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An Ultrastructural Study on the Glochidium and Glochidial Encystment on the Host Fish

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＝國文要約＝

Glochidium larva의 構造와 宿主魚類에서의 被囊形成에 關한 微細構造的 硏究

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Anodonta grandis (Eulamellibranchia)의 glochidium larva形態와 宿主魚類에 부착하였을 경우 宿主의 上皮組織이 glochidium larva를 중심으로 被囊을 形成하는 過程을 走射電子顯微鏡(SEM)을 이용하여 觀察하여 본 바 아래와 같은 結果를 얻었다.

Glochidium의 모양은 그의 貝殼을 닫았을 경우 삼각형에 가까우며 크기는 평균 0.45 mm×0.4 mm이다. 두 개의 貝殼은 크기가 서로 같고 길이 120 μm 넓이 7 μm의 靱帶에 의하여 함께 결합되어 있다. 각 貝殼은 1개씩의 hook을 가지고 있는데 그 크기는 16 μm이며 그 표면에는 많은 spine이 2~3列로 크게 돌출하여 있다. 각 貝殼의 前端部 즉 hook의 基部를 포함한 그 주변의 넓은 부분에는 변방으로 가면서 점차 더 작아지며 결국 소멸되는 많은 수의 spine들이 존재한다.

貝殼의 外表面에는 수많은 突起들이 부위에 따라 모양과 분포양상을 달리하여 존재하고 있다. 外表面에는 上記의 突起 외에 수많은 크고 작은 niche들이 존재하는데 이들은 貝殼의 內層이 가지고 있는 수많은 hole 때문에 얇은 膜性外層이 안쪽으로 함입한 탓이다. Mantle cell들은 貝殼의 내부 대부분을 덮고 있으며, larval thread는 mantle의 ventral plate 중앙으로부터 突出하고 있으며 그 직경은 균일하게 2.65 μm이고 부속 구조물은 가지고 있지 않다.

Hair cell들은 세가지 유형이 있는데 이들간의 기능의 차이는 알 수 없다.

宿主魚類에 대한 人工感染實驗을 수행한 本 硏究에서 宿主의 上皮組織이 부착한 glochidium larva를 둘러싸 완전한 일차적인 被囊을 형성하는데는 약 3~4時間이 소요되었는데 그 方法은 부착부위 上皮細胞의 급격한 增殖에 의한 것이 아니라 주변 上皮細胞들의 활발한 移動에 의한 것이었다.

INTRODUCTION

The young larvae of fresh-water mussels pass their early stages of development inside the marsupial gill pouches of the female. Here they progress to a simple larval bivalves, so called a glochidium. Eventually, the glochidia are discharged from the gills for further processes of development. The glochidia then attach to the gills or fins of appropriate fishes where they become encysted in the tissue, and

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they also become virtual ectoparasites. During the period of this parasitic stage, the glochidia undergo metamorphosis process. After this process, the juvenile mussels emerge from their cysts and become independent.

This investigation was carried out to find out the fine structures of the glochidium and the successive stages of the early glochidial encystment of a fresh-water mussel, *Anodonta grandis*, on the guppy.

MATERIALS AND METHODS

The fresh-water mussel used in this study was *Anodonta grandis*, subfamily Anodontinae, family Unionidae, order Eulamellibranchiata.

A gravid mussel from the tank in which the living mussels were kept, was opened in a small quantity of dechlorinated water and the marsupia were quickly cut loose. The marsupia were transferred into a large Petri dish containing dechlorinated tap water and then rapidly cut opened. The glochidia were shaken out into the Petri dish and collected according to each purpose of this study.

For morphological study of the glochidium, many of the glochidia collected were completely anesthetized to open their valves with menthol. The well relaxed glochidia were fixed with 2.5% glutaraldehyde for 2 hours and washed with phosphate buffer. The glochidia were dehydrated in a graded series of alcohol-amyl acetate mixture. The dehydrated glochidia were dried with the critical point dryer.

In the mean time, many of the anesthetized glochidia were immersed in 10% potassium hydroxide to dissolve their internal flesh masses, including the mantles and the adductor muscles, for overnight and washed with double distilled water. To clean the glochidial shells, the ultrasonic cleaner was also applied. But the sonications in any level gave severe damage to the shells. The glochidial shell valves well cleaned with the double distilled water were air dried.

