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MINERALIZATION AND ARCHITECTURE OF THE LARVAL SHELL OF *Haliotis discus hannai* Ino, (ARCHAEOGASTROPODA)

by

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(with 1 text-figure, 2 tables, and 5 plates)

Abstract

Mineralization process of the larval shell was studied in the recent archaeogastropod, *Haliotis discus hannai* Ino, with electron microscope in order to investigate the mechanism of biomineralization during early ontogeny. Prior to shell mineralization, invagination of ectoderm occurs and a thin periostracum is formed. The periostracum does not mineralize itself. Small organic spherules are secreted on the periostracum and crystal nuclei appear heterogeneously within the organic spherules. Crystal nuclei grow into flaky and granular crystallites which aggregate to form spherulites and fill up the organic spherules. These aggregations of crystallites are joined with one another and form a thin mineralized layer.

Morphology of larval shell is almost completed throughout the planktonic veliger life. The protoconch consists of minute granular architecture, in which crystallites are arranged without particular orientation. After the larvae sink to bottom, inner layer of protoconch and peristomal shell are accreted. The inner layer of the protoconch consists of intersecting blocks which are composed of acicular crystals. The architecture of the inner layer is similar to the crossed-blocky structure in the early stage of pleurotomariids. The nacreous layer is absent in the protoconch.

Introduction

Molluscs acquire mineralized shells during the early stage of ontogeny. In contrast to the many studies on biomineralization of adult shells (Wilbur & Simkiss, 1968; Wada, 1964, 1968, 1970; Watabe, 1965; Wise, 1970; Mutvei, 1970; Erben, 1972a; Uozumi & Togo, 1975), only few studies have been reported on the mechanism of mineralization of larval shells. The minerals of larval shells of some pelecypods have been shown to be different from the adult shells (Watabe, 1956; Stenzel, 1963, 1964; Iwata & Akamatsu, 1975), and the larval shell structures were also different from the adults (Iwata & Akamatsu, 1975). Watabe (1956) reported that the prodissoconch I of *Pinctada martensii* consisted of dahllite, a phosphate mineral. The dissimilarity of the structure in the larval and adult shells is also known in some neogastropods (Togo, 1974, 1977; Bandel, 1974) and archaeogastropods (Erben & Krampitz, 1972b; Batten, 1975).

Mineralization of larval shells of neogastropods occurs at the post-larval stage (Fretter & Pilkington, 1971; Togo, 1974; Bandel, 1974), but the exact time and mechanism of mineralization of larval shells in archaeogastropods are not known. This paper reports the results of the electron microscope study of the protoconch of the recent archaeogastropod, *Haliotis discus hannai* Ino.

Materials and methods

Larval specimens of the abalone, *Haliotis discus hannai* Ino, were obtained from the Hokkaido Institute of Mariculture, Shikabe Village, Hokkaido, where the abalones culture is maintained at 20°C throughout the year. This species of abalones is widely distributed along
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the Pacific and the Japan Sea coast, where warm current prevails, and also near Sakhalin area. Adult abalones were caught from the neighboring sea and the spawning was induced by raising water temperature. Specimens ranging from 15 to 26 hours after spawning were collected at every 30 minutes, and 2, 4, 11, 16, 30–40 days, and about 4 months old larvae were also collected.

The larvae were anaesthetized by a quick heating on a glass slide, and their development was observed under the optical microscope. A few specimens were observed under the polarized microscope in order to check mineralization of larval shells. Preparations for the transmission electron microscope (TEM) and the scanning electron microscope (SEM) were as follows: Larvae were fixed with 1% glutaraldehyde buffered with sea water, pH 7.0, and post-fixed with cacodylate-buffered OsO_4 at 4°C for one hour. After dehydration with ethanols, the specimens were embedded in epoxy resins. Thin sections were obtained by ultramicrotomy using a diamond knife, and stained with uranyl acetate. In order to examine initial mineralization, periostracum of some specimens were separated from the soft body with a needle and a small blade under the optical microscope. Pieces of the periostracum were mounted on the grids coated with formvar. Some specimens were shadowcasted with Pt-Pd, and others were observed without any treatment. Observation was carried out with JEM-120 U. For SEM observations, the larval specimens were fixed and dehydrated as mentioned above, and coated with gold in a vacuum evaporator. Some specimens were dried using critical point dehydration. Observations were also carried out on fractured shell surfaces. Identification of minerals of larval shells was carried out by X-ray diffraction and electron diffraction. About 3000 larval shells were analyzed by powder diffraction method. Elemental analysis (EPMA) was also carried out by SMU-3-SDS wave length X-ray spectrometer on JEOL JSM-U3.

Results

Mineralization process of the larval shell

Pre-mineralization stage

A summary of processes of the development and shell mineralization in *Haliotis discus* is shown in Table 1 and 2. The fertilized eggs of *Haliotis discus* are spherical, and about 230μ in diameter. The cleavage of eggs develop into the trochophore larvae in 15–16 hours. The trochophore larvae bear apical tufts and protrochal girdles. They are positively photoactive and rotate intermittently in a counterclockwise direction. They soon hatch out from the egg capsules and move upward. The trochophore larvae develop into veliger larvae in 18 hours after spawning. They possess vela on the ventral part of the body, and continue to rotate. At about the same time, a part of ectoderm becomes invaginated and shell gland secretes a very thin transparent membrane which covers the invaginated region (Pl. 1, fig. 1). This membrane is periostracum and it has no particular ornamentation. The periostracum is eosinophilic, but not stained with PAS. Under the TEM it is about $300\text{\AA}$ in thickness and structureless. Crystalline materials were not observed in it or within the "extra shell-gland space", the space between the periostracum and the shell gland.

