The Role of Sand Particles in the Ovipository Circumstance of *Tubifex*[1)]

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(*With 5 Text-figures and 4 Tables*)

In a previous paper the manner of oviposition in *Tubifex* was studied in connection with the secretory activity of the clitellar epithelium of the worm (Hirao, 1965a). In that process, the first indication of oviposition was shown to be the adherence of sand particles to the clitellar surface. Further it was found that the deposition of formed cocoon tube is not successful in the sand-free circumstance, with the scattering of the eggs from the tube (Hirao, 1965b). The eggs thus freed from the cocoon cease their development at early cleavage stages as reported by early workers (Inase, 1960a, b; Lehmann, 1948, 1956, *etc.*).

In order to know why the sand-free circumstance prevents the oviposition of the worm, the detailed processes of oviposition and cocoon deposition were studied by placing the worms in various experimental circumstances. The results will be described and discussed in relation to the secretory activity of clitellar epithelium.

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 methods for cultivating the worms in the laboratory were the same as those previously described (Hirao, 1965a). In order to find out the conditions suitable for the successful oviposition, petri-dishes of 5 cm in diameter with well water were prepared, and the bottom of each dish was covered with the following several kinds of substances respectively, *i.e.* sand, sea sand A (0.2 mm in diameter), sea sand B (1 mm), glass fragment A (0.2 mm), glass fragment B (1 mm), glass fragment B′ (2 mm), emery A (0.15 mm), emery A′ (0.5 mm) filter paper, gause, wire netting A (16 mesh/inch), wire netting B (50 mesh), absorbent cotton and glass wool. The petri-dish containing only well water served as control of these conditions. One worm near the oviposition (*cf.* Hirao, 1965a) was separately placed in the petri-dish thus prepared and was allowed to deposit eggs in a dark room (room temp. 20–23°C). Observations were performed with a red light focused on worms (Hirao, 1965b). Water in petri-dishes was renewed twice a week, and the occurrence of oviposition was observed for one cocoon per one worm.

For the histological observations of the clitellar epithelium, worms were fixed in Bouin's

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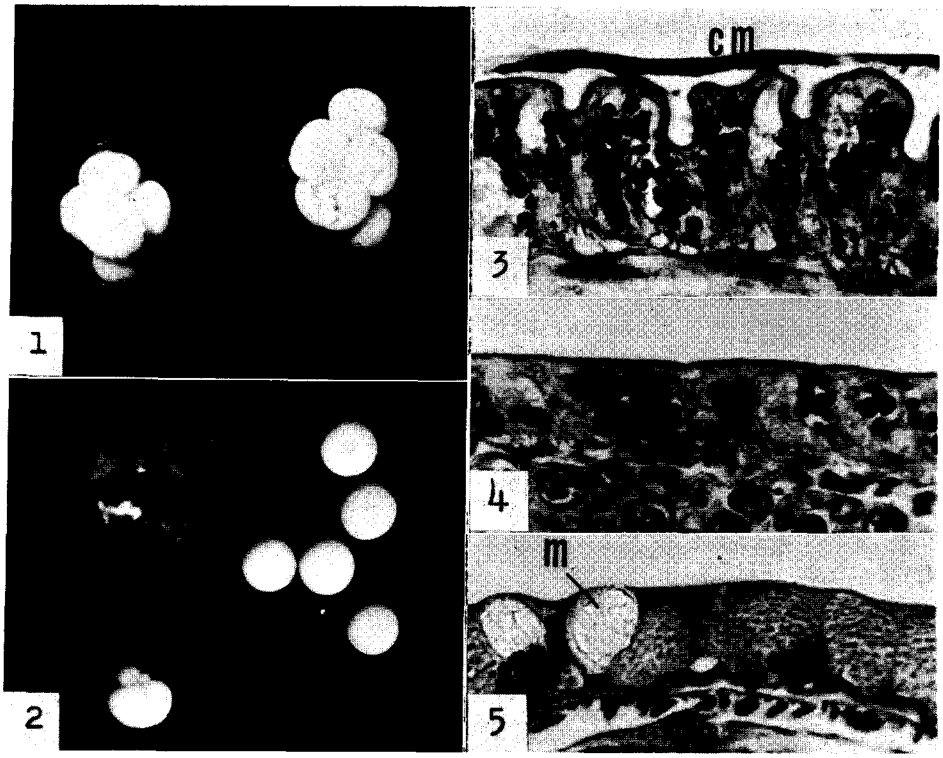
fluid. Three-5μ thick sections were prepared with the ordinary paraffin method as previously described (Hirao, 1965a) and were stained with Heidenhain's Azan stain.