For the experimental study of the glochidial encystment on the guppy, lots of glochidia were transferred to the bottom of a fish globe containing 15 cm deep dechlorinated tap water at 20℃.

A total of 20 guppies supplied from the aquarium were allowed to swim arround in the fish globe which were contained with the glochidia for 5 to 10 minutes. The fishes infected with several glochidia were transferred to the beakers 200 ml in capacity of 2 fishes per beaker.

To examine successive stages of encystment, two fishes per every 30 mintes were caught and fixed with 2.5% glutaraldehyde solution.

The fins or some other parts of the fishes infected with the glochidia were carefully removed from the fishes under the steroscope, washed with phosphate buffer solution, and dehydrated in a graded series of alcohol-amyl acetate mixture. The dehydrated specimens were dried with the critical point dryer.

All the above completely dried specimens were stored in dust-free containers until the gold coating was applied and the specimens were observed with the scanning electron microscope (JEOL JSM-U3).

RESULTS

1. Morphology of the Glochidium

The shape of the glochidium is apparently triangular and its size is 0.4~0.5 mm×0.38~0.42 mm when closed. The two glochidial shell valves are of the same size, kept together by a ligament of 120 μm in length and 7 μm in width (Fig. 8, 16). Each of the two valves has a hook 16 μm long at the internal end (apex) of the valve. The hook is studded with many spines on its superior face and a large area at the apex of the valve surrounding the base of the hook is provided with numerous small spines which become progressively smaller towards the periphery of the area (Fig. 1, 2).

The external surface of the glochidial shell valve is covered with numerous small processes showing

successive change in the shape and in the pattern of distribution by part. The external surface of the membrane that covers the apex (presumptive ventral margin) is coarse (Fig. 3), otherwise the dorso-lateral regions of the shell valve are less coarse with a fine network-like structure of the processes (Fig. 6). The external surface of the central region of the valve is relatively smooth with very loose reticular structure of the processes (Fig. 4). The external surface of the dorsal side (posterior), including the ligament and its surrounding area, is somewhat coarse due to the processes that vary in size and scattered around in even density. Some of the processes in this region are connected with neighboring processes (Fig. 5). The glochidial shell valve has two layers. One is outer thin membrane bearing the processes mentioned above which has numerous niches, and the other is inner layer bearing numerous holes which are the origin of the niches (Fig. 7, 8).

The mantle cells line the glochidial shell valves. A larval thread, without any accessory structure and 2.66 μm in diameter, emerges from a canal located at center of ventral plate of the mantle (Fig. 9, 10).

Three types of the hair cells are observed in the mantle. Each of the shell valves has a very specialized hair cell with a bunch of hair about 50 μm apart from the canal, the larval thread gland (Fig. 10, 11). Some other hair cells with around 10 hairs are situated in the posterior ventral plate. There is a group of hair cells with a lot of hairs in the posterior margin of the mantle (Fig. 10).

Near to the posterior margin of each of the mantle, a large pit was found. When the glochidial shell valves are closed, the pits located in the mantles one for each are facing each other (Fig. 10).

2. Glochidial Encystment

When the glochidia attach themselves to the host fishes, their hooks interlock the fish tissues such as the fins, the gills, the scales, the jaws and the buccal cavity. According to the experiment, the glochidia chose to be attached to the fins usually deep from the margin (Fig. 16~21). About thirty minutes after attachment, it was observed that the epithelial cells of the host fish were attacking the attached glochidium in waves (Fig. 13). During the period of early encystment, the epithelial cells of the host fish actively migrated toward the attached glochidium and began to cover it (Fig. 13, 17). At this stage,the epithelial and cellular connective tissue became loose. The cells lost their mutual connections and became separated (Fig. 15). The shapes and outer surface patterns of the epithelial cells became very irregular (Fig. 13~16). When the epithelial cells stopped migrating and became stable, the over all shapes of the cells and their normal arrangement were recovered.

