ULTRASTRUCTURE AND MINERALIZATION OF THE SHELL OF
*Lingula unguis* Linne, (INARTICUALTE BRACHIOPOD)

by

Keiji Iwata

(with 6 text-figures, 1 table, and 14 plates)

Abstract

Ultrastructure of the shell of *Lingula unguis* Linne, an inarticulate brachiopod, was studied mainly by electron microscopy to investigate mineralization of the shell. *Lingula* has a chitino-phosphatic shell, which consists of the periostracum, alternations of organic and mineralized layers, and the punctae. The main part of the periostracum shows a striped pattern almost perpendicular to the shell surface and covered by a triple-layered membrane. Showing an elaborate reticulated pattern the organic layer is made up of fine chitinous fibrils. The mineralized layer thins out antero-posteriorly and laterally. Not uniformly mineralized, it shows different grading zones of crystallites at different parts of the shell, as distinguished in the following: amorphous particles or minute granular crystallites; acicular crystallites which are arranged subparallel or intersected at a low angle; coalescent acicular crystallites which intersect irregularly. The organic matrix of the mineralized layer consists of fine proteinous fibrils without periodicity. Cytoplasmic projections of the epithelial cells intrude the punctae, and the distal ends of them adjoin the inner wall of the punctae by a net of fine threads.

X-ray diffraction analysis revealed that *Lingula* shell consisted of carbonate fluorapatite which was similar to francoilite. Detected from an electron probe analysis were Ca, P, Mg, F, S, and Na. Amino acid analysis of the shell showed a large amount of alanine and glycine, as well as the characteristic presence of hydroxyproline. A histochemical test of specimens for an organic matrix showed a similarity to collagen, but the ultrastructure of it differed from the typical collagen fibrils.

Introduction

Among recent and fossil invertebrates inarticulate brachiopods possess phosphatic shells except those which have calcium carbonate shells, namely, obolellids, trimerellaceans, and cranieans. From a fossil record it follows that phosphatic mineralization of the shells in inarticulate brachiopods has continued since the Cambrian. Although inarticulate brachiopods flourished particularly in early Paleozoic, many of them declined or disappeared by late Paleozoic Era. Some descendants of Lingulida and Acrotretida (Lingulidae and Discinidae), however, have survived to date since the Ordovician. *Lingula* is well known as a living fossil in light of its long phylogenetic history, having a wide distribution especially in warm waters. The infaunal habit of lingulids is recorded, as seen from strata of various ages since the Silurian (McKerrow, 1978). *Lingula* spends a planktonic life during their early ontogeny, its soft body being covered by a chitinous membrane. Subsequently it sinks to the bottom and secretes a chitino phosphatic shell.

A description of the shell structure of *Lingula* was first given by Gratiolet (1860), in which alternations of the corneous (chitinous) and the calcareous layer were observed and short prisms in the latter were described. Alternations were also described by Chapman (1914). Projections of a mantle epithelium extending into the shell were described by
It was demonstrated by Williams and Rowell (1965) that the mineralized layer consisted of extremely fine lamellae which were subparallel or slightly inclined to the shell surface, and pointed out that Gratiolet's prisms were probably mistaken for the punctae which were developed in the mineralized layer. Their suggestion was that many of the punctae were cytoplasmic strands rather than caeca which were found in some punctate articulate brachiopods, and also that alternations of the shell layers had to be formed by a secretory alternation of organic and phosphatic materials at any point of the epithelium. Minerals of the shell of Lingula were studied by Klement (1938), Vinogradov (1953), and McConnel (1963); inorganic composition of the shell was reported by Logan and Hunt (1854), Clark and Wheeler (1922), Vinogradov (1953), and McConnel (1963); amino acid composition of the shell was reported by Jope (1965).

Despite their efforts, details of the shell structure remain poorly known. Accordingly, this study looked into ultrastructure and mineralization of the shell, by electron microscopy in observation of Lingula unguis specimens and analyzing minerals by X-ray diffraction and electron probe method, as well as analyzing the shell protein, whereby a structural basis was obtained for a comparison among recent and fossil inarticulate brachiopods and other invertebrates.

**Materials and methods**

A total of 40 specimens of Lingula unguis Linne (Text-fig. 1) ranging from 1.5 to 3.5 cm in shell length were collected during the lowest spring tide (Oshio), September of 1979, at a point 1.5 km off the sea coast of Sumiyoshi of Uto City, Shimabara Bay, Kyushu, South Japan. It has an infaunal habit and burrows 10–20 cm in the muddy or sandy bottom, making cylindrical, curved, or U-shaped holes. With tough and flexible pedicles it sticks on the base of the holes, making concretions which are cemented by a mucous and sand grains or muds at the distal end of the pedicles. Dead mollusc shells are also used as a ground mass for pedicle fixation. This species dwells in the sediment not in a scattered manner, but rather in a crowded manner. It coexists with some pelecypods such as Meretrix lusoria and Ruditapes philippinarum, and polychaetes. A filter feeder, it has the stomach
abounding in diatoms. It inhabits even in the brackish environment near the mouth of a river in Shimabara Bay, and the fact showing that *Lingula* has a wide physiological tolerance against low salinity. It endures an agitation of the bottom sediment during a storm (typhoon), and also survives for a time under a condition of drying up during low tides. *Lingula unguis* was kept alive in an aquarium until experimental work. For a comparison *Lingula reevei* (Davidson), Oahu Island, Hawaii, was also observed.