Results

The normal ovipository process as observed in the worm placed in well water with sand was described previously (Hirao, 1965a). Briefly speaking, the process is divided into four steps: (1) the secretion of slime and adherence of sand particles, (2) the formation of cocoon tube, (3) the release of eggs into the cocoon



Figs. 1–2. Photographs showing normal cocoon (Fig. 1), and ruptured cocoon tube with eggs freed from it (Fig. 2). ca. ×16.

Fig. 3. Section through clitellar epithelium of worm under cocoon tube formation without sand. Note a number of azocarmine granules unreleased from the gland cells. cm, cocoon membrane. Heidenhain's Azan. ca. ×600.

Figs. 4–5. Sections through clitellar epithelium just after cocoon deposition. ca. ×600. Fig. 4; Worm without sand, showing imperfect release of azocarmine granules. Fig. 5; Worm with sand, showing disappearance of secretory granules from the gland cells. m, mucus cell. Heidenhain's Azan.

tube, (4) the cocoon deposition. The time required for the steps (1)-(2) is about 20 minutes, and for (3)-(4) within 1/2-1 minute.

Oviposition without sand: When the worm is placed in the sand-free circumstance, the worm creeps about unsteadily on the bottom of the petri-dish. It is found that the formation of cocoon does not begin until 3-5th day. Since the cocoon formation usually occurs in sand circumstance within 1st day, there is much delay of the oviposition in this case. However, when the formation begins, the process normally proceeds and the cocoon tube is formed. To describe in detail, the bulk of the clitellar part increases and becomes more opaque owing to the active secretion of slime. Then the anterior and posterior terminations of the clitellum begin to show violent constriction. Soon later, the cocoon tube is formed around the clitellar part and the release of mature eggs into the formed tube begins. In contrast to the short time, *i.e.* within 30 seconds, in the normal process, it takes more than 1 hour. Thus, in some cases, a certain number of mature eggs remain unreleased in the coelom of segment 11 or ovisac. Continuously the worm begins to try to withdraw itself backwards from the cocoon tube. The attempt is made without result. During unsuccessful violent movements of the worm, the surface of the cocoon tube ruptures in some point. Thus almost all of the eggs contained in the tube are thrown out through the rupture. This movement of the worm for deposition of the cocoon tube continues for 5–6 hours, and finally the worm casts off the ruptured tube in front of the body. The cocoon thus deposited is lacking in the plug-like process at each end (Figs. 1 and 2). Most of the eggs freed from the ruptured cocoon suffer mechanical damage by the violent movements of the worm. The few survived eggs barely develop into 2–4 cell stages but undergo cytolysis soon thereafter. Comparison of the ovipository processes in the conditions with and without sand is shown in Table 1. The delays in the cocoon tube formation, the release of eggs into the formed tube and in the deposition of tube are clearly shown.

It was reported in the previous paper that three kinds of glandular cells (*A*, *B* and *C* cells[2)]) composing the clitellar epithelium show secretory activity during the course of the oviposition. Their secretory products form the cocoon tube and the plug-like processes of the deposited cocoon. The histological observations were made on the clitellar epithelium of the worms undergoing cocoon formation in the sand-free circumstance. Figure 4 shows the glandular cells of clitellar epithelium of the worm fixed just after the deposition of the ruptured cocoon. It is clear that the secretion of *A* and *C* cells is imperfect and a large number of the azocarmine granules remain in the gland cells (Figs. 3 and 4). The sections of the cocoon mem-

2) *A* cell contains only azocarmine granules which participate in the formation of the cocoon membrane. *B* cell contains only aniline blue granules which play a role in the formation of the plug-like processes of the deposited cocoon. *C* cell contains both azocarmine granules and aniline blue granules; the former serves as the material of the cocoon membrane and the latter forms the colloidal fluid of the cocoon in which the eggs are suspended (*cf*. Hirao, 1965a).

brane formed in the sand-free circumstance reveal that its thickness is more irregular than that of the normal cocoon. It was further noticed that the secretion of *B* cells is also imperfect and the aniline blue granules are not released completely.

