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## “ORGANIC MEMBRANE-SHELL” AND INITIAL CALCIFICATION IN SHELL REGENERATION

by

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(with 43 figures and 2 tables)

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### *Abstract*

Initial mineralization of the organic membrane-shell utilized in shell regeneration in mussel was studied by electron probe microanalysis and electron microscopy. The organic membrane-shell, not-fully mineralized regenerated shell, is composed of three different layers: laminated, brown and conchiolin membranous layers in regeneration order. The organic matrix of the first and third are very similar, in electron microscopic and histochemical features, to the conchiolin matrix, closely associated with the mineralization of the shell. While the second may be homologous to the periostracum, outermost non-calcified layer of the mussel shell.

Initial mineralization of the organic membrane-shell takes place within the conchiolin membranous layer, through the following process: Deposition of particles (nucleus of mineralization?) → Aggregation of particles → Coating of aggregated body with amorphous organic materials (= formation of spherule) (calcium accumulation) → Enlargement of its size (calcium concentration and beginning of mineralization?) → Transformation into double-disk body (accumulation and growth of acicular calcium carbonate crystals) → Exposure of preformed acicular crystals to the spaces between conchiolin membranes → Final formation of crystalline aggregates, structurally organized into well-ordered sheet-like layer (nacreous layer).

### Introduction

Until recently, there have been many electron microscopic studies relating to the growth, structure and architectural construction of inorganic crystals, within the calcified shell of molluscs, but few relating to the initial calcification of the molluscan shell. Almost all of the studies of initial calcification utilizing regenerated shell, as well as those concerned with the mantle tissues have been carried out with the light and polarization microscope to determine the microscopic structure and the presence of inorganic crystals.

The present discussion has been confined to a study, with electron microscopy and electron probe microanalysis, of newly regenerated shells, not-fully mineralized, “organic membrane-shell”, of blue mussel, *Mytilus edulis*. Especially we have noticed the onset of mineralization in the organic membrane-shells as a model for the mechanism of calcification of the molluscan shell. We will describe in this paper the ultrastructure and component of the organic membrane-shell. And we will set out to confirm by electron microscopy, the presence of organic spherules (calcium-accumulating bodies) in newly regenerated shell, to determine the life of the organic spherules during shell development, and to show clearly that initial shell mineralization is associated with spherules.

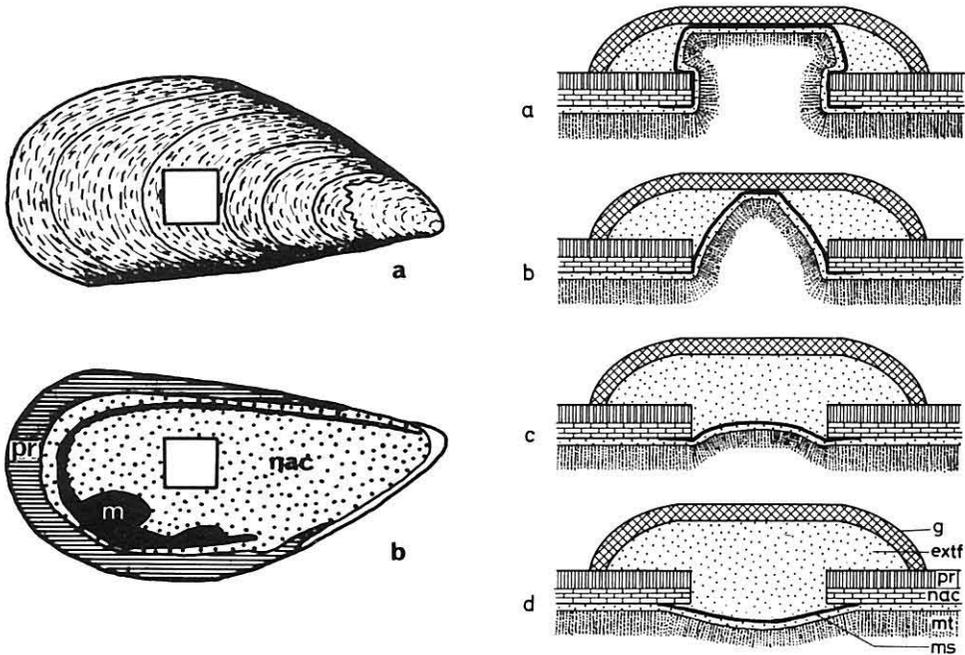


Fig. 1 (Left) Shell of *Mytilus edulis* showing the position of the opening. a: outer view; b: inner view.

Fig. 2 (Right) Diagrammatic vertical sections showing the regenerated portions of *Mytilus edulis*. a and b are types of regenerated shells attaching to the glass slide. c and d are types of regenerated shells isolating from the glass slide. extf: extrapallial fluid; g: glass slide; ms: organic membrane-shell; mt: mantle tissues; nac: nacreous shell layer; pr: prismatic shell layer.

#### Materials and methods

Specimens of *Mytilus edulis* Linne ranging 10–15 cm in shell length were obtained during July and August of 1975–1977 from the coast of Ishikari-Bay, Central Hokkaido, and Usu-Bay, Southwest Hokkaido. They were maintained in sterilized sea water in glass aquaria, fitted with coral sand filters and a circulating water system. The temperature of the aquaria was kept between 18°–20°C. Mussels were fed artificially cultured diatoms.

The shell of *Mytilus edulis* has two mineralized layers: an outer prismatic layer, constructed by calcite crystalline aggregates and an inner nacreous layer, constructed by aragonite ones. The present study was carried out in the central region of one valve, developing nacreous layer (Fig. 1), and by removing a portion of the shell and covering the open area with a glass slide: a hole 10 × 10 mm was cut in the central region of one valve using a dental cutting tool; the open area was then covered by a piece of glass slide and completely sealed with dental cement and “Aquaria Epoxy Bond”. After the operation, the animals were immediately returned to a glass aquaria, and cultured over a period of six ~ twelve months. During this period, the progressive degree of regeneration was observed time after time with a binocular-microscope through the glass slide covered opening, and as the occasion arose, the regenerating materials were flayed from the normal shell as the examined

samples for the electron microscope.

Samples were immediately washed in seawater and fixed in 2.5% gluteraldehyde, buffered with seawater (pH 8.0), and then some of them were post-fixed in osmium tetroxide solution. Following fixation and several buffer rinses, the samples were dehydrated in a graded ethanol series and dried by the critical-point-CO<sub>2</sub> procedure to present the ultrastructural architecture of the organic matrix surfaces. All of the specimens obtained were glued to brass stubs and coated with carbon or carbon-gold in vacuum, and examined by a scanning electron microscope (JEOL JSM-U<sub>3</sub>), operated at 15 KV. Analysis was made by SMU<sub>3</sub>-SDS wave length X-ray spectrometer on JEOL JSM-U<sub>3</sub>.

### Results

Regeneration of *Mytilus edulis* was carried out on 60 operated animals, and studied, first of all, by periodically observing an opening in the central region of one valve with a binocular-microscope. The experimental removal of a portion of the shell was followed immediately by replacement through the secretory activity of the mantle. The completion of the "organic membrane-shell", not-fully mineralized regenerated shell, required about 60 days.

The optical microscopic sequence of the regeneration process within 10 × 10 mm includes: 1) Filling the space between the glass slide and the mantle by extrapallial fluid from the mantle epithelium in 2–3 days, 2) the closure of the opening by the protrusion of mantle epithelium, in 2–7 days, 3) the deposition of fibrous materials at the edge of the opening in 3–7 days, 4) the full-formation (completion) of brown membrane along the outer surface of the protruded mantle epithelium in about 20 days, 5) the deposition of crystalline materials within the organic membrane-shell in more than 30 days.

Systematic observations with the scanning electron microscope were then begun in order to follow the sequence of events more closely; i.e. to make clear the process of

| Membranous layer<br>Calcified body<br>Period<br>(in days) | ORGANIC MEMBRANE - SHELL |            |                            |          |                         |
|---|--------------------------|------------|----------------------------|----------|-------------------------|
|   | Laminated m.l.           | Brown m.l. | Conchiolin m.l.            |          |                         |
|   |                          |            | Particle - Aggregated body | Spherule | Flattened - Double-disk |
|   |                          |            | Calcium accumulation       | ?        | Mineralization          |
| 6   | →                        |            |                            |          |                         |
| 15  | →                        |            |                            |          |                         |
| 23  | →                        |            |                            |          |                         |
| 30  | →                        |            |                            |          |                         |
| 41  | →                        |            |                            |          |                         |
| 52  | →                        |            |                            |          |                         |
| 64  | →                        |            |                            |          |                         |
| 100   | →                        |            |                            |          |                         |

Fig. 3 Formation and calcification of the organic membrane-shell in *Mytilus edulis*.

formation of the organic membrane-shell, to monitor the concentration of  $\text{Ca}^{++}$  in or on the organic membrane-shell with time, to determine if the concentration of  $\text{Ca}^{++}$  was initiated at a few special loci or if it occurs uniformly in or on the organic membrane-shell, and to determine if mineralization was initiated at the above-mentioned special loci.

#### Formation of the organic membrane-shell

On day 2–7 after the operation, the mantle of mussel pressed strongly against the opening: in many cases, a part of the mantle protruded from the opening and completely sealed off the internal environment, as illustrated in Figure 2a-c; in a few cases, the mantle did not protrude and became depressed in the open area, and contacted the inside shell surface near the margin of the opening as shown in Figure 2d.

The organic membrane-shell was formed parallel to the outer surface of the mantle epithelium, as a cast of the latter. In some cases, the organic membrane-shell attached to the glass slide covering the opening, and was deformed taking a mushroom-like form, and in other cases, isolated from the glass slide they were rather flatly formed. The mature organic membrane-shell, as mentioned later, became completed after about 60 days, through the process of formation as shown in Figure 3.

The mature regenerated shell is composed of three distinct layers: laminated membranous, brown membranous and conchiolin membranous layers, as illustrated in Figure 4. The second and third layers were successively deposited inside the first. Upon near completion of the organic membrane-shell, this shell became calcified by supporting crystalline growth on or among the conchiolin membranes.

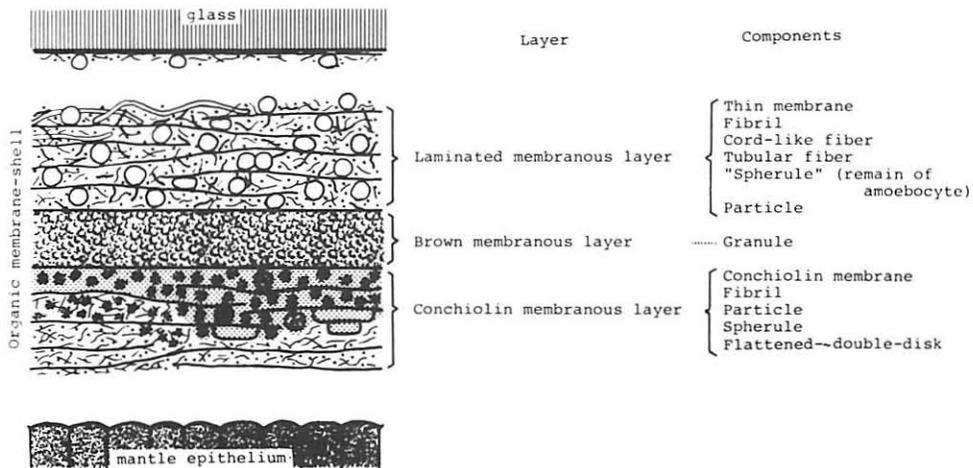


Fig. 4 A diagram of the organic membrane-shell showing its general organization.

#### *Laminated membranous layer*

On day 5–7 after the operation, the laminated membranous layer is first observed through the open area. Scanning electron microscopy showed that this layer is generally

5–50 $\mu$  in thickness and is built up mainly from laminated membranes, laid in layers. Some other kinds of fibers, spherules and particles were also found within the spaces between laminated membranes.

The membrane itself, now in question, is very thin, 0.1 $\mu$  in thickness and is essentially composed of fibrils, which are embedded in and overlain by some kinds of amorphous material, judging from the micrographs, illustrated previously by the authors (Uozumi and Suzuki, 1978, Pl. II, Figs. 4–6). The surface is rather smooth in general, but numerous organic particles, mentioned later, sometimes adhered to it and looks like a pustulous surface.

Fibrous materials, components of this layer, are subdivided into three types: tubular fiber, cord-like fiber and fibril. Tubular fibers are 1.5–5 $\mu$  in diameter, extending to 100 $\mu$  in length, and found commonly on the outermost surface of the laminated membranous layer. Sometimes many tubular fibers congregated with each other and formed a network pattern. Cord-like fibers are 0.15–0.4 $\mu$  in diameter, 5–50 $\mu$  in length and found mainly within the spaces between laminated membranes. They are arranged at random. Fibrils are the finest of all, less than 0.1 $\mu$  in diameter, and develop as a result of filling up the spaces between laminated membranes and between other organic materials. In some cases several fibrils became intertwined and formed rope-like cords. In other cases they became intertwined in a plane and formed a sparse reticulate sheet. Fibrils are very similar, in morphology, to the fibrils which build up the framework of laminated membranes of this layer.

The other kinds of organic materials, which constructed this layer are organic “spherules” and particles. “Spherules” are 0.5–12 $\mu$  in diameter and show a smooth surface texture. They are commonly found on the outermost surface of the laminated membranous layer and within the spaces between laminated membranes, arranged roughly layer by layer. They are normally spherical in outline, but occasionally deformed into the form of flattened disks owing to the fact that they have been put between laminated membranes. Also, they sometimes fuse with each other and become seemingly enlarged in size. When these “spherules” are examined histologically under the optical microscope, some of them, relatively large ones ranging 10–12 $\mu$  in diameter, can be discerned to be the remains of amoebocytes, as reported in detail in another paper (Suzuki and Uozumi, 1979). Organic particles are very small, averaging less than 0.1 $\mu$  in diameter, and found usually on all surfaces of the other organic materials of this layer. Especially they are remarkably found on the surface of laminated membranes and of “spherules”. From the histological and histochemical examination, the substance of the laminated membrane is very similar to that of the conchiolin. It may be said, though somewhat venturesome, that the laminated and conchiolin membranes may be homogeneous with each other in quality and texture, and the particles respectively accompanying both membranes may be the same, too. A comparison of the histological and histochemical data of the membranes, now in discussion, is presented in Table 1.

#### *Brown membranous layer*

The organic membrane-shell is made up of three different layers as stated above, but this shell appears, as a whole, to be a brown coloured shell in the macroscopic and optical

microscopic observations. That is due to the well-development of the brown membranous layer, a component of this shell. The brown membranous layer consists of a black-brown membrane extending only  $20\mu$  from  $5\mu$  in thickness. This membrane begins to form 6 days after the operation, and is almost completed in thickness and expansion on about 20 days. The mature brown membrane, past the long regenerated duration, extending 100 days,

**Figs. 5–6** Oblique fracture of the inner surface of the organic membrane-shell in early stage of shell regeneration. Brown membranous layer (a) is fully developed, and successively thin conchiolin sheet (b) bristling with numerous fibrils on its surface, is beginning to form.

**Fig. 7** Inner (growth) surface of the organic membrane-shell. Conchiolin sheet seems to be a membrane with numerous small pores. Note that such a pattern resembles that of the conchiolin of the nacreous layer, illustrated by Mutvei (1972, Pl. 1, Figs. c,d.)

**Fig. 8** Electron micrograph showing oblique fracture surface of the organic membrane-shell (left), developing at the periphery of the opening, and the inner surface of the normal shell (right). Organic membrane-shell is composed of four layers, outer brown membrane (d) and three inner conchiolin sheets with numerous organic particles (a, b and c). Note that organic particles increase in size, through aggregation.

**Figs. 9–10** Oblique fracture of the inner (growth) surface of the organic membrane-shell showing the sponge-work fibrils and particles attached on the fibrils. a: conchiolin sheet; b and c: sponge-work fibrils filling the spaces between conchiolin sheets. Note that the inner fibrous layers (Figs. 9-c and 10) show a sparse sponge-work in pattern and are studded with minute particles, while the outer (preforming) one (Fig. 9-b) is rather compact with dense aggregated bodies of particles.

**Fig. 11** Slightly oblique fracture of the inner surface of the organic membrane-shell. Aggregated bodies of particles develop on the network fibrils. Note that the aggregated bodies on the inner fibrous layer (a) relatively are smaller in size than those on the outer fibrous layer (b). c: Conchiolin sheet, sandwiched between inner and outer fibrous layers.

**Fig. 12** Highly magnified micrograph of sponge-work fibrils showing the stocking points of particles, junctions of fibrils.

