Light and Electron Microscopical Observations on the Clitellar Epithelium of *Tubifex*

By

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(With 2 Text-figures and 3 Plates)

The freshwater oligochaete, *Tubifex hattai*, deposits several eggs at a time enclosed in a cocoon. The cocoon is important for the embryonic development of the eggs, since eggs freed from the cocoon die in well water (Inase, 1960). According to Hirao (1965), the cocoon is formed by secretory activity in the epithelial cells of the clitellum of a worm undergoing the deposition of eggs. He observed three kinds of granular cells in a light microscopical study of clitellar epithelium and suggested the possible functions of these cells in the process of cocoon formation. Our knowledge of the ultrastructure of the oligochaete integument has been accumulated during the past several years (Coggeshall, 1966; Krall, 1968; Goodman and Parrish, 1971; Potswald, 1971; Burke, 1974). Mucous cells of the body wall have been carefully investigated in several species of oligochaetes (Richards, 1974). In spite of these interesting reports, precise information on the epithelial cells of the clitellum has not hitherto been available at the electron microscopical level. The present study was undertaken in order to examine the fine structure of clitellar epithelium, particularly of the granular cells, in *Tubifex*. The results will be described in comparison with those obtained by light microscopical observation.

Material and Methods

The freshwater oligochaete, *Tubifex hattai*, collected from the stream running through the campus of Hokkaido University, has been reared and bred for several years in the laboratory. These worms deposited cocoons at 3–5 day intervals. When the cocoon deposition drew near, the ovisac of the worm contained several large oocytes (300–400 μ in diameter) which could be observed through the body wall of the post-clitellar region with the naked eye. The worms with large oocytes in the ovisac were used in the present study.

For light microscopy, the worms were fixed in Bouin's fluid. After fixation, a part including the clitellum was dissected from the worm and treated with the ordinary paraffin method. Serial sections, 6–8 μ in thickness, were colored with

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Heidenhain's azan stain. For the demonstration of mucopolysaccharides, sections were successively treated with alcian blue and periodic acid-Schiff (PAS) reagent according to the method of Mowry (1963).

For electron microscopy, small pieces of the clitellum were dissected from worms in 5% glutaraldehyde buffered with 0.05 M cacodylate or with 0.05 M phosphate (pH 7.4) and allowed to stand for two hours in the renewed fluid. After rinsing in three changes of buffered deionized water, they were post-fixed for one hour in 1% osmium tetroxide dissolved in the same buffer solution. These procedures were all performed at 0°–4°C. The specimens were then dehydrated in a graded series of acetones and embedded in Epon 812 (Luft, 1961). Gold thin sections were cut on a Porter-Blum MT–1 ultramicrotome and were successively stained with 1% uranyl acetate and lead citrate (Reynolds, 1963). They were observed in a JEM 100S electron microscope.

Observations

Light Microscopy

The clitellar epithelium of *Tubifex* was covered with a thin layer of cuticle. Beneath the epithelium were muscle layers of the integument. The presence of basement membrane was obscure. At least three kinds of cells constituted the clitellar epithelium; supporting cells, mucous cells and granular cells. On account of the large volume of granular cells, the epithelium of the clitellum was about 2–3 times as thick as that of the other regions.

The supporting cells were most numerous in the epithelium and were found not only in the clitellar part but also in other regions of the worm. They were characterized by an extremely narrow cell body in the clitellar epithelium and by a centrally located nucleus (Fig. 1A). Unlike the other kinds of epithelial cells, the cytoplasm of the supporting cell contained no light microscopically visible inclusions and appeared orange red in the azan stained preparation.

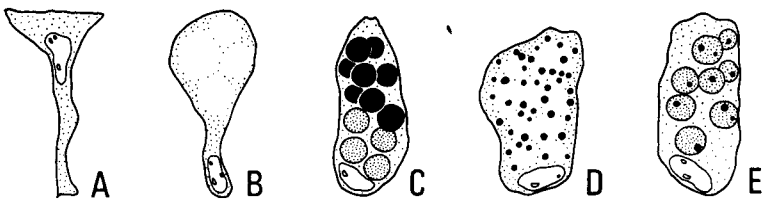

Fig. 1. Diagrammatic drawing of five types of cells in the clitellar epithelium. A, supporting cell; B, mucous cell; C-E, granular cells. *Type I* cell (C) contains two kinds of granules. In *Type II* cell (D), the granules are small in size and surrounded by amorphous materials. The granules of *Type III* cell (E) contain a small number of particles.

