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Scanning Electron Microscope Study of Metacercarial Excystation of the Lung Fluke, *Paragonimus Miyazakii*

(excystation/*Paragonimus*/SEM)

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The process of excystation of metacercariae of a lung fluke, *Paragonimus miyazakii* was studied using scanning electron microscopy. Observations were made of the surface and the internal structures of metacercariae such as cyst wall, tegumental spines, stylets, oral sucker, ventral sucker, sensory papillae, penetration glands, tegument and excretory bladder. The origin of the triple-layered cyst wall is discussed and special attention given to the structural changes which occur in the cyst wall during the excystation. The significance of concretions found in the excretory bladder is also discussed. These concretions filling the excretory papillae are similar to the "calcareous corpuscles" found in cestodes, and may serve to supply energy for the metacercarial excystation and the growth of metacercaria.

A lung fluke, *Paragonimus miyazakii* was first found in the stage of metacercaria by Kamo *et al.* (1) in 1961 from a fresh-water crab, *Potamon dehaani* at Rokuroshi, Iwakuni City, Yamaguchi Prefecture. Since Hayashi *et al.* (2) reported the first case of paragonimiasis in humans in Yokohama City, many such cases have been found in the Kanto District.

The morphology of metacercariae of *Paragonimus miyazakii* has been studied by Kamo *et al.* (1, 3) Komiya *et al.* (4), Hatsushika (5), Maejima *et al.*(6) and Maejima (7). The histology of the cyst wall and the mechanism of excystation were elucidated considerably. In addition, the structure of the surface of metacercariae of trematodes was reported by Lo (8), Mitchell (9) and Kϕie (10), who used the scanning electron microscopy.

There is apparently little documentation on the internal structure of metacercariae of *Paragonimus miyazakii* and the changes which occur in the cyst wall of the worm during excystation. Our study was an attempt to acquire more information on the surface and internal structure of metacercariae, and to observe changes which occur in the cyst wall during excystation.

MATERIALS AND METHODS

Encysted metacercariae were obtained from naturally infected river crabs, *Potamon dehaani*, collected in Hiyoshimura, Kitauwa-gun, Ehime Prefecture.

Some metacercariae were, after being excised from the pericardium of crabs, washed in physiological saline, fixed for 6 hours in 2.5 % glutaraldehyde, washed in 0.1 M phosphate buffer (pH 7.4) and postfixed for 4 hours in 1 % OsO_4 at 4°C. After the postfixation, the specimens were dehydrated through an ethanol series and amylacetate, subjected to critical point drying, and coated with gold palladium and observed under a Hitachi MSM-V scanning electron microscope.

To observe internal structures using a scanning electron microscope some metacercariae were embedded in styrene (11) after being fixed and dehydrated by the above mentioned method. Following polymerization, the specimens were cracked under a stereoscope using the method of Tanaka *et al.* (11).

The other metacercariae were cultured in Tyrode solution (pH 7.2) supplemented with 0.5 % cholic acid at 37°C to initiate artificial excystation. These metacercariae were removed from this solution at 10 minute intervals for a period of 1 hour, then processed successively through fixation, dehydration, embedding and cracking to be observed through SEM.

RESULTS

Metacercariae living in the pericardium of crabs were excised together with tissue of the host, and were observed under SEM. The surface of metacercariae in the pericardium is covered with a membranous substance. The diameter of encysted metacercariae ranges from 400 μ to 500 μ, and the shape is spherical or ellipsoidal. The surface of encysted metacercariae is almost completely covered with a fibrous membrane and studded with blunt projections (Fig. 1).

Cracking an encysted metacercaria we found the worm enclosed in a multilayered capsule which is 12—15 μ in thickness and composed of homogeneous fine matrix. The internal organs observed were an excretory bladder containing numerous concretions, penetration glands and an intestine. The tegumental surface bears spines. The cyst wall is three-layered : an 8 μ thick inner layer of inner wall, a 1 μ thick outer layer of inner wall and a 4 μ thick outer wall. The outer wall is further covered by a fibrous cellular layer, or a "membranous substance", which is about 12 μ thick (Fig. 2).

The cavity between the cyst wall and the parasite is filled with a filamentous mucus substance. The tegument of encysted metacercariae contains abundant vesicles, suggesting that it serves for micropinocytosis. Under the tegument some penetration glands are in the form of clusters of granules. Each granule is about 0.5—1.0 μ in diameter (Fig. 3).

