Jour. Fac. Sco., Hokkaido Univ., Ser. IV,
vol. 17, no. 1, March, 1975, pp. 173-229.

ULTRASTRUCTURE OF THE CONCHIOLIN MATRICES IN MOLLUSCAN NACREOUS LAYER

by

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(with 19 plates)

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I. Introduction

The nacreous layer of mollusc shells is mainly composed of calcium carbonate crystals (aragonite), and always contains small amount of organic materials. Organic materials of mollusc shells decalcified with dilute acid were first called the conchiolin by Frémy (1855), and were recognized to be an insoluble protein – a variety of the scleroproteins (Stary et. al., 1925; Roche et al., 1951). Later, the decalcification method by chelating reagent was adopted in the ultrastructural study of the conchiolin.

The ultrastructure of the EDTA decalcified conchiolin of the molluscan nacreous layer was first investigated by Grégoire et al., (1949, 1955) using an ultrasonic radiation method. They found characteristic reticulate patterns in the fragments of the conchiolin membrane. Later, Grégoire (1957) concluded that this reticulate pattern belonged to the inter-lamellar conchiolin membrane which separates consecutive mineral lamellae. Subsequent work identified three patterns in the ultrastructures of the nacreous conchiolin among three classes of molluscs (nautiloid, gastropod, and pelecypod), based on differences of size, shape, and distribution frequency of the pores (or openings). He also recognized statistically significant differences of the inter-lamellar conchiolin membrane at the class level between Gastropoda and Pelecypoda (Grégoire, 1960). Ultrastructural differences of the inter-lamellar conchiolin membrane among different classes of molluscs was also recognized by Mutvei (1969). Thus, phylogenetical difference of the ultrastructure of the nacreous conchiolin matrices was affirmed.

However, the significance of the minute openings in the inter-lamellar conchiolin membrane was not fully understood. Conchiolin protein of the nacreous layer is known not to be a pure protein, but a conjugated one which is composed of at least three fractions of protein (Grégoire et al., 1955). Although the EDTA soluble protein fraction, soluble nacrin, was already known, it's whereabouts in the nacreous layer is not fully clarified.

In this paper the writer reports the results of observation on the ultrastructure of the nacreous conchiolin by using a new method of decalcification with chromium sulphate.

Acknowledgements

The writer is indebted to Prof. Satoru Uozumi for suggesting this investigation as for constant guidance during the course of the work. Thanks are due to Prof. Makoto Kato and Prof. Masahiko Akiyama for valuable discussion and advice. The writer also wishes to thank Prof. Masao Minato and Prof. Seiji Hashimoto of the Department of Geology and Mineralogy, Faculty of Science, Hokkaido University, for encouragements during the study. Also, thanks are due to Messrs. Shigeshi Ohta, Ietaka Watanabe and Yoshihiro Togo for their kind assistance in the laboratory work. The writer also wishes thanks to Prof. Harry Mutvei of Uppsala University for kind instruction of the decalcification method with chromium sulphate.

II. Materials and methods

The materials available to the present writer comprises the nacreous layer in the following recent mollusc shells.

Cephalopoda:

Nautilus pompilius Linne, Australia

Gastropoda:

Omphalius rusticus colliculus (Sowerby), Otaru, Hokkaido

Clanculus margaritarium (Phillipi), Mexico

Pelecypoda:

Truncacila insignis (Gould), Ohkotsk Sea, Hokkaido

Mytilus coruscus (Gould), Otaru, Hokkaido

Pinctada martensii (Dunker), Ago Bay, Mie Prefecture

Pinna attenuata (Reeve), Kii Peninsula, Wakayama Prefecture

Neotrigonia margaritacea (Lamarck), Australia

The reagents used for decalcifying shell minerals to obtain the conchiolin protein were as follows:

(1) 0.5 M EDTA (ethylene diamine tetra acetic acid) pH 7.4

(2) Chromium sulphate solution pH 3.6

Sundström (1966) in Shiota (1969) was successful in producing effective decalcification of human enamel by using chromium sulphate (III) solution. The preparation of this solution is done by adding 0.5 M NaOH in a 0.5% aqueous solution of crystalline chromium sulphate (III) $[Cr(H_2O)_6 \cdot$

$(SO_4)_3 \cdot 6H_2O]$, and leave for a week to make pH at 3.6. This reagent is considered to act as a kind of acid in decalcifying calcareous minerals in hard tissues by forming hexaquo chromium complex ions $[Cr(OH)_6^{3+}]$, fixing organic matrices by forming a stable covalent bond between chromium and the carboxyl group of the polypeptides of protein and preventing their destruction by simultaneous tannization.

(3) 0.01 N HCl

Demineralization of the nacreous layer was made to obtain the conchiolin with insertion for a few days in 0.5 M EDTA solution and one hour in 0.01 N HCl. Immersion in chromium sulphate solution decalcification was proceeded for a month or more at room temperature. Frequent addition of new solution prevented any excessive lowering of pH causes a remarkable loss of demineralization ability, and products other insoluble precipitates (chemical composition not yet determined) which destroy the structures of the samples.

Preparation of the conchiolin for the transmission electron microscope.