The mucous cells also lay scattered in the epithelium of the entire body wall of the worm. However they were found with high frequency in the clitellar epithe-

lium, especially in the anterior and posterior terminal parts of the clitellum. The clitellar mucous cells were characterized by the typical shape of a goblet cell and by having secretory products which appeared colorless or faint blue in the azan stained preparation (Fig. 1B). The nucleus was located basally in each mucous cell. When the alcian blue-PAS technique was applied, the secretory products in the mucous cell were weakly PAS-positive in some worms but were strongly stained with alcian blue in others.

The granular cells were specific for the clitellar epithelium. They were large columnar cells and had the spheroidal nucleus in their basal part. A great number of granules occupied their cytoplasm. From the staining properties of the granules, these cells were subdivided into three types. Cells of the first type, named *Type I* in this study, contained spherical granules (about 3 μ in diameter) (Fig. 1C). Some of these granules were stained deeply red but others appeared blue in the azan stained preparation. In most worms, these differently stained granules were found to be intermingled within a single cell. In a few worms, however, the red-stained granules were limited in distribution to the apical half of the cell and the blue-stained granules to the basal half. In spite of the difference in the dye-binding property, all of these granules were very weakly positive to the PAS test and negative to alcian blue. Careful observations revealed that the cells of *Type I* were distributed evenly in the clitellar epithelium except for the ventral side of the anterior and posterior terminal parts of the clitellum where they were never found (Fig. 2). Cells of the second type, named *Type II*, contained numerous

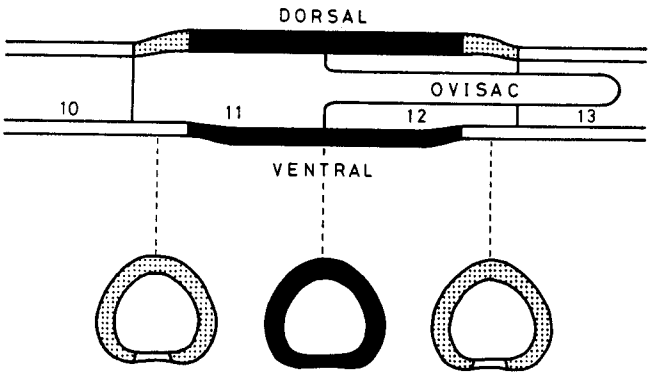

Fig. 2. Diagram showing the distribution of *Types I-III* cells in the clitellum. Dark area, *Types I* and *II* cells; dotted area, *Types I* and *III* cells. Upper, sagittal section of segments 10–13; lower, transverse sections at three levels indicated by broken lines.

fine granules (less than 1 μ in diameter) stained red. The granules were not embedded directly in the cytoplasm but were surrounded by amorphous materials which appeared faint blue (Fig. 1D). Both the granules and the amorphous materials were very weakly positive to the PAS test and negative to alcian

blue. The cells of *Type II* were distributed evenly only in the intermediate part of the clitellum. They were entirely absent in the terminal parts (Fig. 2). Cells of the last type, named *Type III*, contained spherical granules (about 3 μ in diameter). Unlike the cells of *Type I*, all of these granules appeared blue and there were no granules stained red (Fig. 1E). However observations at high magnifications revealed that several particles stained red (less than 1 μ in diameter) were embedded in each granule. A further piece of evidence indicating the peculiar property of granules in cells of *Type III* was that they were strongly positive to the PAS test. They had no affinity to alcian blue. The cells of *Type III* appeared to be much fewer in number than those of *Types I* and *II*. They were found on the dorsal and lateral sides of the anterior and posterior terminals of the clitellum but never detected on the ventral sides or in the intermediate region (Fig. 2). The distribution of cells of *Type III* therefore did not overlap with that of *Type II*.

Electron Microscopy

As described already, the outermost layer of the body wall was cuticle. It was 1.2–1.5 μ thick in the clitellum and was closely in contact with the underlying epithelial layer (Fig. 3). The cuticle was composed of outer and inner layers. The relatively thin outer layer was a compact gathering of filamentous materials having a medium electron density. Its outer border was loaded with regularly spaced particles showing a high electron density (Fig. 4). The particles were elliptical in shape and were oriented with their long axes perpendicular to the outer surface of the layer. The inner thick layer was composed of an electron lucent substance and filamentous materials of a medium electron density. The latter were embedded loosely and unevenly in the former.

The epithelial layer was found in firm contact with the overlying cuticle layer. There was a space, 0.1–0.2 μ in width, between the epithelial and underlying muscle layers (Fig. 5). It was filled with filamentous materials but no indication of the presence of basal lamina was detected. Nervous elements were often seen near the base of the epithelial cell, but no synaptic connections were observed, so far as the present study was concerned.

