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Phosphatic Shell Formation in Atremate Brachiopods[1]

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SYNOPSIS. The atremate brachiopods are unique in that they possess shells of calcium phosphate. In *Lingula adamsi* and *Glottidia pyramidata*, the shell mineral is (CO_3+F)-containing apatite and is crystallo-chemically similar but not identical to the mineral francolite. The shell of *Glottidia* consists of a thin periostracum, a mineralized thick primary layer, and alternating mineralized layers and less mineralized chitin layers. The basic unit of the crystals is the spherulite. Proteinaceous and glycosaminoglycan (GAG) matrices are present in the primary and mineralized layers. The GAGS in the chitin layer are morphologically different from those of the other layers. The GAGS are intimately associated with the apatite crystals.

Shell formation appears to be mediated by three different types of cells in the outer epithelium. The cells primarily involved in the mineral formation are characterized by many vacuoles with electron-dense granular inclusions containing Ca, P, and S. The connective tissue at the anterior edge of the mantle also contains fine granules with Ca, P, and S. Those granules are considered to be a mineral reserve for shell formation. Some problems of the mechanisms of shell formation are discussed.

INTRODUCTION

The majority of skeletal structures of invertebrates are composed of calcium carbonate. The inarticulate brachiopods are unique in that those belonging to the Superfamily Lingulacea (Order Atremata) and Discinacea (Order Neotremata) possess calcium phosphate shells instead of carbonate shells. In spite of the rare occurrence of this mineralogy, only few studies (Gratiolet, 1860; Blochman, 1900; Chapman, 1914; Williams and Rowell, 1965; Williams and MacKay, 1979; Iwata, 1981*a*, *b*, 1982) have been published concerning the structure and formation of these shells. In this paper, we will summarize some crystallographic characteristics of shell minerals of the atremates, *Lingula adamsi* and *Glottidia pyramidata* as well as the structure of shell and mantle epithelium of *G. pyramidata* and discuss some problems of the shell formation.

SHELL COMPONENTS

An average of 50% of the shell weight is composed of inorganic components, the majority of which is calcium phosphate (Von Klement, 1938; Vinogradov, 1953). The shell mineral of *Lingula* was reported to be dahllite, a carbonate containing OH-apatite, by Von Klement (1938) and Vinogradov (1953), while it was identified as francolite, a carbonate containing F-apatite, by McConnell (1963), Lowenstam (1971), and Iwata (1981*a*, *b*). We have analyzed the shell of *L. adamsi* and *G. pyramidata* by x-ray diffraction, electron probe and infrared (IR) spectral analyses (LeGeros *et al.*, unpublished). The X-ray diffraction patterns of both species are similar to the mineral francolite and no other mineral phase is present. The crystallinity of the shell apatite is greater than that of human dentine but less than that of mammalian enamel or fish enameloid (LeGeros and Suga, 1980; LeGeros, 1981). The lattice parameters in *Glottidia* are: a = 9.394 and c = 6.890 (±0.003Å); and a = 9.402 and c = 6.880 (±0.003Å) in *Lingula*.

The average F content determined by electron probe analysis is 1.64 wt % of whole shells and 2.58 wt % of highly calcified layers. The F/Ca ratio is nearly constant (0.033 ± 0.002) throughout the shell layers. The IR spectra show absorption bands due to CO_3^{2-}, PO_4^{3-}, OH^-, and N-H groups. The spectra also demonstrate the structural incorporation of F in apatite. The carbonate content varies approximately 2.2 wt % in *Lingula* to 3.6 wt % in

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FIG. 1. Dorsal (at the left) and ventral (at the right) valves of *Glottidia pyramidata*. Scale bar = 2 cm.

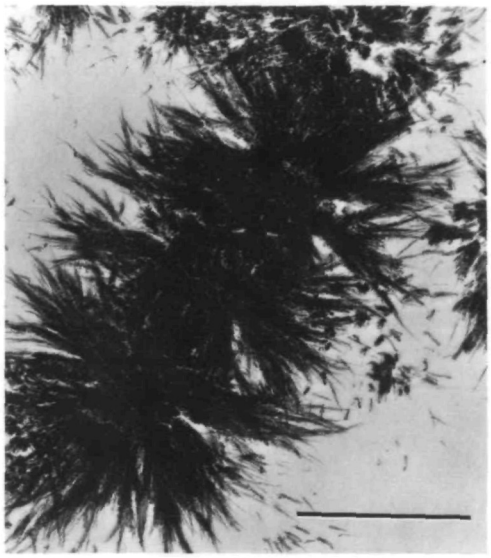

FIG. 2. Aggregates of apatite spherulite in the mineralized layer of *G. pyramidata*. Each spherulite consists of acicular crystallites. TEM. unstained. Scale bar = 1 μm.