Table 1 Embryology and development of soft body in *Haliotis*

Time after spawning	Embryology and features of soft body
0 day	Fertilized eggs
12–13	Trochophore larvae with apical tufts, protrochal girdles and shell gland
16	Hatching
18	Veliger larvae with velum
19	Invagination of shell gland
21–22	Torsion
40–45	Cephalic tentacles, foot, muscle, and operculum formed. Transition from planktonic to benthonic life
5–6 days	Retardation of shell gland and loss of operculum. Accretion of first whorl.
10–16 days	Creeping larvae (juvenile snail). Development of mantle?

Table 2 Various stages of shell formation and mineralization in *Haliotis* during early ontogeny

Time after spawning	Shell formation and mineralization
18 hours	Formation of periostracum
19	Beginning of secretion of organic spherules
21	Initiation of mineralization (nucleation)
21–22	Formation of flaky crystallites
23–24	Crystal growth & aggregation of crystallites
40–45	Completion of planispiral protoconch & thickening of shell layer
5–6 days	Outer shell layer of the protoconch completed.
10 days	Accretion of adult whorl (peristomal shell). Inner shell layer of the protoconch being deposited.

Formation of organic spherules and nucleation stage

This process includes formation of crystal nuclei within organic matrices. It occurs in a short period, but the process can be observed successively due to the different stages of mineralization in different parts of shells and individuals. 30 specimens were observed. After a few hours – 19–21 hours after spawning – the periostracum covers wider space of the dorsal part and a bowl-like larval shell is formed. Under the optical microscope, small spots in greenish color are clearly observed through the periostracum (Pl. 1. fig. 2), and these spots increase in number with the lapse of time (Pl. 1. fig. 2–3). These spots are at first observed on the posterior region of the larval shell, which becomes the first whorl of the protoconch at the later stage. These spots are very small and it is not easy to distinguish each of them under the optical microscope. These spots are not stained well by eosine, hematoxyline, and PAS. Under the TEM it was evident that these green spots were roughly rounded organic bodies (Pl. 2. fig. 1). From now on, these organic bodies will be called organic spherules. The organic spherules range from 0.5 to 1.5 μ in diameter, and none of them exceed 2 μ . They are deposited at first in an isolated state, and more or less of irregular in form. Those in

more advanced stage of development possess a rounded morphology, and their peripheries are serrated. Undeveloped organic spherules are observed as confetti-like bodies under SEM. Advanced organic spherules are fused together and boundaries of neighboring spherules are often obscure. Surface of organic spherules is slightly uneven and minute pores are observed. These organic spherules increase contrast by staining with uranyl acetate and ruthenium red.

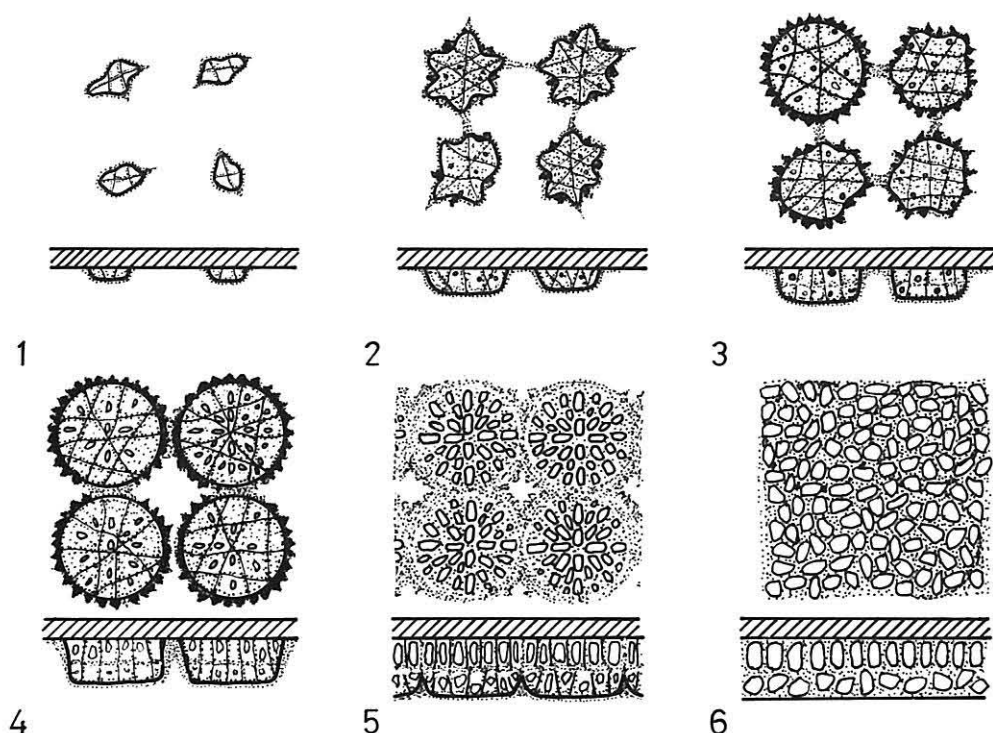
In thin sections, organic spherules are observed as roughly rectangular envelopes, and contain thin filaments and coating materials. These filaments and coating materials form a network within the spherules (Pl. 3. fig. 1). The larval shell of 24 hours after spawning showed electron dense particles of 50 to 200Å on the filaments and coating materials. These electron dense particles are deposited on the middle part of the organic spherules and then settle on the inner surface of the periostracum (pl. 3. fig. 2). Smaller particles give only vague halo by electron diffraction analysis, but particles of more than 200Å in diameter produces diffraction spots. Smaller electron dense particles are regarded as precursors of crystallites — crystal nuclei.

