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The Fine Structure of the *Tubifex* Egg before and after Fertilization

By

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(With 3 Plates)

In the sea urchin and *Nereis*, sperm penetration excites the cortical ooplasm which leads to dramatic changes in the organization of the egg surface; these changes involve the breakdown of cortical structures (granules or alveoli) and a modification in the organization of the egg envelopes (Austin, 1968).

Only a little is known about the cortical change in the fertilization of the *Tubifex* egg. Shimizu (1976) observed in a light microscopic study that the egg membrane is closely in contact with the ooplasmic surface in the ovisac egg but is separated following fertilization. He revealed further that the affinity to hematoxylin of the ooplasm is transitorily decreased in the process of membrane separation. Unfortunately no structural components responsible for these changes have hitherto been detected in the eggs of *Tubifex* (Hirao, 1968; Shimizu, 1976). The present investigation was undertaken in order to make clear the structural changes of the egg surface in this worm.

Materials and Methods

The freshwater oligochaete, *Tubifex hattai*, was collected from the stream running through the campus of Hokkaido University. The worms near oviposition were readily detected by observing the immediately posterior segments of the clitellum under the dissecting microscope. Through the transparent body wall, 6–8 eggs were recognized in the ovisac of these worms (Hirao, 1964). The ovisac eggs used were obtained by cutting a small part (8–10 segments which included the ovisac of the worm) in Inase's culture solution (Inase, 1960). Newly deposited cocoons were collected by the method of Hirao (1965), checking the time-lapse after oviposition.

The small part of the worm containing the ovisac eggs and the deposited cocoons were immersed in 5% glutaraldehyde. In the case of the deposited cocoons, a perforation was made on the membrane of the cocoon with a pair of watchmaker's forceps for the rapid penetration of the fixative into the eggs. Care was taken in order to avoid injury to the eggs. After 2 hours, the eggs were freed

from the surrounding tissues or the cocoon, and fixation was continued for a further 6–8 hours in renewed 5% glutaraldehyde. The eggs were then rinsed overnight in 6 changes of 0.2 M sucrose. After each egg was halved or quartered with a sharp razor, the egg segments were postfixed in 1% osmium tetroxide for 2 hours. The fixatives and 0.2 M sucrose employed were all made with 0.1 M sodium cacodylate-HCl buffer solution, pH 7.4, and the immersions were performed at 0°–4°C. After dehydration through ethanol, the materials were embedded in Epon 812 (Luft, 1961). Silver or gold thin sections cut on a Porter-Blum ultramicrotome were successively stained in a half-saturated aqueous solution of uranyl acetate and in lead citrate (Reynolds, 1963), and were observed in a Hitachi HS–7 electron microscope.

Results

1. *Ripe Eggs*

It has been reported of the meiotic events of the *Tubifex* egg that the breakdown of the germinal vesicle takes place in the ovisac of the mature worm, where the nucleus of the egg reaches the metaphase of the first meiotic division (Hirao, 1968; Shimizu, 1976). In this state, the ripe eggs are released from the body and fertilization occurs in the cocoon formed by the clitellar epithelial secretion of the worm (Hirao, 1968); thereafter the process of nuclear maturation proceeds further. Such eggs as are found in the ovisac, possessing the first meiotic metaphase spindle, are termed *ripe eggs* in this paper.

The ooplasmic surface of the ripe eggs possessed microvilli (Figs. 1 and 2). Their distribution on the surface was almost homogeneous, but the distance between nearest neighbours was not constant. Each microvillus was approximately 0.2 μm in length and 0.1 μm in diameter, and contained a few thin-filaments running parallel to its long axis.

The vitelline membrane covering the egg cell proper was constructed from a loose network of fibrillar materials arranged approximately parallel to the egg surface (Figs. 1 and 2). The contour of the external limit of the membrane appeared very indistinct because of the low density of the fibrillar materials. In general, however, the vitelline membrane appeared one-layered and was approximately 0.15 μm thick.

The microvilli of the ooplasmic surface perforated the vitelline membrane; the distal portion of each villus seemed to end at the external limit of the membrane (Fig. 2). The fibrillar materials composing the vitelline membrane appeared to be closely attached to the surface of the microvilli and showed a peculiar pattern of arrangement in the surrounding region of the latter. That is to say, the fibrillar materials appeared to extend radially from the microvilli. Accordingly the network of the fibrillar materials in the membrane were of high density in distribution at the intermediate region between the neighbouring microvilli (Fig. 4).