Glottidia (LeGeros *et al.*, unpublished). However, the variation may be due to the individuals and therefore may not be species specific. These results show that the shell mineral of *Glottidia* and *Lingula* is (CO_3+ F)-containing apatite and crystallochemically similar but not identical to the mineral francolite.

The organic component consists of varying proportions of protein and chitin (Kelley *et al.*, 1953; see also Hyman, 1959; Jope, 1977). In addition, glycosaminoglycans (GAGS) and lipids have been histochemically detected in *Glottidia* (Pan and Watabe, unpublished). The amino acid composition varies by the species (Jope, 1977; Iwata, 1981*b*, 1982). Generally, alanine is the most abundant (16–33%), followed by glycine (15–29%), aspartic acid (7–12%) and glutamic acid (5–7%). The level of hydroxyproline is high (approximately 2%) among invertebrates, but no collagen has been detected in the shell (Jope, 1977; Iwata, 1981*b*, 1982).

SHELL STRUCTURE

The atremate shell consists of dorsal and ventral valves, is bilaterally symmetrical and somewhat oval in shape (Fig. 1). The shell is very light in weight and in *Glottidia* and *Lingula revi* comprises only 15–20% of the total weight of the animal (Hammen, 1977). The outer surface of the shell is covered with a thin periostracum and marked with numerous growth lines. The mineralized and chitin layers develop alternatively over the innerside of the periostracum (Gratiolet, 1860; Chapman, 1914). Ultrastructural observations of shell structures have been carried out in *Lingula unguis* by Iwata (1981*a*, *b*) and *Glottidia pyramidata* by Iwata (1982) and Pan and Watabe (unpublished). In this paper, we will primarily discuss the *Glottidia* structure based on our own observations.

The basic crystalline component in all the layers is the spherulite, which is several μm in diameter when fully grown and consists of acicular crystallites 50–5,000 Å long and 400–500 Å wide (Fig. 2).

Immediately adjacent to the periostracum is a thick mineralized layer measuring approximately 40–50 μm in thickness in shells which are 2 cm long. This layer which we shall call the "primary layer" consists of aggregates of spherulites embedded in an organic matrix (Figs. 3, 4, 5). The spherulites are less densely packed in regions away from the periostracum. The mineralized layers that follow are almost uniform in thickness (a few μm) and alternate with chitin layers which vary from several μm to 30μm in thickness (Figs. 3, 5). In each mineralized layer, the spherulites are

closely fused together to form a solid single sheet and the organic matrix is hardly discernible in both SEM and TEM micrographs without decalcification. The major portion of the chitin layer is a chitinous organic matrix, in which crystalline rods made of aggregates of spherulites are embedded (Figs. 5, 6). These rods often branch out and form "X" figures, which are oriented obliquely to the shell surface. The mineralized and chitin layers are not always deposited parallel to each other, but cross-beddings of those two types of layers frequently occur.

At least two types of organic matrices are present in the primary and mineralized layers. One is a protein matrix in the form of fibers, approximately 50 Å in diameter and is demonstrated by simultaneous decalcification and staining of thin sections with uranyl acetate (Fig. 7). The other is the GAG matrix which becomes evident in thin sections of shells fixed with glutaraldehyde containing acridine orange (Shepard and Mitchell, 1981) (Figs. 8, 9).

The GAG matrix is also fibrous, 50 Å in diameter and forms a fine network surrounding the crystals (Fig. 8). Treatment of sections with uranyl acetate makes the GAG network more electron dense and reveals that the fibers are intimately associated with the acicular units of the spherulites by filling their interstices (Fig. 9). The spacial relationship between the protein and GAG matrices has not been resolved; however, it appears that the protein component is at the peripheral regions of the GAG networks (Fig. 8).