Formation of crystallites and aggregation stage

The following process involves crystal growth, aggregation, accumulation of crystallites, which lead to form a foundation of the shell layer. In 24–26 hours after spawning, the dorsal part of the soft body becomes much more depressed and “extra shell-gland space” is enlarged. At this stage, the organic spherules increase greatly in number and spread over a half of the dorsal part (Pl. 1. fig. 3–4). Under the optical microscope, the green spots becomes darkened and turn to opaque. These spots show a wavy extinction under the polarized light. Mineralization of larval shells can be recognized in the specimens of 26 hours after spawning by means of a polarizing microscope.

As already described, crystalline particles are formed at the stage of 24 hours after spawning (Pl. 3. fig. 2). Observation of pieces of the separated periostracum, on which organic spherules are deposited, revealed that crystalline particles were deposited heterogeneously within the organic spherules (Pl. 2. fig. 2, Pl. 3. fig. 3). About 200 organic spherules were observed at the initial stage of crystallization. However, it is not clear whether the crystalline particles are confined to a definite region of the organic spherules at the moment of initial deposition. Crystallites are found not only in the central part of the spherules, but also in the intermediate region and peripheral region of the spherules. Direct deposition of crystallites on the periostracum was not observed.

Soon after the initiation of crystallization (25–26 hours after spawning), crystalline particles grow into flaky crystallites of 300 to 600Å in size, and begin to aggregate like dendritic, snow crystal-like manner (Pl. 3. fig. 4–5). At this stage, chains of flaky crystallites are arranged in radial direction and branching disposition can also be observed. At more advanced stage, these aggregations of crystallites show a characteristic, spherulitic pattern (Pl. 3. fig. 6) and give several electron diffraction rings. Neighboring mineralized spherules are fused together, and gradually make up regional platform. Finally, crystallites fill the organic spherules and mineralized spherules are completely fused. Consequently, outermost shell layer is formed (Pl. 4. fig. 1). However, crystallites do not fuse to make a



Text-figure 1 Stylized diagram of the mineralization and aggregation process in the larval shell of *Haliotis*. Horizontal and profile views of the organic spherules are illustrated.

larger single crystals, but still remain as polycrystalline aggregations. Boundaries between each neighboring mineralized spherule are observed as narrow electron dense zones; however, these boundaries are not very clear in some cases. During these processes, flaky crystallites grow into granular ones, and most of them do not exceed 0.1μ in length. Elemental analyses of mineralized spherules showed a concentration pattern of Ca as aggregation of crystallites proceeds (Iwata, 1978). Electron diffraction analyses of crystallites proved that they belonged to aragonite, and existence of other minerals could not be ascertained. X-ray diffraction analysis of larval shells of 2 days also showed that the shells were composed of aragonite. Aggregation process of crystallites within the organic spherules is illustrated in text-figure 1.

Aggregations of crystallites accompanying the organic spherules continue to develop laterally and vertically, and mineralized layers are accumulated on the inner surface of the protoconch (Pl. 1. fig. 5). Paralleling to crystal accumulation, coiling of the protoconch advances anteriorly, as the visceral mass expands. Planispiral morphology of the protoconch is completed in 40–45 hours after spawning. Mineral deposition continued almost throughout planktonic veliger life. The initial part of coiling of the protoconch is, however, not yet fully mineralized in some specimens of 45 hours after spawning, and mineral deposition still continued inwards.

Ultrastructural architecture of the larval shell

After torsion the larvae possess a pair of tentacles, stigma, foot, visceral hump, and operculum. Protoconch reaches about 300μ in shell length, and planispiral shape is completed. At a late stage of the veliger life, the shell gland becomes regressed and disappears, and the mantle appears at the dorsal region of the body. In about 10 days the larvae lose their opercula and vela. The larvae sink to the bottom of the aquaria and begin to creep around.

The surface of the protoconch of *Haliotis discus* does not show a perfect smoothness, but an indistinct series of spots arranged in curved lines can be often observed (Pl. 5. fig. 3). Such an ornamentation may be related to the relict pattern of the mineralized spherules which are deposited during early veliger stage. No growth increments were observed on the surface of the protoconch, which suggests a continuous deposition of minerals during planktonic period. The peristomal shell (first teleconch) is accreted around the periphery of the planispiral protoconch after the animal develops into the benthonic juvenile snail. A marked difference of ornamentation is observed between protoconch and peristomal shell, and the boundary of the two shells shows a sharp gap indicating an interruption of growth (Pl. 5. fig. 3). Growth increments are easily observed on the peristomal shell, and, in addition, radial ribs showing a meandering zig-zag pattern are also observed. Peristomal shell encloses the protoconch at a later stage, forming a low spire (Pl. 1. fig. 6–7).