A narrow interstice between the ooplasmic surface (at the base of the microvilli)

and the internal surface of the vitelline membrane was observed in the ripe eggs. Although its appearance might be due to the artifact of preparation, the space was devoid of any inclusion other than some fibrillar materials extending from the vitelline membrane (Fig. 2). The width of this space varied from portion to portion, ranging from 0.1–0.15 μm.

The cortical ooplasmic layer, ranging from 2–3 μm in thickness, consisted of a finely particulated matrix. This layer was devoid of yolk granules and lipid droplets but contained numerous mitochondria, endoplasmic reticula, and ribosomes (Figs. 1 and 2). Most of the mitochondria were oval and were inclined to form clusters. Each mitochondrion had well-developed cristae running parallel to the long axis which oriented randomly in the ooplasm. As for the endoplasmic reticula, many of them were smooth-surfaced, but some were granulated. These reticula mostly ran parallel to the ooplasmic surface (Figs. 1 and 2).

Other membraneous inclusions found in the cortical layer were minute vesicles and multivesicular bodies (Fig. 3). They were distributed throughout the ooplasm but were very small in number. The multivesicular bodies contained many vesicles which morphologically resembled minute vesicles found freely in the cortical ooplasm. The wall of the multivesicular body appeared trilamellar and was frequently ruptured on the side of the egg surface, thus the interior of the body appeared to continue to the circumferential ooplasm (Fig. 3). The walls of the contained vesicles, as well as those of the minute vesicles distributed freely in the ooplasm, also showed a trilamellar structure. This trilamellar structure was reminiscent of the plasma membrane of the animal cells.

Inside the cortical layer, there was endoplasm. It consisted of a finely particulated matrix and contained a considerable number of yolk granules and lipid droplets (Fig. 1). Mitochondria, endoplasmic reticula, and ribosomes were also found in this part but their distribution was restricted to the immediately surrounding region of the nutrient inclusions. No vesicular structures were observed in the endoplasm.

2. *Deposited Eggs.*

a) *10–20 min after oviposition.* When the fresh eggs were freed from the cocoon in the culture solution at 10–20 min after oviposition and observed under the light microscope, no distinct perivitelline space was detected. An electron micrograph of the egg, however, revealed the existence of a perivitelline space measuring about 0.25 μm in width. Unlike the ripe eggs, it contained amorphous materials of low electron-density (Fig. 5). Furthermore, conspicuous changes in the organization of the cortical structures were detected in these eggs. In detail, the vitelline membrane apparently separated from the plasma surface consisted of three layers; inner, middle, and outer (Fig. 5). Since the membrane was a one-layered structure in the ripe egg, the above-mentioned facts indicate that the vitelline membrane of the ripe egg undergoes structural changes during the process of fertilization. Because of such a structural difference, the membrane enclosing

the fertilized eggs was distinguished from the vitelline membrane of the ripe eggs and will be termed the "*fertilization membrane*" in this paper.

The outer layer of the "fertilization membrane" was 0.05–0.1 μm thick and was the lowest in electron-density of the three layers (Fig. 8). This layer had a low-density, fibrillar texture and extended some component fibrils to the underlying layer. The middle layer, approximately 0.2 μm thick, had moderate electron-density and consisted of a granular component and a small number of fibrils extending from the outer layer. The inner layer was 0.02–0.03 μm thick and was composed of electron-dense fine granules. Therefore, this layer was characterized by the highest electron-density of the components in the "fertilization membrane" (Fig. 8). Occasionally it was observed in the eggs of this stage that the "fertilization membrane" was devoid of such an inner layer in some parts, though the outer layers were already established (Fig. 5).

A particular constituent of the "fertilization membrane" was finger-shaped structures which had high electron-density (Figs. 5, 7 and 8). They were an average of 0.2 μm in length and 0.03–0.04 μm in width. The structures were embedded in the membrane directing their axis toward the exterior of the egg. Their distal ends reached the medium level of the middle layer of the membrane; the other end jutted out into the narrow perivitelline space. Occasionally the inner leaflets of the surface membrane of the finger-shaped structures came closely into contact, which gave rise to the appearance of "*pentalamellar*" structures (inset of Fig. 8).