The chitin matrix in the chitin layer can only be faintly shown by staining sections with Pb and uranyl acetate. However, the acridine orange method shows that GAGS are present in this layer as short fibers or in an almost globular form 50 Å in diameter (Fig. 10). Thus, GAGS in the chitin layer appear to be structurally different from those in the mineralized layer. Whether or not they are chemically different is unknown. As in the mineralized layer, GAGS surround crystallites of spherulites and their aggregates (*i.e.*, rods). The structural relationships between the protein, chitin, and GAG components have

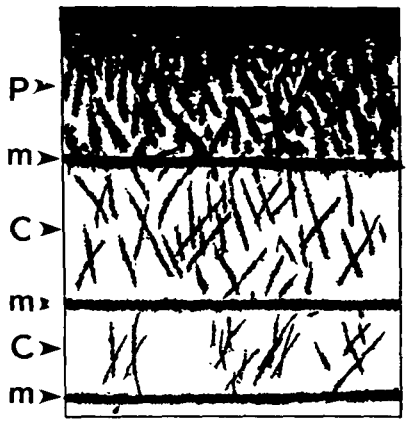

FIG. 3. Schematic diagram of shell layers in *G. pyramidata*. p, primary layer; c, chitin layer; m, mineralized layer.

not yet been resolved. Iwata (1982) reports organic fibers, 3,000 Å thick, crossing one another at 50–90° angles in the organic (chitin) layer of *Glottidia*. Those fibers are reported to be partially or fully filled with crystallites. However, we have not been able to confirm the presence of such fibers.

The close association of the GAG matrix with the apatite crystals may suggest its significant roles in crystal formation and growth. In molluscan shell formation and regeneration, nucleation sites of $CaCO_3$ crystals have been shown to contain sulfated acid mucopolysaccharides (GAGS), and the GAGS are implicated in the induction of crystal nucleation (Abolins-Krogis, 1958; Wada, 1964; Saleuddin and Chan, 1969; Crenshaw and Ristedt, 1975, 1976; Iwata, 1975). In mammalian bone and cartilage formation, the roles of GAGS (proteoglycans) are in dispute. They may inhibit either initiation or growth of the apatite mineral phase, or they could bind Ca^{2+} ions which could later be released by degradation of GAGS, leading to the initiation and precipitation of minerals (see Hascall and Lowther, 1982). Proteinaceous matrices are also important in biological calcification (see Wilbur and Simkiss, 1979; Watabe 1981; Kingsley and Watabe, 1983; Weiner *et al.*, 1983). No information is available concerning the extent to which the protein matrix exerts effects on min-