The larval shell of about two days after spawning consists of one shell layer. This shell layer is structureless under the optical microscope, and is about 2.5μ at the thickest part. The shell layer consists of minute granular crystallites which are mostly less than 0.1μ in length. These crystallites are roughly vertical or slightly inclined to the shell surface in the outermost region, but preferred orientation of the crystallites is not recognized in other part of the shell layer. Granular crystallites do not make up ordered blocks, and neither prismatic nor spherulitic sectors are evident. The shell layer is characterized by a fine, homogeneously granular texture (Pl. 4. fig. 2). The larval shells of 4 and 11 days after spawning show similar structure, but thickness of the shell layer increases to 4μ at the maximum. Granular architecture of this protoconch may be distinguished from the prismatic or acicular structure of protoconchs in fissurelliids, scissurelliids, and pleurotomariids (Batten, 1975; Erben & Krampitz, 1972b). It is also distinguishable from the homogeneous layer of adult pelecypods, such as Veneridae and Nuculanidae (Taylor, Kennedy, & Hall, 1969) on account of different grain size and arrangement of minerals. And it is also different from the

Explanation of Plate 1

Figs. 1–7 Optical micrographs. Scales 100μ .

Fig. 1 Veliger larvae after invagination. Periostracum formed. 18 hrs. after spawning.

Fig. 2 Organic spherules deposited. 21 hrs after spawning.

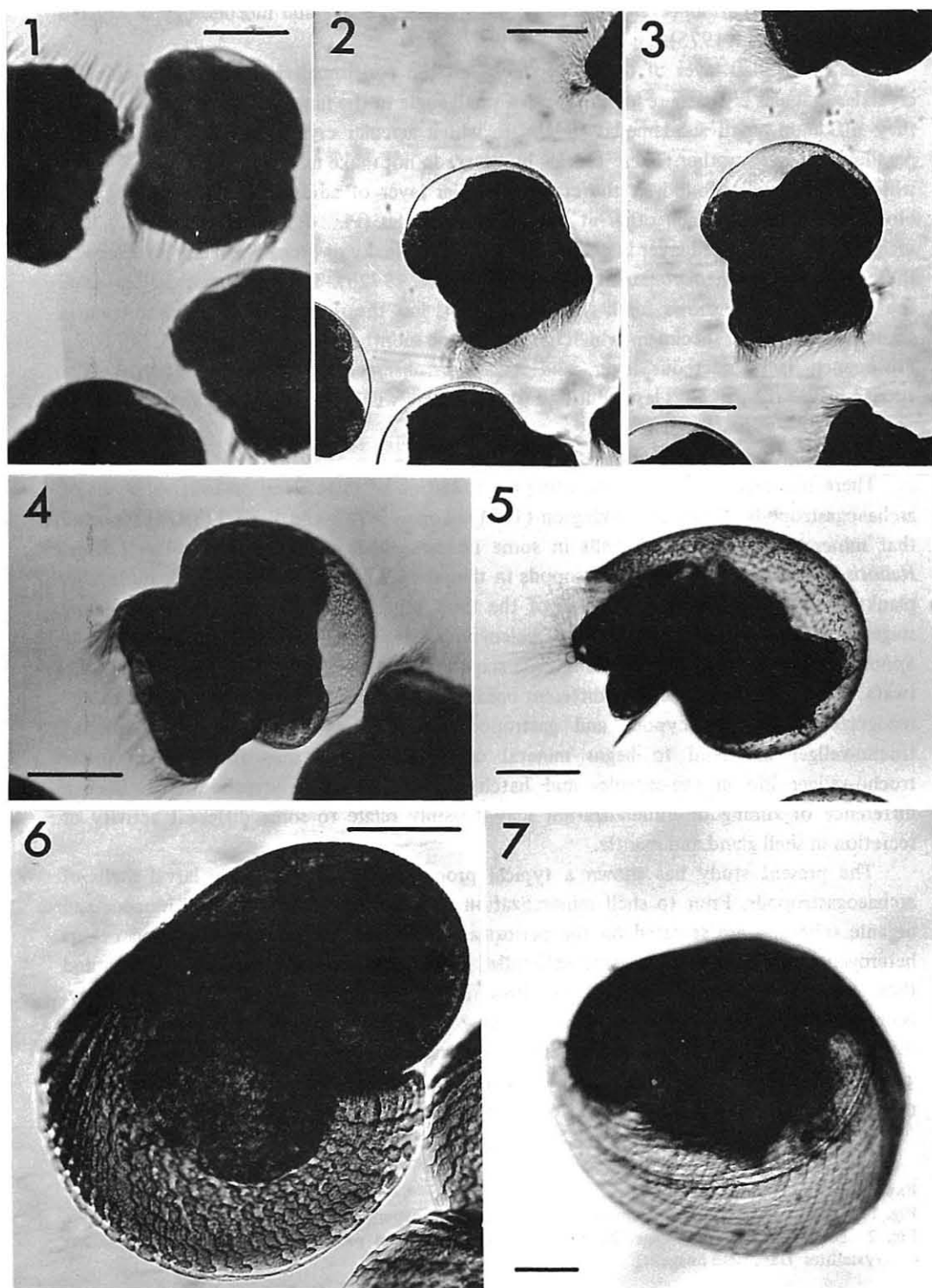
Fig. 3 Larval shell is partly mineralized. 27 hrs after spawning.

Fig. 4 Bowl-like protoconch is formed. 35 hrs after spawning.

Fig. 5 Planispiral protoconch is almost completed. 46 hrs after spawning.

Fig. 6 Peristomal shell (first whorl) is accreted to the protoconch. A creeping juvenile snail of 11 days after spawning.