The other conspicuous change occurring in the structure of the egg surface following fertilization was the decrease in number of the microvilli. They almost disappeared from the ooplasmic surface. In some regions of the egg, however, the microvilli remained unchanged on the ooplasmic surface. They were found to be embedded in the egg membrane. Unlike the case of the ripe egg, the distal ends of the microvilli did not reach the outermost limit of the membrane but were located at the medium level of the middle layer. Furthermore, they were constricted at an intermediate part along their long axis (Figs. 6 and 7). These microvilli had a similar electron-density to the finger-shaped structures of the "fertilization membrane" but were evidently wider than the latter.

As for the ooplasm, a slight decrease in the thickness of the cortical layer was observed. A number of mitochondria, endoplasmic reticula and ribosomes were found in the cortical ooplasmic layer as in the case of the ripe eggs. The multivesicular bodies and small vesicles were also detected. In these eggs, there appeared two new kinds of membraneous structures; a Golgi complex and a sac-shaped structure (Figs. 9 and 10). The Golgi complex, not well-developed, was frequently observed in the cortical ooplasm and was composed of 5–6 stacks of cisternae and several small vesicles. The sac-shaped structures were also observed frequently in the ooplasm and possessed a wall resembling the plasma membrane of the animal cells in their triple-layer construction. They were inclined to gather into groups. The above-mentioned cellular organelles except the Golgi complex

and the sac-shaped structure were also found in the interstices among yolk granules and lipid droplets.

b) *40–50 min after oviposition.* When the fresh eggs 40–50 min after oviposition were observed under the light microscope, the perivitelline space could be readily recognized.

In these eggs, the three-layered constitution of the "fertilization membrane" was more evident than in the preceding stage (Figs. 11 and 12). The entire inner surface of the membrane showed high electron-density indicating that the inner layer was already established in these eggs. The boundary between the inner and the middle layer was clear but that between the middle and the outer layer was indistinct; probably owing to the extending of the fibrillar components of the outer layer to the middle layer. Numerous finger-shaped structures were found embedded in the "fertilization membrane" as in the preceding stage. However, the trilamellar membrane limiting these structures was hardly seen.

There were no microvilli on the ooplasmic surface. Thus, no appreciable structure directly connecting the "fertilization membrane" with the ooplasm could be found in these eggs. The perivitelline space was 0.5–1.0 μm in width. It contained particles of approximately 0.03 μm diameter, and the amorphous materials found in the preceding stage.

The thickness of the cortical layer further decreased in the eggs of this stage. A small number of microfilaments first appeared in the cortical ooplasm (Fig. 14). Small multivesicular bodies were frequently found in these eggs but the general organization of the ooplasm was nearly the same as that at the preceding stage.

Discussion

The present observations showed that the fine structure of the vitelline membrane enclosing the ripe egg underwent drastic changes upon fertilization. Similar structural changes of the membrane after fertilization have been reported in many animal eggs. It has been reported in the sea urchin (Anderson, 1968; Ito, 1969; Inoue and Hardy, 1971) and *Xenopus* (Grey *et al.*, 1974) that the cortical ooplasmic layer of the ripe eggs possesses granules, namely cortical granules, which show a positive reaction to the polysaccharide test (PAS). They break down upon fertilization, and the materials discharged from the granules induce the structural changes in the vitelline membrane. In the *Nereis* egg, the cortical alveoli are released upon fertilization, and their contents pass through the vitelline membrane to make up the periovular jelly (Pasteels, 1966). The cortical granules have been described in surf clam, lamellibranch, and echiuroid eggs (*Spisula*: Rebhun, 1962; Longo and Anderson, 1970; *Mytilus*: Humphreys, 1962, 1967; *Urechis*: Gould-Somero and Holland, 1975). In these eggs, however, the granules do not react to sperm penetration and do not break down upon fertilization. Concomitant with this fact, no structural transformation of the egg membrane seems to occur in these animals. It has recently been reported that

the eggs of gastropods, *Nassarius reticulatus* and *N. obsoletus* (*"Ilyanassa"*) do not possess cortical granules (Schmekel and Fioroni, 1975; *cf.* Taylor and Anderson, 1969). In this case, the vitelline membrane does not show any structural change (Schmekel and Fioroni, 1975). Therefore the structural transformation of the vitelline membrane occurring at the time of fertilization seems to be attributable to the materials constituting the cortical granules.

