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Cocoon Formation in *Tubifex*, with its Relation to the Activity of the Clitellar Epithelium¹⁾²⁾

By

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(With 2 Text-figures and 1 Plate)

Most of our knowledge concerning the oviposition of Oligochaeta originates from observations of terrestrial earthworms (Foot, '98; Foot and Strobell, '02; Grove, '25; Grove and Cowley, '26, '27). With fresh water oligochaetes, however, little information is available on the same subject. Because of the cocoon formation being indispensable to the normal development of *Tubifex* eggs (Lehmann, '56; Inase, '60a, b), it seems necessary to know the processes of its formation and its structure.

The conditions necessary for the occurrence of normal oviposition in *Tubifex hattai* have been presented in the previous paper (Hirao, '65). The present paper deals with observations on the manner of the cocoon formation and its deposition. The results of histological observations concerning the structure of the cocoon and its relation to the activity of the clitellar epithelium will also be described.

Material and method

The material used was the fresh water oligochaete, *Tubifex hattai*, which was collected from the stream running through the campus of Hokkaido University. The worms to be used within a week after collection were placed in glass vats and were cultured with running water (10–15°C), while those reared for a longer period were placed in a refrigerator at 5–7°C with constant aeration. In both cases, a small amount of sterilized sand was placed at the bottom of the glass vats which contained the worms.

For the observations of ovipository behavior, a glass container of special design was prepared. It consisted of two sheets of glass, 10 cm long by 8 cm high, which were separated by narrow strips of glass, 0.5 cm in thickness, along the bottom and sides. The container was filled with well water and sterilized sand, and 5 or 6 mature worms introduced. The observation container was placed horizontally on the stage of the binocular microscope for observation of ovipository behavior. The observations were performed in a dark room (room temp. 20–25°C), with a red light focused on the worms to be observed (*cf.* Hirao, '65).

1) This paper is dedicated to Professor Sajiro Makino, Zoological Institute, Hokkaido University, Sapporo, in honor of his sixtieth birthday, June 21, 1966.

2) Contribution No. 727 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

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Because the worms are very sensitive to slight vibrations of the water, care was taken during the observation to avoid any vibration of the container.

For the histological observations of the clitellar epithelium and cocoons, worms and cocoons were fixed in Bouin's fluid. Serial 3-5 μ sections were made by the ordinary paraffin method. Most of the sections were treated with Heidenhain's Azan stain, but stainings with Delafield's hematoxylin and eosin or Heidenhain's iron hematoxylin were also used. For the detection of polysaccharides, a successive staining with alcian blue and periodic acid-Schiff reaction (alcian blue-PAS) was employed.

Observations

Manner of cocoon formation and its deposition in the fresh worm: The worms near the beginning of oviposition possess fully grown oocytes arranged in the coelom of segment 11 and ovisac (Hirao, '64). The eggs located in these parts can be seen through the swollen body wall of the clitellum with the naked eye. Worms of such appearance begin ovipository behavior soon after being placed at room temperature. In mature worms the bulk of the clitellar part increases slightly more than in immature ones. When placed in the observation container, the worms dive deeply in the sand leaving the posterior portion of the body mildly twisting in the water.

The ovipository behavior can be divided into two successive phases, *i.e.* the cocoon formation around the clitellum and the release of the formed cocoon from the worm. The first indication of cocoon formation is noticed by a marked increase of the bulk and of the opaqueness of the clitellar part (Fig. 1a). To the surface of the swollen clitellum the sand adheres, indicating the occurrence of active secretion of slime (Fig. 1b). Soon later, a membranous structure separates from the surface of the clitellum. This is the cocoon membrane described below. Then the body wall of the worm begins to show violent constriction which spreads in a wave-like fashion starting from the posterior end of the clitellum towards the anterior part of worm. The wave of this constriction is repeated several times. Violent movements of both longitudinal and circular muscles are observable throughout the segments of the body. Accompanied with these movements, the eggs in the coelom of segment 11 and ovisac are found rotating in random directions. The flows of coelomic fluid are also observable. During these movements the cocoon membrane gradually takes a distinct tubular structure, forming a tube called the "cocoon tube" (Fig. 1c). The time required for the formation of the cocoon tube is about 15 to 20 minutes.