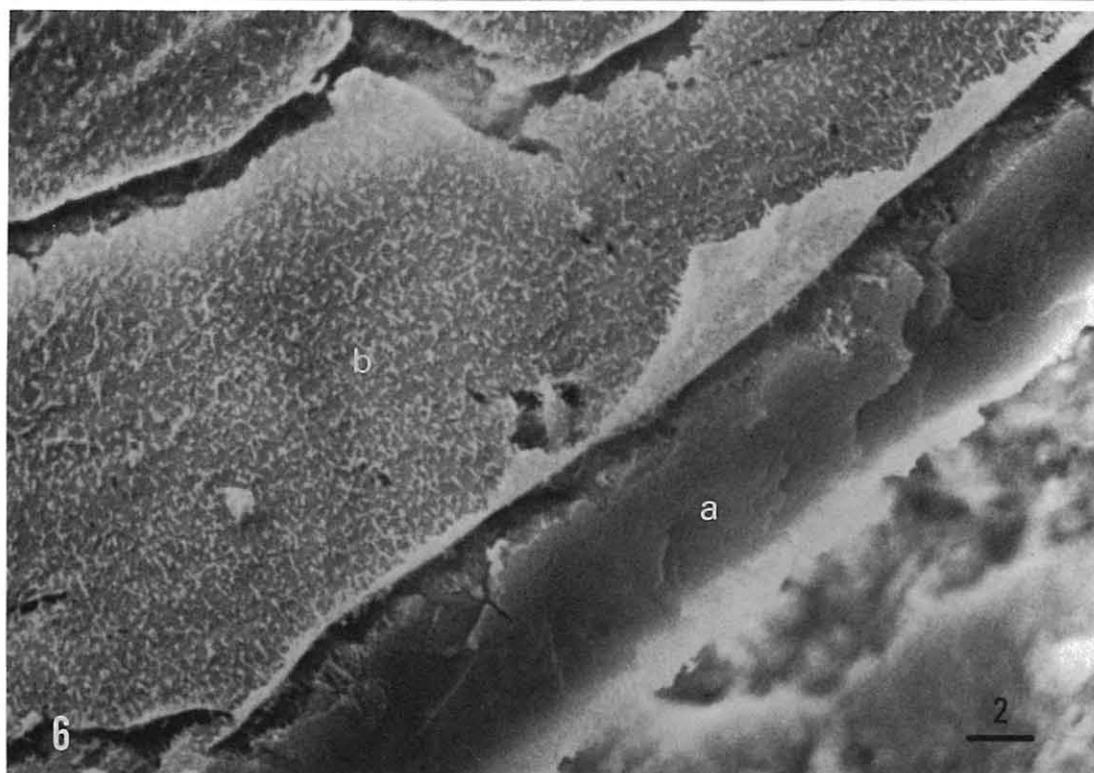
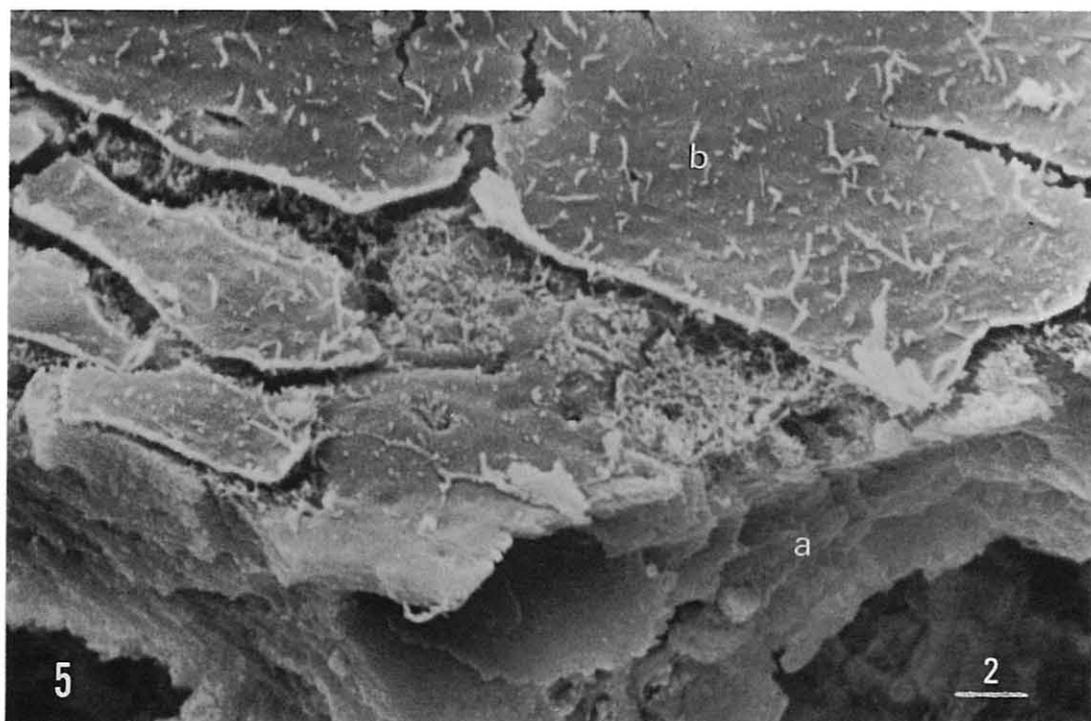
**Fig. 13** Secondary surface, the last-formed conchiolin sheet artificially peeled from the organic membrane-shell, revealing the mature aggregated bodies of particles, approximately  $2\mu$  in diameter. Note that they are not yet coated or embedded with some other organic materials.

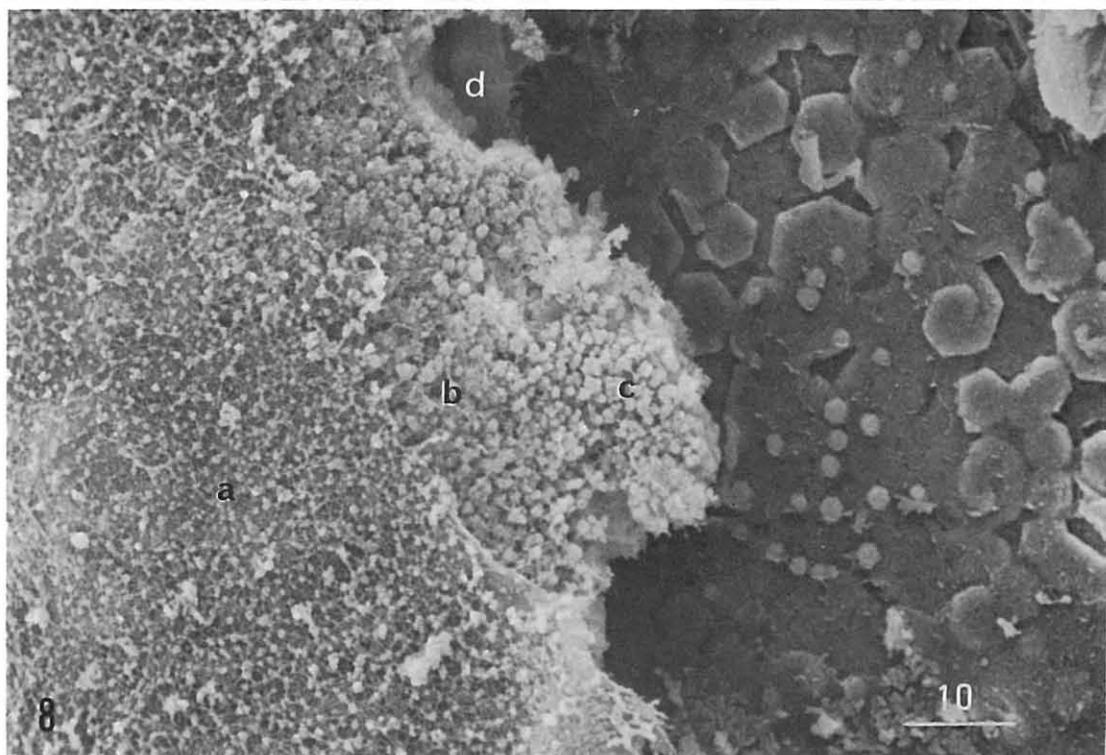
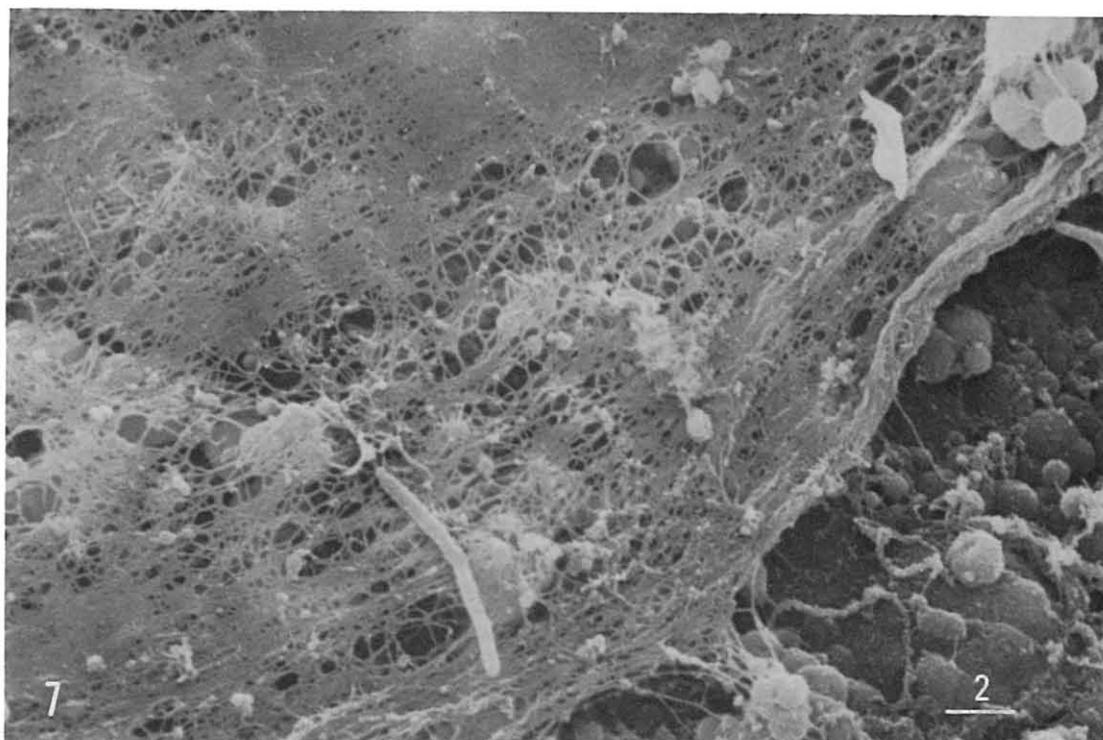
**Fig. 14** Inner (growth) surface of the organic membrane-shell showing the ill-developed fibrils and immature particles on the waved conchiolin sheet.

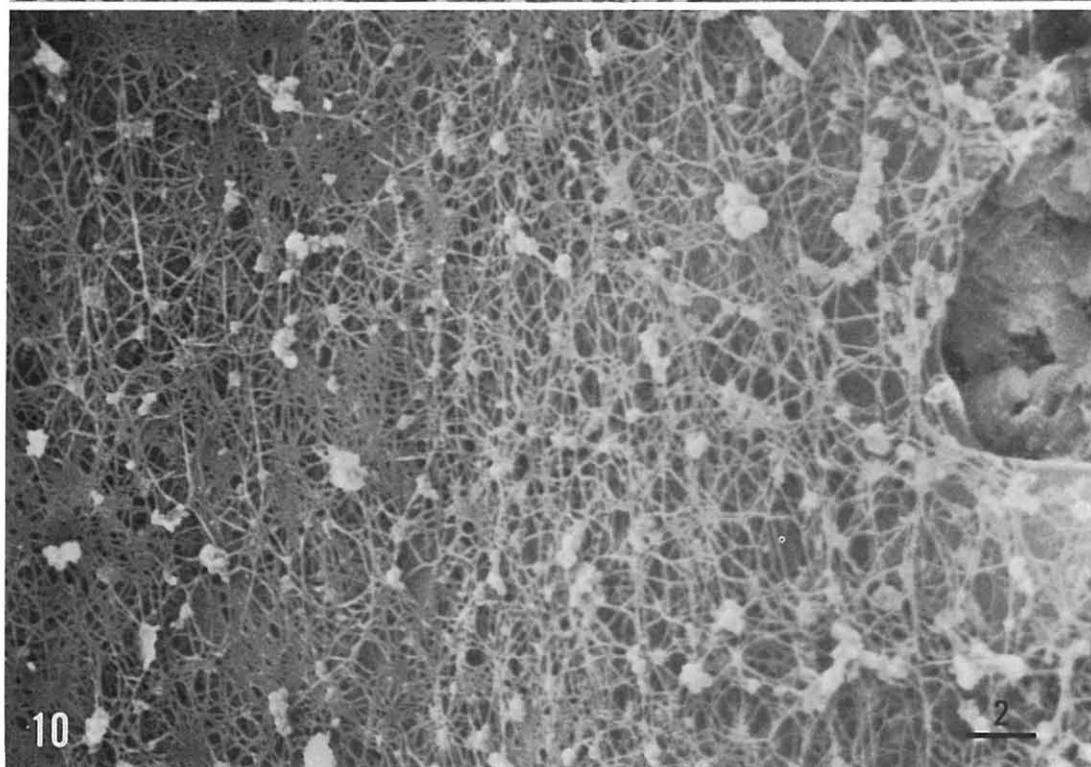
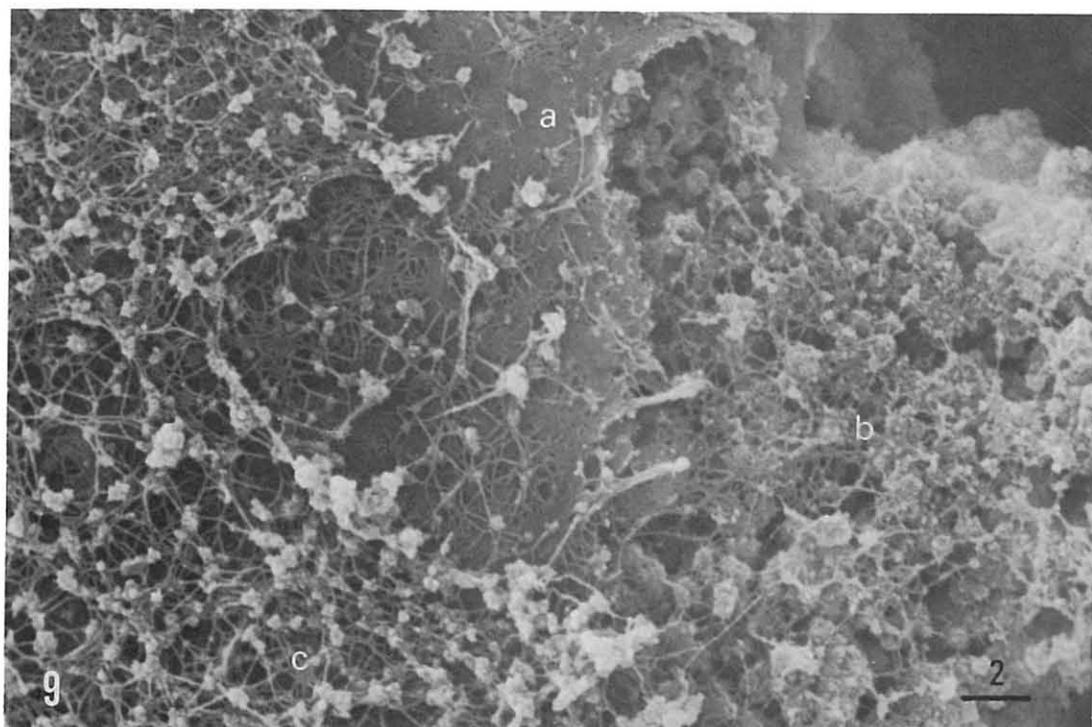
**Fig. 15** Oblique fracture of the inner (growth) surface of the organic membrane-shell showing the last-formed conchiolin sheet (a), enlarged aggregated bodies of particles (b), brown membrane (c) and laminated membranous layer (d). Note that fibrils lie between the aggregated bodies of particles.

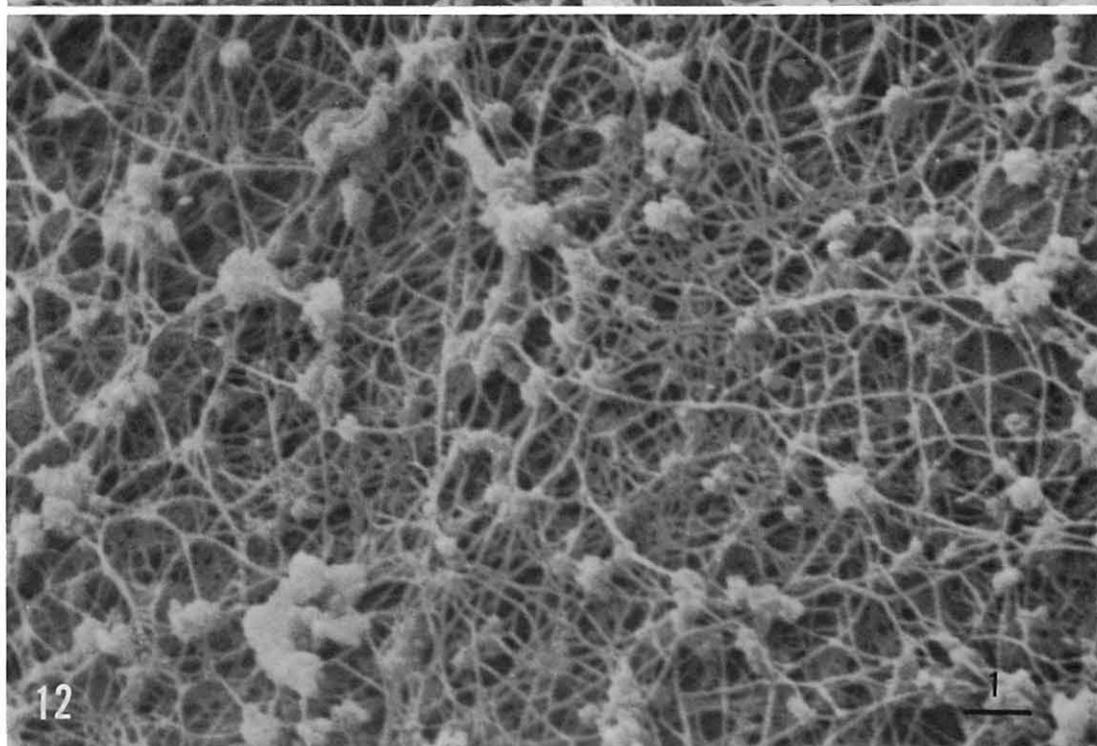
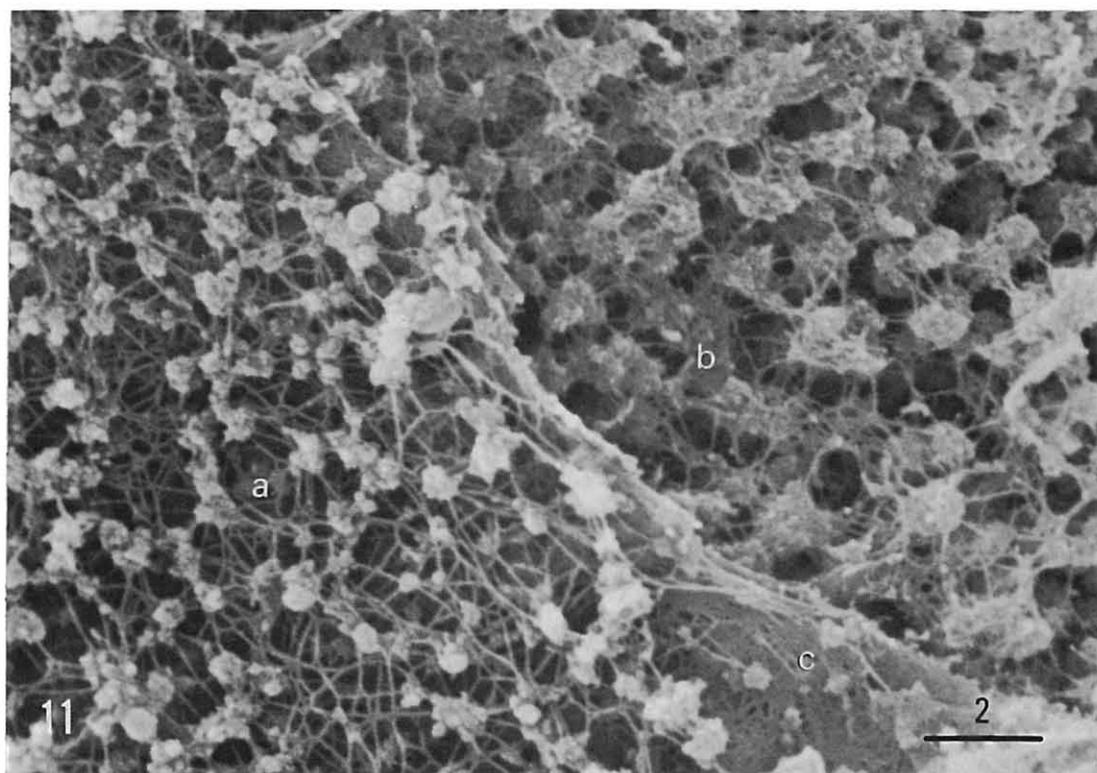
**Fig. 16** Immature particles on the network fibrils. Inner (growth) surface of the organic membrane-shell. Note that the network fibrils appear to rest directly on the layer of globular aggregated bodies of particles, but a conchiolin sheet lies, in fact, between the fibrils and the aggregated bodies of particles.