In the light microscopic studies, Hirao (1968) and Shimizu (1976) reported that the ripe eggs of *Tubifex* are devoid of the PAS-positive cortical granules. The present observation supports these results electron-microscopically. Since the vitelline membrane of the ripe egg undergoes the fine structural changes after fertilization, a particular mechanism should underlie these structural changes. As discussed below, the finger-shaped structures embedded in the "fertilization membrane" are probably remnants of the microvilli. Their outer ends terminated at the medium level of the middle layer of the membrane. In the ripe eggs, the distal ends of the microvilli reached the external surface of the membrane. Unless any retraction of the microvilli occurs upon fertilization, it may be assumed that the outer part of the membrane of the developing eggs, beyond the level of the distal ends of the finger-shaped structures, was formed by the accumulation of some extrinsic substances on the external surface of the vitelline membrane following fertilization.

Immediately after oviposition, the microvilli still connected the ooplasm with the membrane in some regions of the egg, where the membrane was devoid of the electron-dense inner layer. Since the outer and the middle layers of the membrane were seen in every part of these eggs, the inner layer was seemingly formed after the establishment of the middle and outer layers. When the direct connection of the membrane and the ooplasm by the microvilli was lost, no portions of the inner surface of the egg membrane devoid of the inner layer were observed. This fact suggests that the materials forming the inner layer are different from those of the other layers in their origins: The middle and outer layers are possibly formed by the addition of extrinsic materials to the pre-existed membrane surrounding the ripe egg. On the other hand, the inner layer of high electron-density is presumably formed with materials derived from the cortical cytoplasm of the egg.

As the source of the extrinsic materials participating in the formation of the middle and the outer layers of the membrane, the viscous environmental fluid of the fertilized eggs in the cocoon, the cocoon fluid, should be considered. According to Hirao (1965), the cocoon fluid is formed by granular secretion from the clitellar epithelial cells of depositing worms. A preliminary study showed that the cocoon membrane is impermeable to colloids, such as gum arabic, and the cocoon fluid contains some protein (unpublished).

Numerous finger-shaped structures were found embedded in the membrane of the fertilized eggs. The fact that these structures possessed a trilamellar limiting membrane strongly suggests that they are the tips of the microvilli separated from

the egg cell proper. This may be supported by the following findings: 1) The number and distribution of the finger-shaped structures in the membrane quite closely resembled those of the microvilli on the ooplasmic surface of the ripe egg. 2) The ooplasmic surface of the developing egg no longer possessed microvilli. 3) Some microvilli were found in the region where the separation of the egg membrane was in progress. In this case, the microvilli were torn off at the constriction and separated from the ooplasmic surface. In an electron microscopic study of the *Nereis* egg, Pasteels (1966) found that the microvilli elongate and separate from the ooplasmic surface after fertilization and form thin tubules; they exist in the perivitelline space, the egg membrane, and even in the jelly formed after fertilization. In the process of fertilization in the surf clam, *Spisula* eggs, the microvilli are separated from the ooplasmic surface together with the vitelline membrane (Rebhun, 1962). Judging from these facts, it may be safe to say that the finger-shaped structures in the "fertilization membrane" of the *Tubifex* egg correspond to the tips of the microvilli previously existing on the ooplasmic surface of the ripe egg.

After fertilization, the space between the egg membrane and the ooplasmic surface widened. Probably due to the connection of the egg membrane and the ooplasmic surface through the microvilli, the perivitelline space was not observed under a light microscope just after oviposition. After a lapse of time, the perivitelline space became clearly visible when all of the microvilli were torn off from the ooplasmic surface. The enlarged perivitelline space contained unknown amorphous materials. Shimizu (1976) reported in a light microscopic study of the *Tubifex* eggs that the materials in the perivitelline space are PAS-positive. It is unclear whether these PAS-positive materials are the same as the amorphous materials revealed in the present observation. It is known that in sea urchin and amphibian eggs the elevation of the egg membrane following fertilization is due to the colloid osmotic pressure of the perivitelline fluid; the colloid in the perivitelline space originates from the cortical granules which are embedded in the cortical ooplasm of the ripe egg and break down upon fertilization. As mentioned already, the cortical ooplasmic layer of the *Tubifex* egg was devoid of granules which break down following fertilization. Therefore the mechanism, specific for *Tubifex*, should be considered from the point of view of the enlargement of the perivitelline space. In his light microscopic study in the cortical change of the *Tubifex* egg, the author reported that the hematoxylinophilic property of the cortical ooplasm decreased in the process of membrane elevation (Shimizu, 1976). However, the intensity of the PAS-reaction was not altered in this process. Further, any structural components of the cortical ooplasm responsible for the change in the hematoxylinophilic property were detected in the present electron microscopic study. The amorphous materials in the perivitelline space, however, might participate in the enlargement of the space. There is a possibility that these materials are parts of the microvilli, because the volume of the tips of the *microvilli* (finger-shaped structures) decreased in the process of the enlargement of the perivitelline space.