The identification of the cell types of the clitellar epithelium was easily accomplished in the electron microscopical observations. Owing to the large volume of granular cells, the thickness of clitellar epithelium considerably increased. Furthermore the large granular cells compressed the supporting and mucous cells, which seemed to induce the elongation of the latter cells.

The supporting cell in the clitellar epithelium was identified by the absence of large granular inclusions in the cytoplasm (Fig. 3). The basal part of the cell was extremely narrow and contained loose bundles of tonofilaments. The apical part of the cell was fan-shaped and projected microvilli from the surface. As seen in Figure 4 some of the microvilli run through the thickness of the cuticle layer and the tops of them reached the outermost limit of the worm body. The elongated nucleus was found in the neck of the fan-shaped apical part of the cell together with

several well-developed Golgi complexes and many mitochondria. The cisternae of rough endoplasmic reticulum were not noticeable in any part of the cytoplasm.

The mucous cell also exhibited a slightly elongated shape and was adjacent to supporting cells. The spheroidal nucleus was found in the basal part of the cell. Unlike supporting cells, the apical surface of the cell was deeply concave and a small number of short microvilli were detected only on the rim of the concavity. Careful observations revealed that the concavity opens externally through a narrow slit running through the overlying cuticle layer and is filled with fine filamentous materials of low or medium electron density. Just below the concavity secretory granules were located in the cytoplasm. They were membrane-bounded and closely assembled. Each granule was polyhedral or spheroidal in shape and measured roughly 4 μ along its longest axis. As shown in Figure 6, the granules were filled with fine filamentous materials of low or medium electron density which resembled the contents of the concavity, that is, the apical pocket of the mucous cell. In some cases, the secretory granules contained electron lucent particles. Through the fusion of the granular membrane in several small portions, the contents of the granules were often connected with those of immediately adjacent ones. Besides secretory granules, the mucous cell also possessed a number of membranous organelles such as rough endoplasmic reticulum and Golgi complexes in the cytoplasm. Mitochondria were found scattered throughout the cytoplasm but were few in number.

Except for the internal structure of contained granules, the organization of granular cells was nearly the same in all the types of cells distinguished in the light microscopy. They were large and columnar in shape and contained a spheroidal nucleus near the base of the cell. It is worth noting here that the granular cells resembled in some respects the mucous cells. To describe in detail, the apical surface of the granular cells was also deeply concave and a small number of short microvilli were observed only on the rim of the concavity. Regardless of the type of cell, the concavity contained filamentous materials of low electron density and opened externally through a narrow slit found in the overlying cuticle layer (Fig. 7). Furthermore the granular cells were always adjacent to supporting cells, thus they never directly contacted other granular cells and mucous cells. The bulk of the cytoplasm was occupied by a number of granules. Many large cisternae of rough endoplasmic reticulum and several mitochondria were detected in the intergranular and peripheral cytoplasm. Several well-developed Golgi complexes accompanying many secretory vesicles and small granules were observed in the perinuclear or central cytoplasmic regions.

The granular cells could be subdivided electron microscopically from the observations of the granular structure. In one type of cell (*Type A*), the granules were spherical or spheroidal in shape, measuring less than 4 μ in diameter. Each granule had a boundary membrane. As shown in Figure 8, the large granules showed an unusual internal structure. A sheet of uneven electron density, about 300 Å thick, spiralled regularly through an electron lucent matrix for 40–50

revolutions leaving a gap of about 70 Å in width. Such an internal structure was always observed in granules over about 3 μ in diameter. These granules may therefore be regarded as fully-grown. On the other hand, the small granules were more or less irregular in shape and had an internal structure different from that of the large granules. There was no spiral of a sheet. Instead of this, filamentous bundles of a high electron density were laid at regular intervals or randomly in the electron lucent matrix (Figs. 8 and 10). Since there were intermediate types of internal structure of large and small granules, it may be thought that the small ones are in the process of formation. The cells containing these large and small granules were also characterized morphologically by rough endoplasmic reticulum (Fig. 9). Cisternae of rough endoplasmic reticulum were filled with medium electron dense materials and formed bulges in many places. In each bulge, a small number of cylindrical pieces of a relatively high electron density were observed. As for the Golgi complexes, they were rather undeveloped in these cells as compared with those found in other types of granular cells.