Ultrathin sectioning methods employed here for the transmission electron microscope were essentially the same as those described in the previous paper (Uozumi and Iwata, 1969). In the present study the nacreous layer was decalcified in an aqueous solution of Sundström's chromium sulphate. The conventional decalcification method using chelate reagent (EDTA) was simultaneously tried to compare with the results of the chromium sulphate method. A portion of the decalcified sample was dehydrated successively by soluble epoxy resin (Durcupan) and embeded in styrene or epoxy resins. They were cut into thin sections with a glass knife, using a Leitz ultramicrotome. Thin sections were stained with uranyl acetate and studied in a transmission electron microscope JEM 120 U (Accel. voltage 60–80 Kv, objective aperture 20 μ). The other parts of the nacreous conchiolin were washed in redistilled water after decalcification, fixed with OsO_4 (4°C), and disintegrated with fine needles by tearing off large pieces of the consecutive inter-lamellar conchiolin membranes under an optical microscope (hand tearing method). Other parts of the conchiolin were dispersed by ultrasonic waves within the medium of redistilled water to compare with the results of the former method. The conchiolin membranes were then directly collected on a formvar coated grid. They were evaporated with Pt–Pd at low angle (20–30°). Some of the conchilin membranes were stained by uranyl acetate and phospho tungstic acid (pH 7.0).

The presence of inorganic materials was checked by dark field observation,

selected area electron diffraction and electron probe micro analysis. The decalcified conchiolin was mounted on the formvar coated grid, then evaporated with carbon, and contaminating inorganic materials from the sample were analysed with an electron probe microanalyser (JSM-U 3). The analysis was carried out at 15 Kv.

Half-decalcified aragonite crystals, dispersed by ultrasonic waves in the medium of redistilled water, were mounted on the formvar coated grid and evaporated with Pt–Pd. They were then observed under the transmission electron microscope (Accel. voltage 120 Kv).

For measurement of thickness and size under electron microscope the shadow cast distance of polystyrene particles (DOW Chemical Company ϕ: 870 Å in diameter) were used.

Preparation of the nacreous layer for the scanning electron microscope.

Specimens were cut to expose the nacreous layer, polished with Dp-diamond paste, and then etched with 0.5 M EDTA for several minutes. They were coated with evaporated carbon and gold and were examined in a JSM-U 3 (Japan Electron Optics Lab. Ltd.). Accel. voltage used was 20 Kv.

III. Ultrastructure of the nacreous conchiolin.

The nacreous layer is one of the representative shell layers in molluscs. This layer is found among three classes of the mollusca – Cephalopoda, Gastropoda, and Pelecypoda. The nacreous layer is always composed of aragonite and a small amount of organic materials, the conchiolin. The micro- and ultra-structure of this layer has been investigated by many authors (Bøggild (1930); Oberling (1964); Grégoire (1957, 1961, 1962); Wada (1956, 1957, 1958, 1963, 1964, 1968, 1970); Watabe (1963, 1965); Mutvei (1964, 1969, 1970); Travis et al., (1967); Kobayashi (1964, 1968, 1969, 1971); Erben (1968, 1972) and Taylor et al., (1969, 1970).

Aragonite crystals deposited in this layer are usually crystallized as polyhedral tabular forms (Pl. I. Fig. 2). Such morphology is dissimilar to other shell layers composed of aragonite such as crossed-lamellar layer etc., (Uozumi, Iwata, Togo, 1972). As already pointed out by several authors, aragonite in this layer has predominantly hexagonal shapes, being elongated along the a-axis. Each tabular aragonite crystal consists of sublamellar tablets (about 200 Å in thickness) which are displaced relative to each other along the a-axis (Pl. I. Fig. 3, 4).

Tabular aragonite crystals are stacked one on top of the other as walls in

most pelecypods, but in some pelecypods, gastropods and cephalopods the crystals are stacked in columns (Pl. I. Fig. 1, 5). The difference in the stacking arrangement of aragonite crystals in different species of molluscs are stated by several authors and details are ommited in this article.

Each aragonite crystal is embeded in the organic (conchiolin) matrices. Organic materials of the molluscan nacreous layer is a variety of scleroprotein showing strong resistivity against dilute acid, alkali and chelate solution, and proteases such as pepsin, trypsin, and pronases. From most biochemical studies since Frémy (1855) it is known that in the nacreous conchiolin about 15 amino acids are contained and the chief component of which is comprised of glycine and alanine (Grégoire et al., 1955; Akiyama, 1967; Tanaka et al., 1960; etc.,). It is also known the nacreous conchiolin is not a pure protein, but a conjugated one which has three protein fractions (Grégoire et al., 1955). Among the different fractions the nacroin fraction mainly consists of glycine and alanine and contains small amounts of polysaccharide as chitin (Grégoire et al., 1955; Foucart, 1968, in Hunt, 1970). The other protein fractions (soluble and insoluble nacrin) are mainly composed of proteins, which have a slightly different amino acid composition from the nacroin.

The molecular structure of the nacreous conchiolin is not fully understood, but anti-parallel or cross-β conformation and helical structures are found from X-ray diffraction and infra-red spectral analyses (Travis et al., 1967; Hotta, 1968).

(1) Ultrastructure of the decalcified nacreous conchiolin in chelating reagent and dilute acid.

The nacreous layers of the three classes of molluscs were decalcified with conventional decalcification reagents, EDTA and HCl, and ultrastructures of the nacreous conchiolin were observed under the transmission electron microscope.

The basic construction of the nacreous conchiolin has already been described (Uozumi and Iwata, 1969). The conchiolin matrices in this layer are composed of two membranes, the inter-lamellar conchiolin membrane, and the inter-crystalline conchiolin membrane. (Grégoire, 1957). The former is in contact with the tabular plane of aragonite crystals and separates the stacking mineral lamellae, and the latter connects the side walls of each mineral lamellae and separates vertically adjacent crystals (Pl. I. Fig. 1,5 Pl. X. Fig. 2 Pl. XIII. Fig. 1 Pl. XVI. Fig. 1,2 Pl. XVII. Fig. 1,2 Pl. XVIII. Fig. 1,2 Pl. XIX, Fig. 1,2,3).

