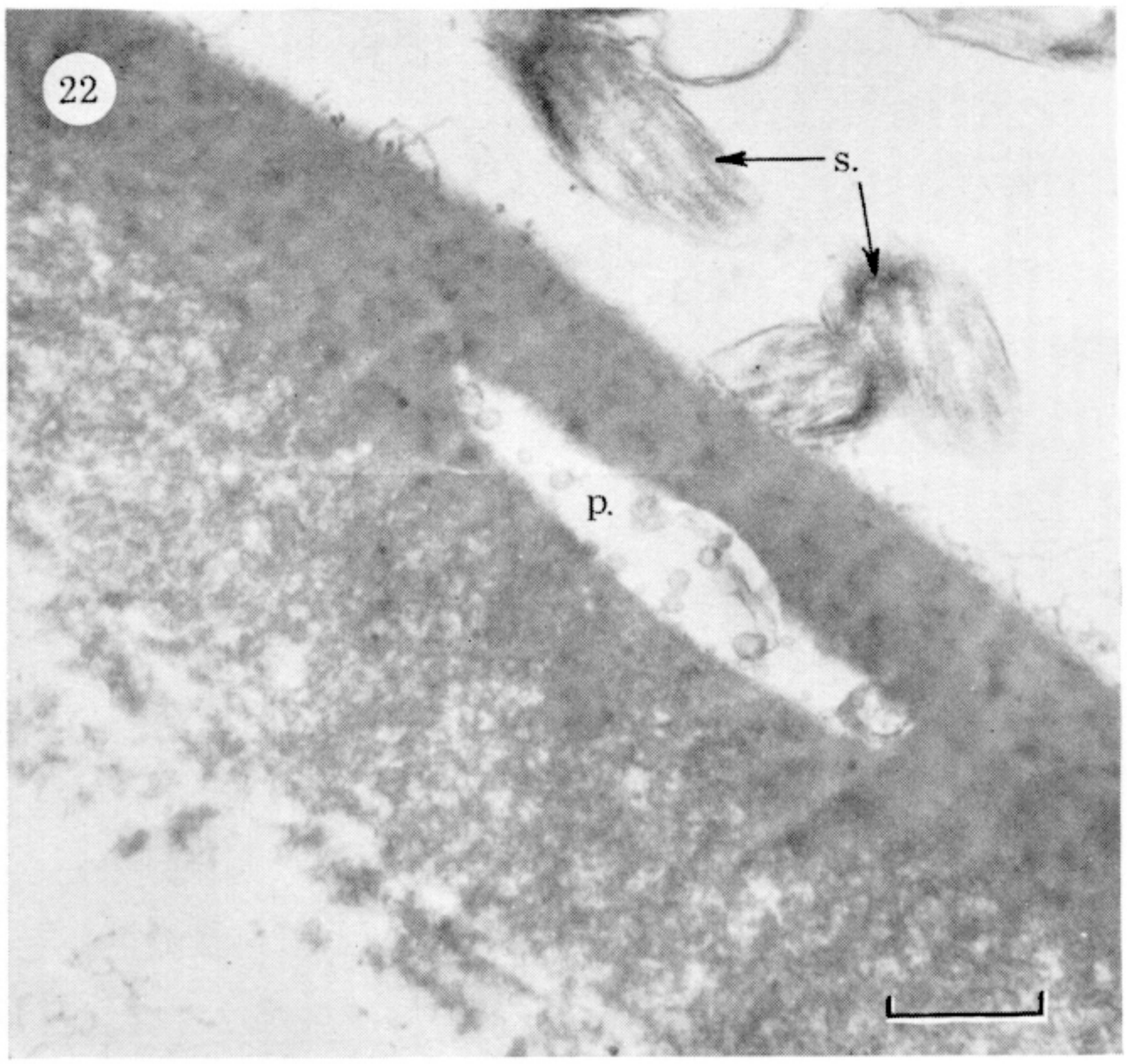


FIGURES 13–19. For description see p. 153.