Optical microscopy and scanning electron microscopy

A shell was separated from the soft body and embedded in epoxy resins. A thin section was observed under the binocular and polarized microscope. Some sections were demineralized with 0.5M EDTA, and stained with hematoxyline, eosine, van Gieson, Azan, Maroly dyes for examination of a histochemical property of organic materials. Polished specimens were observed under the reflective microscope.

A block of a shell specimen embedded in epoxy resins was cut by a diamond wheel and polished with alumina powder. After demineralization by EDTA for 10 minutes or removing organic materials with 10% hypochlorite solution for 10–20 minutes, specimen was coated with gold in an evaporator and observed under the scanning electron microscope (SEM), JEM U3. Fractured and etched specimens and internal surfaces which were untreated or treated with hypochlorite solution were also observed. Some of the polished specimens were coated with carbon, and a line profile analysis of Ca-Kα and P Kα by an electron microprobe analyzer (EPMA), SMU_3-SDS, was carried out in a part of the shell (See Pl.3, Fig.1) at 15 KV and 25 KV.

Transmission electron microscopy

Without separating a mantle tissue a shell was fixed with 1% glutaraldehyde in cacodylate buffer at pH 7.4 for one hour at 4°C, and post-fixed with 1% OsO₄ in the same buffer for one hour. After dehydration with ethanols this sample was embedded in epoxy resins, and then thin sections were obtained by ultramicrotomy, using a diamond knife. Some specimens were demineralized with 0.5M EDTA and embedded in epoxy resins similarly. Prior to observations some sections were stained with uranyl acetate and lead citrate, and others were not stained. Thin sections were subjected to a test to see if organic materials are digested by chitinase, pronase, and collagenase, using transmission electron microscope (TEM), JEM 120 U.

Identification of shell minerals

The shell of *Lingula* was powdered in agate mortar after immersion in 10% hypochlorite solution for one hour to have excess organic materials removed, and then analyzed with an X-ray diffractometer. A shell specimen was heated in an electric furnace at about 500°C for one hour and a powdered specimen was also analyzed for a comparison with it.

Amino acid analysis

After removing the periostracum and muscle fibers, thick mineralized layers of the central part of the shell (3.5 cm) were demineralized with EDTA and analyzed with an
automatic amino acid analyzer after hydrolysis with 6 N HCl, 104°C, for 24 hours under a reduced condition. An unmineralized shell margin and a dead shell (2.5 cm) were also analyzed for a comparison.

**Results**

**Ultrastructure of the periostracum**

The shell of *Lingula* is covered by an organic periostracum (Text-fig.1-c,d), the color of which is green, darkens as the shell grows. Black or brown iron minerals often adhere on the periostracum. It was well stained by eosine and picric acid. It was not digested by pronase, chitinase, and collagenase. Its thickness varies with the part of the shell, about 10μ in the central part (Pl.I, Fig.1). At the shell margin the thickness of the periostracum decreases below one-third (Pl.5, Fig.2). The periostracum was observed structureless under the optical microscope (Pl.1, Fig.1) and scanning electron microscope (SEM), but under the transmission electron microscope (TEM) a characteristic light and dark striped pattern was observed in the main part of it (Pl.6, Fig.2). These stripes, composed of fibrils of less than 100 Å and cementing materials, are arranged nearly perpendicular to the shell surface, to some extent with a periodicity of about 500 Å. However, in some parts they are branched and bending (Pl.6, Fig.2). The striped pattern was clearly observed in a fully hardened periostracum from the apex to the vicinity of the shell margin, but became obscure from the shell margin to the periostracal groove (Pl.5, Figs.1,3) which lay in the inner epithelium of the mantle. The main part of it is covered by a triple-layered membrane of about 700 Å in thickness (Pl.5, Fig.2), and amorphous organic materials (probably mucopolysaccharide) often adhere on it. Vacuoles, mucin inclusions, and mineralized materials were not observable in it. The inner surface of it is very often undulated. Williams (1968) reported that the periostracum of *Notosaria* and *Waltonia*, which were articulate brachiopods, consisted of mucopolysaccharide, an outer fibrillar triple layered membrane, mucoprotein, an inner fibrillar triple layered membrane, vacuoles, and mucin inclusions. The periostracum of *Lingula unguis* differs from those periostra of articulate brachiopods in ultrastructure and construction. Such a striped pattern of the periostracum is uncommon in brachiopods and other invertebrates.