As mentioned by Mutvei (1969) and Iwata (1969) inter-lamellar and

inter-crystalline conchiolin membranes are different in thickness and ultrastructure. Namely, the inter-lamellar conchiolin membrane has a thickness of about 200 Å and this is characterized by a reticulate pattern. The inter-crystalline conchiolin membrane is thinner and without any particular structure.

In the present study ultrastructures of the inter-lamellar conchiolin membranes were observed.

Ultrastructure of the inter-lamellar conchiolin membrane were also studied by Grégoire (1949, 1955, 1957, 1960, 1961, 1962) and Mutvei (1969). It is known that the inter-lamellar conchiolin membranes do not show the same contrast of electron density throughout the membrane. Portions of high electron density which form the framework of the conchiolin membranes are called the trabeculae by Grégoire et al., (1955). The thinner areas contain minute openings or pores of various sizes and forms, and are called inter-trabecular areas by Mutvei (1969).

From a study of the substructures of the nacreous conchiolin protein, Grégoire et al., (1955) showed bundles of polypeptidic fibrils buried in the trabeculae portions and called these fibril protein fractions the nacroin. The protein coating of the nacroin fibrils the above authors gave the name of nacrosclerotin. Later Florkin (1966) proposed to call them the insoluble nacrin. Grégoire et al., (1955) also indicated the existence of the EDTA or borate buffer soluble protein fraction, and called this one the soluble nacrin. The inter-lamellar conchiolin membrane, therefore, is considered to be the doubly coated membrane intercalated with the nacroin fibrils.

Pelecypoda

Truncacila insignis, Pinctada martensii, Pinna attenuata, Mytilus coruscus, Neotrigonia margaritacea

Decalcified inter-lamellar conchiolin membranes of the above mentioned genera were studied.

In *Truncacila insignis* the thickness of the inter-lamellar conchiolin membrane is about 150 Å and a perforated pattern is observed in this membrane. Minute openings in the inter-trabecular areas are mostly 200–300 Å in diameter. The forms of these openings are irregular – cocoon forms, small ellipsoid forms and various angular forms are observed. The portion occupied by the openings in this membrane per unit area is about 10%. This rate is slightly larger than in other pelecypods. Arrangements of the trabeculae are nearly along the long axes of the crystal scars (this term was proposed by Mutvei, 1969). These are crystal outlines on the inter-lamellar conchiolin membrane partitioned by the inter-crystalline conchiolin membrane, but all of them are not strictly parallel (Pl. II. Fig. 1,3). Protuberances on the

trabeculae are smaller than in gastropods and cephalopods. The nacroin fibrils in the trabeculae are about 50 Å in width.

In *Pinctada martensii* the thickness of the inter-lamellar conchiolin membrane is slightly thicker than in *Truncacila insignis.* The morphology of the openings in the inter-trabecular areas are more rounded, and slightly similar to the gastropod pattern. The distribution of the openings is typically sporadical in this species. The sizes of the openings are 200–500 Å in diameter, and are therefore larger than in other pelecypods. These openings are scattered at random. The portion occupied by the openings in this membrane is less than 10% (about 8%). The trabeculae are wider than in other pelecypods and the protuberances on them are larger than in *Truncalcila insignis.* Arrangements of the protuberances on the trabeculae roughly trend along the long axes of the crystal scars. Nacroin fibrils are about 50 Å in width, but thick ones of about 100 Å in width are often observed (Pl. III. Fig. 1). The reticulate ultrastructure of this species is slightly similar in *Unio* and *Anodonta.*

In *Pinna attenuata* inter-lamellar conchiolin membranes are about 150 Å in thickness. Openings in the inter-trabecular areas have irregular forms, like in *Truncacila insignis*, and their sizes are 100–300 Å in diameter. The portion occupied by the openings is smaller than in *Truncacila insignis.* The trabeculae are wider and the protuberances on them are coarser than in *Truncacila* and *Neotrigonia* (Pl. III. Fig. 2).

In *Mytilus coruscus* the inter-lamellar conchiolin membranes are about 150 Å in thickness. The openings in the inter-trabecular areas are very minute, mostly less than 100 Å in diameter, and the trabeculae are narrower than in other pelecypods (Pl. IV. Fig. 2). At lower magnification these openings are not clearly seen (Pl. IV. Fig. 1). Grégoire (1957) called such an elaborate pattern of the inter-lamellar conchiolin the tight pelecypod pattern. This pattern is greatly different from the conchiolin patterns in cephalopods and gastropods.

In the ultrathin section of this conchiolin minute openings are vaguely observed (Pl. XVI. Fig. 1). Portion occupied by the openings could not be precisely measured. Such tight pelecypod patterns are also observed in *Modiolus, Septifer, Laternula,* and *Entodesma,* but in the latter two genera the inter-trabecular areas are more narrower and openings are difficult to distinguish.

In *Neotrigonia margaritacea* the inter-lamellar conchiolin membranes are about 150 Å in thickness. Openings in the inter-trabecular areas are mostly 100–200 Å in diameter. They are irregularly shaped as in *Truncacila insignis,* and compared with *Truncacila* the portion occupied by the openings is fairly smaller (about 5%) in this genus. The trabeculae are rather flat and protuberances on them seem to be elaborately aggregated than in other pelocypods (Pl. V. Fig. 2).

In ultrathin section the inter-lamellar conchiolin membrane is undulated owing to the developement of minute openings (Pl. XVI. Fig. 2). Nacroin fibrils can not be observed in the "openings" region in the inter-trabecular areas of this conchiolin membrane.

Gastropoda

Omphalius rusticus

One genus of Gastropoda was studied.