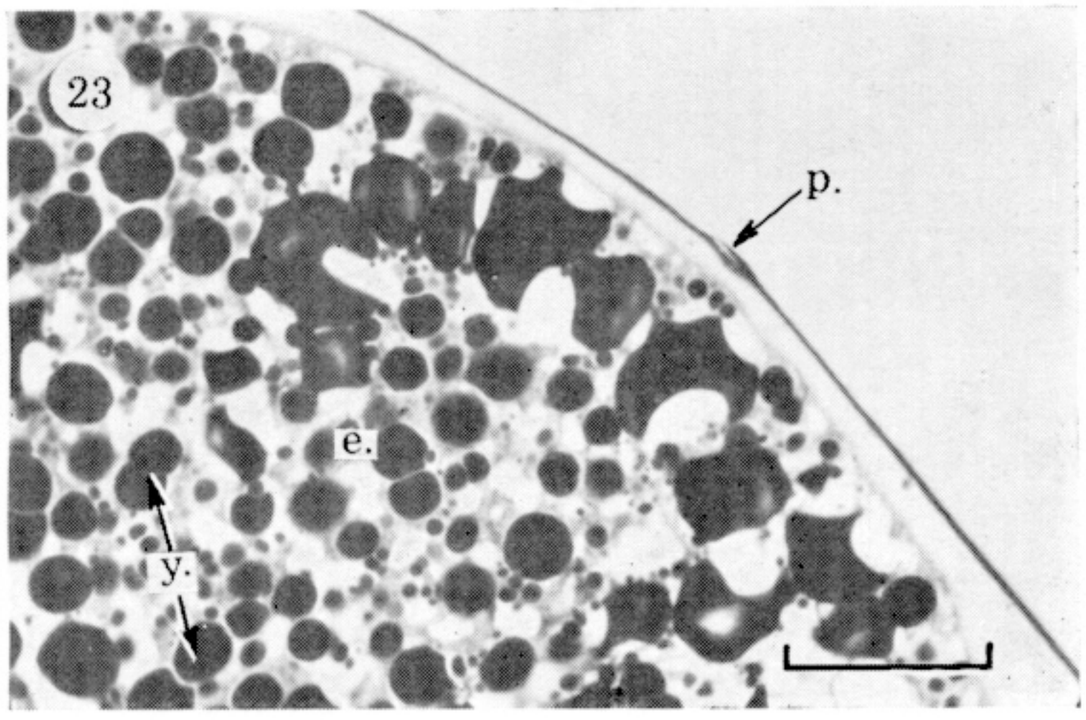
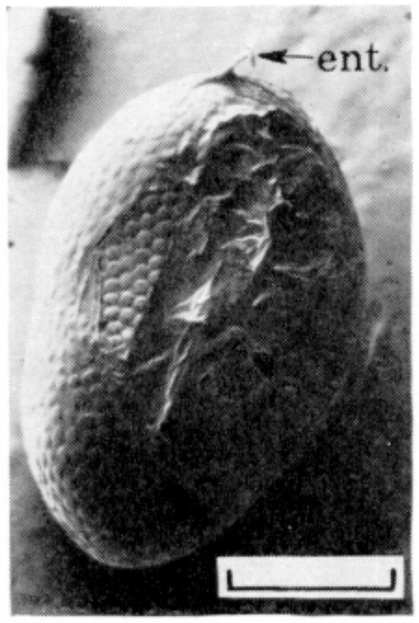


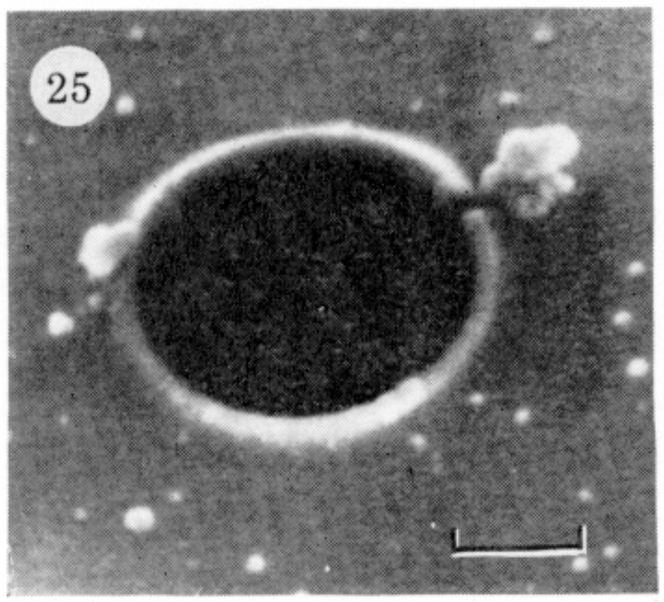
FIGURE 20. *B. hameri*: scanning electron micrograph showing the tip of a penis with its array of setae. Three of the four rows of setae that extend down the penis can also be seen. Scale bar, 50 μm.