In another type of granular cell (*Type B*), the granules were polyhedral or nearly spheroidal in shape (Figs. 3 and 11). The largest granules measured 4–5 μ along their longest axis. Each granule was bounded by a membrane and was often combined with closely adjacent granules by means of the fusion of boundary membrane. Inside the membrane consisted of many spherical bodies usually of high electron density and a filamentous matrix of low electron density. The dense bodies, about 0.8 μ in diameter, appeared to lack a boundary membrane and were generally located along the rim of the granule (Fig. 11). The internal structure of small granules contained in this type of cell was almost the same as that of the full-sized ones, though the dense bodies in one granule were fewer in number. As in the case of the cells of *Type A*, cisternae of rough endoplasmic reticulum swelled in parts and contained medium electron dense materials. The Golgi complexes were well-developed, and the ends of their cisternae often contained electron dense materials (Fig. 12).

In another different type of granular cell (*Type C*), the granule was rather spherical in shape and was less than 3 μ in diameter. It was also bounded by a membrane. The matrix of the granule was homogenous and showed a medium electron density (Fig. 13). There were a small number of electron dense bodies, about 0.8 μ in diameter, in the matrix. In some of these cells, the internal structure of the granules was somewhat altered. In addition to the electron dense bodies, the granules of these cells contained a large number of small, electron lucent particles in the medium dense matrix (Fig. 14). The internal structure of small granules found in this type of cell was nearly the same as that of the full-sized ones, though the electron density of the matrix appeared low. No appreciable difference in the morphological character of rough endoplasmic reticulum and Golgi complex was detected between these cells and the cells of *Type B*.

When sections were made through the anterior and posterior terminal parts of the clitellum, a peculiar type of granular cell (*Type D*) was occasionally observed

(Fig. 15). The granules of these cells were also nearly spherical in shape and were about 3 μ in diameter. However they were so firmly packed with a large number of medium or high electron dense particles that the interstitial matrix of the granule was hardly visible. The same internal structure was observed in small granules found near the Golgi complex. In this case, however, the electron density of the particles appeared low (Fig. 15). Cisternae of rough endoplasmic reticulum showed a considerable swelling and contained low electron dense materials. The Golgi complexes were not so well-developed as that found in cells of *Types B* and *C*.

Discussion

In the present light microscopical observation it was shown that the clitellar epithelium of *Tubifex hattai* consists of at least three kinds of cells: supporting, mucous and granular cells. The granular cells were subdivided into three types (*I, II* and *III*) based on the staining properties of the contained granules. Using the same materials, Hirao (1965) attempted to determine the cell types of clitellar epithelium and also distinguished three types of granular cells: *A* cell containing azocarmine granules, *B* cell having aniline blue granules and *C* cell possessing both azocarmine and aniline blue granules. Judging from the distribution of the cells in the clitellum and the staining properties of the granules, it may be safe to say that the cells of *Types I* and *III* in the present study correspond to Hirao's *C* and *B* cells respectively. Since the cells of *Type II* were never found in the terminal region of the clitellum, they were not equivalent to the *A* cells described by Hirao. Although the staining property of the granules was not strictly in accord with Hirao's description, it seemed probable that the cells of *Type I* also correspond to a variety of *A* cells. If so, it may be supposed that Hirao (1965) failed to detect the cells of *Type II* in the clitellar epithelium.

By the electron microscopical observation, the granular cells were also subdivided into at least three types by the organization of the inclusions. Large granules found in the cells of *Type A* showed an unusual lamellar structure. Small granules did not exhibit such definite structure but contained filamentous bundles. Considering the size of the granules, it is probable that the structural difference found between large and small granules represents variations in the stage of formation of the granules. The variation may be reflected in the dye-binding properties of the granules themselves. Thus, the cells of *Type I* in the light microscopy may appear to contain two kinds of granules showing an affinity to either azocarmine or aniline blue. Since no dense bodies were detected electron microscopically in these granules, the cells of *Type A* probably correspond to the cells of *Type I* in the light microscopy. In the cells of *Type II*, the granules were stained with azocarmine and surrounded by faint blue materials. If the dense bodies found in the electron microscopical observation of the cells of *Type B* had an affinity to azocarmine and the filamentous matrix of low or medium electron density was stained faintly with aniline blue in the light microscopy, it can be

supposed that the cells of *Type II* correspond to those of *Type B*. The number and size of dense bodies also support this inference. If the same held true for the granules found in the cells of *Type III*, it is likely that these cells correspond to those of *Type C*. The electron dense bodies were few in number in a single granule of the cells of this type. A small number of the azocarmine-particles were detected in a single granule. The matrix of these granules is different in chemical composition from that of the granules found in the cells of *Type II* or *B*, because it was intensively stained with aniline blue and showed a strong positive reaction to the PAS test in the former case but was faintly stained with the same dye and exhibited only a weak PAS reaction in the latter. In the present study, the fourth type of cell was distinguished electron microscopically from the standpoint of granular structure. Since it was observed only occasionally, there are not sufficient data on these cells.