In *Omphalius rusticus* the inter-lamellar conchiolin membrane is about 150 Å in thickness, and is slightly larger than in pelecypods. Typical gastropod pattern is observed in this conchiolin. Rounded and subrounded openings are mostly 200–500 Å in diameter. The portion occupied by the openings in the inter-trabecular areas is larger than in most pelecypods. Openings are relatively uniformely scattered throughout this membrane. Arrangements of the trabeculae and protuberances are not more paralelly orientated than in cephalpods and pelecypods (Pl. IX. Fig. 1,2).

Cephalopoda

Nautilus pompilius

One genus of Cephalopoda was studied.

As already reported by Grégoire (1962), the ultrastructures of the inter-lamellar conchiolin membrane of this genus are not similar in shell wall and shell septa. In this paper only the conchiolin of the nacreous shell wall (in the portion of body whorl) was studied.

The inter-lamellar conchiolin membrane is about 200 Å in thickness and is therefore slightly thicker than the conchiolin membrane of pelecypods and gastropods. Openings in the inter-trabecular areas are mostly of elongated ovoid forms and ellipsoidal forms. The sizes of these openings are variable and some openings attain more than 500 Å in the maximum longitudinal diameter. Openings in the inter-trabecular areas run roughly in direction parallel to the long axes of crystal scars. The width of the trabeculae are wider than in pelecypods and gastropods and on these trabeculae round protuberances are numerously observed. These protuberances seem to be slightly larger than in pelecypods and gastropods. Nacroin fibrils in the trabeculae are about 100 Å in width, and therefore thicker than in most pelecypods (Pl. XIII. Fig. 2,3).

Reticulate patterns of the inter-lamellar conchiolin membrane are clearly recognized in the ultrathin section (Pl. XIII. Fig. 1). Openings are observed in the inter-trabecular areas and most of them have ellipsoidal forms. In the portions of the openings in the inter-trabecular areas nacroin fibrils cannot be observed at high magnification. Reticulate pattern of the *Nautilus* conchiolin is

distinctly distinguished from those of gastropods and pelecypods. Grégoire (1957) called this type of pattern the cephalopod (nautiloid) pattern.

Thus, ultrastructures of the EDTA decalcified inter-lamellar conchiolin membranes are significantly different among the three classes of molluscs – Cephalopoda, Gastropoda, and Pelecypoda. Differences in the patterns is mainly due to the forms and sizes of the openings in the inter-trabecular areas and the width and arrangement of the trabeculae. Minor variations also seem to be reflected in the protuberances on the trabeculae.

In pelecypods reticulate patterns of the inter-lamellar conchiolin membranes are distinguished at the family level, based on the differences of size, form, and distribution frequency of the openings in the inter-trabecular areas.

Though it is not dealt in the present paper, ultrastructural differences of the nacreous conchiolin can not easily be distinguished within the same genus of pelecypods in general cases.

Ultrastructures of the inter-lamellar conchiolin membranes decalcified with dilute HCl were studied at the same time. Only one example is reported here. Dilute HCl decalcified inter-lamellar conchiolin membranes were separated by using the hand tearing method and studied similarly under the electron microscope.

The dilute HCl decalcified inter-lamellar conchiolin membrane in *Nautilus pompilius* is about 150 Å in thickness. This is thinner than in the EDTA decalcified conchiolin membrane, but it shows a similar reticulate pattern characterized by the elongate openings (200–600 Å in diameter). Protuberances on the trabeculae are also observed but they are much reduced and the relief of the trabecular portion becomes remarkably flattened (Pl. XII. Fig. 1). In spite of these differences the basic pattern of the inter-lamellar conchiolin membrane is quite similar to that of the EDTA decalcified conchiolin of the same specimen. Boundaries between the trabeculae and the inter-trabecular areas are sharply distinguished as in the EDTA decalcified conchiolin membrane.

Dilute HCl decalcified inter-lamellar conchiolin of other pelecypods and gastropods are also similar to the EDTA decalcified ones in ultrastructures, except in minor differences.

(2) Ultrastructure of the decalcified conchiolin in chromium sulphate

Observation results of the ultrastructures in the inter-lamellar conchiolin membranes decalcified with chromium sulphate solution are described in this section.

As mentioned above, the EDTA decalcified inter-lamellar conchiolin membranes show a characteristic reticulate ultrastructure but in the inter-lamellar conchiolin membranes decalcified with chromium sulphate solution the ultrastructures are significantly different.

Pelecypoda

Neotrigonia margaritacea, Pinctada martensii

In *Neotrigonia margaritacea* the inter-lamellar conchiolin membrane isolated by the hand tearing method the openings in the inter-trabecular areas are never observed and the boundaries between the trabeculae and the inter-trabecular areas can not be distinguished. Minute protuberances are attached on the large portions of this membrane and aggregations of dense granules are often sporadically seen (Pl. VII. Fig. 2). Such granules were never observed in the EDTA decalcified conchiolin membranes. The thickness of this membrane is not greatly different from the EDTA decalcified conchiolin membrane. Significant difference of the contrast was not found in this conchiolin membrane after positive staining with uranyl acetate.

The inperforate structure of this conchiolin membrane is easily distinguished from the EDTA decalcified conchiolin. (See Pl. VI. Fig. 1,2). Electron diffraction study does not reveal any contaminations of inorganic materials.

In *Pinctada martensii* the inter-lamellar conchiolin membrane isolated by the hand tearing method has minute scattered openings (Pl. VIII. Fig. 1). The Sizes of these openings are mostly less than 100 Å in diameter and their forms are irregular. Compared with EDTA decalcified conchiolin (Pl. III. Fig. 1), the sizes and forms of these openings are extremely different. In some portions of this membrane openings can not be observed. Dense granules are seen sporadically on this membrane (Pl. VIII. Fig. 2).