Morphological changes of the cyst wall during the artificial excystation are shown in Figures 4 and 5. In the cyst of the specimens treated with artificial intestinal juice for 30 min several ellipsoidal pores were observed in the inner

layer of the inner wall (Fig. 4). In specimens treated with the same juice for 60 min the inner and outer walls of the cyst were degenerated and a split had occurred between the inner and the outer wall (Fig. 5). The activated metacercaria broke the weakest part of the degenerated inner and outer walls and came out through the outermost membranous substance. Thus the excystation had terminated. On the surface of the membranous substance, we observed ramified vessels which may have originated from the host (Fig. 6).

Excysted juvenile metacercariae are ellipsoidal, about 1 mm in length and about 400 μ in width. A round oral sucker of about 90 μ in diameter is situated in the front ventral face. A depression posterior to the oral sucker is assumed to be the genital pore. A ventral sucker of about 100 μ in diameter is situated near the middle of the body. A cloaca is seen at the near end of the body as a shallow excavation (Fig. 7).

Tegumental surface of an excysted metacercariae is smooth over most of the ventral face and resembles ground paved with stone, but it is densely covered with spines which are 0.4—0.6 μ in diameter and 1.2—1.4 μ in length on the dorsal face and around the ventral sucker. These spines are single-haired and similar to fangs (Figs 8—9). The oral sucker is a muscular, circular, protuberance with a deep pit in the center. Stylets of about 10 μ long are arranged in 8 to 10 rows around the oral sucker (Fig. 10).

The ventral sucker is also a muscular, circular protuberance and is situated in the anterior part of the body. The pore in the center of the ventral sucker is larger and deeper than the pore of the oral sucker. Many sensory papillae of about 10 μ in diameter are distributed around the ventral sucker. These papillae bear no cilia and exhibit no characteristic features. About ten openings of about 1 μ in diameter are present on the surface of the ventral sucker. Such may be openings of the penetration glands (Fig. 11).

In the cross section of a metacercaria, the ventral sucker was observed as a muscular layer of about 10—20 μ in width and contained numerous vacuoles and vesicles (Fig. 12). In the parenchymal layer we observed numerous granules 1—2 μ in diameter which were conglomerated into clusters and situated adjacent to the ventral sucker. These are penetration glands. The pores observed in the ventral sucker may be their openings (Fig.13). A cross section of a metacercaria revealed an excretory bladder filled with excretory papillae of about 10 μ in length (Fig. 14). The membrane covering the excretory papillae is very thin and has some pores, excavations and grooves. The superficial layer of the tip of the excretory papillae bears microvilli (Fig. 15). Excretory papillae are filled with numerous globular concretions of 1—2 μ in diameter. At the base of each excretory papilla, there is a complex network and numerous concretions in the papillae are separated into groups by a thin septal membrane (Figs. 16—18).

DISCUSSION

It has already been ascertained by Kamo *et al.* (3) and Hatsushika (5) that

the metacercarial cyst consists of four layers, an inner and an outer layer of the inner wall, the outer wall and the outermost membraneous substance. The origin of these layers remains unclear. By comparative histochemical examinations of the cyst walls of *Paragonimus westermani* and *Paragonimus miyazakii* Kamo *et al.* (3) explained that the membranous substance of the metacercarial cyst of *P. miyazakii* is originated from the host tissue and is of a different nature from the outer wall of the cyst of *P. westermani*. Our observations using SEM also revealed that the outermost membranous substance has quite a different structure from the other three layers which are composed of homogeneous substance. Cellular structures such as nuclei and mitochondria are observed in a cross section of the outermost membranous substance. Capillary vessels presumably originating from the crab, run over the surface of this substance. This membranous substance is absent in the metacercariae parasitized in the system of crabs other than the cardiovascular related areas.

Lo *et al.* (8) observed the encystment of metacercariae of *Allopodocotyle lepomis*, using SEM, and found that the hemocytes attached to the surface of the cyst wall subsequently formed pseudopods and flattened themselves to complete the capsule formation. Mitchell (12) observed fibroblast strands, microfilament, vesicles, mitochondria and collagen fibers in the outermost cyst wall of *Posthodiplostomum minimum*, using TEM. It was concluded that the outermost cyst wall is formed by fibroblastic cells and the products.