Table 1. Comparison of ovipository processes of worm placed in conditions with and without sand

Steps of oviposition	Conditions	
	without sand	with sand
Slime secretion	apparent	apparent
Tube formation	3-5 days	20 minutes
Egg release	over 1 hour	within 30 seconds
Tube deposition	5-6 hours	within 60 seconds

Oviposition in some modified circumstances: In order to know the factors for the successful oviposition in the petri-dish, the several kinds of substances were used as a substratum of the dish. In the first experiment, the bottom of each petri-dish was covered with a sheet of filter paper, gause, wire netting A, wire netting B, a small amount of glass wool, and absorbent cotton respectively. A single worm just before the beginning of oviposition was put into these petri-dishes with well water. In parallel with these sets, the worms were cultivated in the dishes with and without sand which served as controls. The occurrence of normal cocoon deposition was observed day by day for 15 days. The results obtained are shown in Table 2. As will be seen, all the cocoons deposited in sand are normal and contained the developing eggs, whereas all cocoons deposited in water without sand are ruptured. In most experimental sets, worms fail to deposit normal cocoons, except for the cases of gause, glass wool and absorbent cotton where a small number of normal cocoons are deposited. Close observations on the ovipository behavior reveal that the normal cocoons obtained in the experimental sets are produced only when the worms happened to pass through the narrow spaces formed by fibers of these substances. Additional observations proved that the normal cocoon deposition is unsuccessful, if the quantity of sand is extremely small. These observations

Table 2. Number of cocoons deposited by the worms placed in the petri-dishes containing various substances in combination with water

No. of cocoon	Filter paper	Gause	Glass wool	Absorbent cotton	Wire netting A	Wire netting B	Water (con.)	Sand (con.)
Normal (unruptured)	0	2	2	3	0	0	0	12
Ruptured	12	10	10	10	10	10	12	0

may suggest that the presence of solid substances which may contact not only with ventral, but also with dorsal and lateral surfaces of the worm is necessary for the normal deposition.

The substances used in the preceding experiment were cut into small pieces of about 1 cm^2 and were piled up in the petri-dishes, so that the worms were able to creep in the depth of these substances. The results of these experiments are summarized in Table 3. As shown in the table, the number of the normal cocoon apparently increases in all the experimental sets as compared with Table 2. Among these sets, the increase is most evident in the dish with filter paper. Remarkable increase in the number of the normal cocoon in filter paper seems to be caused by the fact that each piece of filter paper swells up by absorption of water, resulting in the close contact of them with the worm. These findings indicate with the results presented in Table 3 that the close contact of both dorsal and ventral body wall with solid substances is necessary for the normal cocoon deposition. Apparent increase in the number of normal cocoon in filter paper further suggests that the substances indispensable for the normal deposition are not necessarily as hard and heavy as sand particles.

Table 3. Number of cocoons deposited by the worms placed in the petri-dishes piled up with various substances in combination with water

No. of cocoon	Filter paper	Gause	Glass wool	Absorbent cotton	Wire netting A	Wire netting B	Water (con.)	Sand (con.)
Normal (unruptured)	11	4	4	4	4	5	0	14
Ruptured	2	10	12	11	10	10	12	0

Further support of these considerations was obtained in the following experiment where the determined sizes of sea sand, glass and emery particles were used for the preparation of the ovipository circumstances. The results obtained

Table 4. Number of cocoons deposited by the worms placed in the petri-dishes containing various sizes of substances in combination with water

Size of substances* / No. of cocoon	Sea sand A	Sea sand B	Glass fragment A	Glass fragment B	Glass fragment B′	Emery A	Emery A′	Water (con.)	Sand (con.)
Normal (unruptured)	0	14	0	12	12	0	0	0	15
Ruptured	13	0	11	0	0	10	11	13	0

*A, A′; less than 0.5 mm in size: B, B′; more than 1 mm in size are shown in Table 4. It is clear that the presence of particles measuring 1-2 mm in size causes the normal cocoon deposition, whereas worms placed with particles less than 0.5 mm in size always deposit ruptured cocoons. However, it should be noted here that, in the latter case worms were entirely unable to creep between the closely accumulated small particles, though in the former case worms were observed to creep in the depth of the particles. It may thus lead to the conclusion that the close contact of substances at the body wall of the worm is an important factor for the successful cocoon deposition.