In the ultrathin section of this conchiolin openings cannot be observed in any regions of the inter-lamellar conchiolin membrane (Pl. XVII. Fig. 1,2), and boundaries between the trabeculae and the inter-trabecular areas can not be distinguished.

Gastropoda

Omphalius rusticus

The inter-lamellar conchiolin membrane of *Omphalius rusticus* was studied using the same techniques as described above.

In this conchiolin membrane no openings can also be observed (Pl. X. Fig. 1). This is also true from the study of the ultrathin section of this sample (Pl. X. Fig. 2), but slight differences in the contrast are shown as patches

throughout the membrane. Portions of low electron density are not rounded, but irregularly shaped. It is not certain that thick portions of this membrane strictly correspond to the trabeculae. Boundaries between the trabeculae and the inter-trabecular areas are difficult to distinguish. Minute protuberances are observed on the membrane and aggregations of dense granules are also often observed (Pl. X. Fig. 3). This feature is easily distiguishable from the structure of the EDTA decalcified conchiolin of the same species (See figures of Plate IX.). Inorganic materials were not detected in this conchiolin under electron diffraction. However, in the conchiolin isolated by weak radiation with ultrasonic waves, round openings (300–500 Å in diameter) are clearly observed in the inter-lamellar conchiolin membrane (Pl. XI. Fig. 1,2). This ultrastructure is very similar to the reticulate pattern of the EDTA decalcified conchiolin membrane. Boundaries between the trabeculae and the inter-trabecular areas are distinct.

Therefore, organic materials filling the inter-trabecular areas are probably removed by weak vibration during the suspension process.

Cephalopoda

Nautilus pompilius

In *Nautilus pompilius* the inter-lamellar conchiolin membrane does not show distinct differences in the thickness throughout the membrane. The membrane is very flat and openings cannot be observed in any parts of the membrane. Boundaries between the trabeculae and the inter-trabecular areas are difficult to distinguish. Transparent elevations as described by Mutvei (1969) in the central part of the crystal scars were not observed.

Minute protuberances are seen and aggregations of dense granules are often observed on this membrane. Significant differences of electron density corresponding to the boundaries between the trabeculae and the inter-trabecular areas was not clearly observed after positive and negative staining with uranyl acetate and phospho tungstic acid. Therefore, adherance of the simple granules within the inter-trabecular areas cannot be imagined (Pl. XIV. Fig. 1,2).

Openings of the inter-lamellar conchiolin membrane cannot be recognized in the ultrathin section even at high magnification. (Pl. XIX. Fig. 2,3). However, after radiation by ultrasonic waves elongated and ovoid openings can be clearly observed and boundaries between the trabeculae and the inter-trabecular areas are easily distinguished (Pl. XV. Fig. 1,2). After a few seconds of radiation by ultrasonic waves openings in the inter-trabecular areas are partially filled with organic materials and the character of the inter-trabecular areas becomes visible (Pl. XV. Fig. 1,2). The reticulate pattern of this

inter-lamellar conchiolin membrane after radiation by ultrasonic waves is very similar to those of the EDTA decalcified conchiolin membrane (See Pl. XIII. Fig. 2,3).

No organic materials were detected in this inter-lamellar conchiolin membrane from electron diffraction and electron probe micro analyses. Therefore, it is clear that filling materials in the inter-trabecular areas is some kind of organic materials.

In addition, ultrastructural study of the ultrathin section of this conchiolin matrices showed other types of organic materials between the spacing enclosed by the inter-lamellar and inter-crystalline conchiolin membrane (Pl. XVIII. Fig. 1,2). These organic materials are connected with the inter-lamellar conchiolin membranes as extremely thin membranes. The thickness of these membranes is less than 50 Å. Their arrangement is nearly parallel to the inter-crystalline conchiolin membrane in some places, but all of them are not always similar.

The organic materials are considered to belong to the intra- crystalline conchiolin membrane and were never observed in the EDTA decalcified conchiolin, although some of them might include contaminated organic materials within the aragonite crystals.

Such organic materials were not clearly observed in *Pinctada martensii* and *Omphalius rusticus,* but existence of the intra-crystalline conchiolin matrices is suggested.

From the above mentioned results it is clear that the ultrastructures of the nacreous conchiolin decalcified with chromium sulphate are quite different from those decalcified in EDTA and dilute HCl. Reticulate patterns characterized by the minute openings in the inter-trabecular areas are not found in chromium sulphate decalcified nacreous conchiolin.

IV. Conclusion and consideration

The ultrastructure of the nacreous conchiolin has been investigated in the three classes of molluscs (Cephalopoda, Gastropoda, and Pelecypoda). The conventional decalcification method using chelating reagent (EDTA). dilute acid (HCl), and a new technique using chromium sulphate were employed. Ultrastructural differences were compared mainly on the inter-lamellar conchiolin membrane.

Based on the observations under the electron microscope, the following conclusions are apparent.

(1) In all the specimens decalcified with dilute HCl and EDTA, the inter-lamellar conchiolin fragments appear perforated owing to the presence of minute openings in the inter-trabecular areas. Such minute openings were

recognized not only in the conchiolin membrane dispersed by ultrasonic waves, but in the ultrathin sections and hand teared materials. In the "openings" in the inter-trabecular areas nacroin fibrils could not be observed.

(2) Reticulate patterns of the inter-lamellar conchiolin membrane are easily identified among the three classes of molluscs. Differences in the nautiloid, gastropod, and pelecypod patterns in the inter-lamellar conchiolin membrane were confirmed by the diversity of form, size and distribution frequency of minute openings. In pelecypods minor structural variation was also recognized among different families. Such results agrees with those of Grégoire (1960).