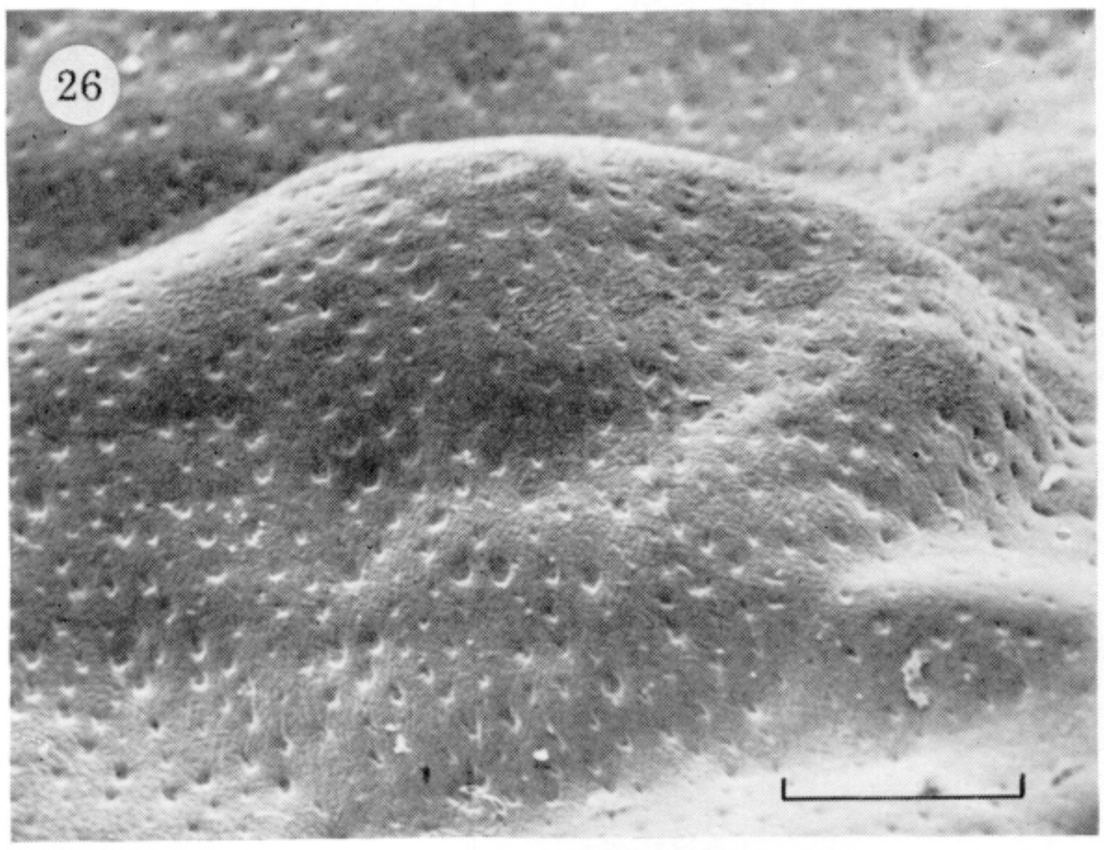
FIGURE 21. *B. balanoides*: (*a*) 1 μm Araldite section through an early egg mass, showing the mass of surrounding sperm. Scale bar, 100 μm. (*b*) Higher magnification, showing the ovisac wall and sperm. Scale bar, 20 μm.