**Alternation structure**

The shell of *Lingula* lacks a myostracum and consists of thin consecutive alternations of the mineralized and the organic layers (Pl.1, Figs.1-4; Pl.2, Fig.1; Pl.3, Figs.1-3). Similar alternations were also observed in the shell of *Lingula reevei* (Pl.2, Fig.2). However, the apex and the shell margin of the *Lingula* shell were not mineralized (Pl.1, Fig.2), and no alternations were observed. In a strict sense this shell cannot be divided into the outer (primary), the middle (secondary), and the inner (tertiary) shell layer. The thickness of the shell is the largest near the central part of the shell which the body cavity occupies, and decreases towards the apex and the shell margin. The mineralized and organic layer are deposited subparallel to the shell surface, but they slope down to the direction of the apex and the shell margin. Thickness of the alternations varies vertically and horizontally, and the
mineralized layer thins out anteroposteriorly and laterally. Some of the mineralized layer begin from various regions of the shell (Text-fig.2; Pl.1, Fig.1; Pl.3, Fig.1). Therefore, shell layers are deposited as a thin lenticular body and overlap vertically. Nevertheless, some of the mineralized layers extend considerably wide (beyond a half of the shell) to the growth direction. It is often not easy to trace a continuity of the mineralized layer under the optical microscope, because some of them are fairly thin especially at their margins. The number of alternations increases as the shell grows. Under the SEM, TEM, and EPMA, 6-7, 12-14, 16-17 pairs of alternations were observed in several specimens of 1.5, 2.5, 3.5 cm in shell length respectively. *Lingula reevei* of 3.5 cm in shell length has 16 alternations, which are structurally similar to *L. unguis*. Alternations were also found in *Glottidia*, but details were different (Iwata, unpublished).

![Text-fig. 2 Stylized section of Lingula shell.](image)

**Text-fig. 2** Stylized section of *Lingula* shell.

- o.l.: organic layer
- p.l.: phosphatic layer
- p.: periostracum
- ep.c.: epithelial cells
- p.gr.: periostracal groove
- s: seta.

**Inner surface of the shell**

From the vicinity of the apex to the central part of the shell the inner surface is heavily mineralized in all specimens studied. In large specimens the mineralized zone occupies about two-thirds of the inner surface (Text-fig.1-a,b). Outside of this widely mineralized zone several pairs of the mineralized and the organic bands are exposed. In untreated specimens alternations of these bands were not so much clearly observed, but 4 or 5 alternations were vaguely observed in the specimens 2.5 and 3.5 cm in shell length. After treatment with hypochlorite solution for one hour, 4, 12, 11 alternations were observed in specimens of 1.5, 2.5, and 3.5 cm in shell length. In the specimens of 3.5 cm the decrease of alternations must be caused by burying of a few layers in the shell. The inner surface treated with hypochlorite solution does not always show the true number of alternations due to removal of organic materials. It is clear, however, that not only the central part but at least a few of alternations are actively formed at the peripheral region of the shell. Observations of the inner surface show a concentric accretion to the periphery and an inward deposition of shell materials at the overall region from the vicinity of the apex to the body cavity. Alternations exposing
themselves at the periphery of the inner surface reflect that the outer epithelium of the mantle is divided into a few zonations of cells which secrete phosphatic and organic layers simultaneously.

Ultrastructure of the organic layer

Consisting of only organic materials, the organic layer is usually called chitin layer. It was observed almost structureless under the optical microscope (Pl.1, Figs.1-4; Pl.2, Figs.1-2). It was stained well with eosine. It was stained red with Azan and Maroly dyes and yellow with a van Gieson dye. Therefore, it is clear that this layer is composed of acidophilic organic materials. The organic materials were not attacked by organic solvents and weak acids, and it is fairly difficult to attack it by chitinase and collagenase. Therefore, “chitin” may be masked by other organic materials such as scleroprotein. Under the SEM this layer was a structureless compact layer without any treatment by chemical reagents, and it was difficult to observe a fine structure under the SEM.

Free from any staining this layer was also structureless under the TEM (Pl.7, Fig.1). After staining it could be clearly distinguished from the organic matrix of the mineralized layer as an electron dense layer (Pl.7, Fig.2; Pl.12, Figs.1-3; Pl.13, Fig.1). This layer consisted of organic fibrils of less than 100 Å in width, and showed a reticulate pattern (Pl.6, Fig.1). Organic fibrils were observed as bundles of fine filaments of about 30 Å from negative staining of phosphotungstic acid (Pl.6, Fig.3). The reticulated pattern was caused by many openings of 100-400 Å in diameter (Pl.6, Fig.3). Circular or somewhat elongated, the openings were distributed irregularly in the organic layer except punctae regions. They were not exactly vacant, and one or two organic filaments were bridged across the openings (Pl.6, Fig.3). Such a reticulated ultrastructure of this layer is different from the organic matrix of the mineralized layer and cuticular tissues in arthropods, which are composed of chitin-arthropodin fibrils. A similar pattern was observed in the organic layer of L. reevei and partly in Glottidia (Iwata, unpublished). A boundary between this layer and the mineralized layer showed a straight and discrete line, but an inner boundary was undulated and showed a gradual transition into the mineralized layer (Pl.7, Fig.2; Pl.12, Figs.1-2). Coalescent acicular crystallites were often deposited in the outermost region of the organic layer (Pl.7, Fig.1). The punctae intrude in this layer, as described below concerning the details.