In the experiment study, it took about three to four hours to complete the early cysts on the guppy but their stages of encystment were sometimes different even in neighboring glochidia (Fig. 19, 20).

DISCUSSION

There are two well marked types of glochidia in the Unionidae as long been known. One is provided with stout hook on the ventral margin of the valves and the other is in quite different shape and entirely hookless (Lefevere and Curtis, 1910). The mussels belonging to the genus *Anodonta* have glochidia provided with hooks.

The structures of the glochidia of the *Anodonta* species have been described by Surber (1912, 1914), Arey (1924), Wood (1974a), Giusti *et al.* (1975) and Tompa (1979). Wood (1974b) made a very detail report on the light microscopic structure of the glochidium of *Anodonta cygnea*. An ultramicroscopic study on the glochidium of *Anodonta cygnea* by Gisuti *et al.* (1975) presented some new features of the glochidial shell valves.

Present study focused on the fine external structures of the glochidium of *Anodonta grandis* and on

the process of the glochidial encystment.

The shape of the glochidium of *Anodonta grandis* is apparently triangular and unequilateral as described on the other species belonging to genus *Anodonta* by Brondiewicz (1968), Wood (1974a) and Giusti et al (1975).

The two glochidial shell valves are of the same size about 4.6 μm in length and about 4 μm in height, kept together by a ligament 120 μm in length. As the description on the glochidium of *Anodonta cygnea* by Giusti *et al.* (1975), the external surfaces of the glochidial shell valves of *Anodonta grandis* are covered with a great number of processes so called calcium carbonate crystals by Tompa (1979). But the shapes and the pattern of distribution of the processes seem to be different from those of *Anodonta cygnea*. In the region of the ligament a lot of small processes are distributed at random. Unlike the glochidial shell valves of *Anodonta cygnea*, there is not a place where the processes are parallel in rows.

Besides the region of the ligament, the processes formed certain types of reticular structures, coarse, fine or loose, by region (Fig. 3~6).

The surface of the glochidial shell valves have numerous niches scattered all over (Fig. 8). As mentioned on those of *Anodonta cygnea* by Giusti et al., (1975), the niches are formed by the pellicle covering the holes of the internal layer.

The posterior region (presumptive dorsal region) of the glochidial shells shows the same basic pattern of arrangement of the ligament and margins as the adult clam shells.

The larval thread is even in diameter (2.65 μm) from the base to the tip, and it does not have any accessory structure on its surface. Wood (1974a) stated that the larval thread of the glochidium of *Anodonta cygnea* was found to be a non-cellular structure which was periodic acid Schiff (PAS) positive, and a mucoid nature. A transmission electron-microscopic study on the thread would help us to understand its nature.

The larval thread is positioned on its base in the middle of the mantle, ventral center to the hinge line of the shell valves. The thread seems to be in a suitable position to keep the balance of the glochidial body when pulled.

A total of three types of hair cells were observed in the glochidial mantle. A considerate study on the function of the hair cells existing in the mantle of the glochidium of *Anodonta cygnea* by Wood (1974a) concluded that the hair cells have chemical sensitivity. From the present study, a question was raised whether the above three types of the hair cells have the exactly same functions.

On the method of cyst formation, some eariler researchers such as Young (1911), Lefevre and Curtis (1912) and some others, stated that direct proliferation of the cells of the host tissue provides the material for the cyst that encloses the attached glochidium.

Opposing the opinions of the above workers, Arey (1932a) asserted that the process of cyst formation is one of cell migration, whereby neighboring host cells assemble and actively push forward over the invader until wound is closed and the glochidium is covered. Arey (1932b) insisted the above theory based on an actual observation of encystment stages which did not show the presence of more than the ordinary number of random mitoses seen in control, uninfected tissue. To the theory of encystment through cell multiplication, Arey (1932b) also presented several counterevidences: (1) The cyst may be composed of several thousand cells; (2) the time required for the formation of a cyst under favorable conditions is three to four hours; (3) the mitotic cycle is relatively slow and consumes several hours in cold-blooded vertebrates.

The results, obtained from the SEM observation in the present study, entirely support the theory of cell migration in the method of cyst formation.