Considering these findings the membranous substance of *Paragonimus miyazakii* may have its origin in endothelial cells of vessels of the host. These endothelial cells cover the entire outer wall of metacercarial custs and form a fibrous coat during the growth of metacercariae.

The origin of the outer and the inner wall is still unclear. Most investigators consider that the outer wall originates in the host tissue and the inner wall is secreted by the parasite. We observed numerous vesicles in the tegument of encysted metacercariae and a secretory mucus substance on the tegumental surface. We thus assume that some secretory substance is excreted through the tegument and forms the inner wall of the metacercarial cyst.

Laurie (13), in his study of experimental encystment of *Himasthla quissetensis*, observed an outer wall formed by granules which were released from the tegument, and an inner wall formed by scrolled rods originating in subtegumental cell bodies. The same findings were reported by Cable and Schutte (14) and Harris *et al.* (15). Mitchell (12) observed the interface zone between the outer wall and the inner wall of the metacercarial cyst of *Posthodiplostomum minimum* by TEM and stated that this zone appeared to be comprised by degenerating host endothelial cells.

Morphological changes during artificial excystation were observed in our study. At first, vesicular pores occur in the inner layer of the inner wall, and degeneration is soon apparent over the entire inner wall. Finally, the activated metacercaria breaks out through the weakest part of the cyst. This process seems to be related to the fact that the parasite has the ability to

synthesize mucopolysaccharidase, as it was evidenced in trematodes, and that the inner wall is mainly formed of various mucopolysaccharides and keratin.

A topographical observation was made on the surface of an excysted juvenile metacercaria. Sensory organs, which are generally regarded as tango-receptors, rheoreceptors, or chemoreceptors surround both the ventral and the oral suckers. These are of a simple papillary type. Sensory organs of a pit-type or a papilla-type with cilia were not observed. The lack of stylets around the oral sucker is reportedly one of the characteristics of metacercariae of *Paragonimus miyazakii*, but in our observations, well-developed stylets were found around the oral sucker. The excretory bladder is situated in the area between the offspring of the intestine and the end of the body, displaying the shape of letter I. It opens into the cloaca at the end of the body. In light microscopic preparations, numerous black granules were observed with high reflections, in the excretory bladder. We defined these as concretions filling the excretory papillae of the excretory bladder. The concretions in the excretory papillae are similar to the "calcareous corpuscles" of cestodes and such have been described by other workers (von Brand *et al.* (16), Chowdhury *et al.* (17), Yamane *et al.* (18)).

Bennett and Threadgold (19) discerned the same concretions in the excretory bladder of newly excysted juveniles of *Fasciola hepatica*. Mitchell and Crang (9) examined components of these concretions, by X-ray microanalysis. They stated that the concretions appear to be layered from a central core and show an abundance of calcium, and, in some cases, magnesium. They assumed that these concretions and higher fatty acids contained within may be significant in supplying the metacercaria with energy, or they may serve to buffer organic acids. In the case of *P. miyazakii*, it is assumed that the excretory bladder serves as a storage place for the concretions which supply energy for the excystation.