The mature eggs located in the coelom of segment 11 and ovisac are released through a pair of ventrally opened female pores to the formed cocoon tube. During the release, several contracting movements occur throughout the segments of the body. The release of eggs results in a remarkable swelling of the cocoon tube, which is fixed to the body surface at the anterior and posterior ends (Fig. 1d). The cocoon tube is firmly adhered to by relatively heavy sand particles, so that when the worm begins to withdraw, it remains stationary and is released from the body

of the worm (Figs. 1e-g). The time taken for the backward movement of the worm to deposit the cocoon is extremely short, *i.e.* within 10 seconds. Immediately after deposition, the anterior and the posterior ends of the tube exhibit a marked depression (Fig. 1g). Soon after, the deposited cocoon takes an oval shape with plug-like processes on both ends which correspond to the anterior and the posterior ends of the cocoon tube (Fig. 1h). The average time required for the whole process of cocoon deposition after the completion of the cocoon tube is 30-60 seconds. The clitellum of the worm after cocoon deposition exhibits a reduction in bulk and takes a color close to that of the other segments. No eggs are found in the coelom of segment 11 and the ovisac.

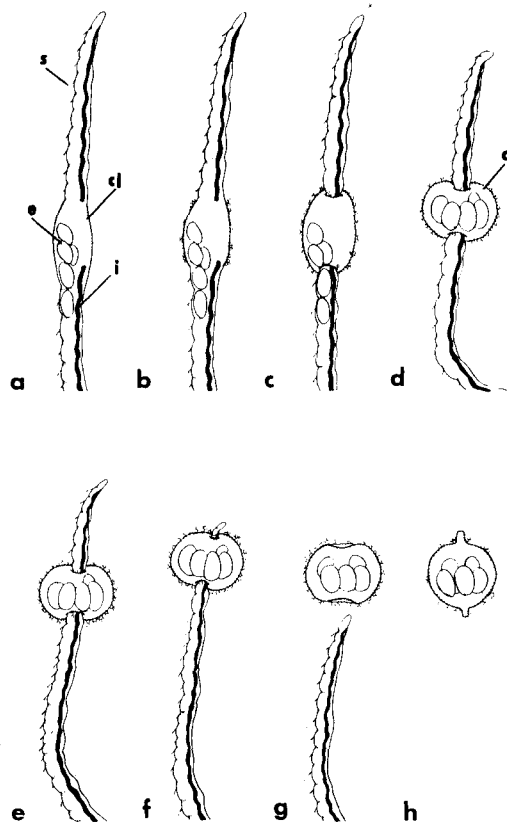


Fig. 1. Semi-diagrammatic illustration showing the successive steps of cocoon formation and deposition. c, cocoon tube; cl, clitellum; e, egg; i, intestine; s, seta.

About 2-4 hours after the cocoon deposition, sand particles adhering to the cocoon surface fall off, indicating the disappearance of the adhesiveness of the surface.

The cocoon thus freed from sand has a transparent and well-polished surface, so that the eggs contained in the cocoon can be observed through its membrane. Besides the eggs, the cocoon contains a colloidal fluid. The number of eggs present in a cocoon is usually 7-8, maximum 18 and minimum 1. The size of a cocoon containing 7-8 eggs measures 1.4 mm and 0.9 mm in long- and short axis, respectively.

Histological studies of clitellar epithelium in relation to cocoon formation: The clitellum of the present material occupies segments 10 to 11, as previously described (Hirao, '64). Except at the caudal end of the worm, the epidermis is composed of supporting cells and mucus cells. Between the supporting cells, mucus cells stained homogeneously with aniline blue are found here and there.

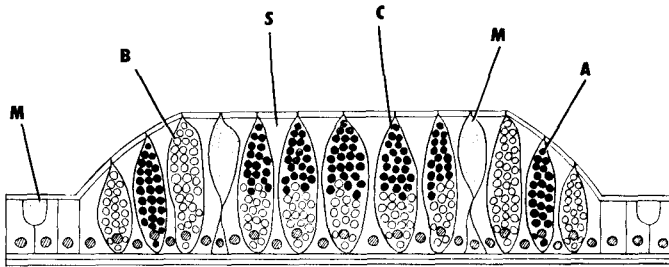


Fig. 2. Semi-diagrammatic illustration of clitellar epithelium, showing four kinds of gland cells. A, A cell with azocarmine granules; B, B cell with aniline blue granules; C, C cell with azocarmine and aniline blue granules; M, mucus cell; S, supporting cell.