Summary

Fine structures of the vitelline membrane and the cortical region of unfertilized and fertilized eggs of a freshwater oligochaete, *Tubifex hattai*, were studied with the electron microscope.

No cortical components comparable with the cortical granules in sea urchin eggs could be detected in the ripe eggs. Except for the microvilli on the ooplasmic surface, the structure of the cortical ooplasmic layer did not undergo any drastic change at the time of fertilization. The microvilli which traversed the one-layered, fibrillar vitelline membrane in the ripe egg in the ovisac decreased in number in the fertilized eggs (10–20 min after oviposition). After fertilization, the vitelline membrane was transformed into a three-layered one (*"fertilization membrane"*), of which the outer and middle layers were of medium electron-density and the inner layer was highly electron-dense and the thinnest of the three. Numerous finger-shaped structures resembling the tips of the microvilli were embedded in the membrane. They had no connection with the ooplasm. Such ooplasmic organelles as mitochondria, endoplasmic reticula, and ribosomes did not change in distribution, morphological structure, or quantity during the early period of development studied. The Golgi complexes and the sac-shaped structures were not observed in the ripe eggs but appeared after fertilization.

The mechanism of the structural transformation of the vitelline membrane and the enlargement of the perivitelline space following fertilization were discussed.

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Explanation of Plates XI-XIII

Fig. 1. Section through the peripheral region of the ripe egg. Fibrillar vitelline membrane (VM) is traversed by microvilli (m). Finely granulated cortical cytoplasm includes mitochondria (Mt), endoplasmic reticula (ER), and ribosomes but excludes yolk granules (Y) and lipid droplets (L). Arrows indicate the ooplasmic surface. × 21,000 (bar=1 μm).

Fig. 2. Higher magnification of the peripheral region in Fig. 1. The distal portion of the microvilli (m) ends at the external surface of the fibrillar vitelline membrane (VM). Note the fibrillar components radially extending from the distal surface of the microvillus. ER, endoplasmic reticulum; Mt, mitochondria; asterisk, perivitelline space. × 66,800 (bar =0.25 μm).

Fig. 3. Multivesicular body located in the cortical cytoplasm of the ripe egg. Note the egg-surface side of the body, where the content of the body continues to its circumambient cytoplasm including mitochondria (Mt) and endoplasmic reticula (ER). × 28,000 (bar= 0.5 μm).

Fig. 4. Tangential section of the ripe egg. The section intersects at approximately the medium level of the microvilli (m). × 44,000 (bar=0.25 μm).

Fig. 5. Section through the peripheral region of the fertilized egg about 15 min after oviposition. Note the diminishing of most microvilli from the ooplasmic surface (arrows). ER, endoplasmic reticulum; FM, "fertilization membrane"; G, Golgi complex; Mt, mitochondria. × 22,400 (bar=1 μm).