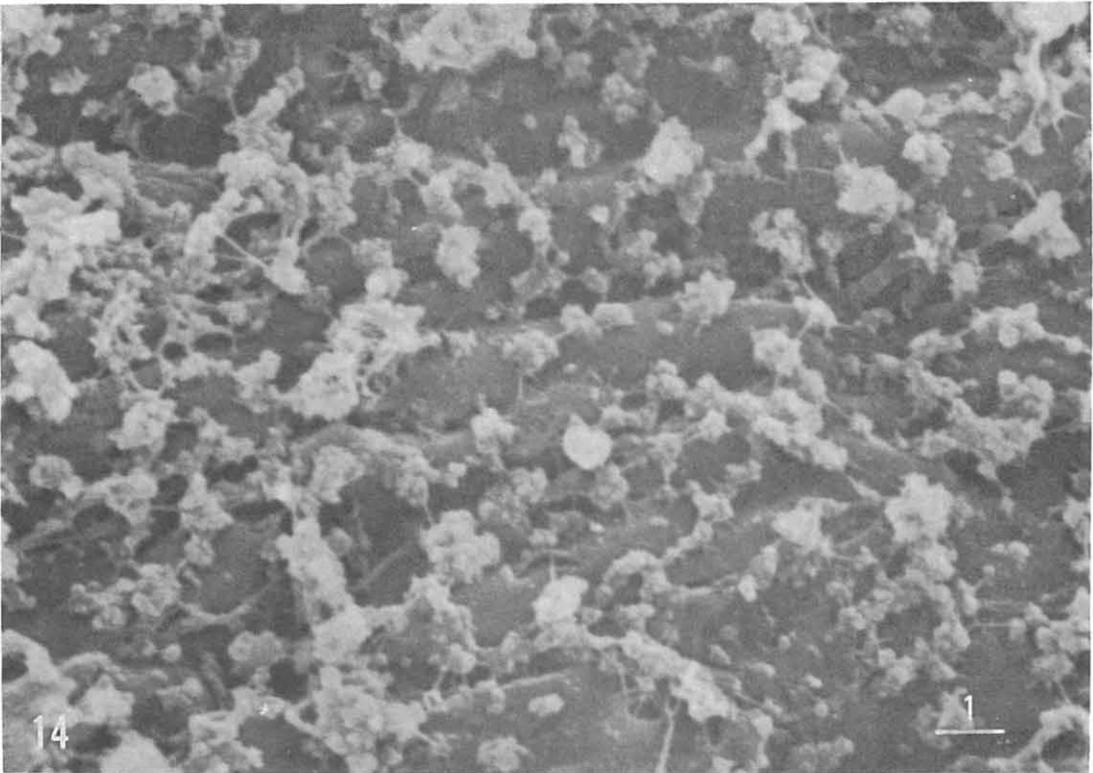
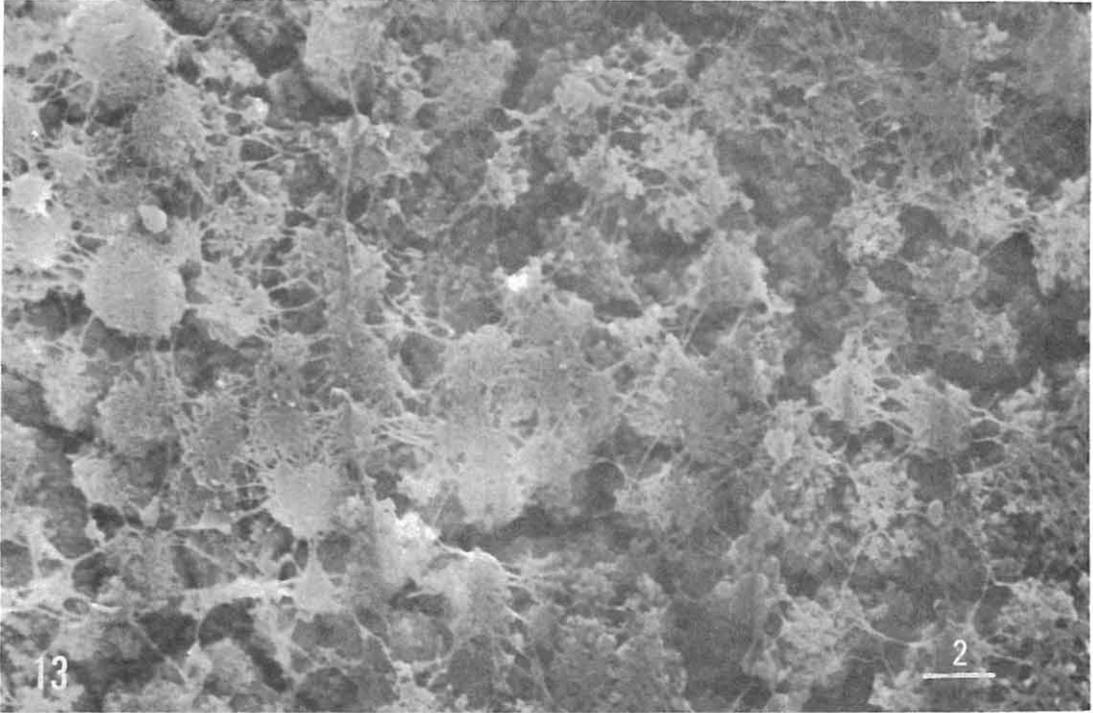
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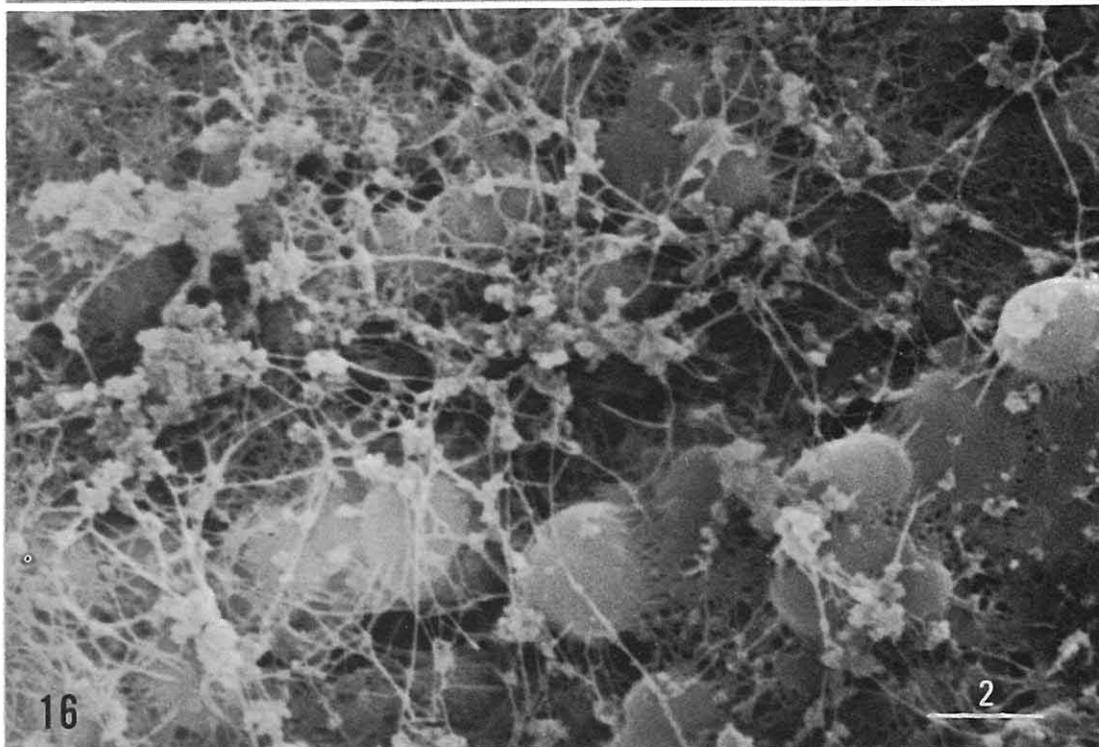
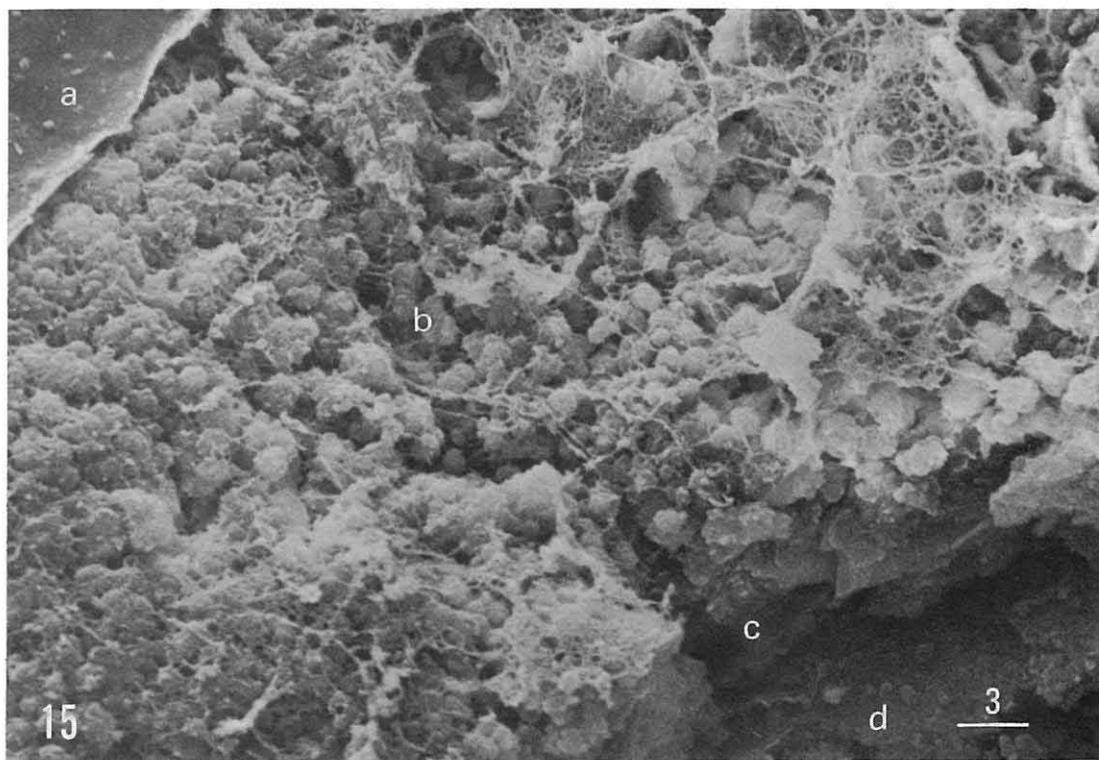












appears to be homogeneous and structureless in the fracture surface of cross section. On the contrary, the immature one, on day 23 after the operation, shows two different textures according to portions; granular and structureless. From the scanning electron microscopy, it is clear that the granular texture portion is composed of minute granules,  $0.1-0.2\mu$  in diameter. Moreover, it is noticed that the granular portions, now in question, can be observed to turn gradually into structureless portions within the same membrane. From the foregoing evidence, this membrane might be primarily composed of minute granules, stated above, and increase in thickness and expansion with further accumulation and fusion of granules. Finally this membrane is transformed with maturation into the membrane with a homogeneous and structureless texture. Meanwhile, mineral depositions or calcified grains can not be entirely found within this membrane. Very probably, this membrane seems to be not wholly associated with the mineralization of the organic membrane-shell. This membrane is very similar to the periostracum, outermost non-calcified layer of mussel shell, in the histological and histochemical examination.

#### *Conchiolin membranous layer*

Upon completion or near completion of the brown membrane, other membranes, fibrils and other particles are further deposited inside it (Figs. 5, 6). The conchiolin membranous layer is made mainly of these organic materials in the inner parts of the organic membrane-shell. In detail, the conchiolin membranous layer consists mainly of the laying down of a succession of organic membranes, each welded partly to the other (Fig. 8). And

**Table 1** Comparison of the histological and histochemical reactions of the organic matrices in the organic membrane-shell and normal shell of *Mytilus edulis*.

| Materials<br>Methods              | ORGANIC MEMBRANE-SHELL     |                        |                             | NORMAL SHELL |                       |                      |
|-----------------------------------|----------------------------|------------------------|-----------------------------|--------------|-----------------------|----------------------|
|                                   | Laminated Membranous Layer | Brown Membranous Layer | Conchiolin Membranous Layer | Periostracum | Outer Prismatic Layer | Inner Nacreous Layer |
| Haematoxylin-Eosin                | Purple                     | Red                    | Purple                      | Red          | Purple                | Purple               |
| Azan-Mallory                      | Reddish purple             | Red                    | Blue                        | Red          | Blue                  | Blue                 |
| Van Gieson                        | Red                        | Yellow                 | Red                         | Yellow       | Red                   | Red                  |
| Verhoeff                          | Dark purple                | Colorless              | Dark purple                 | Colorless    | Dark purple           | Dark purple          |
| Methyl green-Pyronine             | Red                        | Colorless              | Red                         | Colorless    | Red                   | Red                  |
| Periodic acid-Shiff               | +                          | -                      | +                           | -            | +                     | +                    |
| Metachromasia with Toluidine blue | +                          | -                      | +                           | -            | +                     | +                    |
| Ninhydrin-Shiff                   | +                          | ±                      | +                           | ±            | +                     | +                    |

fibrils, particles and mineral deposits are scatteringly found within the spaces between the above-mentioned membranes.

The membrane now in question is smooth in surface, but at its broken edges, the fibrils constituting this membrane can be observed sometimes as a network pattern as shown in Figure 7. This observation under the aid of the electron microscope indicates that the membrane is made up mainly of fibrils, about  $0.1\mu$  in diameter, which are embedded in and overlain by some kinds of amorphous materials. Such construction with a sparse reticular framework appearance is very similar to that of the interlamellar conchiolin membrane which is an organic matrix of calcified shell layer of molluscs, already reported by Uozumi and Iwata (1969). Also, this membrane closely resembles in ultrastructural appearance the laminated membrane, outer layer of the organic membrane-shell. Taking such electron microscopical appearance and histochemical features (Table 1) into consideration, the laminated membrane seems to be a conchiolin membrane itself in quality and texture.

Fibrils beyond  $0.1\mu$  in diameter,  $5-40\mu$  in length, develop in a highly porous sponge-work pattern within the spaces between conchiolin membranes (Figs. 9, 10). And particles attached on the fibrils, formed larger spherical bodies (spherules), by aggregation (Figs. 11, 12). An aggregated body became successively transformed, with the advance of calcification, into the following forms: flattened- and double-disks. During this course, each aggregated body became progressively mineralized until spherulitic structures finally resulted. This will be described later in more detail in connection with the calcification of regenerated shell. However, the nature of the particles has not yet been resolved at the present time, and much more definitive work is obviously need on the inorganic – organic chemistry as well as the ultrastructure of the particle.

#### Calcification in the organic membrane-shell

Mineralization in the regenerated shell is clearly observed from the outer morphological appearance about 30 days after the operation. Crystalline bodies having a double-disk form develop within the spaces between the conchiolin sheets in the inner layer of the organic membrane-shell and are made up of minute acicular crystals. These double-disk bodies are the first crystalline ones that crystals are identified with certainty on the appearance of the mineralized materials (usually in surface only). However, these bodies do not seem to represent the initial stages but relatively advanced stages in the calcification of the organic membrane-shell. Probably the first steps in mineralization occur simultaneously with the formation of the flattened-disk matrix or just before it, and calcium in many forms may be concentrated in varying degrees in some parts of the flattened-disk matrix. In this case, mineral deposits may be indistinguishable within the flattened-disk, below certain concentrations, particularly if they are not crystalline.