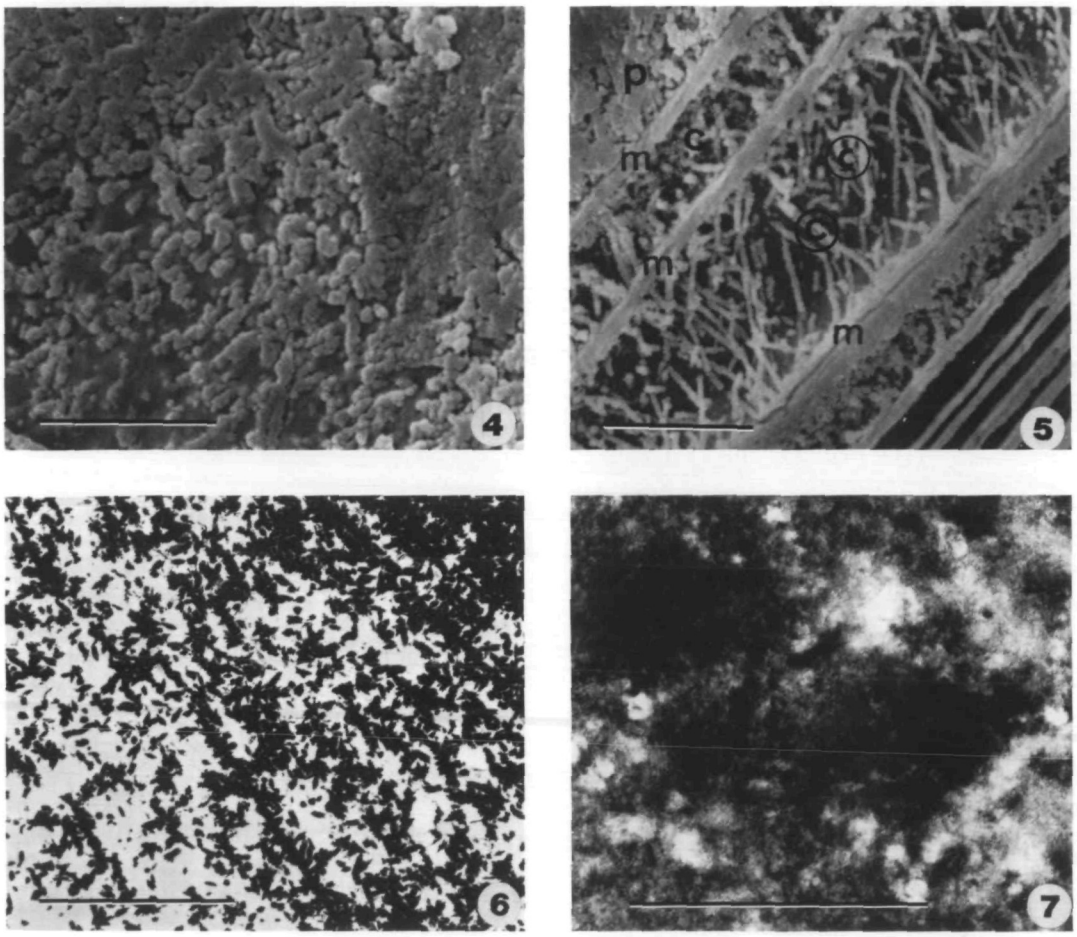

FIG. 4. SEM micrograph of a primary layer of *G. pyramidata* consisting of aggregates of spherulites. Scale bar = 10 μm.

FIG. 5. Primary layer (p), chitin (c) and mineralized (m) layers of *P. pyramidata*, SEM. Scale bar = 20 μm.

FIG. 6. TEM micrograph of a chitin layer, showing spherulites at various growth stages and aggregates of spherulites forming rods. Unstained. *G. pyramidata*. Scale bar = 5 μm.

FIG. 7. Fibrous protein matrix in the mineralized layer of *G. pyramidata* revealed by decalcification of a thin section with uranyl acetate. TEM. Scale bar = 1 μm.

eral formation in brachiopods. Experimental investigations are needed to elucidate the functions of GAGS and protein matrices in mineralization of the brachiopod shells.

ULTRASTRUCTURE OF THE MANTLE

Williams (1977) reported in detail the ultrastructure of atremate mantle, especially that related to periostracum formation. Accordingly, we will limit our discussion to the structure relative to the calcareous shell layer formation in *Glottidia*. The *Glottidia* mantle consists of an inner and outer epithelium and connective tissue (see Williams, 1977). The outer epithelium is directly associated with the calcareous layer formation and the following three types of cells have been identified (Pan and Watabe, unpublished).

1. Columnar cells

The cells at the anterior edge of the mantle are columnar with a basally situated