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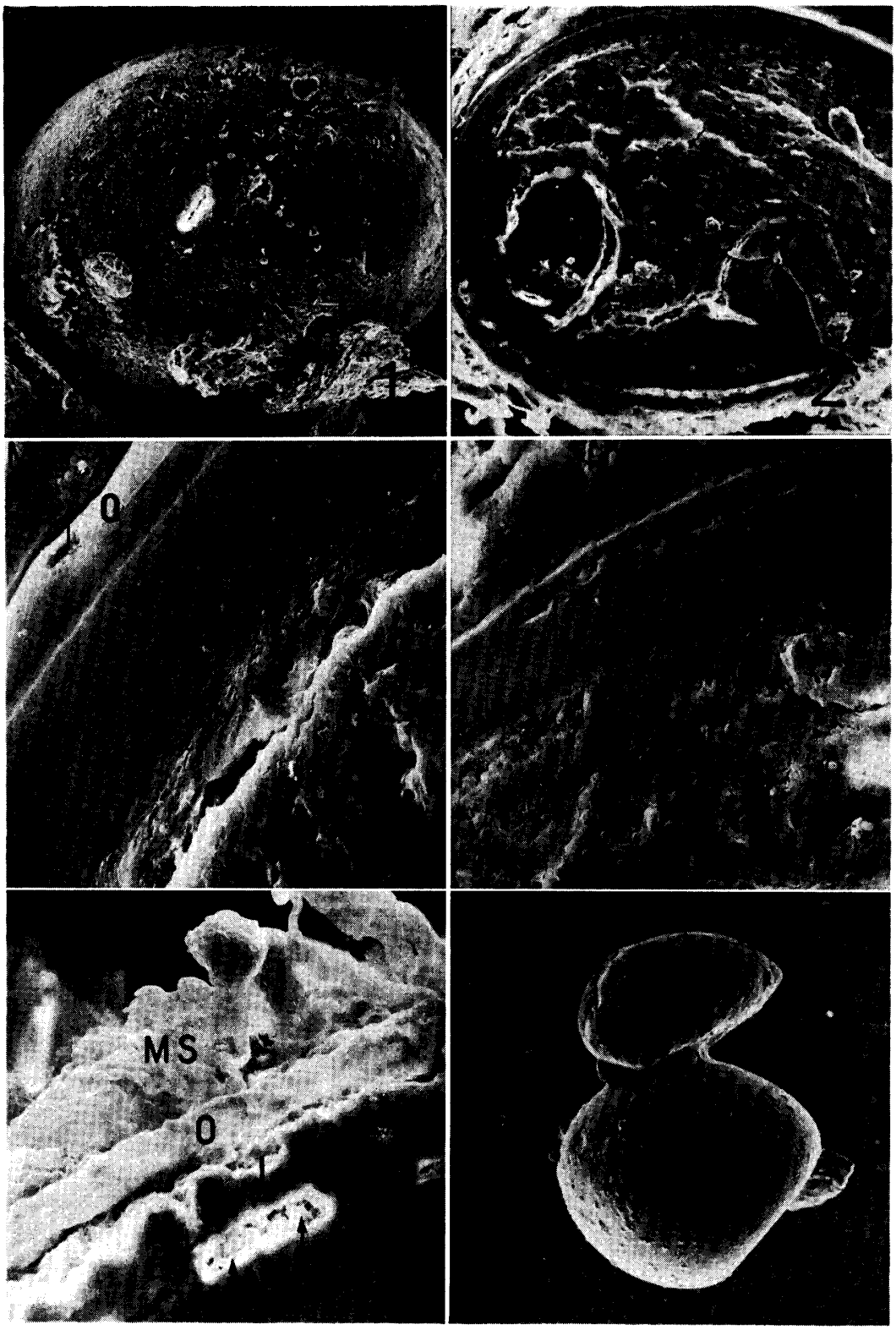
EXPLANATION OF FIGURES