The careful observation of disk formation indicates that the flattened-disk is formed by enlargement in size and transformation in morphology of the spherules, and the spherule itself is made by the aggregation of particles. Particles, averaging  $0.1\mu$  in diameter, are irregular in form without any evidence of a gross surface structure in the scanning electron microscope examination (Fig. 14). They are found to be attached on the fibrils which are distributed within the spaces between the conchiolin sheets in the conchiolin membranous

layer (Figs. 11, 12, 16). They locate occasionally on the junctions of fibrils, aggregate with each other, and finally form the spherical aggregated bodies, up to  $2\mu$  in diameter (Figs. 13, 15). In a few cases particles are tied in a row on fibrils and form club shaped aggregates. These aggregates have a pustulous surface in their early stage, but with further growth, they acquire a smooth outer surface, since they are covered by or embedded in some kinds of amorphous organic materials. Thus, they form regular spherules (Figs. 17, 19). We have analyzed the spherules in the intermediate stage of maturation, with an electron probe microanalyser, and we only found a signal for a calcium when the spherule contained tiny, discrete crystallite. No significant signal was recorded from spherules which are small in size (below  $10\mu$  in diameter) and smooth in surface, as reported already by Uozumi and Ohta (1977). When spherules reached a larger size which exceeded  $10\mu$  in diameter (Fig. 18), they became transformed into the flattened-disks in form, and swelled up towards mantle epithelium, forming a semi-dome or spindle shape as illustrated in Figure 29. In this stage, they acquired a rough surface texture as shown in Figure 22. Namely, it is remarkable that the outer surface of flattened-disks is not only with pustulous, but is covered by a sparse fibrous sheet, framework of conchiolin sheet (Fig. 28). Thereafter, through rapid lateral growth, they reached about  $30\mu$  in diameter,  $5\mu$  in height, and underwent a gradual change in form: they became transformed from flattened-disks into double-disks, as shown in Figures 24, 26 and 29. In the earliest stage of double-disk formation, their interior spaces are rather porous, filled by sparse sponge-work fibrils with numerous particles attached to them (Fig. 27). Furthermore, the double-disks continued to grow larger in size, coalesce progressively with each other, reach large sizes which may exceed  $100\mu$  in diameter and  $30\mu$  in height, and break out on the covered fibrous conchiolin sheet, as does a pimple on the face. Also, the fibrous sheet covering on the growing disks is artificially broken in parts forming an undulated surface, during the procedures required for SEM studies, for example, drying the specimens (Fig. 23). Thus, the interior construction of the disks is exposed, and numerous acicular crystals developing with radial configuration are noted to appear remarkably at the marginal regions of basal parts of the double-disks (Figs. 30, 32, 34). The immature disk itself is not wholly mineralized, but some portions of it, for some purpose, become filled with mineral deposits, varying from scarcely-detectable deposits to completely crystalline structures (Figs. 35, 36). The interior space of the mature ones is filled up wholly with crystals,  $0.1-0.2\mu$  in diameter and variable in length (Fig. 31). Namely, in early stages of mineralization, mineral deposits in many forms may be developed in varying degrees in different disks and in different portions of one disk.

The authors have carefully observed the onset of mineralization at all stages of flattened- and double-disk formation and calcification, but were not able to directly detect the earliest stage of mineral deposition. This result may indicate that the interval between nucleation and growth of the relatively large crystals within disks is extremely short. During this interval, it is an easily observed phenomenon that numerous particles were found to attach on the sponge-work fibrils that inundate within the interior space of immature disks but they appear to decrease with maturation, along with a concomitant increase in crystal number. The foregoing evidence, although incomplete, may suggest that young, seed-like crystals are formed initially in close association with particles, in a random fashion, and

undergo secondary growth within the interior space of disks. In contrast to such a hypothesis based on the examinations of the partly-mineralized disks, there are few observations of the actual process of initial crystal formation within particles. However, the following evidence may be available in the crystal formation in association with particles: In the conchiolin membranous layer, particles are usually confined to the spherule and double-disk with maturation, but in the laminated membranous layer, particles are scatteringly found within the spaces between the laminated membranes. Namely, it seems likely that particles in the latter are still in an immature stage, and any crystalline materials usually can not be found within them. In special rare cases, it can be observed that tiny crystallite in irregular forms come out from particles as shown in Figure 25. This observation indicates that the crystallite within the particle, grows until it perforates the outer wall of a particle and becomes exposed to the space between the laminated membranes. In addition, it is also likely that a particle enables the sequestration and storage of calcium and carbon dioxide during the initial formation of calcite or aragonite crystals.

Within the double-disks, the crystals show occasionally irregular spherulitic habit and grow to relatively larger sizes without any preferred morphological orientation (Fig. 35). Judging from such bizarre crystal habit and their growth to a relatively large size, the crystals within the double-disks may not grow in the closely restricted components of the organic matrix of a disk, but grow rather freely within the relatively wide interior space of a disk. In the meantime, it is noted that crystals develop in different arrangement in different double-disks and in different portions of one disk: crystals develop commonly with radial configuration, but with torsional or parallel ones in some cases (Fig. 36). In general, the crystal growth, as deduced from crystals of comparative mature double-disks, appear to have occurred primarily by an increase in crystal length, and many growing double-disks begin to interlock with adjacent disks at the edges of growing acicular crystals (Figs. 38, 39), thus

**Figs. 17–18** Spherical bodies (=spherules) in which the aggregated bodies of particles have been coated by some organic materials. Inner (growth) surface of the organic membrane-shell. Note that spherules are rather smooth is surface compared with the aggregated bodies of particles. Some of the enlarged spherules (Fig. 18-arrow) may be beginning to concentrate calcium as pointed out by Uozumi and Ohta (1977).

**Figs. 19–20** Spherules in different stages of development: the larger they are in size, the earlier they have occurred. Inner (growth) surface of the organic membrane-shell.

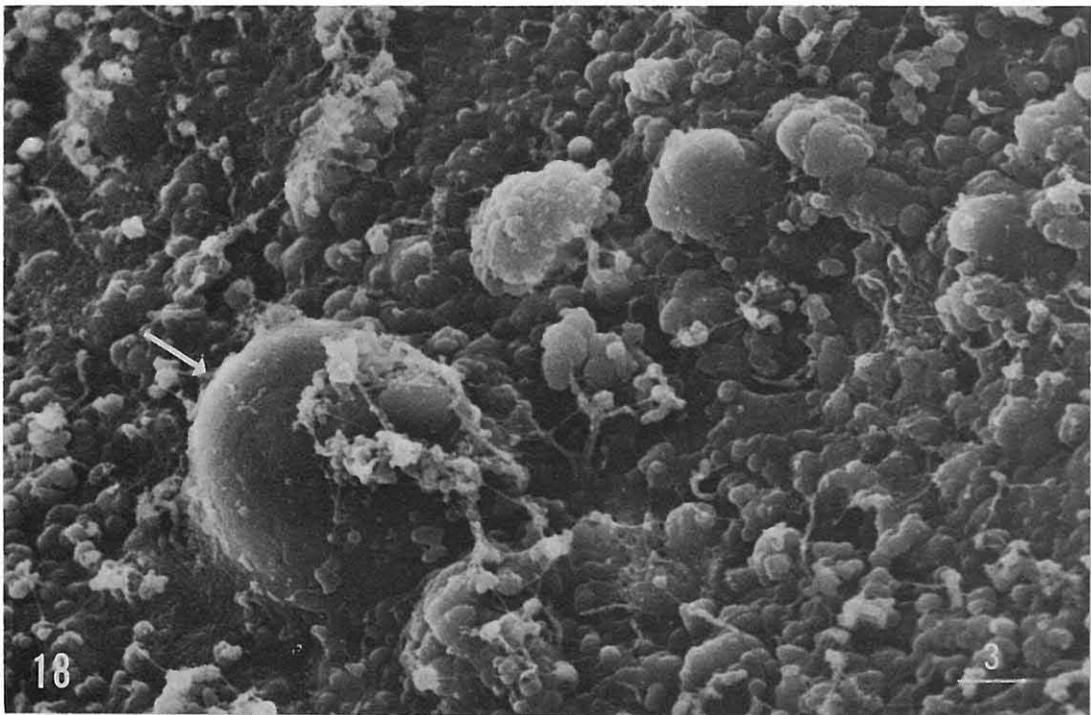
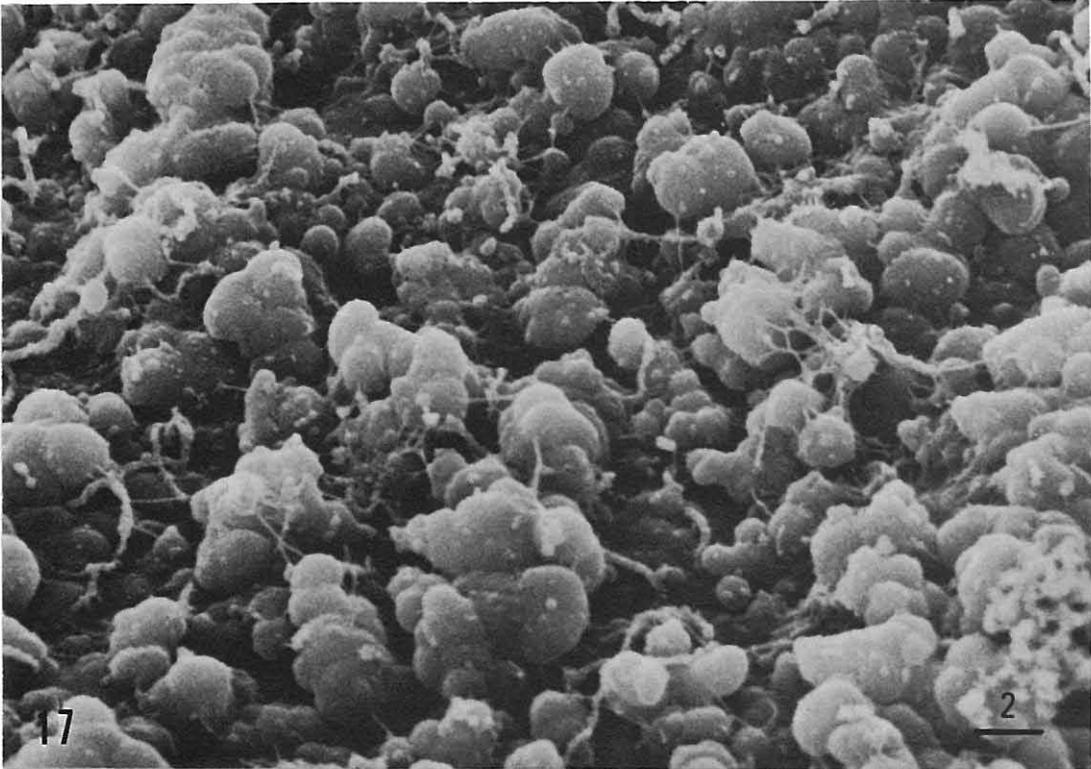
**Fig. 21** Higher magnification of spherules shown in Fig. 20 showing relatively smooth surface texture.

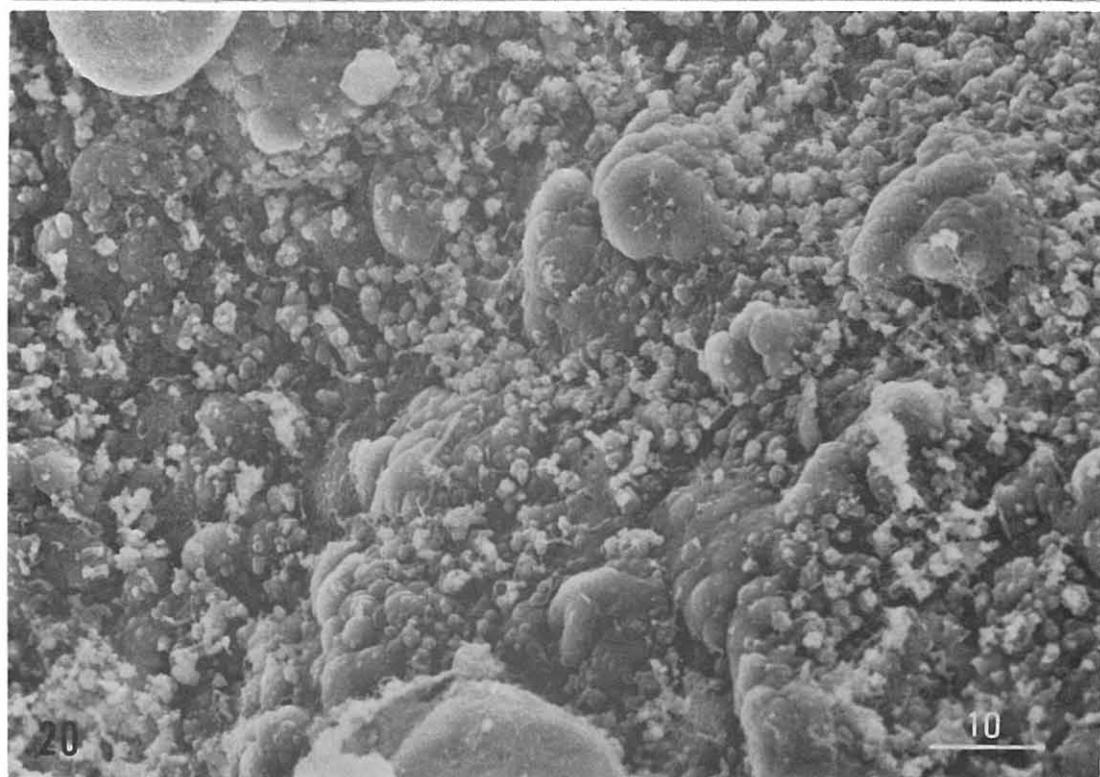
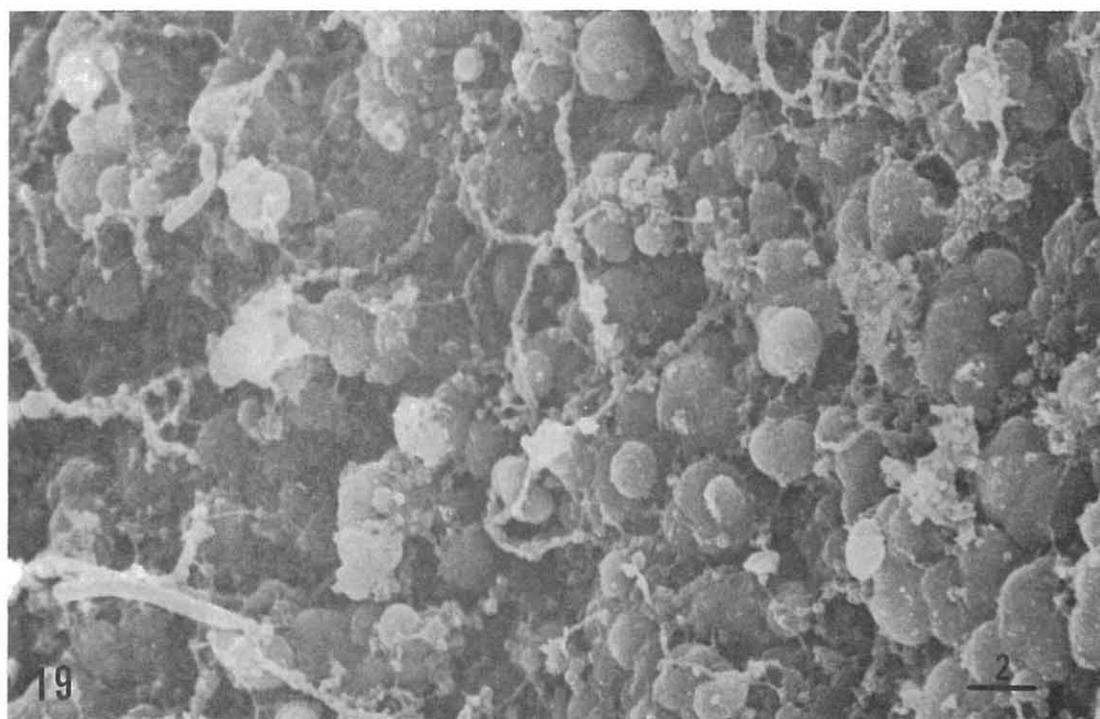
**Fig. 22** Enlarged spherules showing two different shapes: disk and dome shapes. Dome-shaped spherules may be gradually transformed into disk ones, with rapid lateral growth.

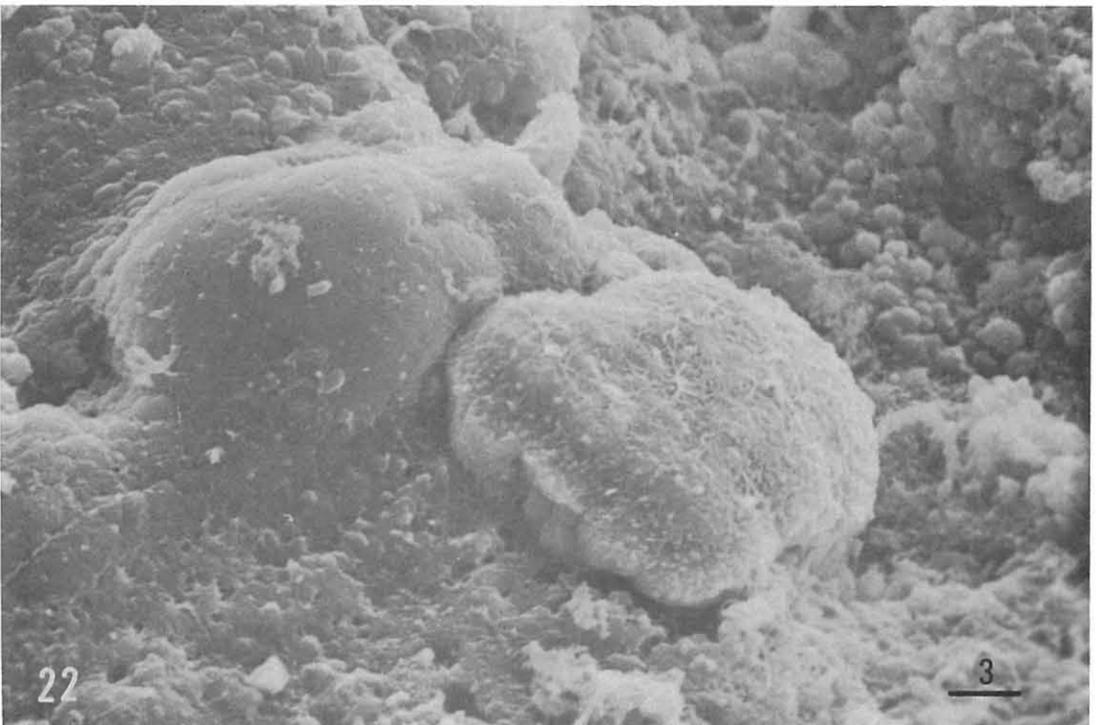
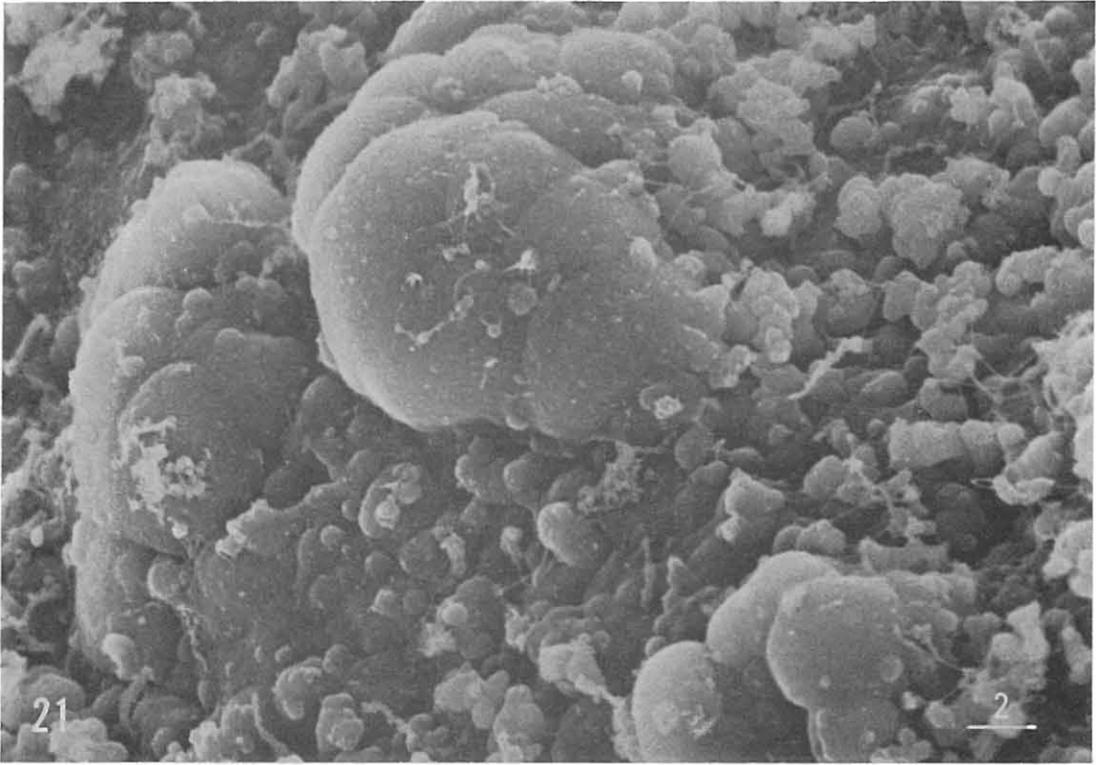
**Fig. 23** Double disks (b) covered by the post-formed conchiolin sheet (a). Inner (growth) surface of the organic membrane-shell. Conchiolin sheet has been artificially removed in parts.