Discussion

The present observations reveal that the cocoon tube formation in *Tubifex* is markedly delayed in the sand-free circumstance, and that the cocoon tube, even though formed, fails to be normally deposited. The occurrence of oviposition without cocoon has been reported in Tubificidae cultivated in water (Ditlevsen, 1904; Storkan, 1925). These authors, however, did not observe whether the cocoon formation is actually prevented in such condition or not. Storkan (1925) noted that the worms cultivated in a mass without mud deposit eggs, some with and some without cocoons. This can be also confirmed in the present material, when more than 10 worms are placed in a clump in the sand-free circumstance. An interesting observation on these worms was that the worms situated in the central parts of a clump deposit normal cocoons, whereas those situated in the peripheral part deposit ruptured cocoons. In this case, the centrally situated worms are likely to have acted upon each other as the substitute for the role of some solid substances in cocoon deposition. Ditlevsen (1904) assumed that the resistance for the fixation of the cocoon tube seems to be necessary in the normal cocoon deposition. In the present study, it is indicated that, if the quantity of sand is too small for the worms to creep in the depth of sand, even sand particles do not assure the occurrence of normal oviposition. On the other hand, when the quantity is large enough to allow creeping of worm in the depth, whatever substances tested may be sufficient as a substitute for sand particles. The point which is deduced from the present results is that some solid substances must be in close contact with the surface of the worms at the time of cocoon deposition.

In this connection, the histological observations on the localization of clitellar gland cells deserve to be mentioned (*cf.* Hirao, 1965a). Among 4 kinds of glandular cells composing the clitellar epithelium, *A*, *B* and *C* cells located in the dorsal and lateral sides are extremely larger than those in the ventral side. Mucus cells, showing uniform distribution in both dorsal and ventral sides, secrete their contents irrespective of the presence or absence of sand. The observation that the secretory activity of *A*, *B* and *C* cells, which supply the substances of the cocoon, is greatly inhibited in the sand-free circumstance may explain the delay of cocoon formation and weakness of the formed cocoon tube. It seems possible that the close contact of the substances with the dorsal and lateral surface of worms may facilitate the secretion of the clitellar epithelium pertinent to the formation of cocoon tube. Therefore in normal oviposition of a seaprate worm in the dish, the role of sand is twofold, *i.e.* stimulation of the secretory activity of clitellar epithelium and the mechanical fixation of formed cocoon tube at the time of cocoon deposition.

Summary

1. The manner of cocoon formation and its deposition in the fresh water oligochaete, *Tubifex hattai,* was studied with the worms placed in various circumstances. Histological observations were also made on the clitellar epithelium in relation to ability of the worm to form cocoon.

2. In the worms placed in the condition without sand, the cocoon formation was much delayed and usually the released cocoon tube ruptured at some point.

3. None of the worms placed in the petri-dish with a sheet of filter paper and wire netting deposited normal cocoons. A few normal cocoons were deposited when the worms were able to successfully creep in the narrow spaces between the fibers of gause, glass wool and absorbent cotton.

4. Worms placed in a small amount of sea sand, glass and emery particles measuring 1–2 mm in size, deposited cocoons. On the contrary, the worms placed in the same particles less than 0.5 mm in size failed to deposit normal cocoons, probably due to the inability to creep in the depth of the accumulated particles.

5. Histological studies of the clitellar epithelium revealed the reduced secretory activity of glandular cells, *A*, *B* and *C* cells, of the worms in the absence of sand.

6. Discussion was offered on the conditions necessary for the cocoon deposition, with the emphasis that the close contact of solid substances with both dorsal and ventral body walls of the clitellar part is necessary for the normal deposition.

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