Ultrastructure of the mineralized layer

A mineralized layer alternates with an organic layer, forming a thin intercalation in which a large number of punctae develop. The mineralized layer was observed almost homogeneous under the binocular and polarized microscope, and it was fairly difficult to distinguish boundaries and orientations of crystallites even at high magnification. Under the SEM it was also difficult to observe details of the crystallites owing to their minuteness (Pl.3, Figs.1-2; Pl.4, Fig.1). After removing an organic matrix with hypochlorite solution, the orientation of the acicular crystallites of B-zone (stated below) could be observed (Pl.4, Figs.2-3). To make a more detailed observation thin sections were observed under the TEM.

From many observations of different parts of the shell it became clear that mineralized layers were not composed of crystallites of the same size, morphology, and orientation.
Terms of A-, B-, and C-zones are described for different styles of depositions of crystallites at different parts of the mineralized layers. The schematic figure of these zones were illustrated in Text-fig. 3. All of these three zones did not always occur in the same layer, and, especially, C-zone lacked in some layers. Usually A- and B-zones accompanied in most layers, but the peripheral part of the outermost layer consisted only of a modification of B-zone. When three zones were present, C-zone always lay on the upper part of the layers and followed A- and B-zones. When A- and B-zones were present, A-zone was deposited above B-zone. From this fact a grading tendency of crystallites is evident from the upper to the inner part of a given mineralized layer.

**A-zone**

This zone was the most prevalent in the mineralized layers and occupied the main part of the layer. This zone consisted of acicular or needle crystallites of 300-400 Å in width, 1000-1500 Å in length, 200-300 Å in thickness. To some extent the crystallites varied in inclinations to the shell surface with the part of the shell (Pl.8, Figs.1-3). They were arranged subparallel to the shell surface or inclined at a very low angle. In a well developed part of this zone the crystallites intersected together at a low angle. However, the intersection of them did not form any particular bundles or blocks and other architectural ordering. In some layers this zone was situated below C-zone and above B-zone. Transition from C- to A-zone was observed very often gradual, but transition from A- to B-zone showed a more or less sharp boundary.

**B-zone**

This zone consisted of acicular crystallites of 500-800 Å in width, 1000-2000 Å in length, 300-400 Å in thickness. They are larger than those of A-zone and are deposited irregularly. Some of them are intersected at various angles (Pl.7, Fig.1; Pl.9, Figs.1-4). At
high magnification the crystallites were observed to be formed by a coalescence of thinner needle crystallites (Pl.10, Figs.1-2). Size and such a lath-like morphology, and irregular mode of deposition of this zone can be easily distinguishable from A- and C-zones. Especially near the shell margin of the outermost layer the crystallites were scattered within organic materials at an interval (Pl.9, Fig.2). A part of this zone often enters the outer region of the organic layer (Pl.7, Fig. 1). At the inner surface in the central part of the shell an irregular deposition of this zone was clearly observed (Pl.4, Fig.3).

C-zone

This zone consists of closely packed grading particles of amorphous crystalline materials and granular crystallites, ranging from less than 100 Å to about 500 Å in diameter (Pl.7, Fig.3). This zone gradually changed into A-zone and the grading tendency of the crystallites was clearly observed. This zone was usually thinner than the other two zones and did not form a single shell layer at all. It was also observed thinning out near the terminal region of the mineralized layer. Deposited on the uppermost region of the mineralized layer, this zone was rarely intercalated in B-zone.

Consequently, A-, B-, and C-zones may be called briefly amorphous particles or minute granular crystallites, acicular crystallites which are arranged subparallel or intersected at a low angle, and coalescent acicular crystallites which intersect irregularly, respectively. In *Lingula reevei* A- and B-zones were present, C-zone not being clearly observed in the present observation. The arrangement of crystallites in A- and B-zones was not completely the same between *L. unguis* and *L. reevei*. Therefore, a slight difference in the mineralized layer may be present between the two species. The ultrastructure of the mineralized layer of *Glottidia* is different from that of *L. unguis* and *L. reevei* (Iwata, unpublished).

Mineralization front

Intracellular mineralization was not observed in this study, while initiation of the mineralization took place within an extra-pallial fluid (Pl.14, Fig.2). Amorphous crystal nuclei were generated in the intermediate region of the extra-pallial space, but they were not easily distinguishable from the precursors of the organic matrix. They grew into minute crystalline particles in an almost amorphous mucous matrix, and further into rod or short needle-like crystallites around a mucous and fairly fine filamentous matrix. Growing crystallites were deposited subparallel or oblique to the inner surface. A coalescence of needle-like crystallites was also clearly observed at the inner surface of the shell (Pl.10, Fig.2).