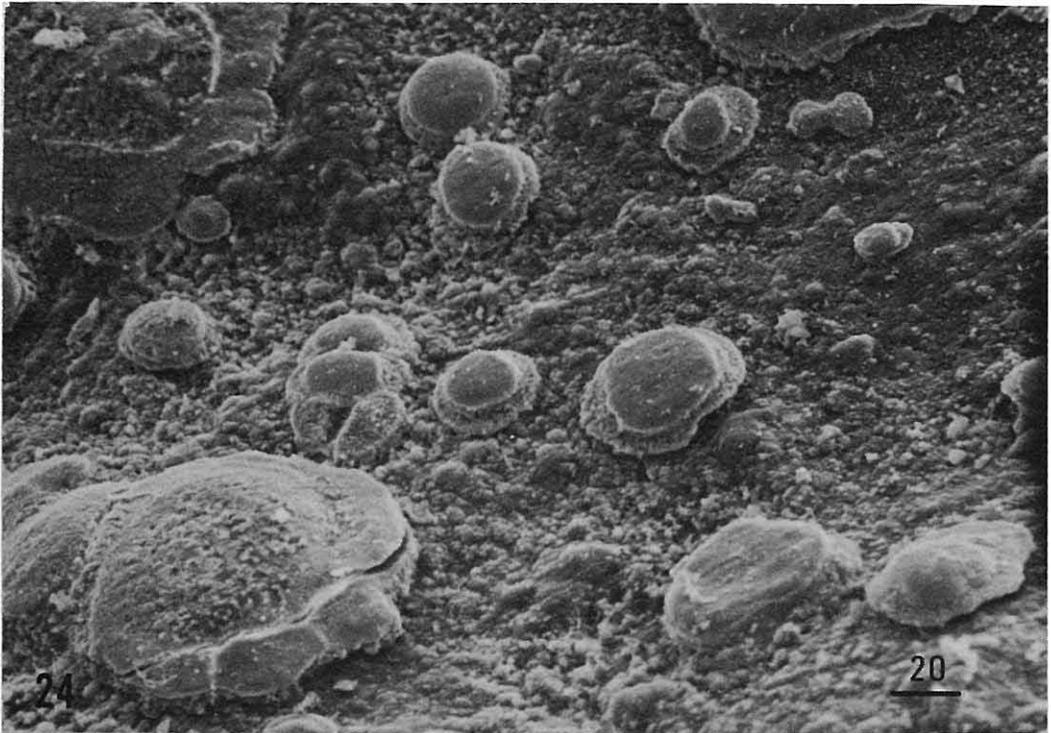
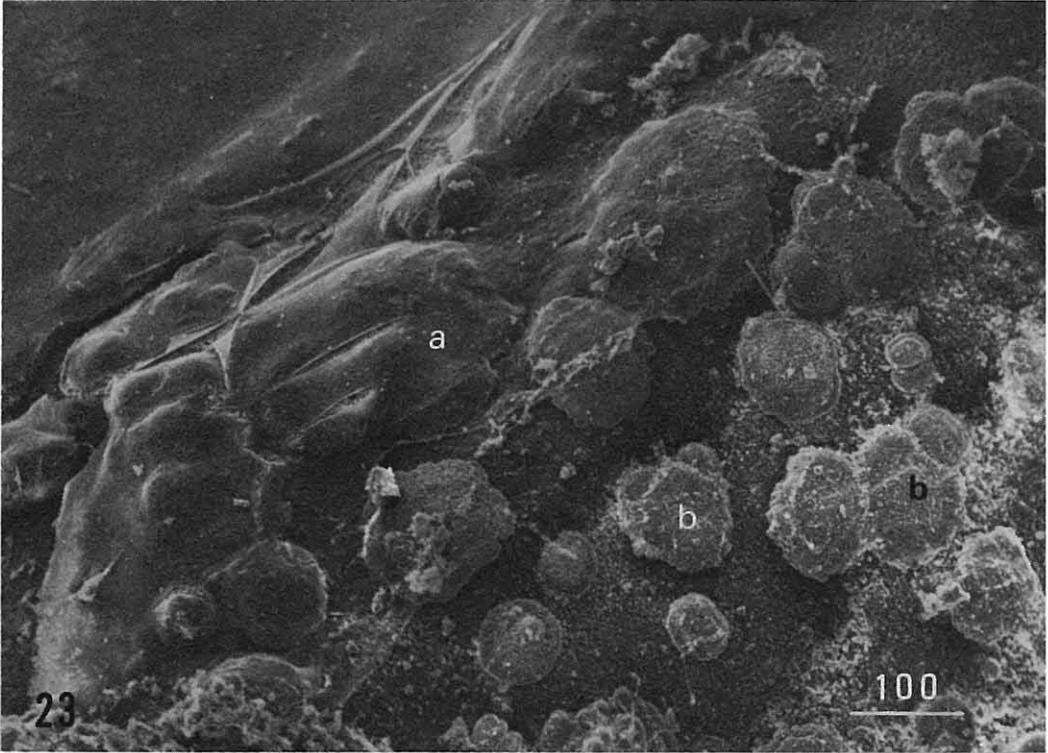
**Fig. 24** Double disks in different stages of development. Inner (growth) surface of the organic membrane-shell.

(Bar scale in microns.)









forming a structure similar to the so-called "complex crossed-lamellar structure", a typical architectural structure of molluscan shell layer (Taylor *et al.*, 1969). In some cases, crystals achieve their maximum length comparatively rapidly and then grow in width and thickness independently or by coalescence with adjacent ones, to their final form which can be best described as flattened anvil-like rods (Fig. 37). The anvil-like rod crystals show an arrangement pattern that is similar to "prismatic structure", architectural structure of the outer layer of mussel shell, as described by Travis (1968). The complex crossed-lamellar structure is usually constructed by acicular aragonite crystals, while the prismatic structure by rod calcite crystals. From the appearances of the crystals and their arrangement pattern, it may be said that there are changes in crystal habit, in crystal arrangement and even in crystal structure, in close association with the advance of mineralization. In this connection, there is other available evidence that in spite of the fact that the organic membrane-shell are created by the secretory activity of the confined mantle epithelium beneath the opening, mineral materials, deposited during the initial calcification inclose two types of crystals of calcium carbonate, aragonite and calcite. The authors will give here only a X-ray diffraction data of crystals within the mature organic membrane-shell (Table 2), and will report in detail in the near future.

Finally, shell regeneration is completed, by deposition of a smooth-surfaced mineralized, sheet-like nacreous layer, inside the mineral depositions with the above-mentioned bizarre habits.

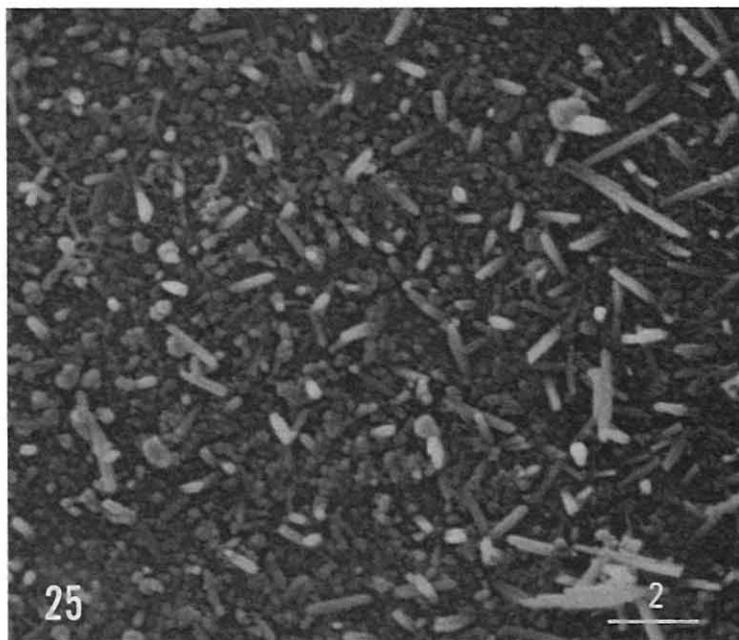
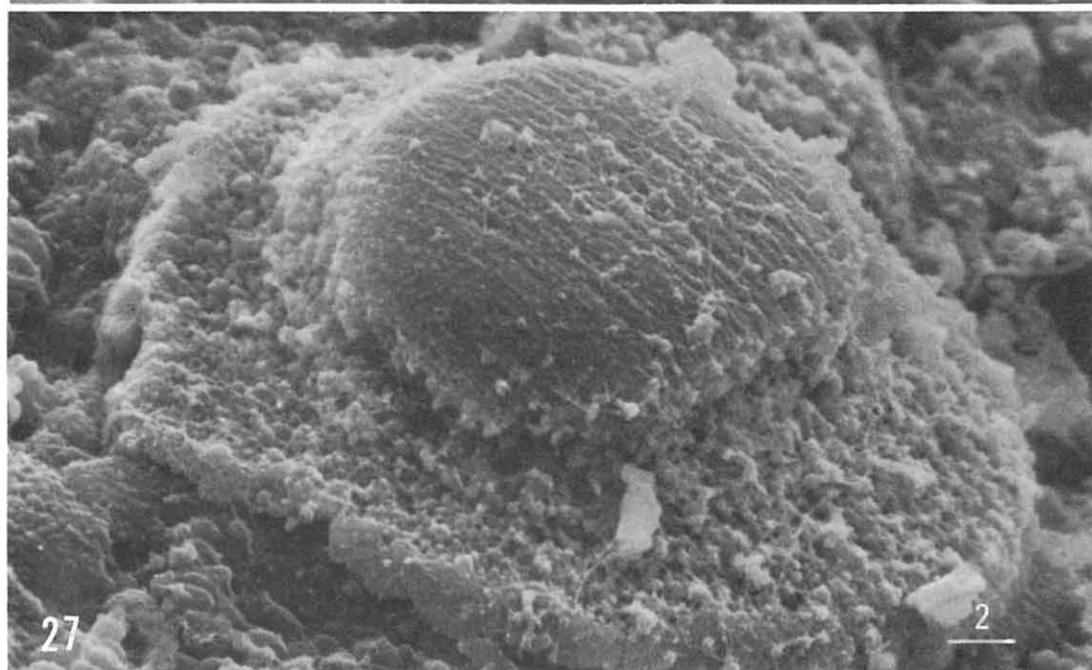
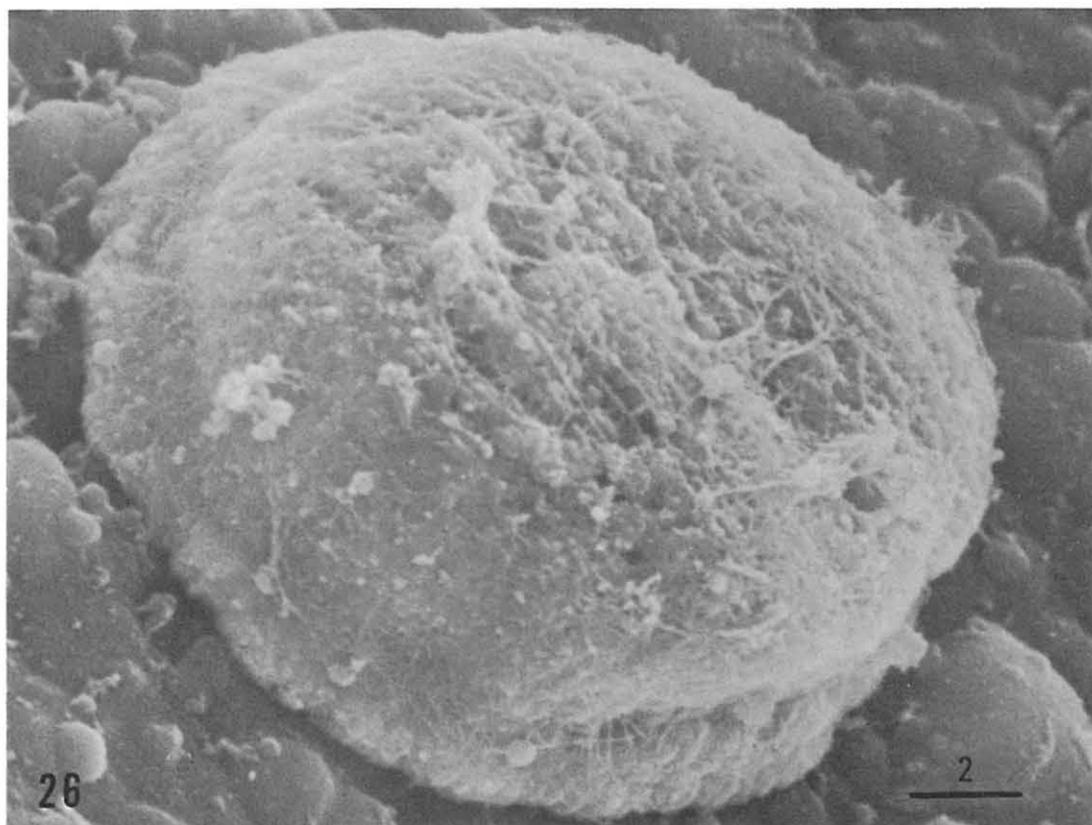
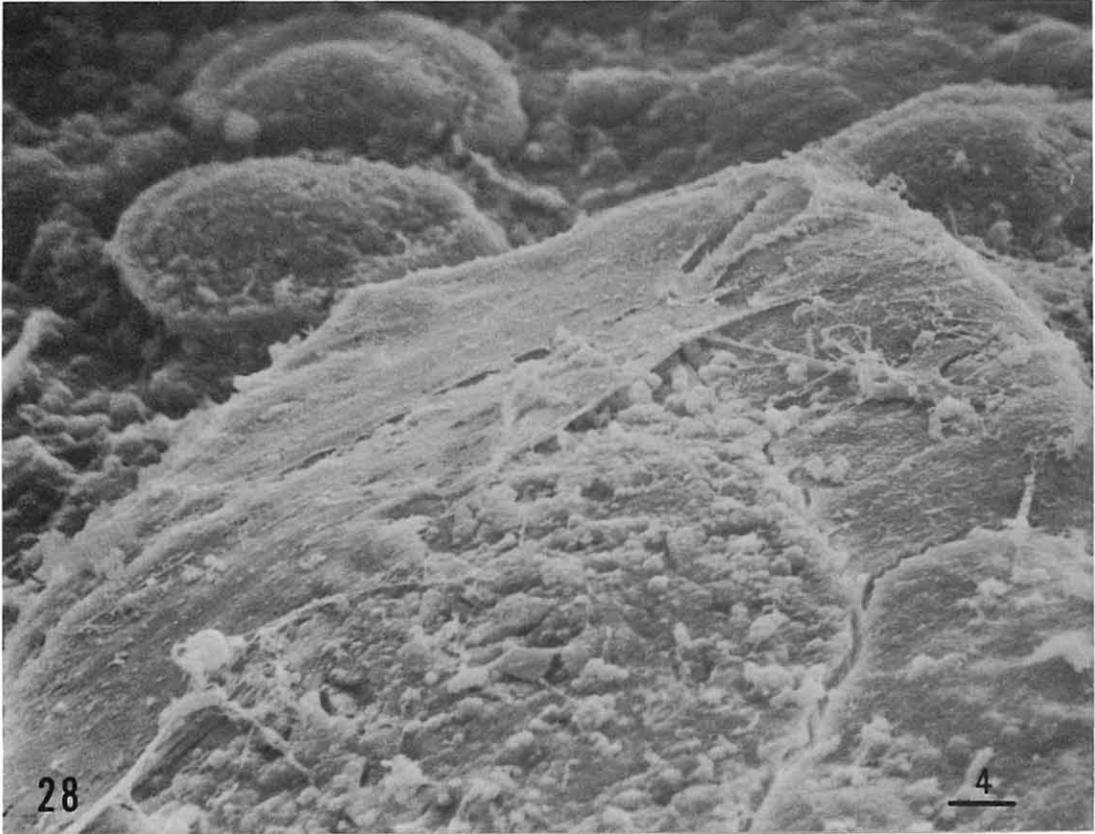


Fig. 25 Crystallites coming out from each particles, presented within the laminated membranous layer in the organic membrane-shell. (Bar scale in microns.)