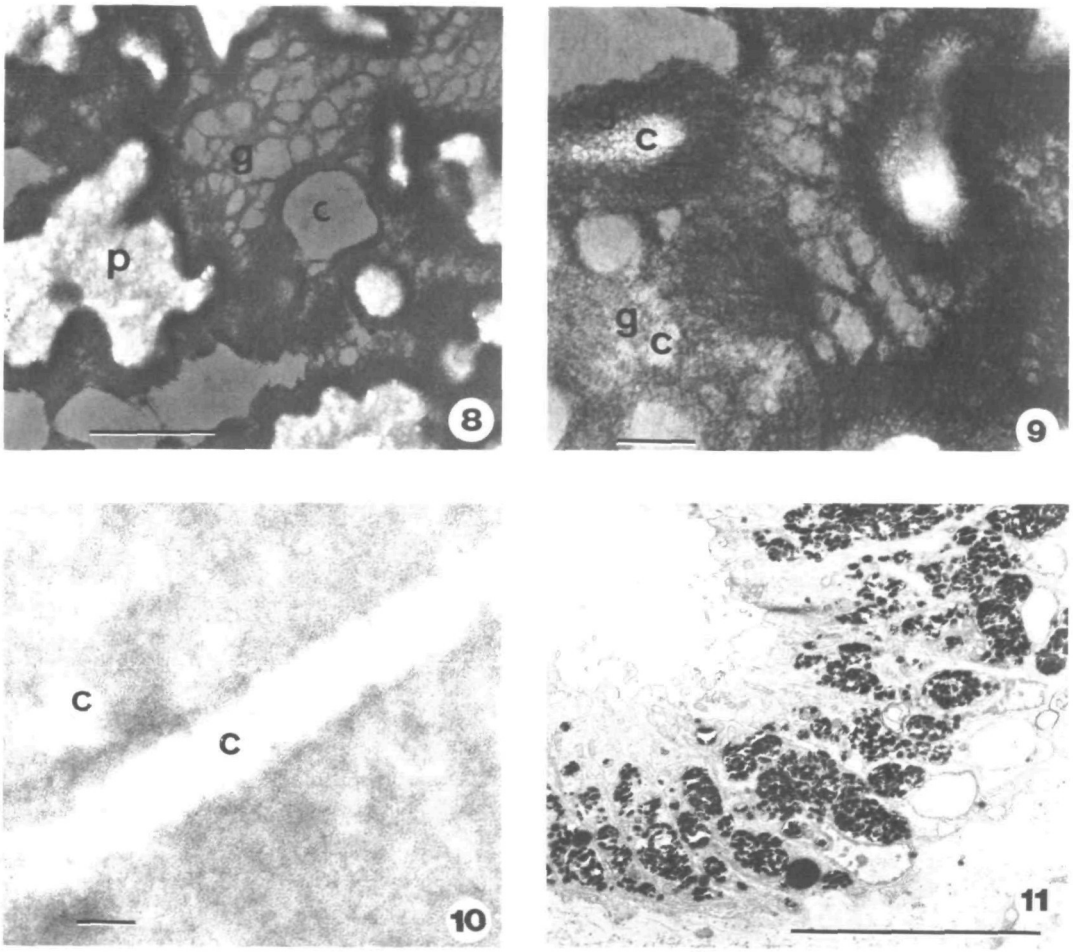

FIG. 8. Glycosaminoglycan matrix (g) and proteinaceous matrix (p) in the mineralized layer revealed by acridine orange method and staining with uranyl acetate. TEM. *G. pyramidata.* Scale bar = 1 μm. c represents areas occupied by apatite crystals before staining.

FIG. 9. Similar to Figure 8 but at higher magnification, showing glycosaminoglycan fibers (g) filling the interstices of acicular crystallites of apatite (c). *G. pyramidata.* Scale bar = 0.2 μm.

FIG. 10. Glycosaminoglycan matrix in the chitin layer revealed by acridine orange method and stained with uranyl acetate. c, areas occupied by apatite crystals before staining. *G. pyramidata.* TEM. Scale bar = 0.1 μm.

FIG. 11. Columnar cells at the anterior edge of the outer mantle epithelium of *G. pyramidata* showing numerous electron-dense granules in vacuoles. TEM. Pb stain. Scale bar = 10 μm.

nucleus. The apical plasma membrane is smooth and almost no microvilli are present. The salient feature of these cells is the numerous electron-dense granules, approximately 0.1–0.5 μm in diameter, enclosed in large vacuoles which occupy the major portion of the cytoplasm (Fig. 11). A number of mitochondria are located at the apical, and at times at the basal, portion of the cells. Not many other organelles are discernible except for ribosomes, glycogen granules and a few GER which are scattered in the cytoplasm.

By X-ray microanalysis the granules are shown to contain phosphorus, sulfur and very frequently, calcium. The granules give distinct patterns by electron diffraction, and appear to be mixtures of varying amounts

of apatite minerals such as brushite, octacalcium phosphate, etc. However, their exact nature is not firmly established. The granules disappear by uranyl acetate staining and/or strong bombardment of electrons under the electron microscope. Some of the granules undergo natural dissolution leaving organic material in the vacuoles (Fig. 12).

The connective tissue underlying these cells is also populated by electron-dense granules, up to approximately 50 μm in diameter, in addition to the collagen fibers which are common to this type of tissue. These granules also contain P, S, and Ca. Similar to those in the epithelial cells, many are dissolved by uranyl acetate staining.

2. Cuboidal cells

The cell decreases in height and becomes cuboidal in the epithelium adjacent to the anterior edge. This is the region which forms the primary shell layer as is shown by isolated crystals at the early stages of spherulite formation (Fig. 13). The large vacuoles which contain granules are also present. Cell organelles are scanty. Only a few mitochondria, GER, and free ribosomes are observed.