The clitellar epithelium is characterized not only by its increased thickness but also by the nature of the cells contained in it. Namely, in addition to the mucus cells, three kinds of gland cells are distinguished by Heidenhain's Azan stain (Fig. 2). The cells of the first type, designated A cells, contain a number of granules which stain with azocarmine. The cells of the second type, designated B cells, contain granules which stain with aniline blue. The cells of the third type, designated C cells, contain two kinds of granules which stain with azocarmine and aniline blue, respectively. In this case the azocarmine granules occupy the upper portion of the cell, and the aniline blue granules the bottom half. These three kinds of gland cells are large in volume, but the supporting cells in the clitellum are extremely narrow in width. The nucleus of the gland cells is situated in the basal part of the cells. The gland cells described above cannot be distinguished in the preparation stained with Delafield's hematoxylin and eosin. With Heidenhain's iron hematoxylin, the azocarmine granules stain deeply, but the aniline blue granules do not stain at all. When the alcian blue-PAS test is employed, the aniline blue granules and mucus cells are strongly positive either with alcian blue or PAS test, but the azocarmine granules stain only weakly.

Except for the mucus cells, the gland cells show a definite pattern of distribution in the clitellar part of the epithelium. A and B cells occupy both the anterior

and posterior terminations of the clitellum (Figs. 4a & b), whereas the intermediate part is predominantly occupied by *C* cells (Fig. 2). Mucus cells are found in every part of the clitellum. At the anterior and posterior terminations of the clitellum, the epithelium of dorsal and lateral sides is considerably thicker and contains both *A* and *B* cells as well as mucus cells. In the ventral side, however, the epithelium is relatively thin and no other gland cells are found except the mucus cells. At the intermediate part, the epithelium of the dorsal and lateral sides is thicker than that of the ventral side. In this part, however, *C* cells and mucus cells are located in each side.

Sections of the clitellar part showing the first indication of cocoon formation revealed that slime stained with aniline blue is present on the clitellar surface. On the other hand, the mucus cells of the clitellum are almost empty. It seems that the contents of the mucus cells are secreted at the first step of cocoon formation. Adherence of sand particles as observed in the fresh material is likely to be due to the secretion of the slime from the mucus cells. In the section of the worm at the next step of cocoon formation, a membrane stained deeply with azocarmine becomes observable beneath the slime described above (Fig. 9). This membrane, *i.e.* the cocoon membrane, gradually increases in thickness and is separated from the clitellar surface. A remarkable change of the clitellar epithelium at this step is that the azocarmine granules are scarcely visible in *A* and *C* cells, indicating the occurrence of active secretion of these gland cells (Fig. 5). These observations provide evidence that the cocoon membrane is formed by the secretion of the azocarmine granules contained in *A* and *C* cells. It must be noticed here that the cocoon membrane attaches firmly to the epithelial surface at both terminations of the clitellum. When the cocoon tube is fully formed, eggs are pushed out through the narrow female pores into the cocoon tube (Fig. 3). Often eggs of a dumb-bell shape are found. The eggs released from the worm possess the metaphase spindle of the first maturation division. In the lumen of the cocoon tube, there is a small amount of precipitate stained faintly with aniline blue (Fig. 7). This is the remnants of colloidal fluid which has been found in the fresh material. At this stage of the cocoon formation the aniline blue granules situated previously in the bottom half of *C* cells are found in the upper part of the cells. Sometimes it was observed that aniline blue granules were secreted from *C* cells to the lumen of the cocoon tube (Fig. 6). Secretion of aniline blue granules also occurs from *B* cells at both terminations of clitellum. The granules from *B* cells can be distinguished from those of *C* cells by their strong affinity to the dye even after secretion.

The membrane of a cocoon immediately after deposition is composed of two layers: *viz.* the inner layer is a membranous structure stained with azocarmine and the outer one is the slime stained with aniline blue (Fig. 9). The colloidal fluid in the cocoon lumen stains faintly with aniline blue. The plug-like processes, consisting of a material deeply stained with aniline blue, are formed by the aniline blue granules in *B* cells (Figs. 8a & b). When subjected to the alcian blue-PAS

test, the processes show a strongly positive reaction. The outer slime layer of the cocoon membrane and the colloidal substance of the cocoon lumen show a moderate reaction, but the cocoon membrane itself stains only faintly. After deposition, the surface of the cocoon becomes smooth losing the outer slime layer with adhering sand particles. In the section, the outer slime layer is not observable and the cocoon membrane consists of only a membraneous structure (Fig. 10).