Ultrastructure of the organic matrix

The mineralized layer includes EDTA insoluble organic materials. Under the optical microscope they were stained blue by Azan and Maroly dyes, and yellow by van Gieson dye respectively. This histochemical attitude suggested a similarity to collagen which was the major organic matrix of the phosphatic hard tissues in vertebrates. The organic matrix of the mineralized layer showed a remarkable histochemical contrast against the organic layer. Under the TEM the organic matrix was easily distinguished from the organic layer owing to
differences in electron density and ultrastructure. The organic matrix consisted of fine fibrils of about 100 Å in width (Pl.11, Fig.1). They seemed to be composed of thinner filaments of about 30 Å. In A-zone they were oriented subparallel or slightly inclined to the shell layer (Pl.11, Fig.1), while in B-zone they were irregularly inclined to the layer. Therefore, it may be said that fibrils of the organic matrix run nearly parallel to the long axes of the acicular crystallites. In C-zone only minute openings of grading size were observed among organic fibrils. The fibrous matrix in any zones lacks an axial periodicity, while is greatly different from collagen fibrils found in the connective tissue of the mantle (Pl.11, Figs.2-3). Small angle dispersion electron diffraction failed to detect an axial periodicity. The collagen fibrils of the connective tissue have an axial periodicity of about 500 Å. The organic matrix was not digested by collagenase, pronase, and chitinase.

Ultrastructure of the punctae and their histological relation to the mantle epithelium

The shell of Lingula unguis abounds in minute tubules or punctae. The punctae are not mineralized and develop nearly perpendicular to the inner surface of the shell. They penetrate the mineralized layer and become slender in the organic layer. They thin out in the middle part of the organic layer. Observed sinuous, the punctae were much taller in the thickly deposited part of the shell near the central part than in other parts (Pl.2, Fig.1). However, any branching of the punctae was not found. The punctae were not found in the apex (Pl.1, Fig.2) and the shell margin which were both unmineralized. In dead shells most of the punctae were observed vacant, but in living shells very thin strand-like materials were often observed under the optical microscope. Undemineralized and demineralized thin sections were observed under the TEM to study the ultrastructure of the punctae and the relation between them and the mantle epithelium.

Text-fig. 4 The relation between punctae and epithelial cells of the mantle.
p.l.: phosphatic layer o.l.: organic layer ex.fl.: extrapallial fluid ep.c.: epithelial cells.
The punctae in the outer and the middle part of the shell were closed; so, their direct relations to the mantle epithelium could not be observed. However, all of them were not vacant, being filled with fine organic threads of about 100 Å, which were observed like webs or networks (Pl.12, Figs.2-3). Rarely, some punctae included organic granules of 0.05-0.2μ in diameter (Pl.12, Fig.1). Meanwhile, the punctae which faced against the mantle epithelium were always open and extended to the organic layer lying above. The punctae thinned out in the middle part of the organic layer and did not penetrate more than two mineralized layers. Most punctae were about 1μ in diameter, but slender ones were also found. From the observation of the mantle-shell interspace it became evident that cytoplasmic projections of the epithelial cells of the mantle intruded the punctae and occupied the interiors of the punctae (Pl.13, Figs.1-2; Pl.14, Figs.1-2). The intruding mode of the cytoplasmic projections was not the same in all punctae and the width of the projections varied in punctae. The straight and the top-heavy types of the projections were observed. Cytoplasmic projections were not always present in all epithelial cells, but lacking in some cells. The epithelial cells of the mantle were columnar shaped and cytoplasmic projections protruded from the central or the near marginal region of the cells. Inside the cytoplasmic projections many small vesicles and granules were observed (Pl.14, Fig.2). Crystalline materials were not observed within the punctae. At the distal ends of the punctae, especially in the organic layer (often in the mineralized layer), webs or networks of fine threads which were very similar to those of the above mentioned closed punctae filled the punctae like “rootlets” or “anchors” (Pl.13, Fig.1), adjoining the cytoplasmic projections.

Mineral identification and EPMA analysis of the mineral

An X-ray diffraction analysis of the mineral in the shell of Lingula unguis showed from the pattern that the mineral was different from hydroxyapatite which was the chief mineral phase of bones and teeth of vertebrate animals, as well as revealing that the mineral consisted of one crystalline phase of fluorapatite which was similar to francolite and that impurities including calcium carbonate, calcium sulphate, and other phases of minerals were not present (Text-fig.5-a,b). The X-ray diffraction pattern of unheated shell materials showed a broader peak (Text-fig.5-a) than a heated one (Text-fig.5-b). Heating caused a change in crystallinity, but did not cause a transformation of fluorapatite into other phases of minerals at about 500°C. The diffraction pattern of heated samples resembled those of fossil lingulids (Iwata, unpublished). Carbon dioxide was released from the mineral dissolved with HCl, but the amount of carbon dioxide seemed small.