3. Squamous cells

Approaching towards the anterior region squamous cells are localized. They are laterally interdigitated and the epithelium has a pseudostratified appearance. Two types of cells are present. One has a relatively smooth apical cell surface, is situated closer to the anterior mantle edge and is associated with the mineralized layer (Fig. 14). Its cytoplasm contains a few GER, mitochondria, lipid bodies, and lysosomes, a number of ribosomes and tonofilaments. Granule-containing vacuoles are present but their number is very few. The second type of cell is located more towards the posterior portion of the mantle and appears to form the chitin layer (Fig. 15). The apical cell surface has many small microvilli in which bundles of tonofilaments terminate. The cells are characterized by well-developed GER, abundant mitochondria, and numerous free ribosomes. Vacuoles with mineral granules reside sporadically within the cells.

PROBLEMS CONCERNING PHOSPHATIC SHELL FORMATION

The atremates must concentrate phosphate as well as calcium in order to form the apatite shells. The major route of Ca ion uptake is probably similar to that of marine molluscs, *i.e.*, directly from the sea water through the body surface (see Wilbur and Saleuddin, 1983). In molluscs, the gill and the mantle are the main organs involved in this uptake (Tanaka and Hatano, 1955). No information is available on Ca uptake in brachiopods; however, the lophophore could be one of the sites of uptake because this organ is shown to accumulate ^{45}Ca in *Glottidia* (Pan and Watabe, unpublished).

Phosphate uptake could be directly from seawater or indirectly from food. If it occurs through the direct route, the atremates must have an extremely high efficiency for phosphate accumulation because the phosphate concentration in seawater is quite low. Regardless of their method of uptake the atremates appear to have the ability to maintain large storage of shell mineral components. The presence of P- and Ca-containing granules in the mantle epithelial cells in the regions of primary and mineralized layer formation; the unstable characteristics of these granules; and their frequent disappearance from the vacuoles all strongly suggest that the minerals in the granules are utilized for the shell apatite formation. The connective tissue underlying the anterior mantle edge epithelium is apparently high in Ca^{2+} and PO_4^{3-} ions because precipitates containing Ca and P are also present in this tissue. The mechanisms of transport of these ions into the vacuoles, their precipitation as granules, dissolution and further transport onto the shell surface and final crystallization as apatite are virtually unknown.

The atremates belong to the few invertebrates which concentrate relatively high amounts of fluoride in their skeletons. Others are some gastropods and arthropods (Vinogradov, 1953; Lowenstam and