The granules of mucous cells bore a comparative resemblance to those described by Coggeshall (1966) for *Lumbricus*. These mucous cells possessed concavity at the apical end. Just beneath the concavity, there were granules consisting of filamentous materials. Similar materials were also found in the concavity which opened externally through a narrow slit found in the overlying cuticle layer. These facts may indicate that the mucous cells release the materials of the granules into the concavity, that is, they are gland cells. The same morphological situation was also observed in other kinds of granular cells. In many oligochaete species, Potswald (1971), Burke (1974) and Richards (1974) also observed secretory products of epidermal cells and detected electron dense bodies in the products which had a morphological resemblance to those contained in the granules of the cells of *Types II* and *III*. From the histochemical studies on lumbricids, Richards (1973) suggested that all cells containing secretory products in the ordinary epidermis belong to the category of mucous cells. Therefore it can be supposed that the granular cells observed in the present study has the function of a unicellular gland. The granular components may represent the secretory products of these cells. It is known that secretory granules of many kinds of cells show characteristic ultrastructures (for example, the cortical granules of the sea urchin and the secretory granules found in the salivary glands of the fly, etc.) (Afzelius, 1956; da Cunha *et al.*, 1973). According to Hirao (1966), contact of the dorsal epithelial cells of the clitellum with a solid body induces the release of the inclusions from the granular cells. Since no synaptic figures of the epithelial cells were observed in the present study, it might be supposed that the nervous system of the worm does not directly participate in the initiation of the release of granules from the cells.

The electron microscopical figures obviously indicate the occurrence of the conjugation of small granules in the manner of the fusion of the boundary membrane. In the process of conjugation, the reorganization of the constituents of the granules is induced and the characteristic structures of the granules are formed. Although the way in which the granules appear in the cells is unknown, the

presence of well-developed Golgi complexes and endoplasmic reticulum in the granular cells might indicate the contribution of these cellular organelles in the granular formation.

Summary

1. Cell types of the clitellar epithelium of a freshwater oligochaete, *Tubifex hattai* were determined. The epithelium consisted of supporting, mucous and granular cells.

2. The granular cells were a characteristic component of the clitellum. From the staining properties and the fine structure of the inclusions, they were subdivided into three types; *Types I*, *II* and *III*. All of them had the morphological nature of gland cells and showed a specific pattern of distribution in the clitellum.

3. Except for the ventral side of the anterior and posterior terminal parts, the cells of *Type I* were found in every part of the clitellum. Their granules have an affinity to either azocarmine or aniline blue, and the larger ones showed a definite lamellar structure at electron microscopical level.

4. The cells of *Type II* were distributed only in the intermediate part of the clitellum. Their granules were stained with azocarmine, and were never directly embedded in the cytoplasm but surrounded by amorphous materials faintly stained with aniline blue. Electron microscopy showed that the azocarmine granules really represent many electron dense bodies suspended in the granular matrix.

5. The cells of *Type III* were found only in the anterior and posterior terminal parts of the clitellum. Even in this part, however, they were not detected on the ventral side. Their granules were clearly stained with aniline blue and contained a small number of azocarmine-stained particles. Electron microscopically the granule consisted of a small number of electron dense bodies and a medium electron dense matrix.

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Explanation of Plates I-III

Fig. 3. Longitudinal section through the dorsal clitellar epithelium showing four cell types: supporting cells (S), *Type A* cells (A), *Type B* cell (B) and *Type C* cells (C). Cuticle (Cu) and muscle layers (M) are also shown. × 4,000.

Fig. 4. Epidermal cuticle of the clitellar region showing outer layer (O) frilled with particles (P) and inner layer (I). Note microvilli of the underlying supporting cells penetrating the cuticle (arrows). × 12,000.

Fig. 5. Electron micrograph showing space between epithelial (E) and muscle layers (M) filled with filamentous materials (arrows). The basal lamina can not be found. × 15,000.

Fig. 6. Granules of a mucous cell which consist of filamentous materials and lucent particles (arrows). × 15,000.