Discussion

With the terrestrial earthworm, *Eisenia foetida*, Grove and Cowley ('26, '27) made extensive studies on the manner of oviposition and its relation to the change of the clitellar epithelium. As for the process of cocoon formation and deposition, similarities are found in many respects between the present material and *Eisenia*; *i.e.* the secretion of slime, the cocoon tube formation, and the backward movement of the worm at cocoon deposition. According to Grove and Cowley ('26), the prominent slime tube is observable extending over several segments of both anterior and posterior ends of the clitellum. Though such a prominent tube has not been observable in *Tubifex*, the secretion of slime actually occurs, as defined by an adhesion of sand particles as well as by histological examinations. The slime tube of *Eisenia* dries up and disappears on exposure to the air. Similarly the slime of the present material disappears 2-4 hours after cocoon deposition. Even though the role of the slime has to be studied in more detail, there are some observations that the adhesion of sand particles to the slime may play a role in the successful deposition of a formed cocoon. That is to say, worms reared in water without sand fail to deposit the normal cocoon (Hirao, '65).

In the present observation four kinds of gland cells are distinguished in the clitellar epithelium from their staining properties as well as their specific distribution. The histological studies of Grove ('25) and Grove and Cowley ('27) on the clitellar epithelium of *Eisenia* and *Lumbricus* revealed that three kinds of glandular cells are distinguishable; *i.e.* mucus cells, large granule cells and fine granule cells. The mucus cells occur also in *Tubifex*. The contents of the large granule cells may correspond to the azocarmine granules of *A* and *C* cells, because they participate in the formation of the cocoon membrane. Similarly the contents of the fine granule cells seem to be comparable to the aniline blue granules of *C* cells in that they are the substance of the colloidal fluid contained in the cocoon lumen. Aniline blue granules found in *B* cells, a material for the plug-like processes of the deposited cocoon, uniquely occur in *Tubifex*. Such processes at both ends of the cocoon of *Tubifex* are not found in the cocoon of the terrestrial forms.

Summary

1. With the fresh water oligochaete, *Tubifex hattai*, observations were made on the manner of the cocoon formation and its deposition. Histological observations were also made on the clitellar epithelium in relation to cocoon formation.

2. The process of cocoon formation and deposition as observed in the fresh material was described. At first the sand adheres to the clitellar surface and the "cocoon tube" is formed, into which mature eggs are pushed out through the female pores. The cocoon deposition is accomplished by the backward movement of the worm.

3. The deposited cocoon takes an oval shape, with plug-like processes at both ends. In a cocoon, there are usually 7-8 eggs which are suspended in colloidal fluid.

4. Histological studies revealed that the epithelium of whole segments is composed of a single layer of cells, except at the caudal end. Most epithelial cells are supporting cells. Between the supporting cells, mucus cells stained with aniline blue are distributed over whole segments.

5. The clitellar epithelium is characterized by its increased thickness and the gland cells contained in it. From their reaction to Azan stain, four kinds of gland cells are distinguishable in this part; *i.e.* *A* cells containing azocarmine granules, *B* cells containing aniline blue granules, *C* cells containing both azocarmine and aniline blue granules, and mucus cells. *A* and *B* cells occupy anterior and posterior ends of clitellum, whereas *C* cells occupy the intermediate part.

6. The cocoon membrane is formed by the secretion of azocarmine granules from *A* and *C* cells, and the colloidal fluid by that of aniline blue granules from *C* cells. Finally the plug-like processes at both ends of the cocoon are formed by the secretion of *B* cells. The adherence of sand around the clitellar surface is due to the secretion of the mucus cells.

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Explanation of Plate XXI

Fig. 3. Section through a female pore of a worm undergoing oviposition. cl, clitellar epithelium; e, egg; p, female pore. Delafield's hematoxylin-eosin. ca. \times 340.