SUMMARY

A scanning electron microscopic study on the glochidium and glochidial encystment of *Anodonta grandis* on the guppy was conducted.

The shape of the glochidium is apparently triangular and its average size is 0.45 mm×0.4 mm when closed. The two glochidial shell valves are of the same size, kept together by a ligament of 120 μm in length and 7 μm in width. Each of the glochidial shell valves has a 16 μm long hook studded with many spines on the superior face. A large area at the apex of the valve surrounding the base of the hook is provided with numerous small spines which become progressively smaller towards the periphery of the area. The external surface of the glochidial shell valve is covered with numerous small processes showing successive change in the shape and the pattern of distribution by part. Besides the processes, there are a number of niches scattered all over the exterior surface. The glochidial shell valve has two layers. One is the outer thin membrane bearing the processes and the niches and the others is the inner layer bearing numerous holes which are the origin of the niches. The mantle cells line the glochidial shell valves. A larval thread, without any accessory structure and 2.65 μm in diameter, emerges from a canal located at center of ventral plate of the mantle. A total of three types of the hair cells are observed.

In present artificial infection of the glochidium to the guppy, it took about three to four hours to complete an early cysts. During the period of encystment, the epithelial cells of the host fish actively migrated toward the attached glochidium and covered it.

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EXPLANATIONS FOR FIGURES

Abbreviations:

S, spine	Po, posterior	P, pit
L, ligament	LV, left shell valve	EC, epithelial cell of the fish
T, larval thread	BH, bunch of hair	An, anterior
H, hook	V, glochidial shell valve	RV, right shell valve
Ho, hole	M, mantle	CB, cell border
HC, hair cell	G, glochidium	

Fig. 1. A lateral view of the apex of the glochidium. The glochidial shell valves are slightly interlocked. The hooks(H) are studded with spines. ×300.

Fig. 2. An apex of the glochidial shell valve studded with various sizes of spines. ×800.

Fig. 3. The external surface of the valve, near the apex (presumptive ventral margin), showing the coarse pattern of the processes. ×10,000.

Fig. 4. The external surface of the central region of the valve showing the rather smooth pattern. The calcium carbonate processes are forming a loose network-like arrangement. ×10,000.

Fig. 5. The external surface of the ligament (L) and its surrounding area. The ligament has dark base in this figure. A number of calcium carbonate processes in various sizes are scattered and they are interconnected sometimes. ×10,000.

Fig. 6. The external surface of the lateral region of the valve showing a fine networklike structure by the calcium carbonate processes. ×10,000.

Fig. 7. The internal surface of the valve with holes (Ho). The holes do not completely perforated through the valve. ×6,000.

Fig. 8. A posterior view of the glochidial shell valves showing the ligament (L) and its surroundings. Numerous niches are seen all over the surface. The names of the parts labelled in this figures indicate the presumptive names those the clam shells would have. ×200.

Fig. 9. An anterior view of the glochidium. The glochidial shell valves with a hook for each are slightly opened so that the mantle (M) with a larval thread (T) protruded a little is visible. ×150.

Fig. 10. The mantle of the glochidium showing the larval threads (T), the specialized hair cells (HC), the mantle pits (P), and a big group of hair cells with a bunch of hair (BH). The mantle cell borders (CB) are also recognizable.

Fig. 11. A very specialized hair cells with numerous hairs about 50 μm apart from the thread gland. This micrograph also showed the tip of the larval thread (T). ×2,000.

Fig. 12. The epithelial cells of the fin of the guppy showing the cell arrangement and surface pattern. The cell borders (CB) are recognizable. ×1,300.

Fig. 13. The epithelial cells of the guppy's fin showing the pattern of the encysting movement. The epithelial cells are moving in waves toward the attached glochidium (G). Thirty minutes after attachment. ×1,300.

Fig. 14. The epithelial cells of the guppy's fin encysting the glochidium. The arrangement of the cells become loose and the cell shapes become very irregular. One hour after attachment. ×2,000.

Fig. 15. The epithelial cells of the early cyst peeled off. The cells show their reverse sides and loose connections. One hour after attachment. ×1,300.

Fig. 16. A glochidium attached to the host fin. The hook accidentally bent outward. Thirty minutes after attachment. ×130.