Fig. 1. A metacercaria which is excised from the vessels of the crab, *Potamon dehaani*, showing the fibrous membrane or blunt projections on the surface. (× 300)
Fig. 2. Cross section of a metacercaria before artificial excystation, showing multilayered capsule, penetration glands, intestine and sucker. (× 300)
Fig. 3. Multilayered capsule of a metacercaria before treatment with artificial intestinal juice, showing membranous substances (MS) with some cellular structure (arrow), outer wall (O), outer layer of inner wall (IO), inner layer of inner wall (II), and vesicles in the tegument (T). (× 2,400)
Fig. 4. Inner wall of the capsule of a metacercaria which was treated for 30 min with artificial intestinal juice, showing several ellipsoidal pores (arrows). (× 2,400)
Fig. 5. Capsule of a metacercaria which was treated for 60 min with artificial intestinal juice, showing degenerated membranous substances (MS), outer wall (O), inner wall (I) and split (arrows). (× 2,400)
Fig. 6. The artificial excystation, showing the perforation of the capsule (C) by the activated metacercaria (M). On the surface of outermost layer of capsule the ramified vessels can be seen. (× 150)
Fig. 7. An excysted juvenile metacercaria. (× 260)
Fig. 8. The surface structure of ventral face of an excysted juvenile metacercaria. (× 4,000)
Fig. 9. The spine of dorsal face of an excysted juvenile metacercaria. (× 4,000)
Fig. 10. The oral sucker and stylets around the oral sucker. (× 1,600)
Fig. 11. The sensory papillae and openings of glands (arrows) around the ventral sucker (VS). (× 1,600)
Fig. 12. Cross section of the ventral sucker, showing the openings (arrow) of penetration glands (G). (× 4,000)
Fig. 13. Cross section of the granules (G) of penetration glands. (× 7,000)
Fig. 14. Papillae in the excretory bladder (EB) and its cross section showing numerous concretions. (× 1,000)
Fig. 15. The surface structure of papillae showing the growth of microvilli (MV) and pores (arrows). (× 25,000)
Fig. 16. Inner structure of papillae (P) in which were packed numerous concretions. (× 3,000)
Fig. 17. Numerous concretions (Cr) were packed in the excretory papillae and separated by the membranous septa. (× 6,000)
Fig. 18. The basis of excretory papilla showing the concretions (Cr), basal membrane (BM) and complex network formation. (× 20,000)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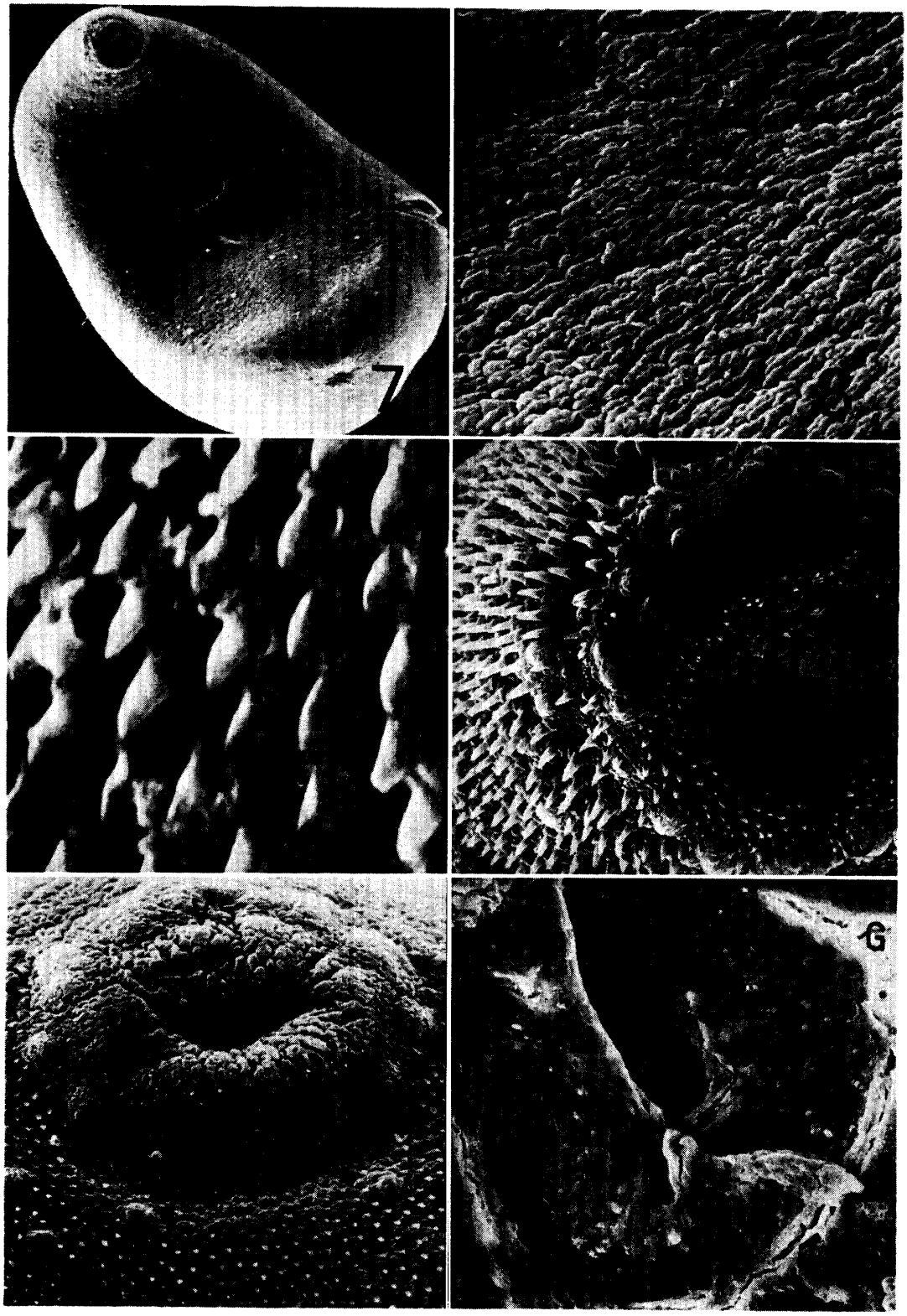