An EPMA analysis of the mineralized layers showed presence of Ca, P, Mg, F, S, and Na. Iron minerals and clay minerals often adhered on the shell surface, but Fe, Al, and Si were not detected from this analysis of shell interiors. A line profile analysis of a part of the shell (See Pl.3, Fig.1) showed 17 alternations of the mineralized and the organic shell layers. While, a pattern of line profile analysis of Ca-Kα and P-Kα was found fairly consistent, and an elemental map of Ca-Kα showed a homogeneous distribution of it in the mineralized layer (Pl.3, Fig.3). It followed from a preliminary quantitative analysis that among impurity elements the Mg was relatively high and the fluoride content exceeded more than 1%.
Amino acid analysis

The shell of Lingula contains organic materials of about 40% of the total in dry weight. About a half of them consists of protein, while the remnants include hexosamine and a small amount of lipids. The mineralized and the organic layers were so thin and firmly combined together that much difficulty was involved in separating and analyzing amino acids contained in each of the layers. This paper showed in Table 1 results of the amino acid analysis of the mineralized layers including thin intercalations of some organic layers (but mostly mineralized layers) near the central part of the shell (living and dead shells) and the unmineralized shell margin of the living shell.

The protein of the shell of Lingula contained such a large amount of alanine and glycine that they occupied more than one-third of the total amino acids. Following them in amount were acidic amino acids (such as aspartic acid and glutamic acid), proline and valine. Characteristically, hydroxyproline was present in amount of 2%, hydroxylysine being
undetected. The amino acid composition of the shell protein in the mineralized layer (Table 1-1) and the unmineralized shell margin (Table 1-3) was fairly similar to the foregoing in major constituents. Between living and dead shells a difference was recognized in amounts of alanine and glycine most likely due to inclusions of punctae in the former, a difference in thickness of mineralized or organic layers, or both as well as in size of two specimens.

**Discussion**

The shell of *Lingula unguis* consists of the periostracum, alternations of the mineralized and the organic shell layers, and the punctae. Previous studies on the shell structure of *Lingula* having been all based on observations under the optical microscope, studies using an electron microscope have not yet been reported. The shell structure of *Lingula* was first described by Gratiolet (1860), who observed alternations of the corneous (chitin) and the calcareous layer and described short prisms in the latter. Chapman (1914) and Blochmann (1900) also described alternations of the two layers; Blochmann (1900) demonstrated that projections of the mantle entered the shell. Williams & Rowell (1965) demonstrated that the mineralized layer consisted of extremely fine lamellae which were subparallel or slightly inclined to the shell surface, and pointed out that Gratiolet's prisms were probably mistaken for the punctae which developed in the mineralized layer. They suggested that many of the punctae were cytoplasmic strands rather than caeca, which were found found in some punctate articulate brachiopods. In spite of these studies, the limited resolution of optical microscopes did not allow a further clarification of details of the shell structure of *Lingula*. 

Text-fig. 6 EPMA line profile pattern of *Lingula* shell. See Plate 3, figure 1.

(a) Analyzed at 15 KV. (b) 25 KV. Left side of this profile showing inner layer of the shell.
Thus, this electron microscopic study led to the following results:

(1) The mineralized layers consists of minute crystallites of calcium phosphate which show differences in size, morphology, and mode of arrangement at different parts of the shell. Distinguishable are three zones of crystallites: amorphous particles or minute grading granular crystallites (C-zone); acicular crystallites, which are deposited subparallel to the shell surface or intersected at a low angle (A-zone); coarser coalescent acicular crystallites, which intersect irregularly (B-zone). Most of the mineralized layers accompany A- and B-zones, but any one or two of the three zones are absent in some parts of the shell. The crystallites tend to show a grading size from the upper (smaller) to the inner (larger) part in most of the mineralized layers.

From this fact it may be assumed that deposition and mineralization of the shell do not continue in the same manner during the ontogenetic period, even in the time of formation of a single mineralized layer. The ultrastructural difference of these zones of the crystallites derives naturally from a fluctuating condition of mineral deposition. Other likely reasons are changes in such factors within extra-pallial fluids as include ionic concentration, pH, mucous precursor of an organic matrix, rate of deposition as well as temperature and other environmental conditions.

(2) Rhythmic alternation of the mineralized and the organic layer is not common in the shells of brachiopods and other invertebrates. Alternation is exemplified by Lingulida, but unknown in articulate brachiopods. Meanwhile, absorption of minerals during ecdysis causes
alternation of the calcium carbonate and the organic layers in cuticular carapaces of crustaceans. Little is known of the genesis of rhythmic alternation of the shell of *Lingula*. Williams & Rowell (1965) suggested that it must be caused by a secretory alternation of the organic and the phosphatic materials at any point of the mantle epithelium. *Lingula* presents no proof of a large absorption of minerals like crustaceans; so, it does not account for rhythmic alternation. It may be explained by a repetition of cycle of secretion of the mineralized and the organic layers from different zones of epithelial cells of mantle epithelium. The cells are lined in concentrical zonings which are roughly parallel to the shell margin. A morphological difference is not noted between the cells secreting mineralized layers and the cells secreting organic shell layers, but a little difference in ultrastructure between them seems present in respects of a quantity of vesicles, endoplasmic reticulum, etc. Clarification of details of a difference in secretory activity calls for a further ultrastructural study of secretory cells.