(3) In the conchiolin obtained after decalcification with chromium sulphate reticulate patterns in the inter-trabecular areas of the inter-lamellar conchiolin membrane could not be observed both in the sample isolated by the hand tearing method and in the ultrathin sections. Inter-lamellar conchiolins do not differ greatly in thickness throughout the membrane. Difinite boundaries of the trabeculae and the inter-trabecular areas are difficult to distinguish. The protuberances are uniformely distributed over the inter-lamellar conchiolin membrane, and aggregations of dense granules are often observed. These dense granules are never seen in the EDTA decalcified conchiolin.

Such features are common among the same species of the three classes.

(4) However, in the chromium sulphate decalcified conchiolin radiation by ultrasonic waves makes the reticulate patterns clear and these are characterized by minute openings in the inter-lamellar conchiolin membrane. And boundaries between the trabeculae and the inter-trabecular areas become distinct. Thus the reticulate patterns of the conchiolin membrane in the cephalopods, gastropods, and pelecypods are quite similar to each of the patterns observed in the conchiolin decalcified with EDTA or HCl.

(5) In the portions of the "openings" in the inter-lamellar conchiolin membrane some kind of protein, which is soluble in EDTA and HCl but insoluble in chromium sulphate, fill or coat the inter-trabecular areas. After vibrating treatment, this protein is seemed to be weakly attached to the trabeculae.

(6) The intra-crystalline conchiolin matrices are not observed in the ultrathin sections of the nacreous conchiolin decalcified with EDTA. Even in the chromium sulphate decalcified conchiolin they are also hard to observe. In some cases a part of the possible intra-crystalline matrices can be observed between the spacing enclosed by the inter-lamellar and inter-crystalline

conchiolin membrane.

(7) The effects of decalcification with chromium sulphate and EDTA or HCl are dissimilar. Chromium sulphate preserves conchiolin protein better than the other methods.

Since Grégoire et. al., (1949) first introduced the demineralization method using chelating reagent, ethylene diamine tetra acetic acid, ultrastructural investigations of the proteineous matrices of mollusc shells have been performed following this method. Mild decalficication by chelate reagent enables perfect demineralization of shells in a comparatively short period. The obtained conchiolin persists the original forms well in most of the specimens except some shells with a low content of EDTA insoluble protein. The nacreous conchiolin contains a high amount of the EDTA insoluble protein fraction. Therefore, the EDTA decalcified nacreous conchiolin has been considered to reveal its ultrastructure in good condition. Besides the insoluble fraction, the soluble protein fraction of the nacreous conchiolin is known and called the nacrin. Other insoluble fractions are called the insoluble nacrin and the nacroin. The nacroin fraction corresponds to the polypeptidic fibrils buried in the trabeculae and the insoluble nacrin corresponds to the protein coating and enclosing the trabeculae (Grégoire et al., 1955; Florkin, 1967). Such soluble protein is not observed in an ordinal preparation of ultrathin sections. The reticulate ultrastructure of the inter-lamellar conchiolin membrane in the molluscs were studied by Gregoire (above cited). Based on the comparative observations of the morphology, size, distribution frequency of openings or pores, and relative surfaces in the inter-lamellar conchiolin membrane cephalopod (or nautiloid), gastropod, and pelecypod patterns were distinguished. Significant ultrastructural differences were also recognized in the different families between gastropods and pelecypods.

Besides these phylogenetical differences other significances were not considered about the existence of the openings in the conchiolin membranes. Whether they are true openings or not has not been determined as yet. Although the existence of the soluble protein fraction was known, whereabout this protein is consealed was not fully clear.

From the above mentioned results it is clear that another kind of protein fills or coats the portions of the "openings" of the inter-lamellar conchiolin membrane. This protein is soluble in EDTA and HCl, so it probably belongs to the hitherto mentioned soluble nacrin. Such soluble protein is well fixed by chromium sulphate and remains in the inter-trabecular areas of the inter-lamellar conchiolin membrane. The exact fixation mechanism is not yet fully understood but probably enabled from the binding effect of the chromium and

the carboxyl group of polypeptides of the conchiolin protein as in human enamel.

Whether these proteins are simple granules or more complicated aggregations of granules is not certain, but it is clear that they are bound loosely to the trabeculae and fill or coat the "openings" region in a non-fibrous state.

If the fundamental structure of the nacreous conchiolin membrane is stably inheritable in the molluscan line, the reticulate patterns observed in the EDTA decalcified conchiolin and the structure of above mentioned filling protein would imply an important phylogenetical significance. It may be inprobable that the incorporation of such protein might reflect accidental mistakes in the formation of the conchiolin matrices. Ultrastructural diversity of the nacreous conchiolin is considered to be due to differences in arrangement of the conchiolin protein fractions and their biochemical composition.

Furthermore, as to the role of the soluble protein an interesting report was offered by Crenshaw (1972). He reported soluble protein from the *Mercenaria mercenaria* shell and demonstrated that the calcium concentrating ability is remarkably higher in the protein fraction. However, it is not yet certain that the soluble protein in the inter-trabecular areas might be related to the calcification in this shell layer. Further biochemical and experimental study will be necessary for a fuller understanding of the significance and role of this soluble protein.

In spite of slow rate of decalcification chromium sulphate preserves the conchiolin protein better than the chelate reagent. This agent can be applied in the demineralization not only of calcified hard tissues of vertebrates which are mainly composed of calcium phosphate, but also of invertebrate shells which are composed of calcium carbonate minerals.