Fig. 7 Creeping snail of about one month after spawning.



prodossoconch of *Patinopecten yessoensis*, on the basis of size and morphology of crystals (Iwata & Akamatsu, 1975).

The inner shell layer of the protoconch and the peristomal shell consists of acicular crystals. Acicular crystals are inclined with a small angle to the inner surface of the shell, and they make up small sub-lamellar blocks, in which acicular crystals are arranged roughly parallel with one another. These blocks, however, do not make true lamellae of higher order which are typically found in the crossed-lamellar layer of adult shells. Neighboring two blocks intersect with each other at various acute angles (Pl. 4. fig. 3, Pl. 5. fig. 1). The architecture of this shell layer is similar to the crossed-blocky or crossed-acicular structure of the protoconch in *Pleurotomaria* (Erben & Krampitz, 1972b). The outermost shell layer of the peristomal shell shows small rod-like element, but the most part of the shell layer is granular even in the specimens which has passed one month after spawning (Pl. 5. fig. 1-2). Protoconch lacks nacreous layer, and it is not observed in one-month-old snail. The appearance of the nacreous layer during early ontogeny will be described elsewhere.

Discussion

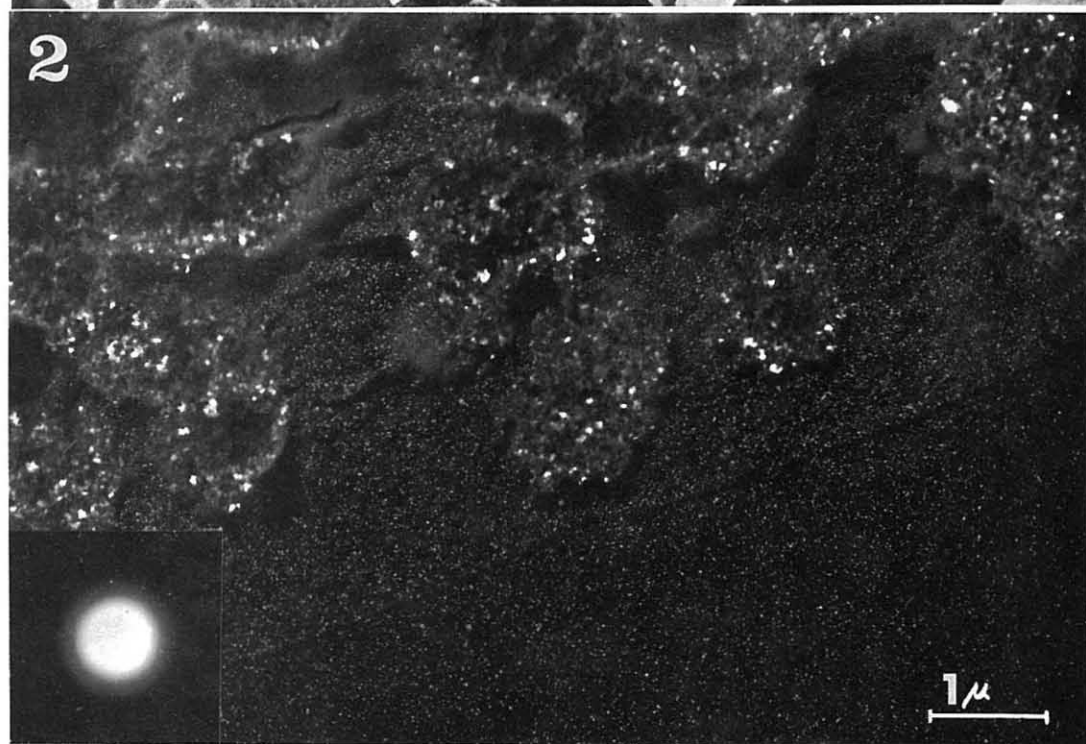
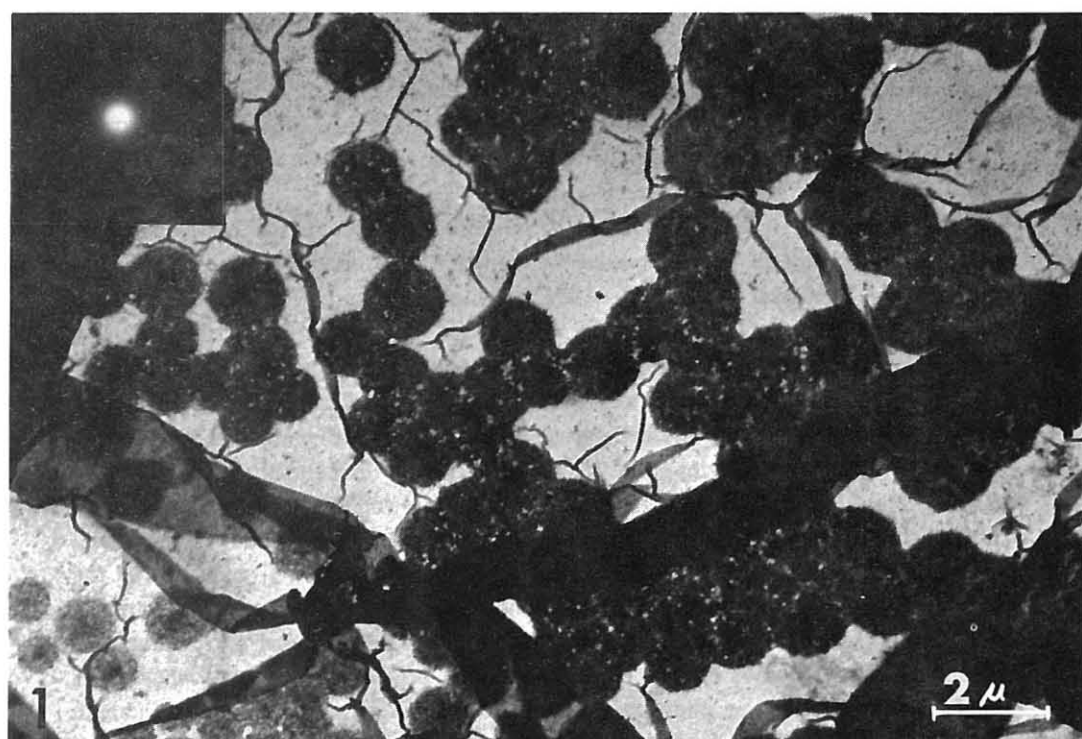
There has been no report concerning the initiation of mineralization in larval shells of archaeogastropods. Fretter & Pilkington (1971), Togo (1974), and Bandel (1974) reported that mineralization of larval shells in some neogastropods occurred at post-larval stage. *Haliotis* is different from these gastropods in that the mineralization takes place during the planktonic stage. However, most part of the thick inner layer is formed at the post-larval stage. Mineralization of larval shells of pelecypods such as *Pinctada*, *Pecten*, *Anadara*, and *Spisula* also takes place during planktonic stage (Watabe, 1956; Iwata & Akamatsu, 1975; Iwata, unpublished). Hence, two different occasions of mineralization of larval shells can be recognized among pelecypods and gastropods. Molluscs which manage free-swimming trocho-veliger life tend to begin mineral deposition earlier than those which spend trocho-veliger life in egg-capsules and hatch out as benthonic juvenile snails. Such a difference of timing of mineralization may possibly relate to some different activity of secretion in shell gland and mantle.