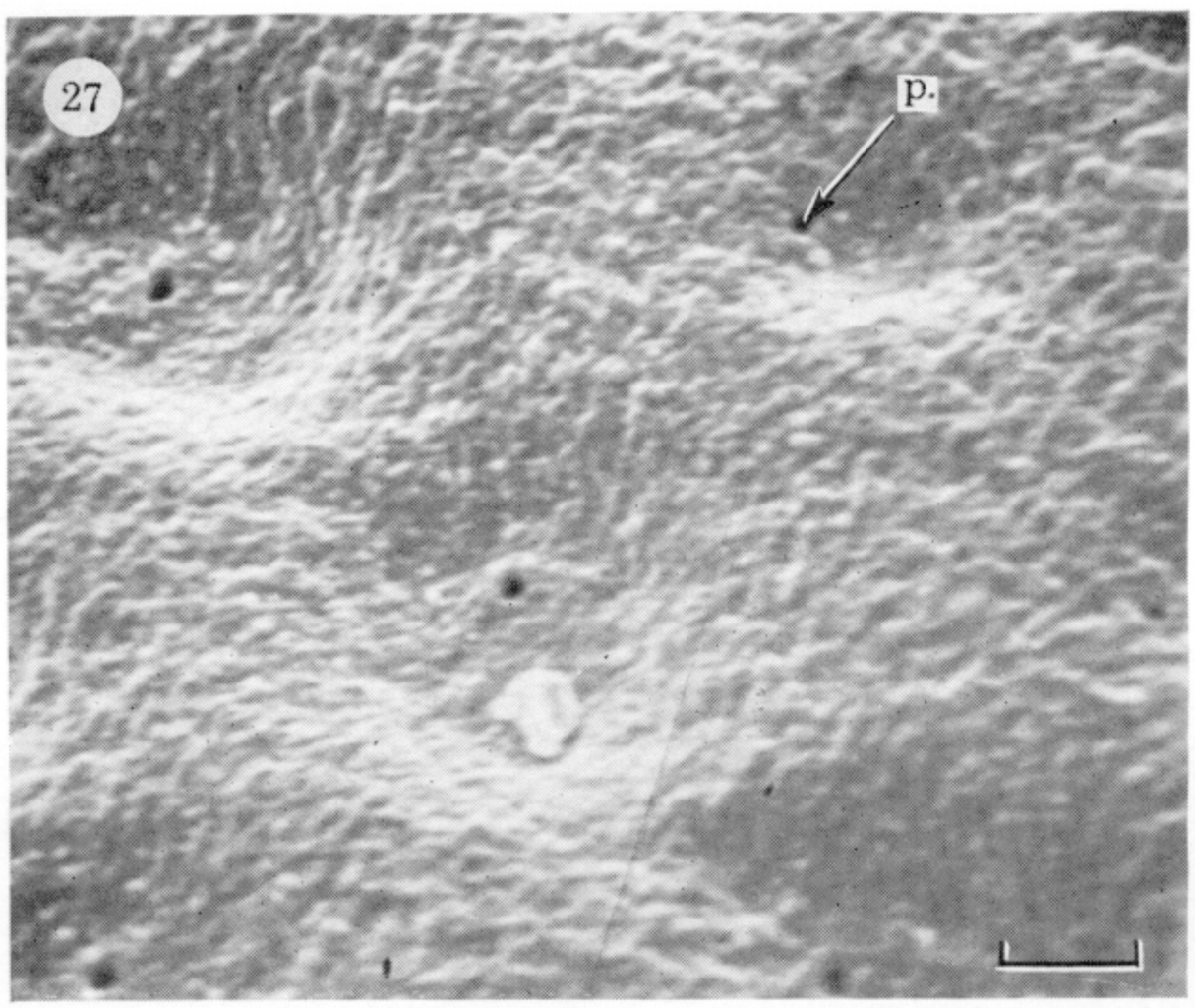
FIGURE 22. *B. balanoides*: electron micrograph of a section across a partially stretched ovisac wall, showing cytoplasmic debris within a pore. Scale bar, 0.3 μm.

FIGURE 23. *B. balanoides*: 1 μm Araldite section through a late egg mass, showing the thinned ovisac wall with a pore and underlying egg. Scale bar, 50 μm.