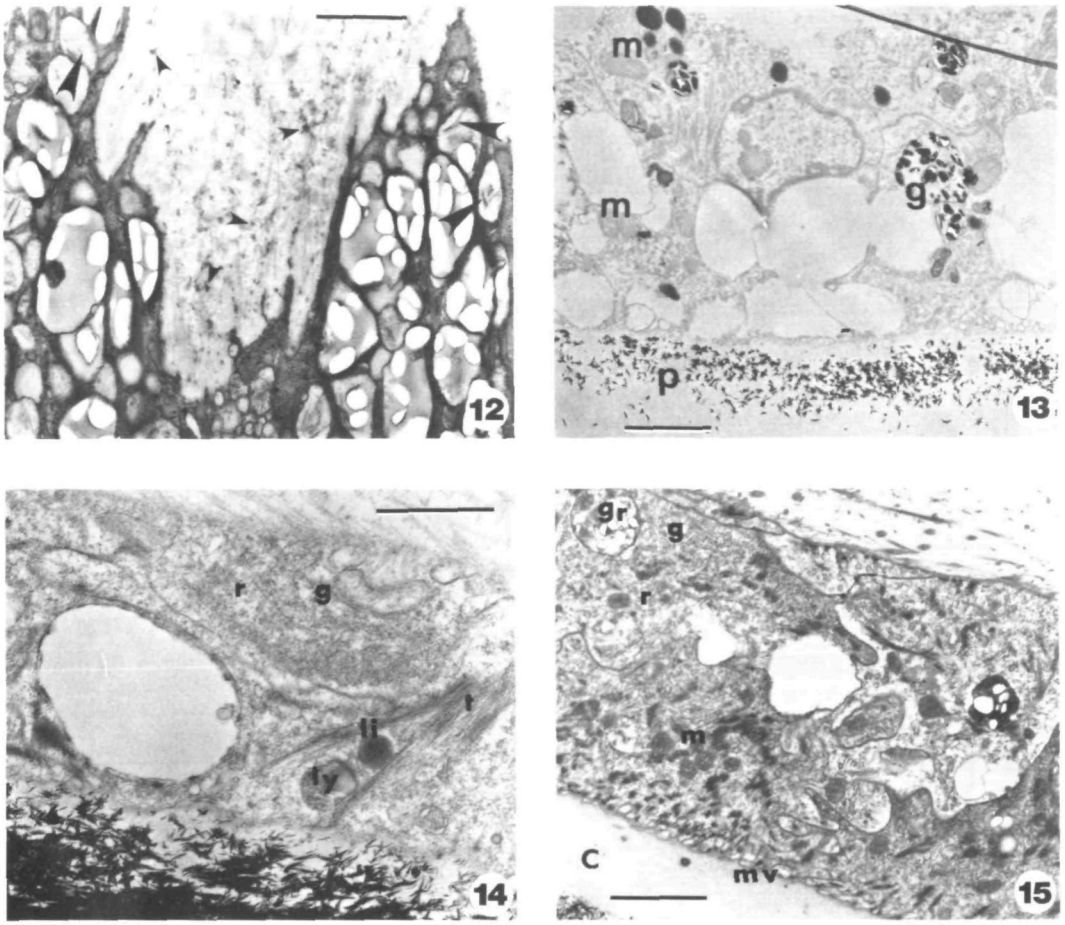

FIG. 12. Basal portion of the columnar cells shown in Figure 11. The section was stained with uranyl acetate and Pb, thus the electron-dense granules have been dissolved leaving empty spaces. Natural dissolution of the granules is evident by the presence of organic matrix (large arrowheads) in the vacuoles. Small arrowheads indicate small electron-dense granules in the connective tissue. Scale bar = 1 μm.

FIG. 13. Cuboidal cells in the mantle epithelium adjacent to the mantle edge forming the primary shell layer (p). g, electron-dense granules; m, mitochondria. TEM. Pb stain. *G. pyramidata*. Scale bar = 2 μm.

FIG. 14. Squamous cells with smooth apical surface forming a mineralized layer. g, GER; li, lipid; ly, lysosome; r, ribosomes; t, tonofilaments. TEM. Pb stain. Scale bar = 1 μm.

FIG. 15. Squamous cells with small microvilli (mv) forming a chitin layer (c). g, GER; m, mitochondria: r, ribosomes, gr, electron-dense granules. TEM. Uranyl acetate and Pb stain. *G. pyramidata*. Scale bar = 2 μm.

Weiner, 1983). The process of fluoride fixation may be different in each example, and in the case of the atremates it could be crystallographic rather than physiological factors which control the fluoride content. This problem needs to be investigated in order to fully understand apatite shell formation in this organism.

The shell surface of *Glottidia* is marked with many growth lines. A specimen, 2 cm in length from the anterior to posterior ends of the shell, has approximately 1,000 such lines (Pan and Watabe, unpublished). However, the number of shell layers is approximately one dozen and no sub-layers are recognized in the shell. Thus, the

periodicity of periostracum layer formation is more frequent than that of the shell layer formation. There are no data available regarding the growth rate of shells in *Glottidia*, and it is rather difficult to obtain an age estimation. However, judging from our culture data, a 2-cm shell may be at least 1 year old. If so, one shell layer would form per month. Even if the shell is 6 months old, which is most unlikely, only two layers would form in a month. This rate is much lower than that of molluscs in which daily or sub-daily layer formation is common (see Rhoads and Lutz, 1980). This extremely low frequency of shell formation may be attributed to the very low rate of metabolism of the atremates (Hammen, 1977). In *Glottidia*, the formation of one layer may continue at an extremely slow rate over a month. Alternatively, the layer formation may take place in a relatively short period of time, followed by a long period of recess until the next layer formation begins. These problems should be investigated in conjunction with other physiological rhythms in these organism.

The *Glottidia* (and also *Lingula*) shell shows an alternate deposition of mineralized and chitin layers. This implies that the epithelial cells of the mantle change their function periodically even on a limited scale. Yet so far, we have not been able to observe these changes in the epithelium.

The problems presented in this review cover only a few of the many aspects of atremate brachiopod shell formation. Other areas needing investigations are: endogenous and exogenous control of shell formation; shell dissolution; structural and chemical transformation of mineral and organic components during and after the shell formation; and incorporation of minor and trace elements into the shell.

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