The present study has shown a typical process of mineralization in larval shells of archaeogastropods. Prior to shell mineralization, periostracum is formed, and mucous and organic spherules are secreted on the periostracum by the shell gland. Nucleation occurs heterogeneously within the organic spherules, followed by the development of flaky, and then, granular crystallites. These crystallites fill the organic spherules in a special way. Namely, a dendritic pattern as seen in snow crystals is observed first, and then, the formation of typical spherulitic pattern of aggregation of crystallites proceeds. Finally, granular crystallites entirely fill the organic spherules. Such an initial process of mineralization may represent a type of mineralization of planktonic larval stage of some

Explanation of plate 2

Fig. 1 A horizontal view of organic spherules. 21 hrs after spawning. Small spherules lacking crystallites.

Fig. 2 Early crystallization stage. 23 hrs after spawning. Bright particles within organic spherules are crystallites. Dark field image.



archaeogastropods. Similar type of mineralization may be present in larval shells of other molluscs and invertebrates. Spherulitic aggregation of crystallites may also show a relatively simple and primitive, but a characteristic type of mineralization among molluscs. Compared to adult shells, the size of crystals of larval shell is small. In the larval shells, crystal growth seems to be restricted considerably due to the narrow space in organic matrices and "extra shell-gland space".

Several hypotheses have been recently proposed concerning the mechanism of biomineralization in adult shells, i.e., epitaxy or template hypothesis (Wada, 1964 & 1968; Crenshaw, 1972; Crenshaw & Ristedt, 1976; Weiner & Hood, 1975), compartment hypothesis (Bevelander & Nakahara, 1969, 1971), and envelope hypothesis (Wilbur & Watabe, 1963; Erben & Watabe, 1974). Organic spherules are probably composed of protein-mucopolysaccharide complex, and they may be regarded as a kind of envelopes. However, it is not yet certain whether active template sites are present in the organic spherules or not. To testify these hypotheses, further ultrastructural and biochemical studies on organic spherules, shell gland, and "extra shell-gland" fluid will be necessary.

The mineral of *Haliotis* protoconch consists of aragonite. A few studies on mineral component of molluscan larval shells have been reported. Togo (1974) reported that the protoconch of *Neptunea* consisted of aragonite, and Stenzel (1964) and Iwata & Akamatsu (1975) reported that prodissoconchs of *Ostrea* and *Patinopecten* consisted of aragonite respectively. However, Watabe (1956) reported that the prodissoconch I of *Pinctada* was composed of carbonate bearing phosphate — dahllite. The existence of phosphate mineral in larval shells of molluscs should be noteworthy in biomineralization from evolutionary point of view. Further mineralogical studies of many larval shells will be necessary to understand a significance of changes of minerals during the course of evolution.

Indirect two structurally different shell layers of the protoconch of *Haliotis* are formed at different stages of ontogeny and they suggest different ways of structural formation. Homogeneously granular architecture does not change immediately into acicular or prismatic one during the planktonic stage, but the transition and differentiation of shell structure may be related to the appearance of mantle at the earliest post-larval or late veliger stage. As pointed out by Erben & Krampitz (1972b), Batten (1975), and Togo (1977), architectural structure of larval shells are different from adult ones and they seem to show less differentiated state of shell structure.