Table 2 X-ray diffraction measurements

| Calcite<br>ASTM (5-586) |     |      | Aragonite<br>ASTM (5-453) |     |         | Organic<br>membrane-shell |     |
|-------------------------|-----|------|---------------------------|-----|---------|---------------------------|-----|
| d(Å)                    | I   | hkl  | d(Å)                      | I   | hkl     | d(Å)                      | I   |
| 3.86                    | 12  | 102  | 4.212                     | 2   | 110     | 3.85                      | 9   |
|                         |     |      | 3.396                     | 100 | 111     | 3.40                      | 36  |
|                         |     |      | 3.273                     | 52  | 021     | 3.28                      | 21  |
| 3.035                   | 100 | 104  |                           |     |         | 3.04                      | 100 |
| 2.845                   | 3   | 006  | 2.871                     | 4   | 002     | 2.87                      | 4   |
|                         |     |      | 2.730                     | 9   | 121     |                           |     |
|                         |     |      | 2.700                     | 46  | 012     | 2.70                      | 32  |
| 2.495                   | 14  | 110  | 2.481                     | 33  | 200     | 2.49                      | 34  |
|                         |     |      | 2.409                     | 14  | 031     | 2.41                      | 5   |
|                         |     |      | 2.372                     | 38  | 112     | 2.38                      | 22  |
|                         |     |      | 2.341                     | 31  | 130     | 2.34                      | 14  |
|                         |     |      | 2.328                     | 6   | 022     |                           |     |
| 2.285                   | 18  | 113  |                           |     |         | 2.28                      | 23  |
|                         |     |      | 2.188                     | 11  | 211     | 2.19                      | 5   |
|                         |     |      | 2.106                     | 23  | 220     | 2.10                      | 14  |
| 2.095                   | 18  | 202  |                           |     |         | 2.09                      | 23  |
|                         |     |      | 1.977                     | 65  | 221     | 1.980                     | 27  |
| 1.927                   | 5   | 204  |                           |     |         | 1.924                     | 9   |
| 1.913                   | 17  | 108  |                           |     |         | 1.913                     | 17  |
|                         |     |      | 1.882                     | 32  | 041     |                           |     |
| 1.875                   | 17  | 116  | 1.877                     | 25  | 202     | 1.875                     | 31  |
|                         |     |      | 1.814                     | 23  | 132,230 | 1.816                     | 13  |
|                         |     |      | 1.759                     | 4   | 141     |                           |     |
|                         |     |      | 1.742                     | 25  | 113     | 1.745                     | 16  |
|                         |     |      | 1.728                     | 15  | 231     | 1.726                     | 11  |
|                         |     |      | 1.698                     | 3   | 222     | 1.700                     | 1   |
| 1.626                   | 4   | 211  |                           |     |         | 1.625                     | 5   |
| 1.604                   | 8   | 212  |                           |     |         | 1.605                     | 10  |
| 1.587                   | 2   | 1010 |                           |     |         |                           |     |
|                         |     |      | 1.557                     | 4   | 311     | 1.559                     | 2   |
|                         |     |      | 1.535                     | 2   | 232     |                           |     |
| 1.525                   | 5   | 214  |                           |     |         | 1.524                     | 6   |
| 1.518                   | 4   | 208  |                           |     |         |                           |     |
| 1.510                   | 3   | 119  |                           |     |         |                           |     |
|                         |     |      | 1.499                     | 4   | 241     | 1.500                     | 3   |
| 1.473                   | 2   | 215  | 1.475                     | 3   | 321     | 1.474                     | 4   |
|                         |     |      | 1.466                     | 5   | 151     |                           |     |
| 1.440                   | 5   | 300  |                           |     |         | 1.438                     | 6   |
| 1.422                   | 3   | 0012 |                           |     |         |                           |     |
|                         |     |      | 1.411                     | 5   | 312     |                           |     |
|                         |     |      | 1.404                     | 3   | 330     | 1.405                     | 10  |
|                         |     |      | 1.365                     | 3   | 242,331 |                           |     |
|                         |     |      | 1.358                     | 3   | 114     | 1.360                     | 3   |
| 1.356                   | 1   | 217  |                           |     |         | 1.353                     | 2   |

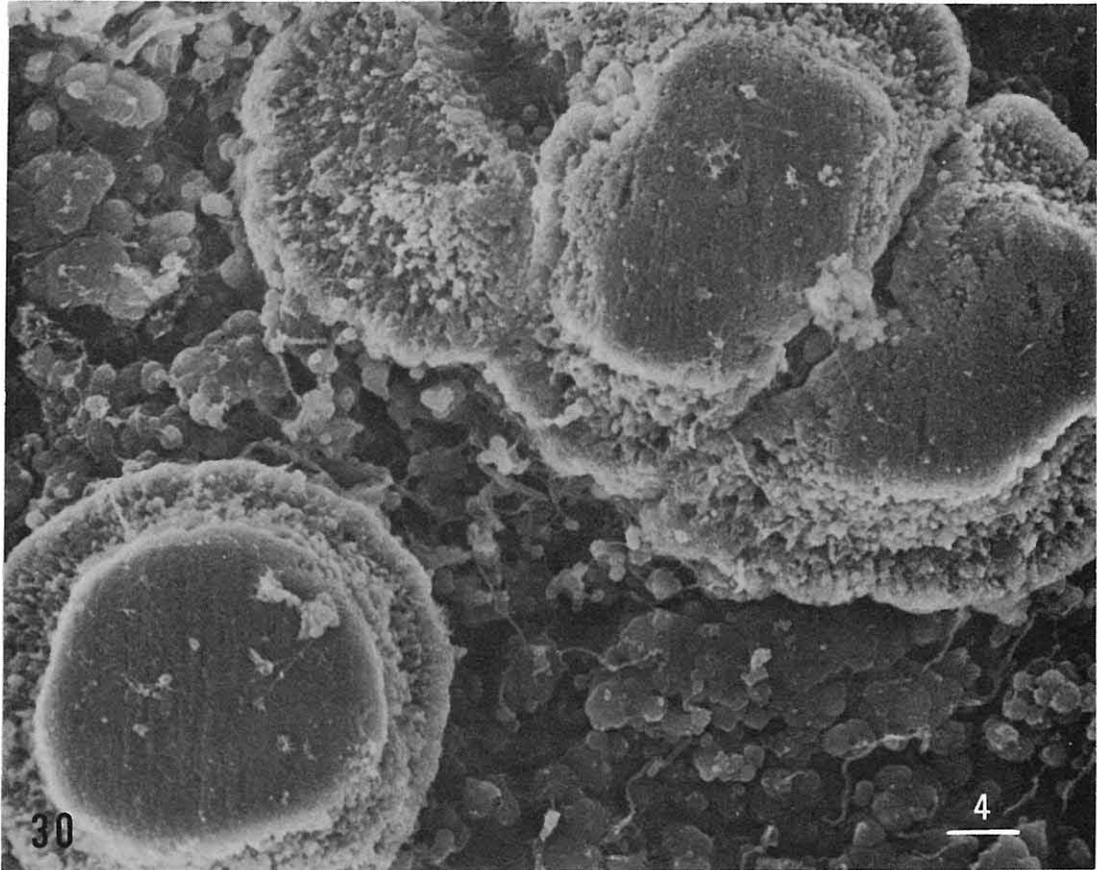
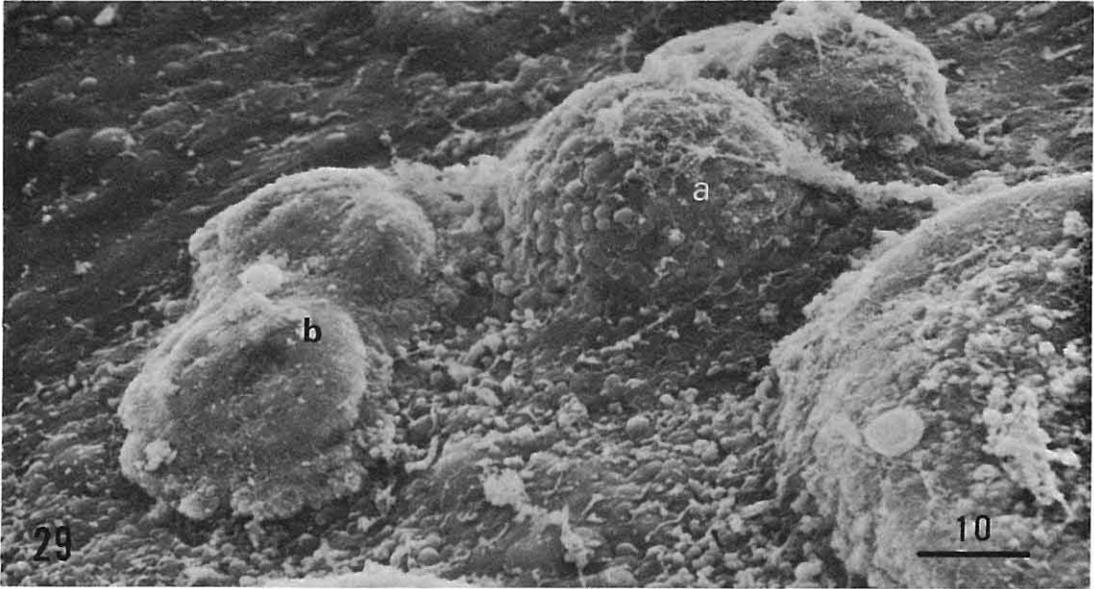




**Figs. 26–28** High magnification micrographs of double-disks. Note that the surface of double-disks (Figs. 26, 28) are covered with the post-formed conchiolin sheet and numerous fibrils, while the marginal region of the basal part of the disk in Fig. 27 shows a pustulous surface without any covering sheet.

**Fig. 29** An immature dome-shaped spherule (a) and a further advanced one (b) which is beginning to be transformed into the form of a double-disk. Inner (growth) surface of the organic membrane-shell.

**Fig. 30** A double-disk and a fused body of three double-disks on the inner surface of the organic membrane-shell. Note that parallel striae developing on the surface of upper disks may not be intimately associated with the interior structure of disks, although a clear understanding of the meaning of such parallel orientation awaits further studies. (Bar scale in microns.)



## Discussion

## Formation of the organic membrane-shell

The organic membrane-shell regenerated in *Mytilus* is composed of three different layers: laminated, brown, and conchiolin membranous layers. The organic substance of the first and the third can be considered homologous to conchiolin, the organic matrix of the mineralized layer of molluscan shell. While the organic substance of the second may be very probably the periostracum itself, it is not in close association with the mineralization of the shell.

Tsujii (1960), Kawaguti and Ikemoto (1962), and Meenakshi *et al.* (1973) have respectively reported that the regenerated shell in bivalves is composed of only one organic membranous layer, made of the same substance as that of periostracum. Also, Wagge (1951) and Abolinš-Krogis (1968) have described respectively that the organic membrane of the regenerated shell in land snails, is conchiolin sheet itself. Their opinions have not always coincided with each other in regard to the organic substance of the regenerated shell, but agree with each other in the point that the organic membrane-shell is composed of a single layer. In contrast to a single layer, it has been reported that the organic membrane-shell is composed of three different layers in *Anodonta* (Beedham, 1965) and of two different layers in *Lymnaea* (Timmermans, 1973). Especially, it is noticed that in Beedham's report, the formation of the regenerated shell begins from the deposition of the conchiolin-like sheet, following with the deposition of a periostracum-like sheet. Such a regeneration process is very similar, even though the experimental animals differ, to that in *Mytilus* which the present studies have clearly indicated. Beedham's "laminae of inner and outer layer type conchiolins" and "periostracal-like conchiolin" may be considered to correspond successively with "laminated membrane" and "brown membrane" in this paper.

The reason for such a discrepancy concerning the number of constituent layers, mono- or multi-layers, may very well be that different stages of regenerated shell formation, under different procedure of experimental methods, were studied with special emphasis.

The sequence by which the organic membrane-shell is formed may be summarized as follows:

- 1) During the relative short period after the operation, the secretory activity of the mantle epithelium is still in a normal state and will be able to continuously produce the organic matrix, which may be conchiolin itself or very similar to it and may be associated with the mineral deposition.

The laminated membranous layer, the outer layer of the organic membrane-shell, is the first deposition in early stages of regeneration, under the normal state of the secretory activity, as stated above. In these stages of regeneration, amoebocytes, ranging 10–12 $\mu$  in diameter, are found in large numbers within the laminated membranous layer. As have been pointed out previously by some authors (Takatsuki, 1934; Wagge, 1951; Dunuchie, 1963; Beedham, 1965), they probably play a restricted secretory role in the earliest stages of regeneration only. And it seems that the major portion of the regeneration process is carried out by the outer mantle epithelium. In this regard, much further work is needed to establish

the degree and route in which amoebocytes play the secretory role in the formation and calcification of the organic membrane-shell.

2) Sequentially, since the open area must be sealed off quickly and firmly from the external environment, the rigid protective membrane may be formed across the inside of the opening, as pointed out previously by the authors (1978). The membrane component which is available to this purpose, may not be homologous to conchiolin but rather to periostracum. Such a membrane is called "brown membrane" in this paper, and it is not surprising that this membrane component may be very similar to that of periostracum from the histological and histochemical examinations.

3) After the formation of the protective membrane, the secretory activity of the mantle epithelium will turn back gradually in normal, and again produce the organic matrix, precursor to the mineralization of a new shell. Such a matrix may be a conchiolin matrix itself or very similar to it. The conchiolin membranous layer, named in this paper, is composed of organic deposits and inorganic materials in this stage of shell regeneration. Finally this layer forms the mineralized sheet-like nacreous layer.

If the above-mentioned assumption concerning the formation of regenerated shell is correct, it is likely that both periostracum-like and conchiolin-like matrix can be produced, according to physiological requirement, by slight differences of the secretory activity of the mantle epithelium.

Beedham (1965) has reported that the epithelium of the outer surface of the mantle in *Anodonta* can secrete, as well as nacre, material which closely resembles that of the periostracum and outer (prismatic) layer. The present studies on shell regeneration in marine bivalve clearly agree with Beedham's observations on shell regeneration in fresh water bivalve.

Apart from the organic matrix, the problems of mineral deposits are present in shell regeneration. Mineral deposits are scatteringly observable within the partly-mineralized

Fig. 31 Fully-developing double-disk composing of clusters of acicular crystals, which take on a radial arrangements, producing spherulite-like forms.

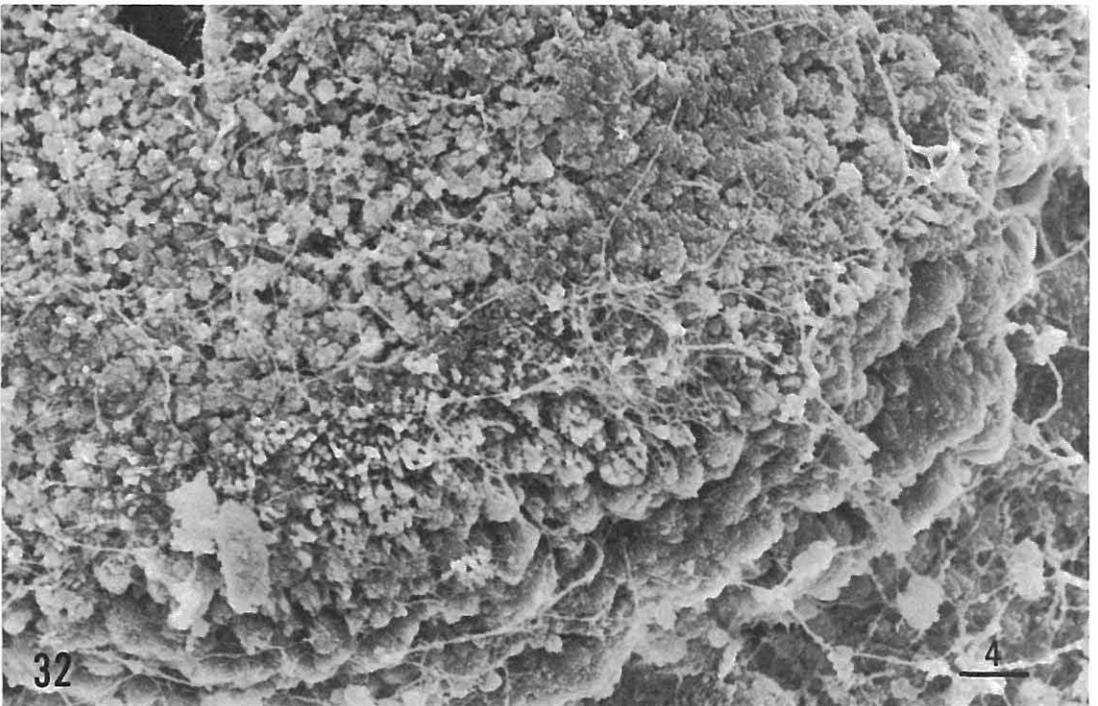
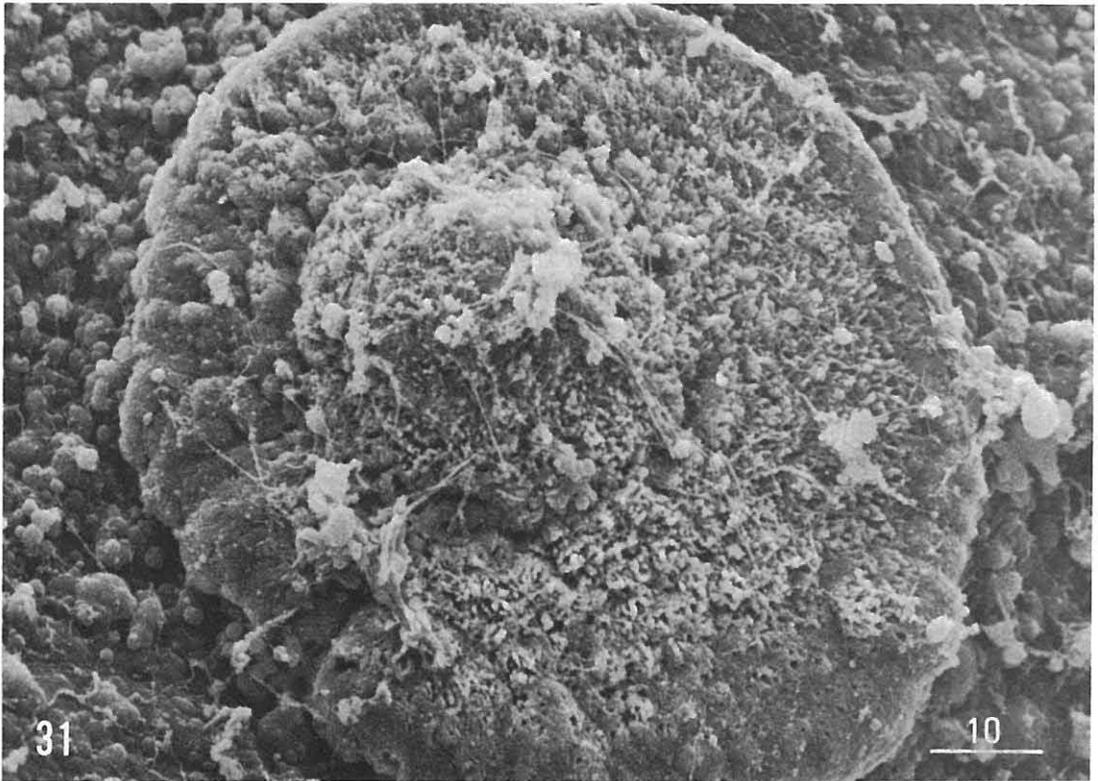
Fig. 32 High magnification micrograph of a fully-developing double-disk, Note that the marginal space of the double-disk has been filled up completely with clusters of acicular crystals.