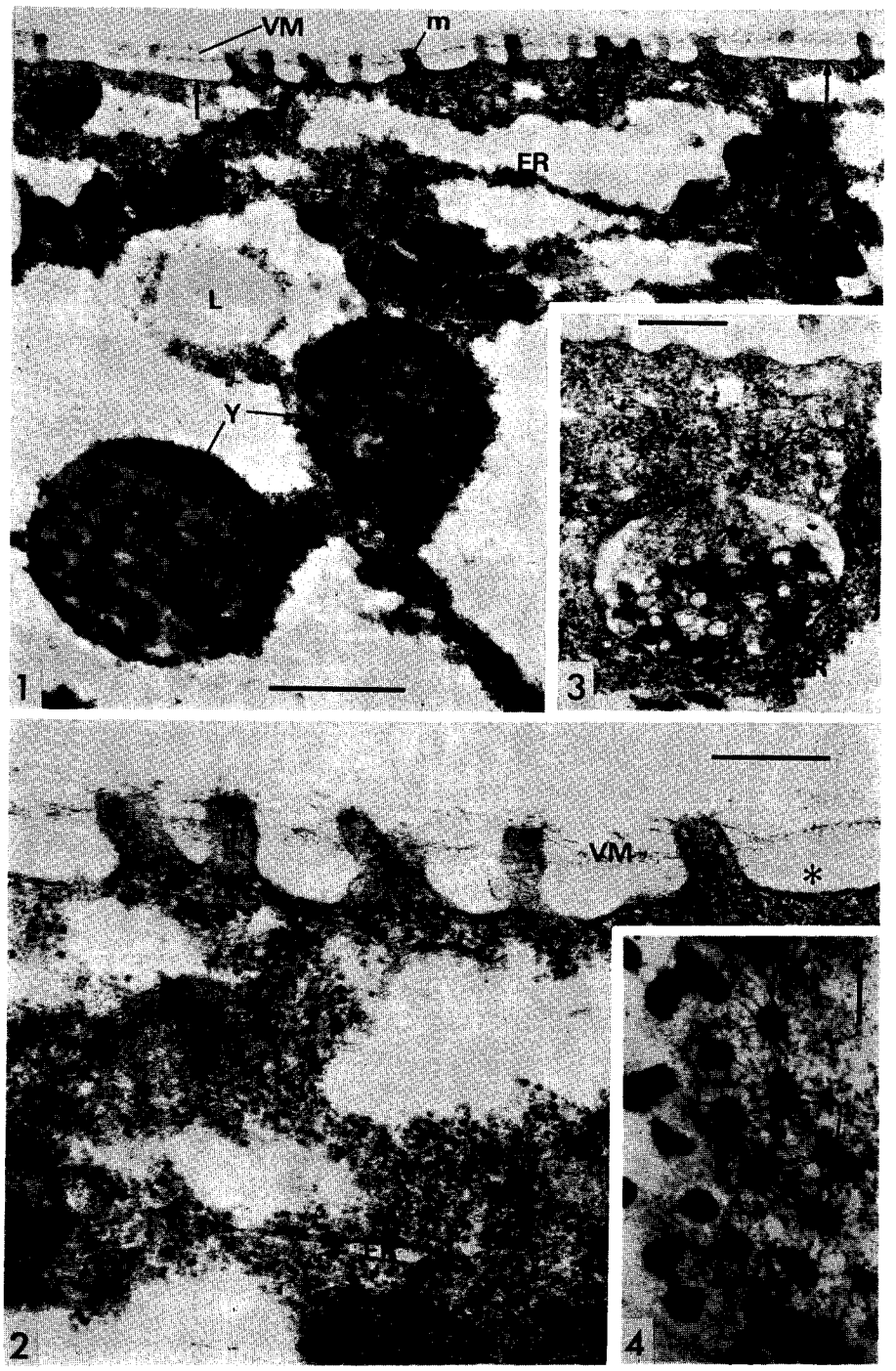
Figs. 6–8. Sections through the peripheral region of the fertilized eggs about 15 min after oviposition. The egg membrane ("fertilization membrane", FM) is composed of three layers: the outer (O), the middle (M), and the inner (I) layers (Fig 8). Some microvilli are found remaining on the ooplasmic surface. Note constriction of villus (arrows in Figs. 6 and 7) and the finger-shaped structures embedded in the "fertilization membrane". The wall of these finger-shaped structures is trilamellar (double arrow-heads in Figs. 7 and 8), and sometimes shows pentalamellar appearance (arrow and inset in Fig. 8). ER, endoplasmic reticulum; Mt, mitochondria. Fig. 6: × 47,700 (bar=0.25 μm); Fig. 7: × 40,000 (bar=0.25 μm); Fig. 8: × 50,000 (bar=0.25 μm); inset of Fig. 8: × 112,500 (bar=0.1 μm).

Figs. 9 and 10. The cortical cytoplasm of the fertilized egg about 15 min after oviposition. Note the Golgi complex composed of four stacks of cisternae and several vesicles (Fig. 9), and sac-shaped structures (Fig. 10). × 48,000 (bar=0.25 μm).

Figs. 11 and 12. Sections through the peripheral regions of the fertilized eggs 50 min after oviposition. No microvilli are seen on the ooplasmic surface. Note amorphous material and particles (arrows) of moderate electron-density in the perivitelline space. ER, endoplasmic reticulum; FM, "fertilization membrane"; L, lipid droplet; Mt, mitochondria; Y, yolk granule. × 23,000 (bar=1 μm).