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Explanation of Plate 3

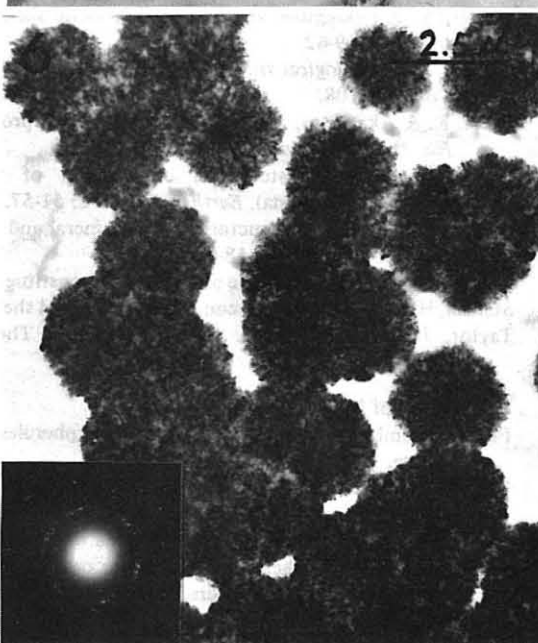
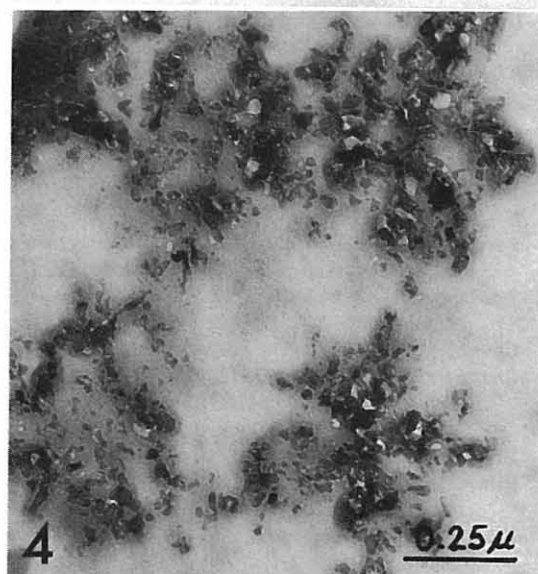
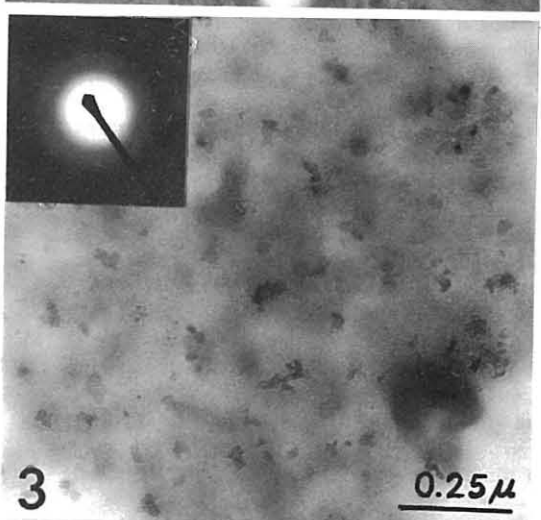
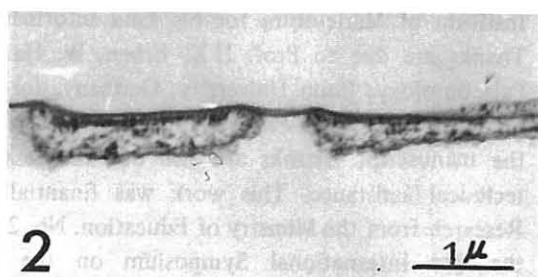
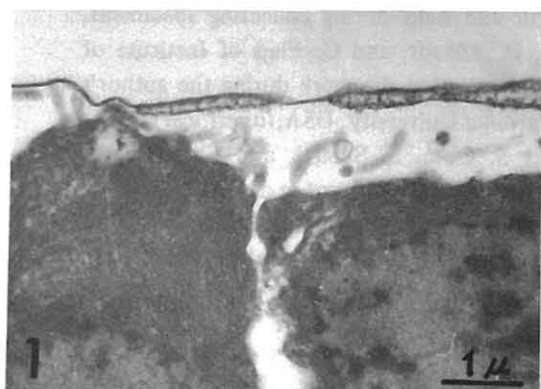
Fig. 1 Thin section of larval shell showing organic spherules.

Fig. 2 Crystal nuclei within organic spherules.

Fig. 3 Flaky crystallites at early stage of mineralization.

Figs. 4-5 Aggregations of flaky crystallites showing a dendritic snow crystal-like pattern.

Fig. 6 Advanced stage of aggregations of crystallites showing a spherulitic pattern.