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Received on Nov. 6, 1974

Explanation of Plates

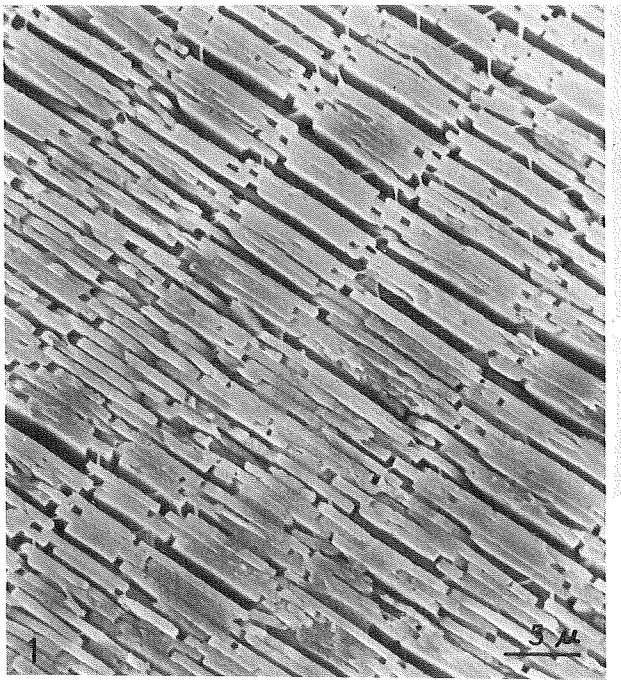
Plate I.

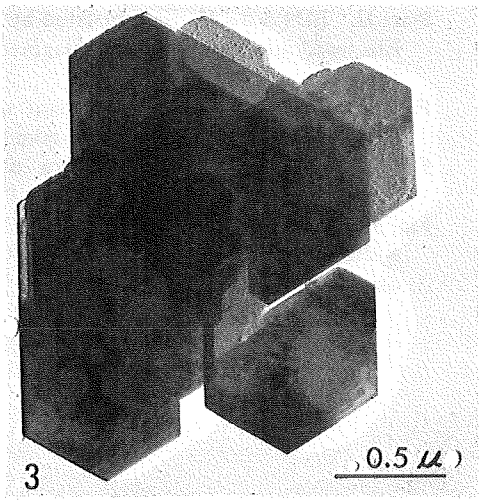
Fig. 1. Scanning electron micrograph of the inner nacreous layer in *Clanculus margaritarium*. Vertical profile.
Aragonite crystals are stacking in columns. X 2,000

Fig. 2. Scanning electron micrograph of the fresh inner surface of the nacreous layer in *Mytilus edulis*.
Tabular aragonite crystals of various hexagonal shapes and sizes are forming. X 1,500

Fig. 3,4. Transmission electron micrographs of the nacreous aragonite, half-decalcified with EDTA and ultrasonic dispersed.
Fig.3 showing sublamellar aragonite tablets in *Truncacila*.
Fig.4 showing sublamellar aragonite tablets in *Nautilus*.
Polyhedral nacreous aragonite seem to be constructed of sublamellar aragonite tablets of about 200 Å in thickness. These tablets are not stacked perfect in vertical direction, but each lamelae displaced lateraly in short distance. Fig.3,4 X 30,000

Fig. 5. Vertical profile of the column-like nacreous layer in *Truncacila insignis*. Scanning electron micrograph. X 5,000


1
5 μ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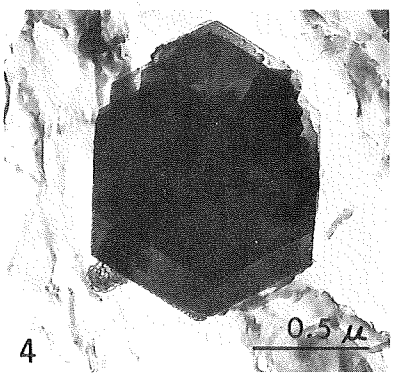
0.5 μ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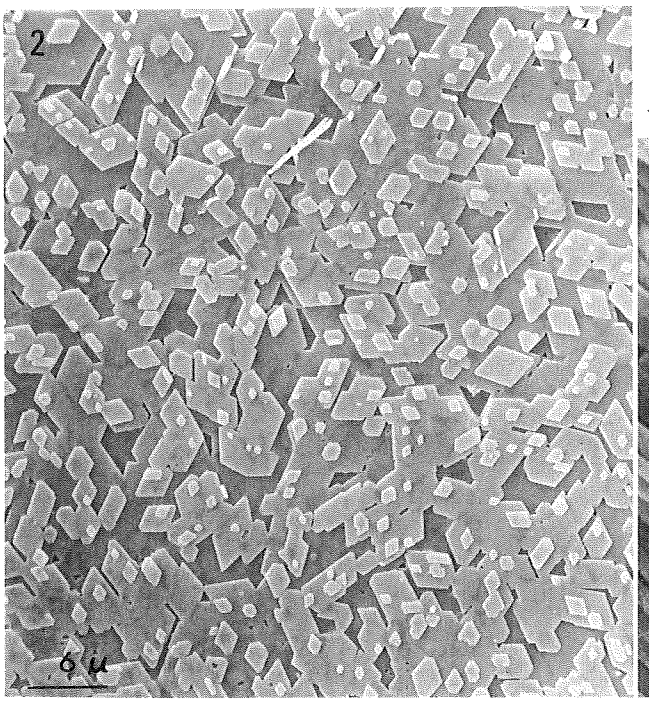
0.5 μ