Planktonic larvae of *Lingula unguis* have unmineralized ("chitinous") protegula (larval shells), and after sinking to the sea, juvenile animals secrete first a mineralized layer. It is yet unknown how and when replacement of shell layers occurs. Functional change of epithelial cells at the general region and the peripheral region must occur more than 15 times throughout the ontogenetic period. From a cultivation experiment by Yatsu (1902) and Chuang (1961), *Lingula*'s longevity is known to be more than 5 years. If a cyclic deposition from secretory cells takes place annually or seasonally (for example, mineralized layer from spring to autumn; organic layer in winter), alternations of shell layers will imply an annual increment or seasonal growth. However, the exact time of replacement of the secretion and how many alternations are formed annually are yet uncertain; information on it is prerequisite to an examination for understanding the genesis and significance of alternations. It is also not easy to answer by what factors a change of secretory activity of epithelial cells is controlled, because Ca metabolic activity and relating physiology of this animal is known poorly. It is probable that hormonal control of secretion which is related to the deposition or termination of mineralization may be involved. Temperature and other environmental conditions may also be involved. A preliminary investigation of the shell ultrastructures of fossil lingulids suggests that relics of alternations are recorded in the specimens of the early Paleozoic in age. Further studies on the formation of this structure in recent species and comparative observations of fossil lingulids will be important for understanding the significance and the evolution of the structure.

(3) Punctae present in the shell of *Lingula* are very minute and clearly differ from those of articulate brachiopods in size and ultrastructure. Blochmann (1900) and Williams et al. (1965) suggested that projections of the epithelium entered the punctae. It is confirmed by the present electron microscopic observation. Slender cytoplasmic projections of the epithelial cells of the mantle protrude into the punctae of the innermost mineralized layer and extend to the organic layer lying above. At the distal ends of these projections webs or networks of fine organic threads are bound to the inner walls of the punctae. These organic webs or networks may serve as anchors or rootlets of the projections of the mantle epithelium. The formation process of the punctae is considered to begin at about the intermediate stage of formation of the organic layer and continue to be formed throughout
nearly all the period of deposition of the mineralized layer. The projections of the mantle epithelium must retreat inwards or may be broken after the formation of the mineralized layer.

Tubules or punctae are also known in the shells of other inarticulate brachiopods (Schumann, 1970), articulate brachiopods (Owen & Williams, 1969), pelecypods (Omori et al., 1969; and others), polyplacophora (Haas, 1972), and monoplacophora (Erben et al., 1968). The functional significance of the tubules and punctae among these invertebrates has been discussed by several authors, but it has not been fully understood. A few hypotheses concerning the function of the punctae and the tubules have been proposed: (a) a respiratory organ of the epithelium (cited in Zittel, 1937; Shibata et al., 1968) or an exchanging organ of materials from the mantle (Schumann, 1970; Shibata et al., 1968); (b) a sensitive organ especially serving for perception of light (Haas, personal communication, 1979); (c) a specialized mantle caecum which stocks mucoprotein and excretes it at the time of injuries of the periostracum (Owen et al., 1969); (d) an organ to serve as an adhesive function between the shell and the mantle (Shibata, 1977 MS). As in Lingula the punctae do not penetrate into the shell, hypotheses of (a)-(c) unaccountable for the function in point. From this study alone it is difficult to determine the functional significance of the punctae of the shell of Lingula. However it is probable that the projections of the mantle epithelium which protrude the punctae seem act as an auxiliary adhesive means between the mantle and the shell. For what purpose such an adhesive assistance is used or whether they are relict organs of other functions are still a matter of speculation. An experimental study of the punctae will be necessary. The punctae of lingulids seeming to date back to the early Paleozoic time, a study is required of an evolutionary history of the punctae.

(4) Klement (1938) and Vinogradov (1953) suggested that the mineral of the shell of Lingula was composed of hydroxyapatite. On the contrary, McConnel (1963) demonstrated that it consisted of francolite-like apatite. Results of this X-ray analysis supports the latter. A further chemical analysis and a crystallographical study will be necessary to understand the chemistry and the crystal structure of this mineral. Apatite minerals in hard tissues among recent and fossil invertebrates may be subdivided into some groups, but details should be studied making a comparison among them.