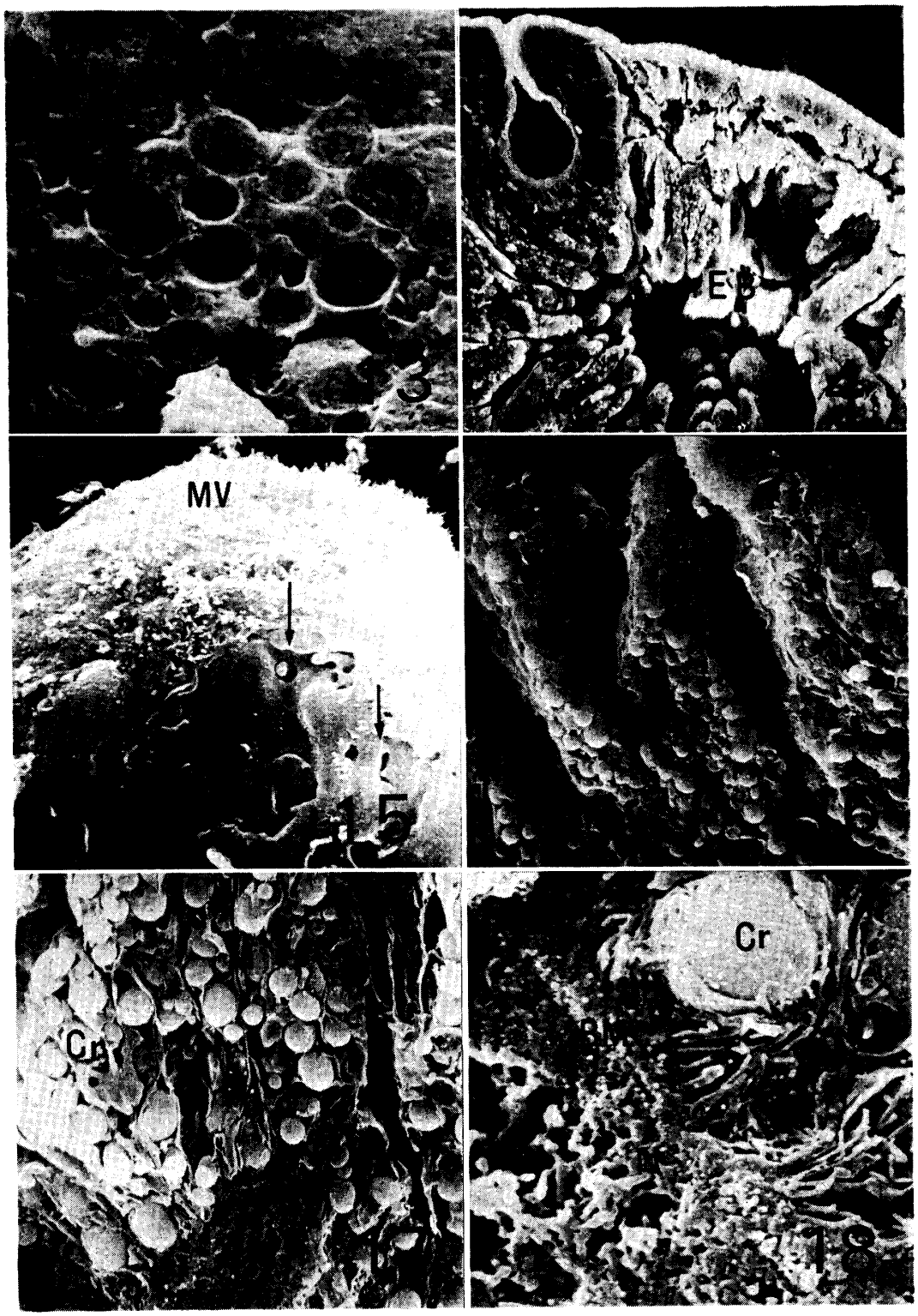
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