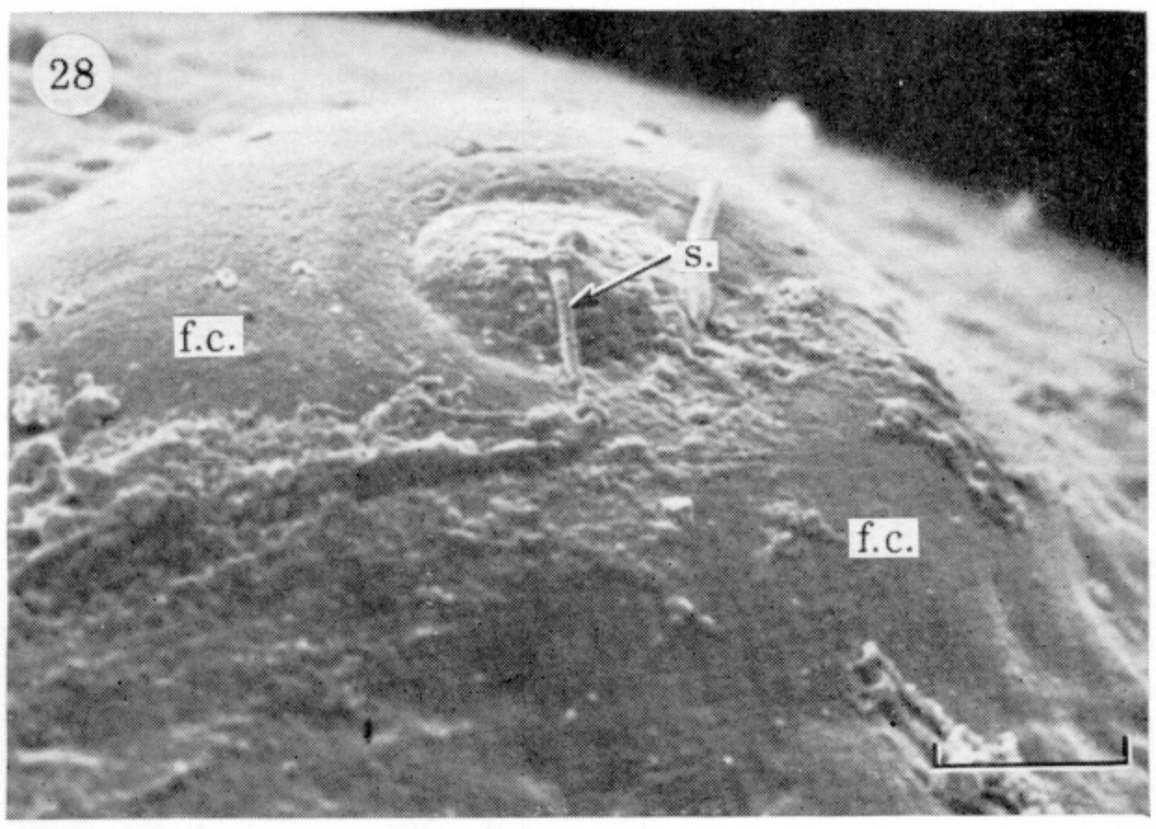


FIGURE 24. *B. balanoides*: scanning electron micrograph showing the areas of contact between eggs and ovisac wall in a fully formed egg mass. Scale bar, 50 μm. Inset: the same whole egg mass (scale bar, 1 mm). The slight damage occurred during preparation.

FIGURE 25. *B. balanoides*: scanning electron micrograph showing a pore in the ovisac wall surrounding a fully formed egg mass. Scale bar, 1 μm.

FIGURE 26. *B. hameri*: scanning electron micrograph showing the pitted appearance of the outer surface of an ovisac fixed during early egg laying. Scale bar, 50 μm.

FIGURE 27. *B. hameri*: higher magnification micrograph of an ovisac fixed during early egg laying, showing the outer ovisac surface with pores within pits. Scale bar, 2 μm.

FIGURE 28. *B. hameri*: scanning electron micrograph showing the fertilization cone thrown up by a fertilized egg. Scale bar, 5 μm.