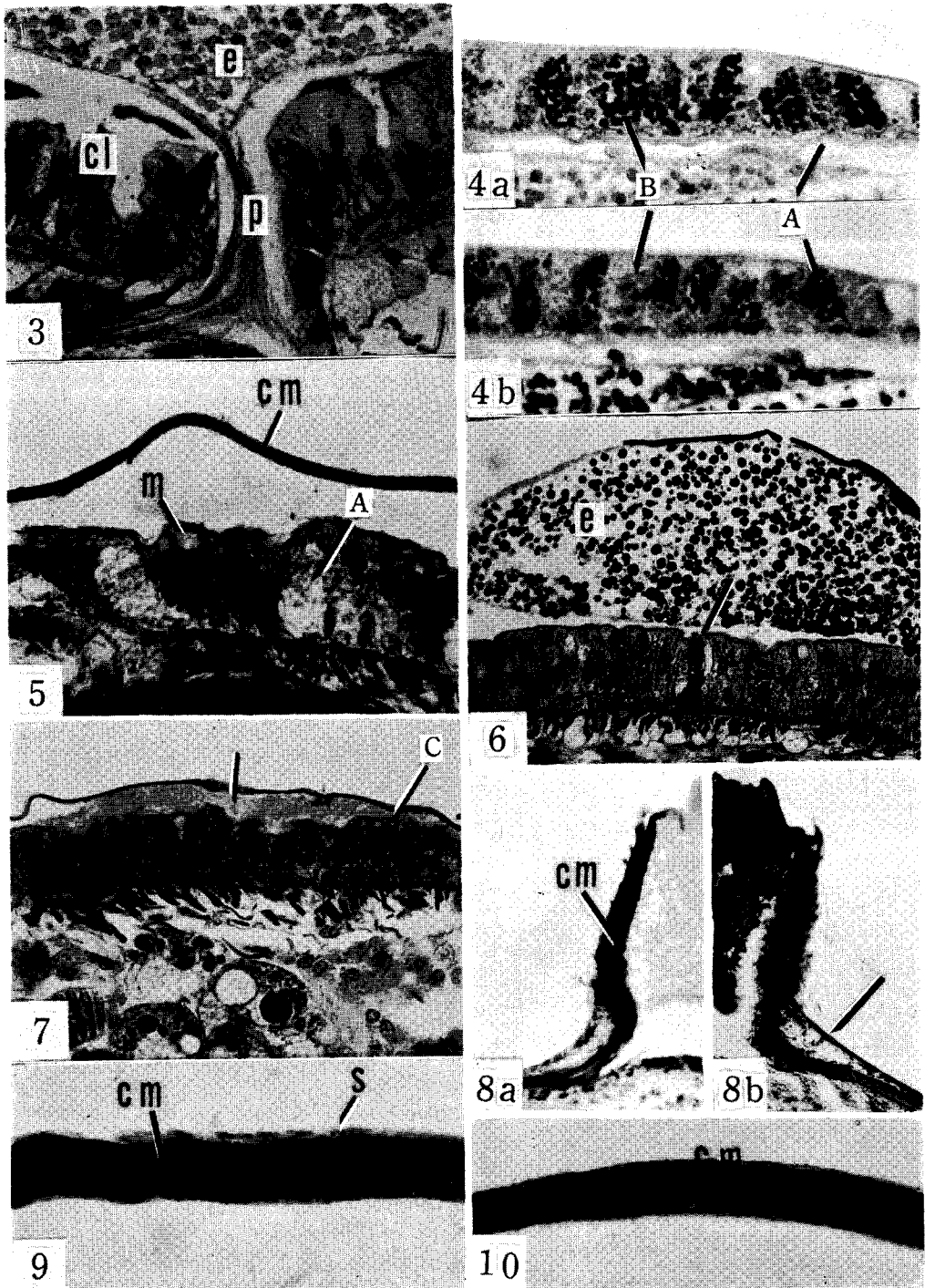
Figs. 4a & b. Photomicrographs of the same section through the termination of a clitellar epithelium before cocoon formation. ca. \times 620. 4a, photographed through blue filter to show only azocarmine granules. 4b, photographed through red filter to show only aniline blue granules. A, A cell; B, B cell. Heidenhain's Azan.

Fig. 5. Section of clitellar portion under cocoon formation, showing formed cocoon membrane (cm). Note A cell (A) and mucus cell (M) which are empty. Heidenhain's Azan. ca. \times 510.

Figs. 6-7. Cross-sections of clitellar portion under cocoon formation. ca. \times 100. Fig. 6; showing an egg(e) released into cocoon tube and the empty epithelial cells. Note secretion of aniline blue granules from C cell (arrow). Fig. 7; showing colloidal fluid (arrow) and aniline blue granules migrated to the upper portion of C cell (C). Heidenhain's Azan.

Figs. 8a and b. Photomicrographs of a section through plug-like process of cocoon. ca. \times 510. 8a, photographed through blue filter to show cocoon membrane (cm) stained with azocarmine. 8b, photographed through red filter to show slime (arrow) and content of plug stained with aniline blue. Heidenhain's Azan.

Figs. 9-10. Sections through deposited cocoon membrane just after deposition (Fig. 9) and 4 hr. after deposition (Fig. 10), respectively. ca. \times 540. Note that the outer slime layer (s) can be hardly seen in Fig. 10. cm, cocoon membrane; s, slime layer. Heidenhain's Azan.



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