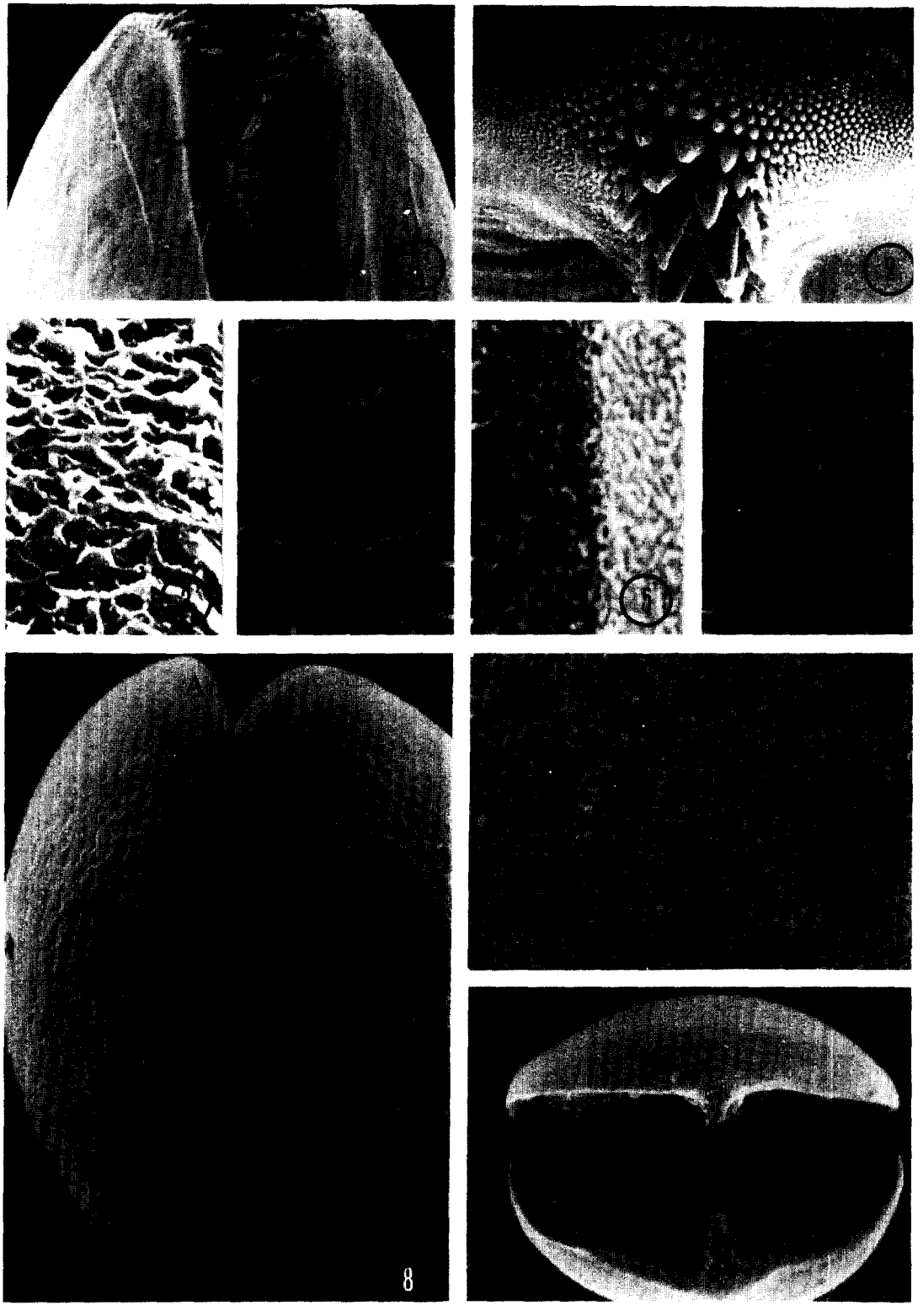
Fig. 17. A glochidium being encysted. About half of the glochidium is covered with the epithelial cells of the fin. One and half hours after attachment. ×130.