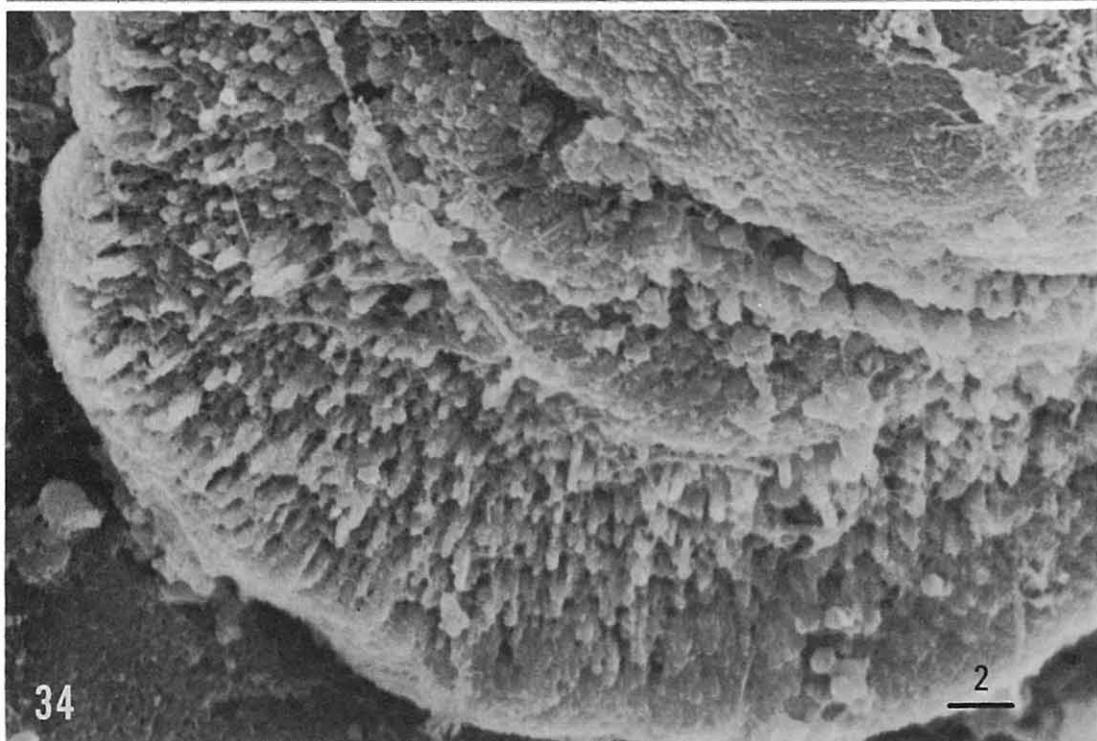
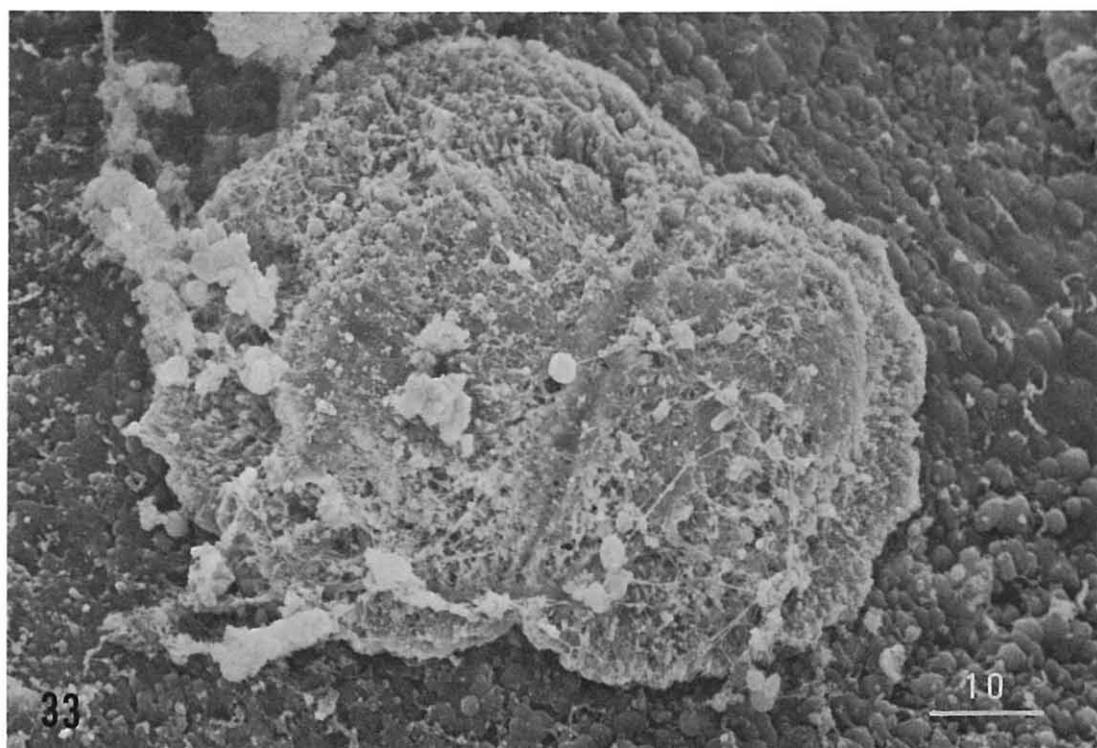
Fig. 33 Coalescent body of two double-disks composed of numerous acicular crystals, which are radial in arrangement.

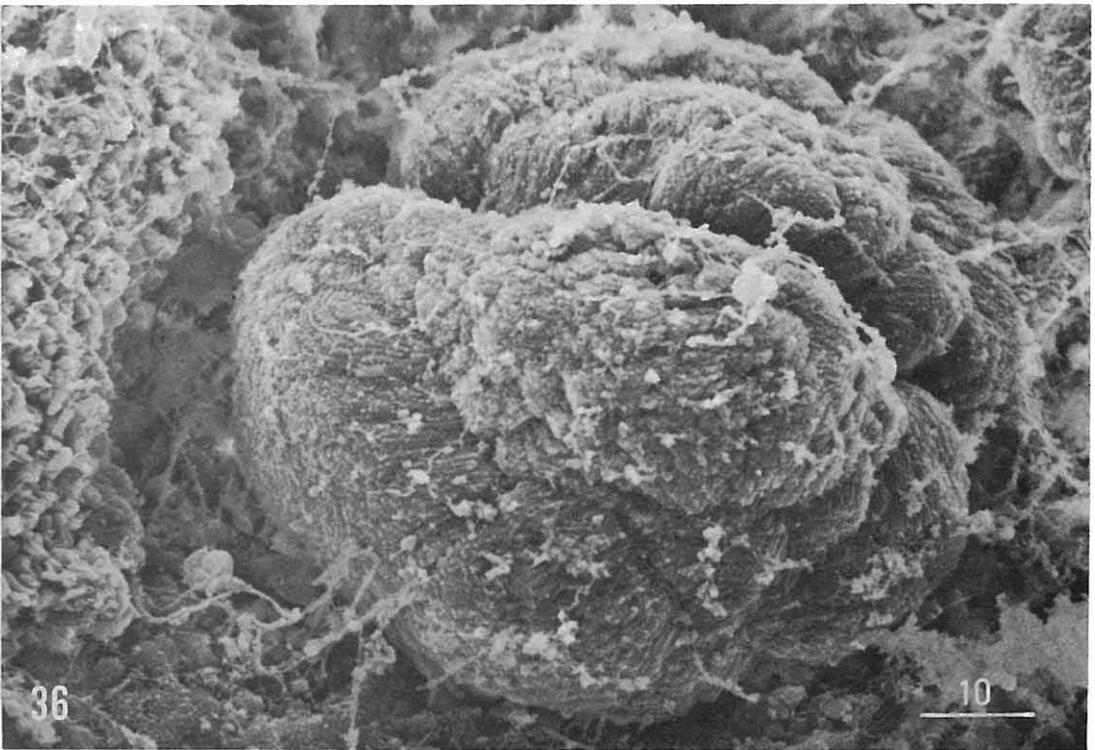
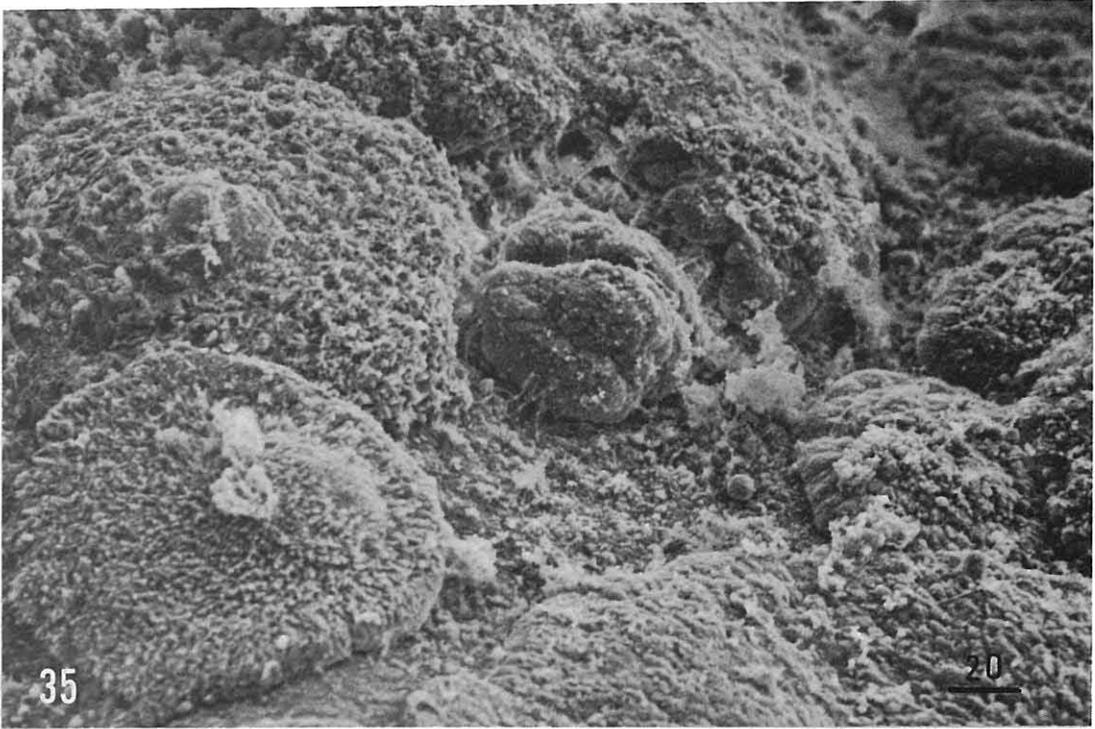
Fig. 34 Marginal region of a fully-developing double-disk occupied with relatively large acicular crystals. Note that mineralization takes place from circumference inwards.

Fig. 35 Fully-developing double-disks in the inner (growth) surface of the organic membrane-shell. Note that the interior space of the disks have been completely filled up with numerous acicular crystals.

Fig. 36 Higher magnification of a double-disk shown in Fig. 35. Note that the interior space of the double-disk is filled up with clusters of minute acicular crystals (approximately  $0.2\mu$  in diameter).  
(Bar scale in microns.)

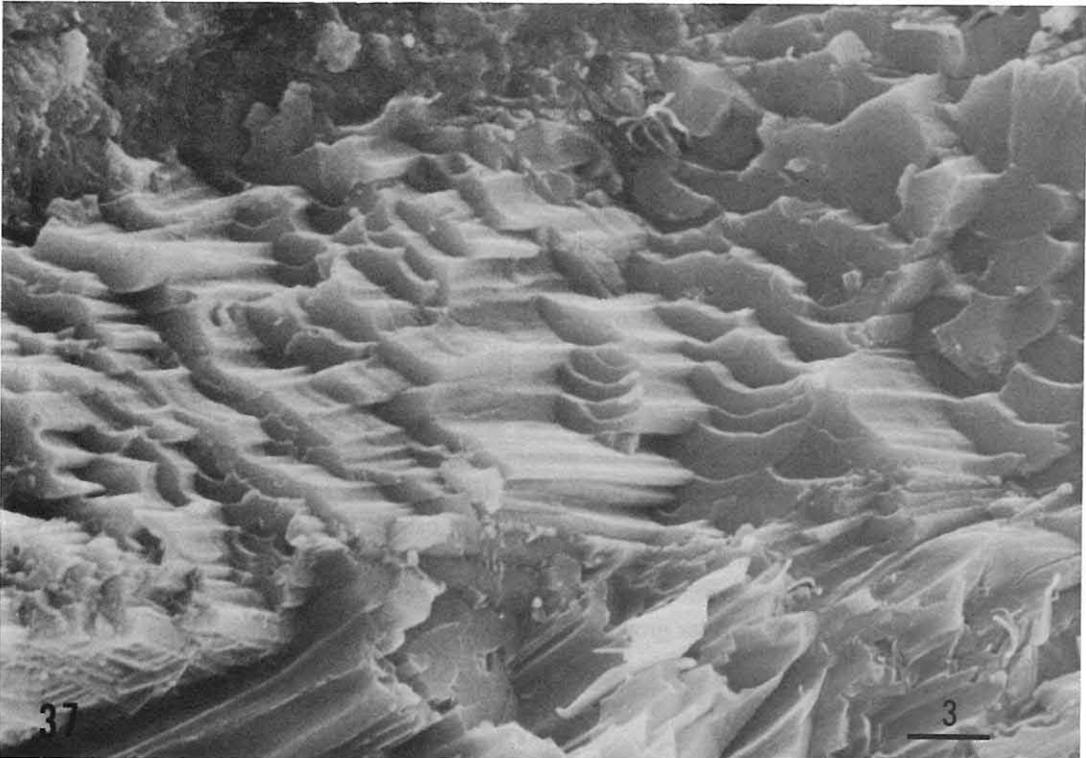






conchiolin membranous layer in early stages of shell regeneration. In some cases, mineral deposits are similar, in appearance, to the anvil-like rod calcite crystals as commonly seen in the outer layer of mussel shell (Fig. 37), or similar to polygonal calcite crystals in the foliated architectural structure (Tayler *et al.*, 1969) of the inner layer of the oyster and scallop shells (Figs. 40–42). In other cases, mineral deposits arranged in the nacreous architectural or spherulitic pattern are observable in this layer. These crystalline aggregates which contain any organic materials may be respectively composed of small hexagonal aragonite or of acicular aragonite crystals.

These crystal structure can be identified, not only by their morphological appearances, as shown by previous investigators but by using X-ray diffraction. From the X-ray diffraction pattern, it appears that the mineral deposits within the organic membrane-shell are mainly composed of two types of calcium carbonate crystals, aragonite and calcite. This evidence may suggest that a restricted region of the mantle epithelium can secrete not only the various organic matrix of all layers of mussel shell, but might be relevant to deposits of simultaneously or successively two different types of calcium carbonate crystals, aragonite and calcite.



**Fig. 37** Architectural structure constructing by flattened anvil-like rod crystals in early stages of the mineralization of the organic membrane-shell. Note that this structure is very similar to that of the outer prismatic layer of *Mytilus* shell. The prismatic layer is usually composed of calcite crystals. (Bar scale in microns.)

### Initial mineralization in the organic membrane-shell

The present studies showed that crystalline bodies having a double-disk form were first present in the conchiolin membranous layer on day 30 of shell regeneration. Although the double-disks were not numerous on day 30, some of them were already mineralized throughout, while others had calcium only at the basal portions of the disk. Judging from such an appearance, they will be in rather relatively advanced stages of calcification of shell regeneration.