(5) The organic materials of the organic layer seems to contain mainly hexosamine and a small amount of protein. On the contrary, the organic matrix of the mineralized layer is supposed to consist mainly of protein. The presence of chitin in the organic matrix is not yet examined. From this result it is very difficult to distinguish the difference of the amino acid composition of the proteins between the mineralized and the organic layer. A histochemical test suggests that the organic matrix of the mineralized layer is somewhat similar to collagen. Large amounts of glycine and alanine, and the presence of hydroxyproline also suggests a biochemical similarity to collagen. The presence of hydroxyproline, however, does not constitute an absolute criterion for collagen, because the amino acid is extractable from elastin and some plants. The fibrils of the organic matrix differ from a typical collagen which has a characteristic periodicity. If the protein of the organic matrix belongs to collagen, it may be grouped into amorphous type of collagen. However, X-ray diffraction and other biochemical analyses of the organic matrix are necessary to determine whether they are true
collagen or belong to other categories of proteins. The presence of a collagen-like protein in the organic matrix of the shell of Lingula, anyhow, is of deep interest in comparison with vertebrates' hard tissues.

The mineralization of the shell of Lingula may be presumably related to the proteineous matrix. Formation of matrix vesicles, envelopes, and compartments was not yet affirmed by the present investigation. Epitaxial (or template) nucleation may be possible in this shell, and a biochemical study on Ca-binding systems of the organic matrix may favor this hypothesis. Mechanism of mineralization, however, must be studied in details. A change of mineralization during an ontogenetic period is also to be studied.

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References


ULTRASTRUCTURE OF LINGULA SHELL


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Explanation of plates,
Plate 1. Optical photomicrographs.
Fig. 1. Outer layers at the central part of the shell.
Fig. 2. Alternations at the apical region.
Fig. 3. Enlarged micrograph of Fig. 2.
Fig. 4. Middle layer at the central part of the shell.

Plate 2. Optical photomicrographs.
Fig. 1. Inner layer at the central part of the shell.
Fig. 2. Alternations of Lingula reevei.

Plate 3. SEM photomicrographs of the specimens of 3.5 cm in shell length.
Fig. 1. Low magnification image of alternations. 2 lines are line profiles by electron microprobe analysis.
Fig. 2. Reflective electron image of the alternating layers.
Fig. 3. Elemental map of Ca-Kα.

Plate 4. SEM photomicrographs.
Fig. 1. Fractured surface of two mineralized layers.
Fig. 2. Inner surface at the central part of the shell. Hypochlorite treated.
Fig. 3. Enlarged micrograph of Fig. 2.

Plate 5. TEM photomicrographs of the periostracum.
Fig. 1. Periostracum of shell margin in the specimen of 3.5 cm in shell length.
Fig. 2. Periostracum and unmineralized portion near shell margin in the specimen of 1.7 cm in shell length.
Fig. 3. Periostracum of near periostracal groove.

Plate 6. TEM photomicrographs.
Fig. 1. An organic layer stained uranyl acetate and lead citrate.
Fig. 2. Matured periostracum. Striped pattern remarkable.
Fig. 3. Reticulated pattern of an organic layer. Stained negatively with phosphotungstic acid.

Plate 7. TEM photomicrographs.
Fig. 1 Undecalcified section. Unstained.
Fig. 2. Decalcified section of two pairs of alternations.
Fig. 3. Undecalcified section of C-zone. Graded deposition of amorphous and minute granular crystallites.

Plate 8. TEM photomicrographs of undecalcified mineralized layer.
Fig. 1. Low magnification image of A-zone.
Fig. 2,3. Enlarged micrographs of A-zone.

Plate 9. TEM photomicrographs.
Fig. 1,2. Outmost mineralized layer (B-zone) of Lingula reevei.
Fig. 3,4. B-zone of L. unguis.

Plate 10. TEM photomicrographs. Undecalcified.
Fig. 1,2. Enlarged micrographs of crystallites of B-zone. Note coalescence of fine needle crystallites.
Fig. 3. Undecalcified section of mantle-shell preparation. Unstained.

Plate 11. TEM photomicrographs.
Fig. 1. Decalcified. Organic matrix of a mineralized layer (A-zone).
Fig. 2,3. Collagen fibers of connective tissue in Lingula unguis.

Plate 12. TEM photomicrographs. Decalcified middle layers.
Fig. 1. A punctum including granules.
Fig. 2,3. Punctae including fine network of organic threads.

Plate 13. TEM photomicrographs. Decalcified sections of mantle-shell preparation.
Fig. 1. Distal end of a punctum. Fine network of organic threads.
Fig. 2. Two cytoplasmic projections from the epidermis.

Plate 14. TEM photomicrographs. Undecalcified section of mantle-shell preparation.
Fig. 1. Two slender cytoplasmic projections from the epidermis.
Fig. 2. Two cytoplasmic projections entering punctae. Mineralization process within extrapallial fluid can be observed.

(In the above-mentioned plates, all figures are taken from Lingula unguis, unless described special notes.)
ULTRASTRUCTURE OF LINGULA SHELL

Plate 3
ULTRASTRUCTURE OF *LINGULA* SHELL

Plate 7