Fig. 18. A glochidium being encysted. Two thirds of the glochidium is covered with the epithelial cells of the fin. Two hours after attachment. ×130.

Fig. 19. The glochidia still being encysted. Most of the glochidial shell valves are covered. Two and half hours after attachment. ×100.

Fig. 20. The early cysts almost completed. Three hours after attachment. ×100.

Fig. 21. A cyst newly formed. A glochidium is completely covered with the epithelial cells of the fin. The cells are yet loosely interconnected in part. Three hours after attachment. ×130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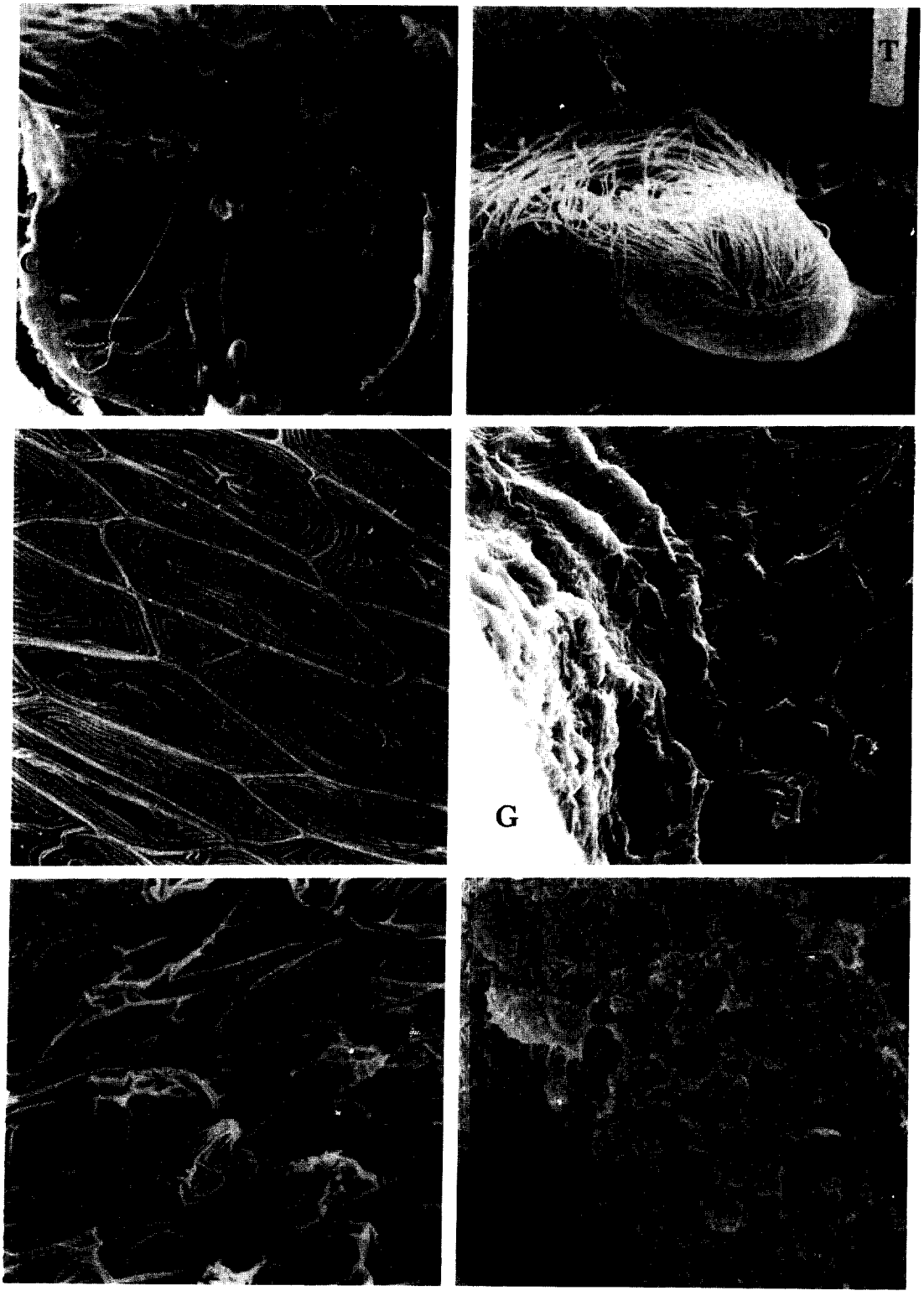
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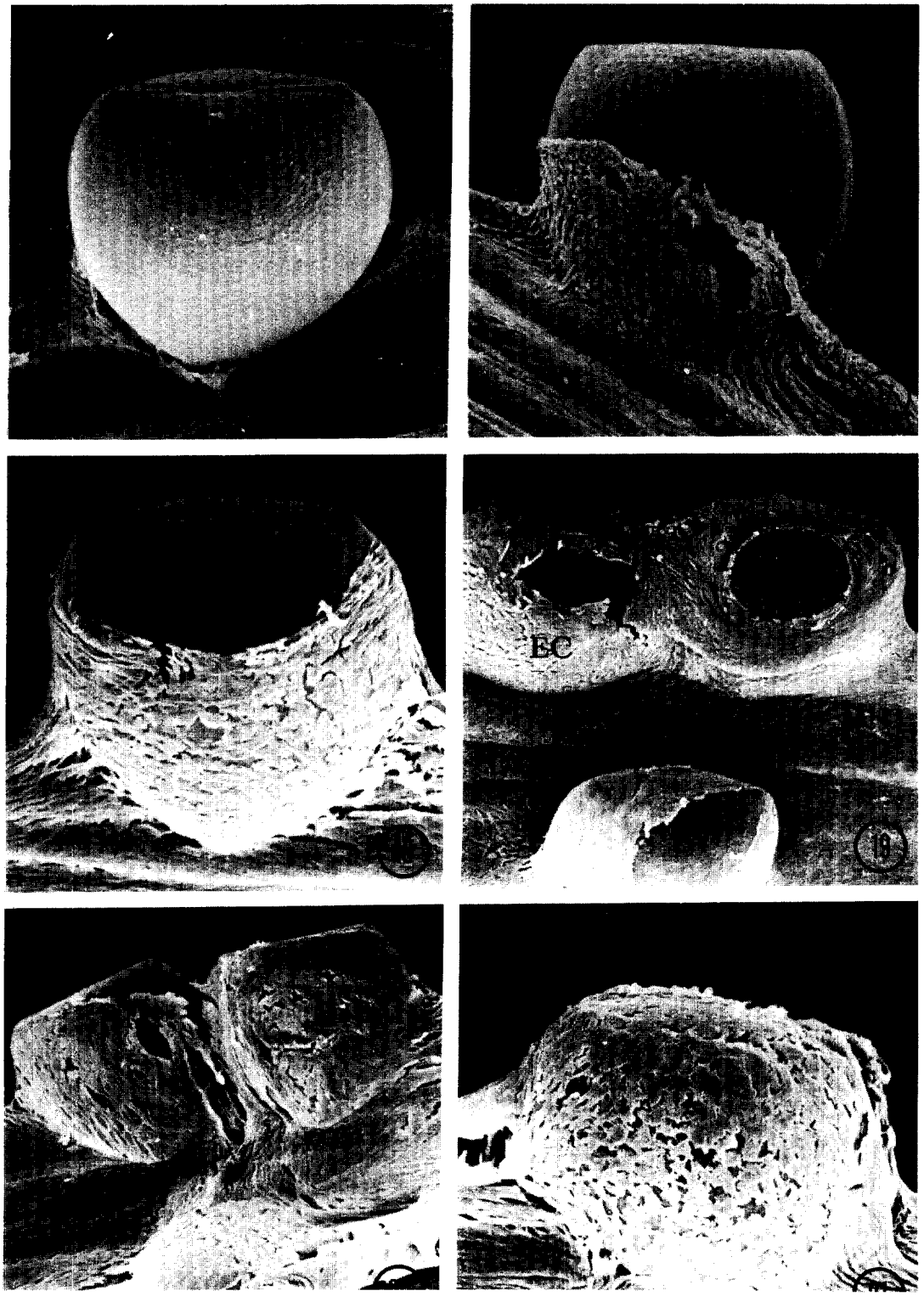
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