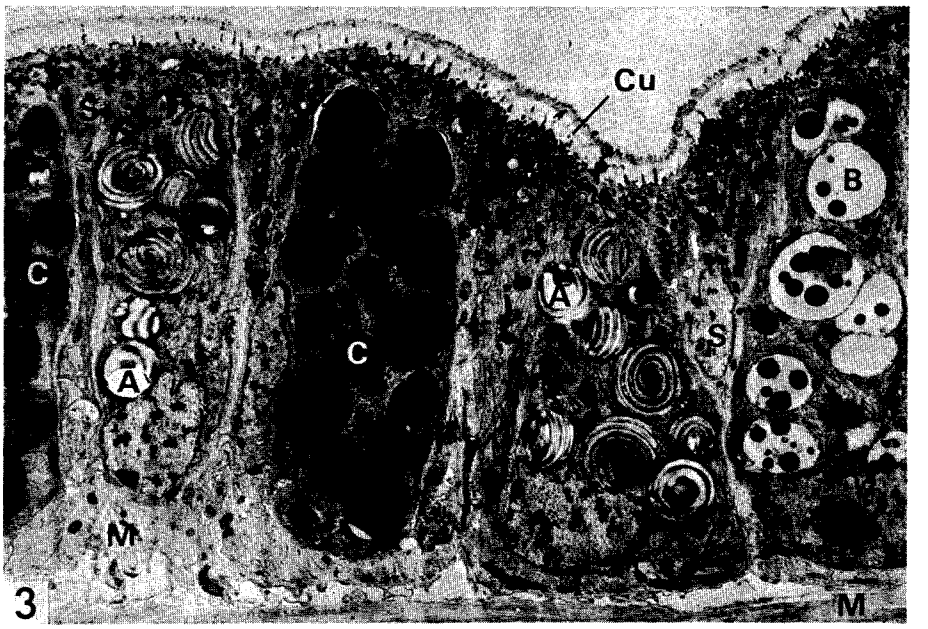
Fig. 7. Section through the apical portion of *Type A* cell. The concavity (Cc), secretory granules (S) and microvilli (M) are clearly seen. Note slit (arrow) running through the cuticle (Cu). × 15,000.

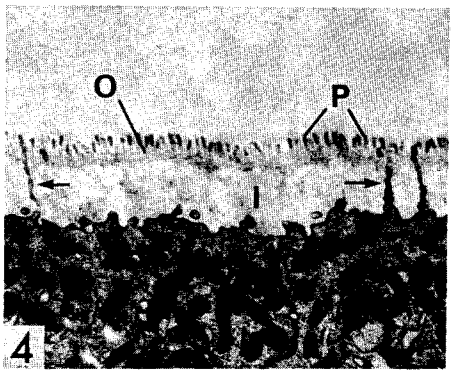
Fig. 8. A large granule of *Type A* cell showing the lamellar structure. In the lower right, a small granule is also seen. × 18,000.

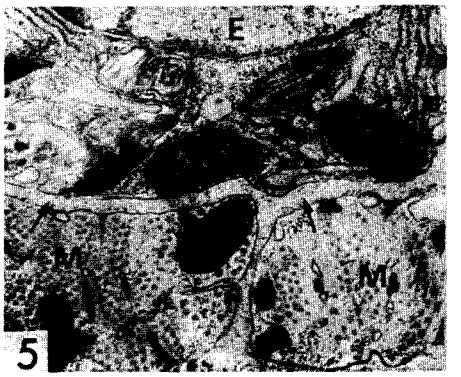
Fig. 9. Electron micrograph of central region of *Type A* cell showing rough endoplasmic reticulum (ER), Golgi complexes (G), secretory vesicles (S) and mitochondria (M). Note bulges of rough endoplasmic reticulum which contain relatively dense materials (arrows). × 25,000.

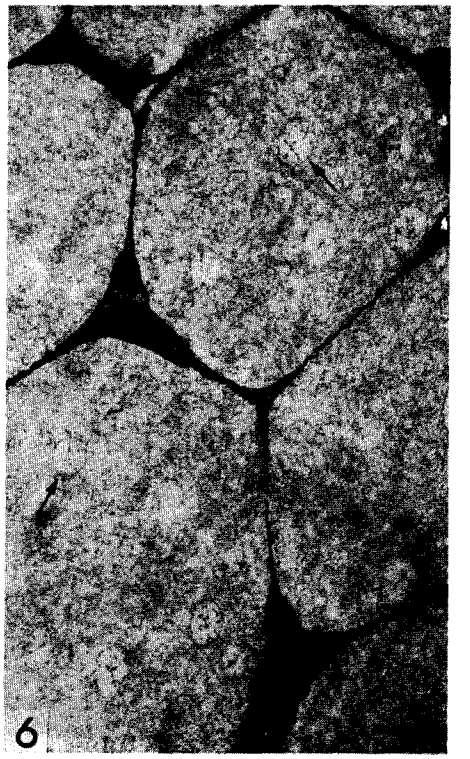
Fig. 10. A small granule found in the region shown in Fig. 9. × 16,000.

Fig. 11. Large granules of *Type B* cell containing many dense bodies (B) in the filamentous matrix. In parts, the matrix shows medium electron density (asterisk). Note the fusion of granular membrane in small restricted areas (arrows). × 15,000.