Fig. 13. Multivesicular body of small size in the cortical region of the fertilized egg 50 min after oviposition. Mt, mitochondria. × 20,000 (bar=0.5 μm).

Fig. 14. Microfilaments (arrows) in the cortical region apposed to the ooplasmic surface. The fertilized egg 50 min after oviposition. Mt, mitochondria. × 44,000 (bar =0.25 μm).



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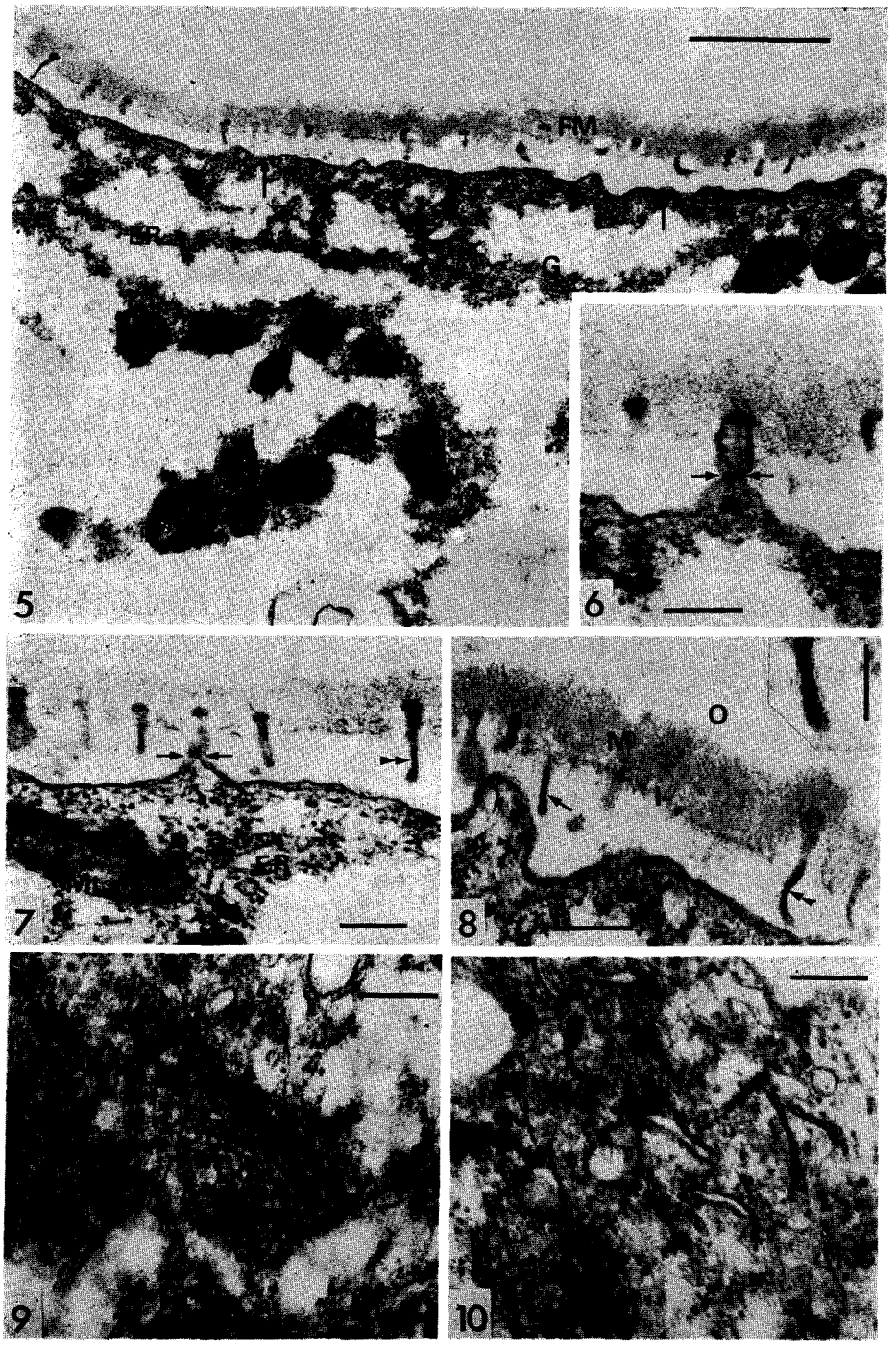