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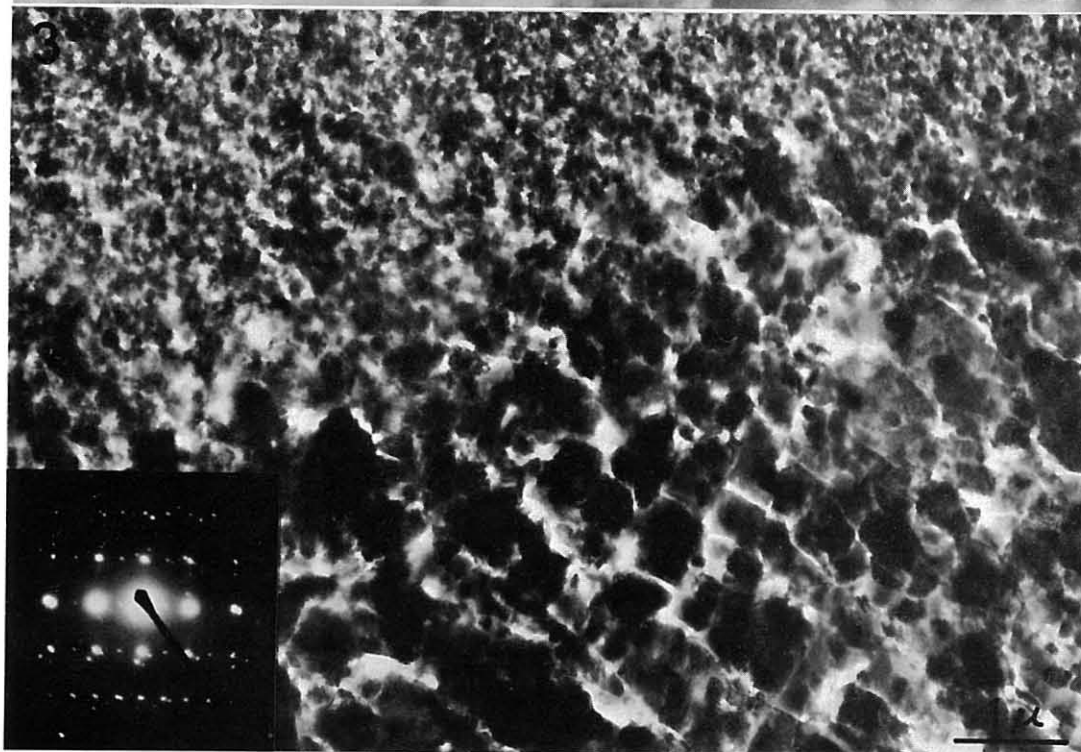
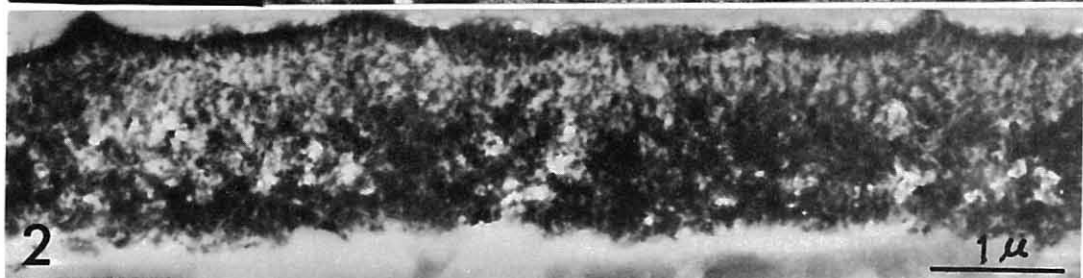
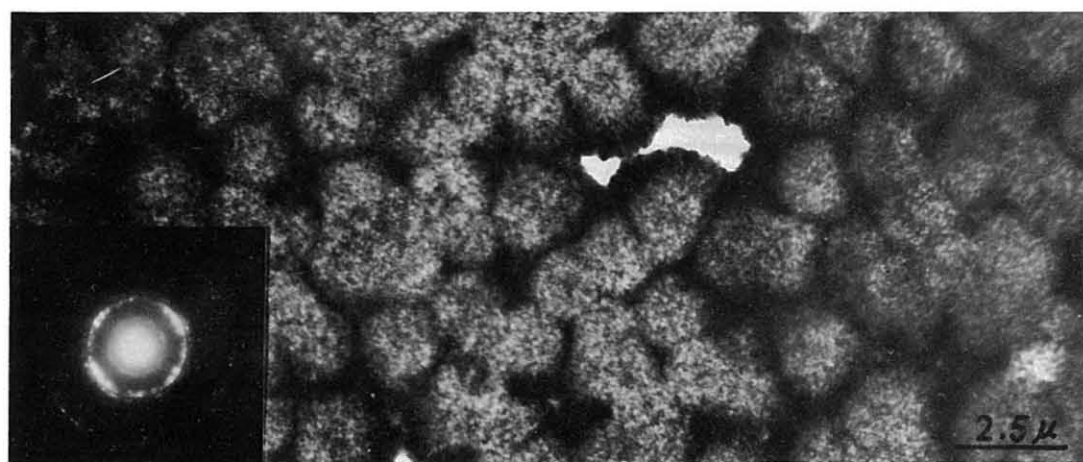
Explanation of Plate 4

Fig. 1 Combination of fully mineralized spherules forming a platform of shell layer. 25 hrs after spawning.

Figs. 2-3 Undecalcified thin section of protoconch cut by a diamond knife.

Fig. 2 Architectural shell structure of the protoconch proper. 43 hrs after spawning, Minute granular crystals depositing without any preferred orientation and ordered blocks

Fig. 3 Architectural shell structure of the outer shell layer (upper half) and inner layer (half below) in the peristomal shell of a creeping snail of 11 days after spawning. Inner shell layer consists of intersecting blocks of sub-lamellae.



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Explanation of Plate 5

- Fig. 1 A scanning electron micrograph showing architectural shell structure of the peristomal shell of one month after spawning.
- Fig. 2 Thin section of shell margin of the peristomal shell. 11 days after spawning. Rod-like crystallites deposited obliquely to the shell surface. Nacreous layer is not yet formed.
- Fig. 3 SEM micrograph of a shell of about 4 months after spawning. Difference of surface ornamentation between protoconch and teleoconch can be observed.