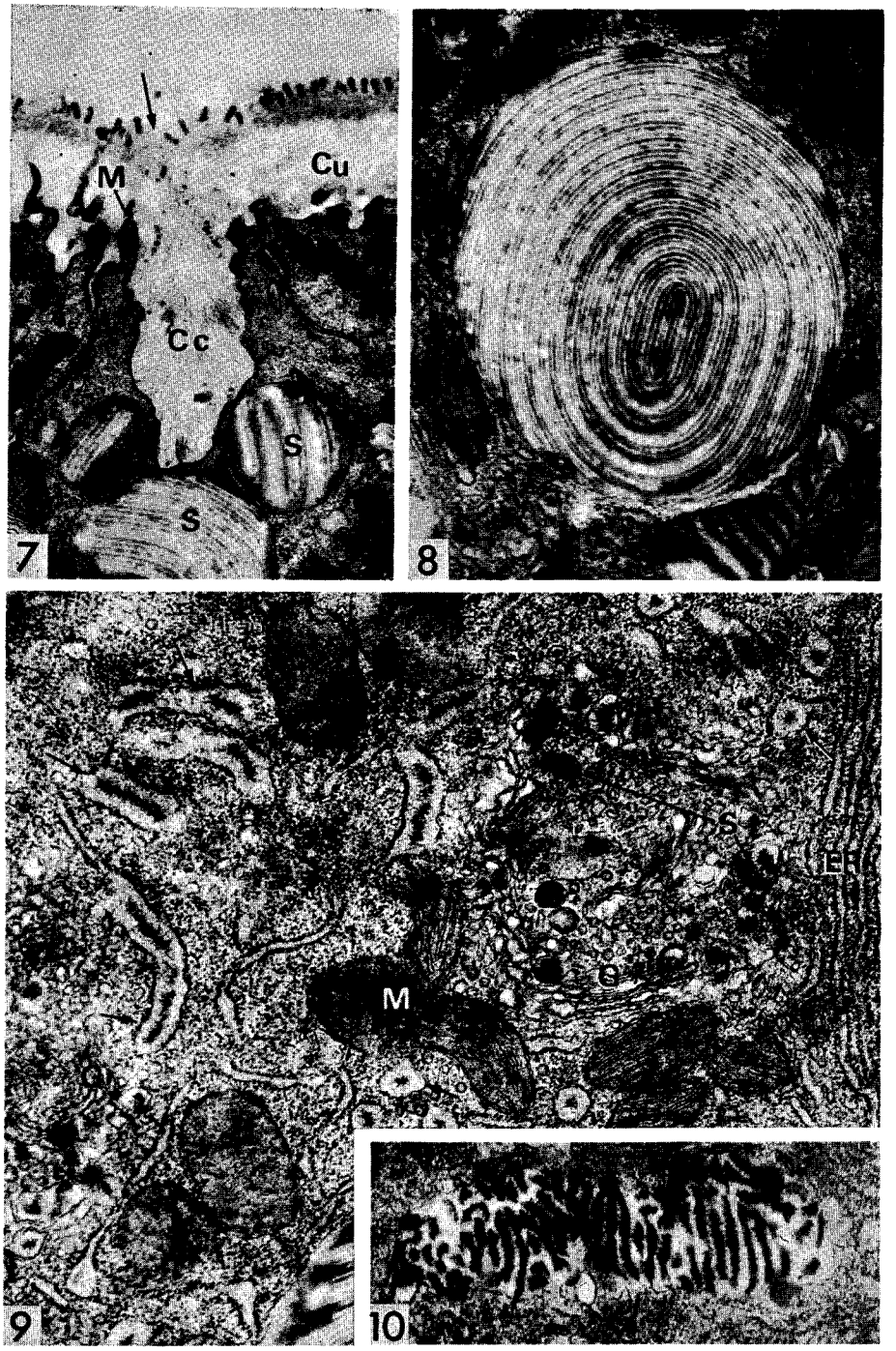








Ch. Suzutani: Clitellar Epithelium of Tubifex



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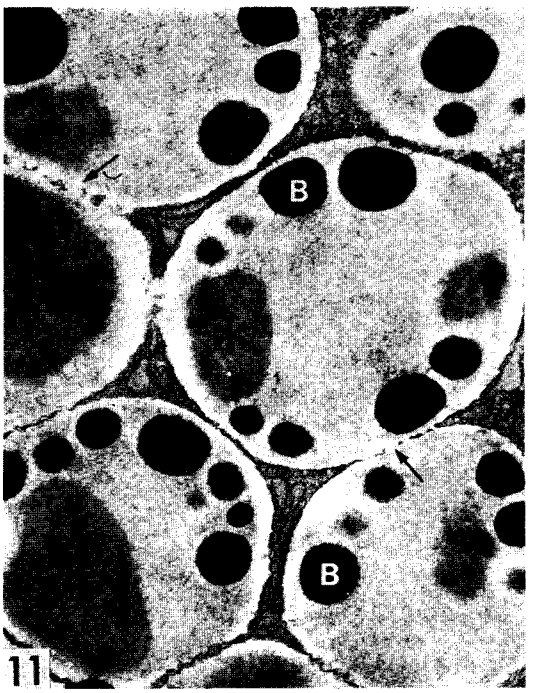

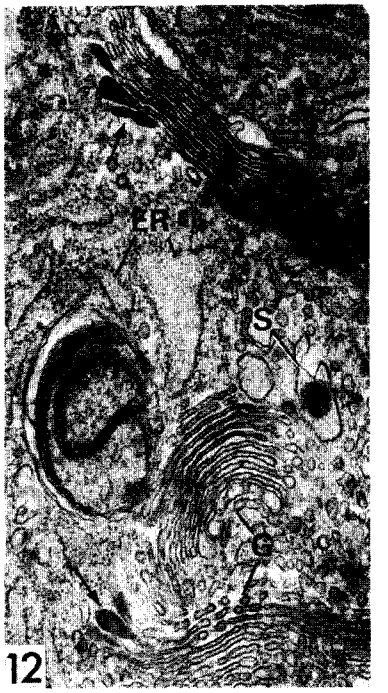

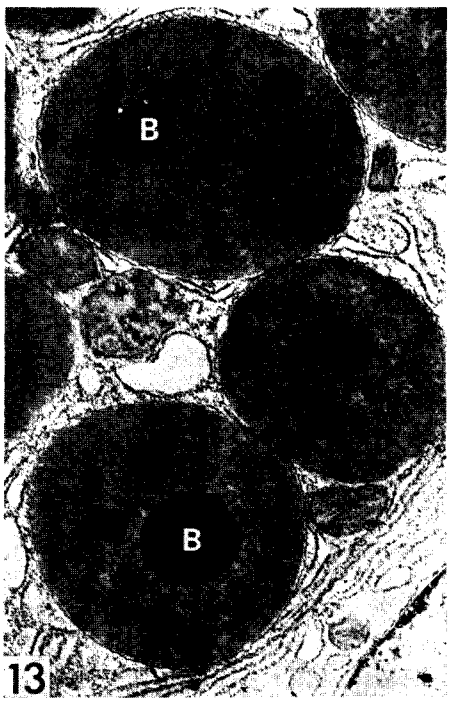

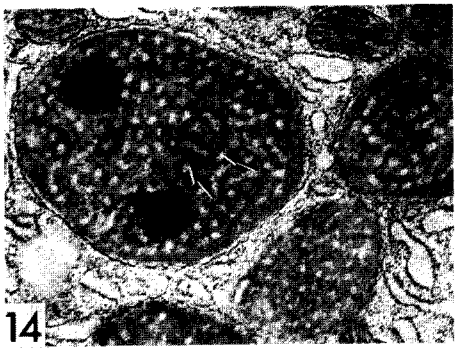

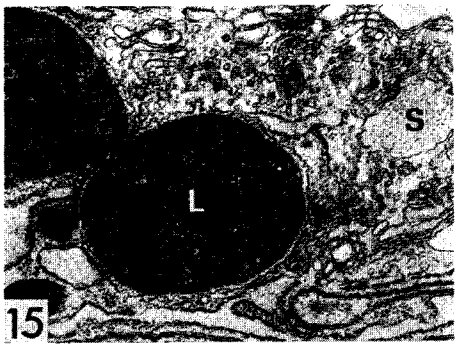

Ch. Suzutani: Clitellar Epithelium of Tubifex

Fig. 12. Electron micrograph of the peripheral region of *Type B* cell showing Golgi complexes (G), rough endoplasmic reticulum (ER) and a secretory vesicle containing a dense body (S). Note dense materials contained in the cisternal ends of Golgi complexes (arrows). × 25,000.

Fig. 13. Large granules of *Type C* cell. A small number of dense bodies (B) are suspended in a matrix of medium electron density. × 15,000.

Fig. 14. Electron micrograph showing peculiar granules found in *Type C* cell. Note a number of electron lucent particles (arrows) in the granule. × 15,000.

Fig. 15. Electron micrograph showing large (L) and small granules (S) in *Type D* cell. Note numerous fine particles occupying the interior of the granule. × 15,000.