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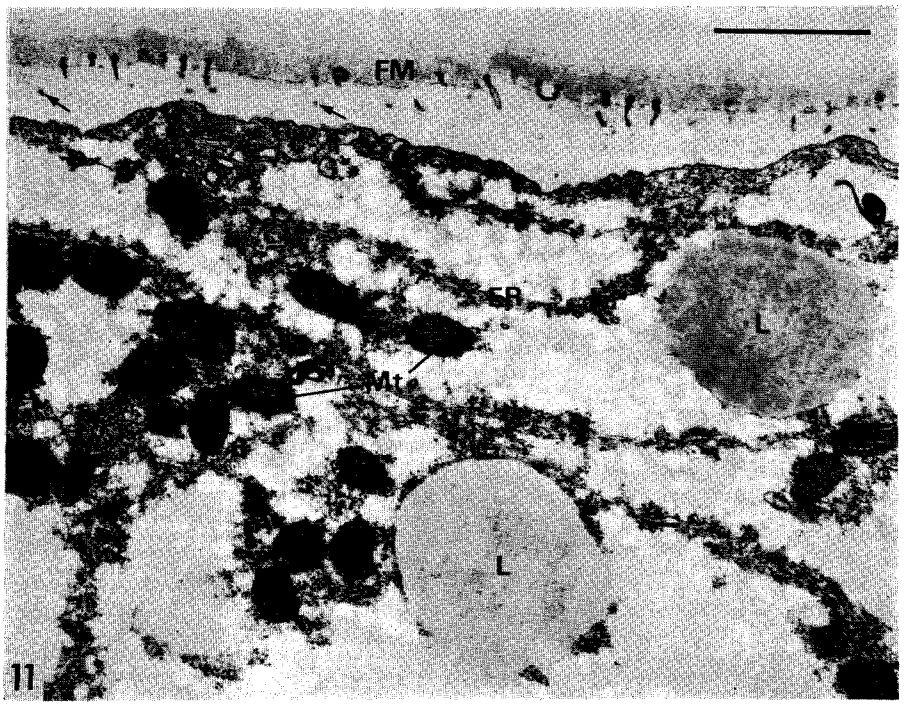

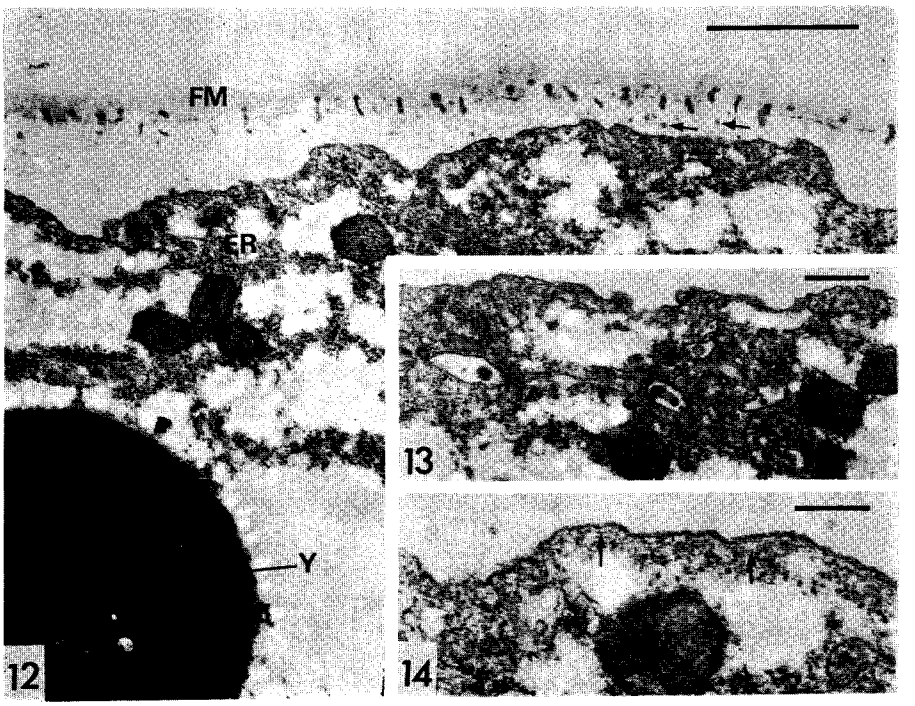

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