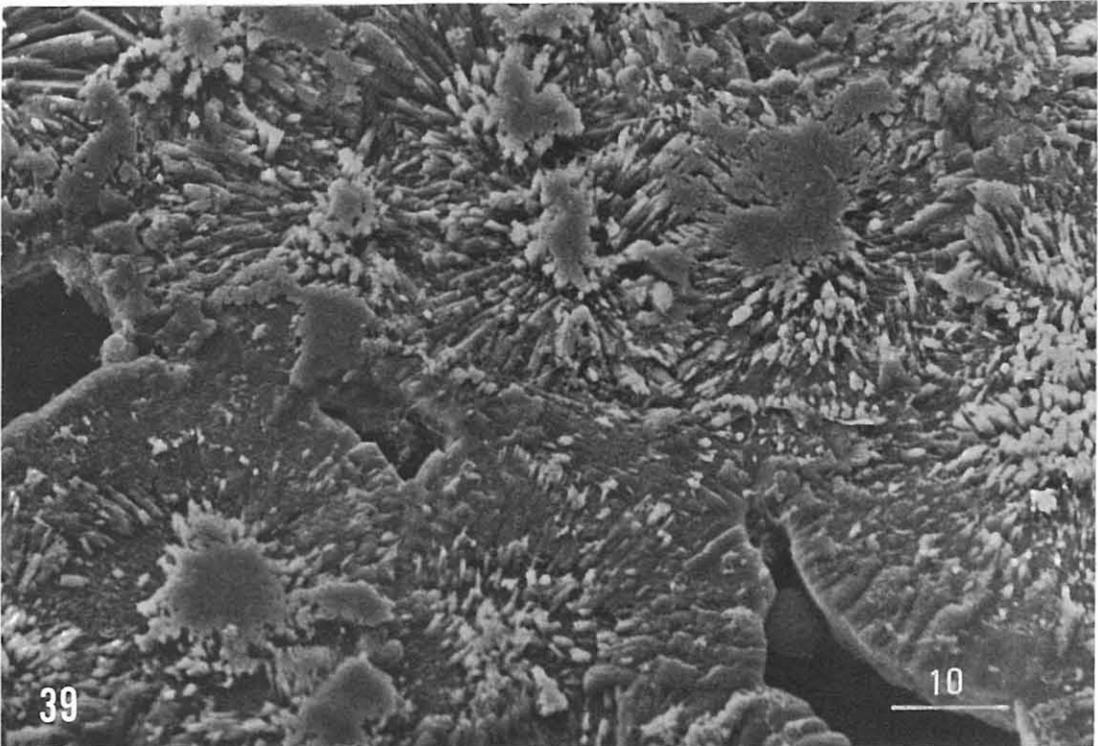
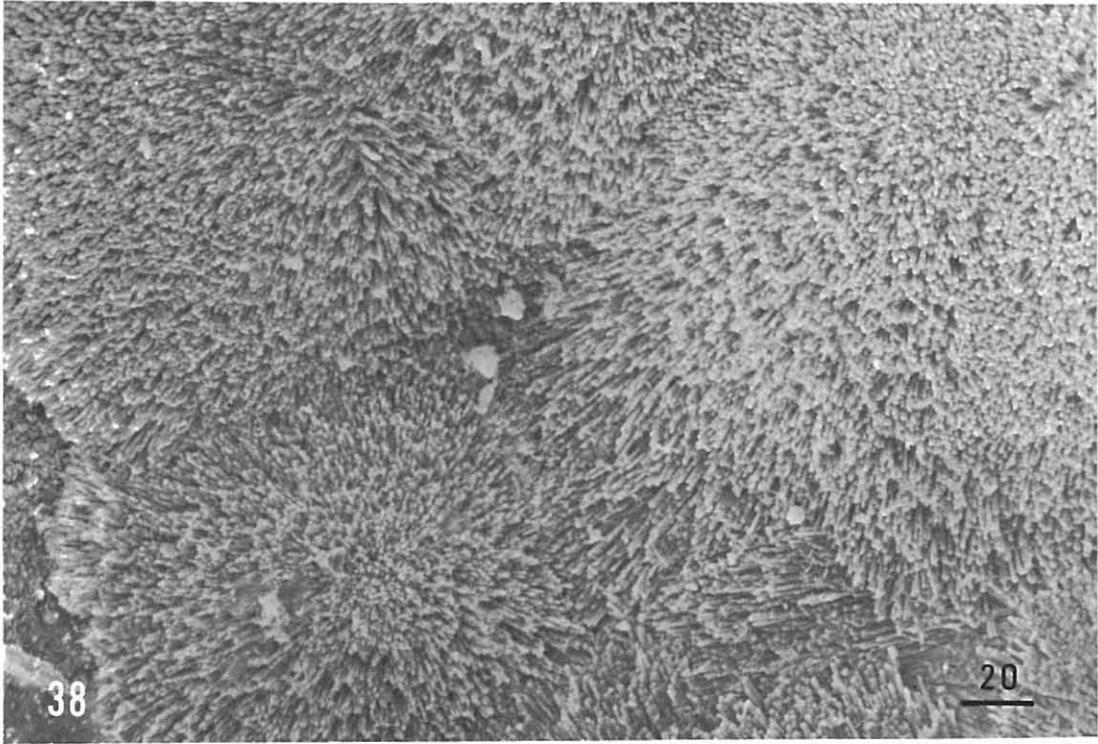
Spherules, made up of double-disks, are commonly seen within the conchiolin layer. They will be in a more immature stage of calcification, and a few of them concentrated in calcium can be traced by electron probe microanalyser. Moreover there are rarely a few containing tiny, discrete pieces of inorganic materials, as have been reported previously by Uozumi and Ohta (1977).

Spherule is constituted by aggregation of numerous particles. Consequently, through the present studies, the particle may be the smallest essential unit associated with calcification. They are found to attach on the fibrils which distribute within the spaces between conchiolin sheets, and rarely a few with accumulated calcium may be seen, but one containing calcium carbonate crystallite has not as yet been found. From the above-mentioned evidence calcification in initial stages of shell regeneration can be subdivided into two phases: concentration of calcium and initiation of mineralization, and growth of calcium carbonate crystallite.

In the first phase, calcium accumulates within the matrix of spherule (and perhaps particle), as judging from the trace of calcium by electron probe microanalyser (Uozumi and Ohta, 1977). The accumulated calcium reacts initially with available carbon dioxide to form calcium carbonate crystals within a particle which is embedded within the organic materials of spherule, or within a larger spherule. When this happens in the latter case, particle seems to act as a catalyzer for the nucleation of crystals within the spherule.

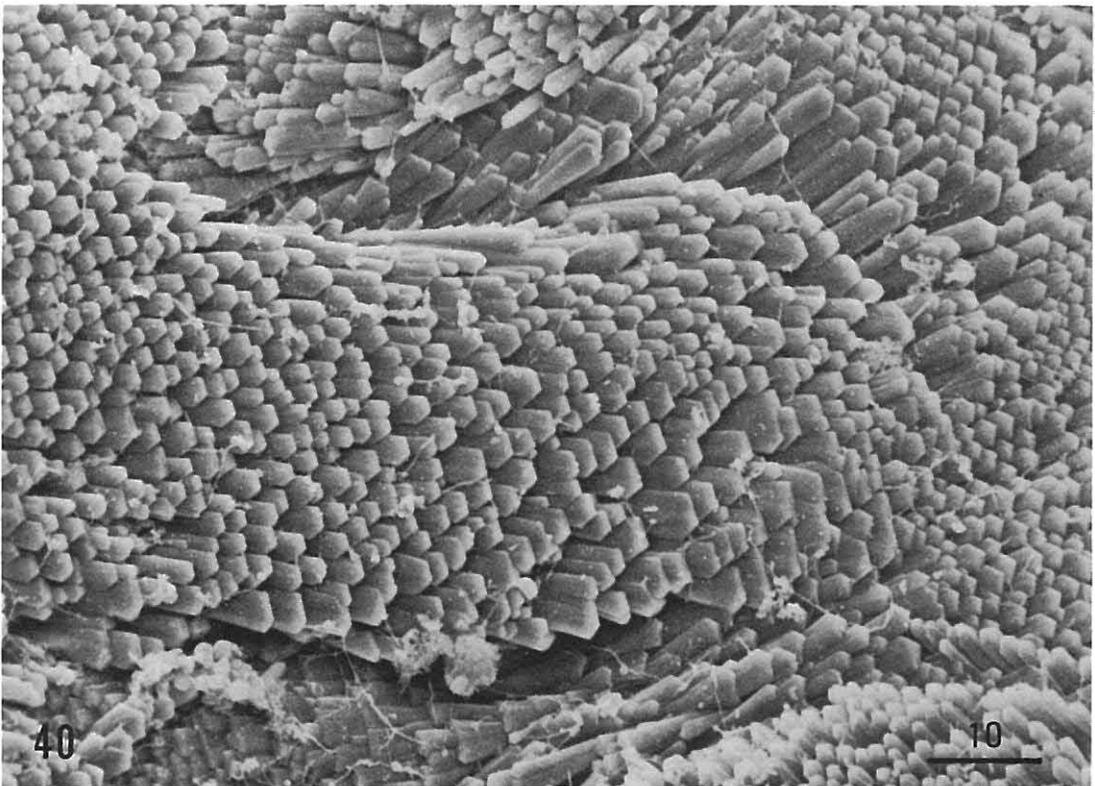
It is unfortunate that the most of our ideas about the initiation and growth of the inorganic phase within particles must inevitably be derived from indirect observation, since the particle size is too small to be seen at all in the optical microscope and not large enough to permit reliable, artifact-free deduction from scanning electron micrographs during the initial calcification of the particle. The present studies, still in progress, will be able to indicate some direct ultrastructural evidence in regard to the initial calcification of the particle within a spherule, but from the observations of calcified isolated particles, presented within the laminated membranous layer, as stated in the foregoing paragraphs, it may be possible that initial mineralization takes place to some extent within a particle and during secondary mineralization, further growth of the young, seed-like crystal takes place within a larger spherule.

Figs. 38–39 Electron micrographs showing double-disks transformed into “regular clusters of radially outgrowing acicular crystals”. In final stages of development, the outline of each disk may be ill-defined. (Bar scale in microns.)



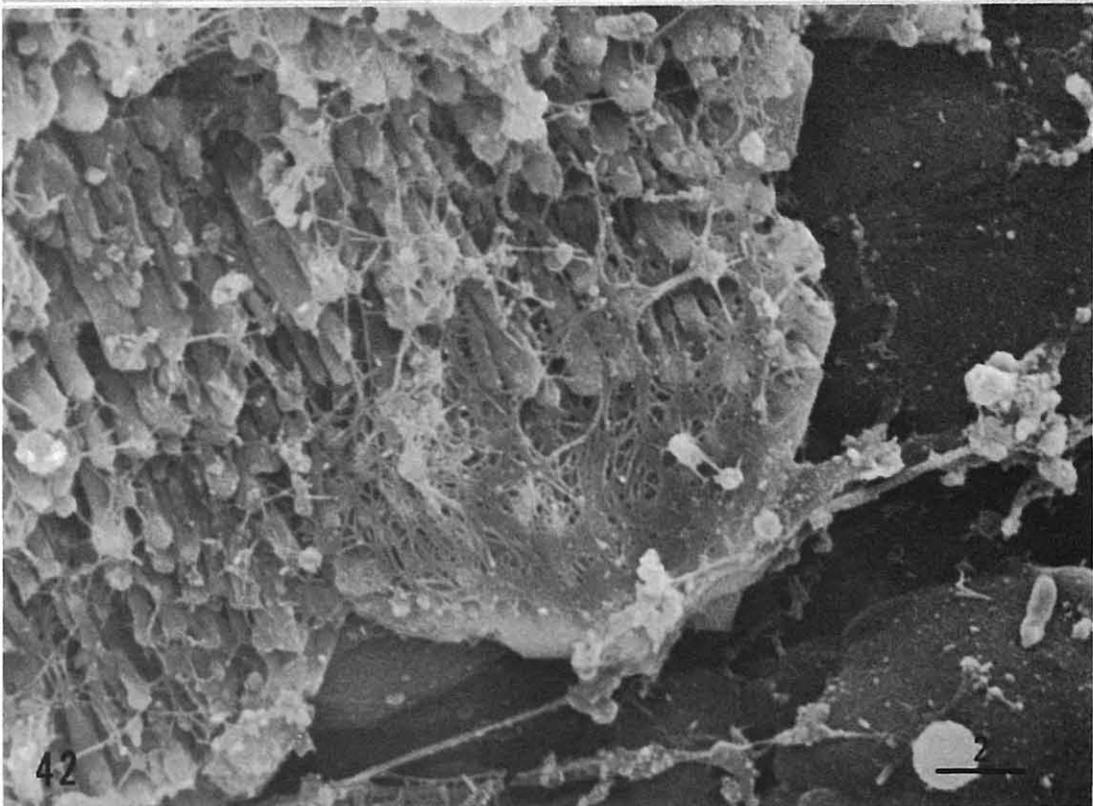
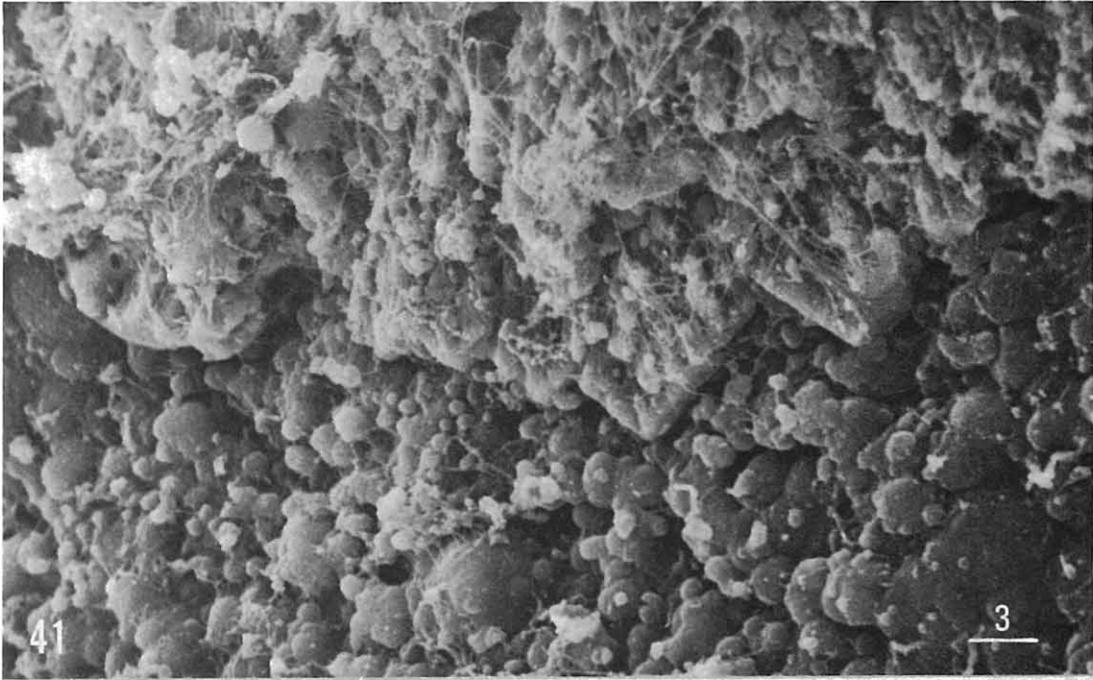
Excepting the present studies, the intimate relationship between organic spherules and young, seed-like crystals is well observed in early stages of calcification of protoconch in the ontogeny of abalone, as reported previously by Iwata (1978), a member of the Biomineralization Research Group in our department.

In the second phase, the young crystal within the particle grows until it perforates the outer wall of the particle. After that, the crystal will continue to grow, especially in crystalline length within the larger spherule, and form an acicular crystal. Acicular crystals might occur almost simultaneously from many particles within a spherule and will grow in concert and will either fuse to form a single crystal or, more likely, will exhibit an intergrowth pattern not visible optically or detectable crystallographically. Growing acicular crystals, as a whole, will show irregular spherulitic or complex crossed-lamellar architectural patterns as seen commonly in molluscan shell (Figs. 38, 39). Also, they become so increased



**Fig. 40** Architectural structure constructing by polygonal crystals in early stages of mineralization of the organic membrane-shell. Note that this structure is very similar to that of the inner foliated layers of the oyster and scallop shells. The foliated layer is usually composed of calcite crystals.

**Figs. 41–42** Periphery of fully-developing double-disk showing a serrate pattern. Note that the marginal region of the disk is occupied with larger acicular crystals (approximately  $0.5\mu$  in diameter) in comparison with crystals (approximately  $0.1\mu$  in diameter) in immature disks, as shown in Figs. 31 and 34. (Bar scale in microns.)



in crystalline length more rapidly along the basal plane of the spherule, that the spherule undergoes a gradual change in form, from spherical to flattened ~ double disk-like. When acicular crystals fill up the interior space of a disk, they will perforate the outer wall of the disk and become exposed to the spaces between conchiolin membranes, lain in layer. Finally they will become transformed into hexagonal tabular form and will continue to grow there, until forming crystalline aggregates, which are structurally organized into well-ordered, sheet-like layers. The change from an acicular crystal to a hexagonal tabular one may seem to be another problem of crystalization, associated with the growth of one crystalline material on a preforming different crystal with the same or similar lattice spaces, and will be discussed in another paper in the near future.

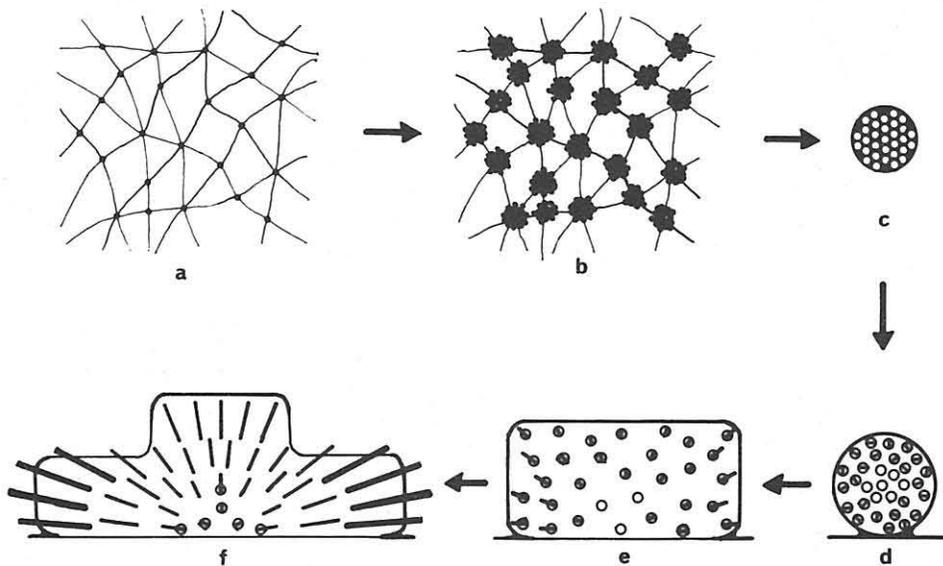


Fig. 43 Steps in the formation of mineralized spherule in the organic membrane-shell of *Mytilus edulis*. a. Deposition of particles on the junctions of fibrils. b. Formation of aggregated bodies of particles. c. Coating of the aggregated body with amorphous organic materials (= formation of spherule) (calcium accumulation). d. Mineralization of the particles within a spherule. e. Exposure of preformed crystals to the interior space of the spherule, and deformation of the outline of the spherule with further accumulation and growth of intra-spherular crystals. f. Final shape (double-disk) of the fully-mineralized spherule, and exposure of preformed crystals to the extra-spherular environment.

With regard to the precursory organic matrix of calcification, as termed “particle” and “spherule” in this paper, previously the terms “b-granule and organic crystal” (Abolinš-Krogis, 1968), “granule and organic crystal” (Wada, 1964), “envelope”? (Bevelander and Nakahara, 1969), and “crystal seed” (Watabe *et al.*, 1958) had been used. However further investigation is needed to decide whether they are homologous or heterogeneous with each other.

### Acknowledgements

This paper is dedicated to Professor Masao Minato at his retirement from Hokkaido University.

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Thanks are due to Dr. Kenji Togari for his kind discussion of the crystallographical problems, to Mr. Shigeshi Ohta for electron micrographs and technical assistance and to Miss Chiaki Sato for her help in the laboratory works. This research was financially supported partly by a grant-in-aid for Scientific Research from the Ministry of Education, No. 154272.

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