2
6 μ

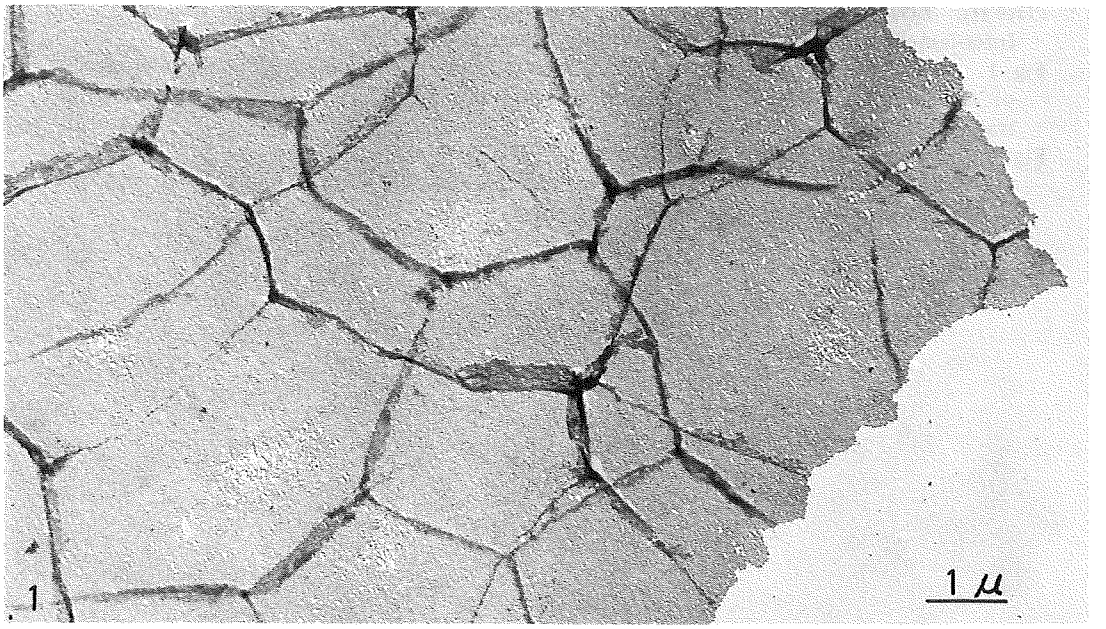

5

Plate II. EDTA decalcified. Isolated by ultrasonic waves.
Electron micrographs show fragments of the inter-lamellar conchiolin membranes in *Truncacila insignis.*

Fig. 1. Polyhedral outline of crystal scars are bordered by the inter-crystalline conchiolin membrane on the inter-lamellar conchiolin membrane. X 10,000

Fig. 2. Lace-like reticulate pattern of the inter-lamellar conchiolin membrane. Minute openings (mostly 200 Å in diameter) of irregular shapes can be observed. X 24,000

Fig. 3. After strong radiation with ultrasonic waves fine nacroin fibrils (about 50 Å in width) exposed from this sheet. Round particles are polystyrene latex of 870 Å in diameter. X 30,000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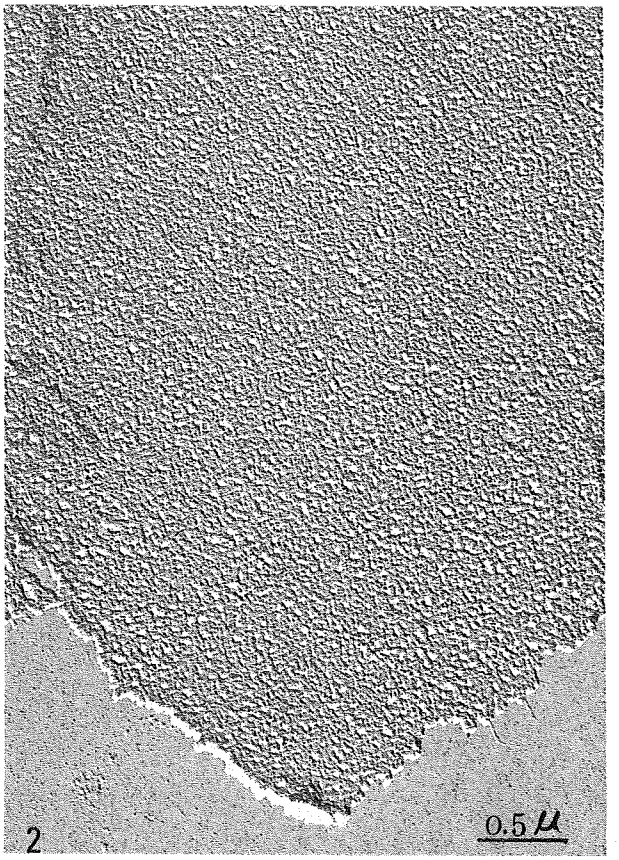
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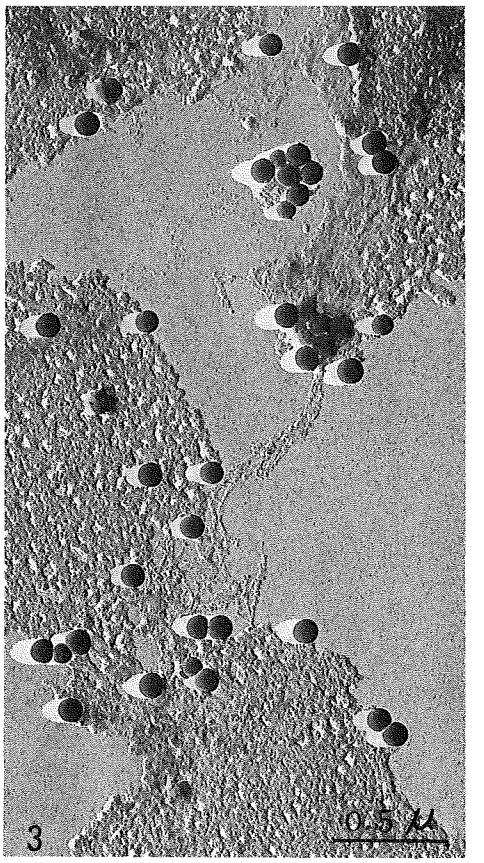
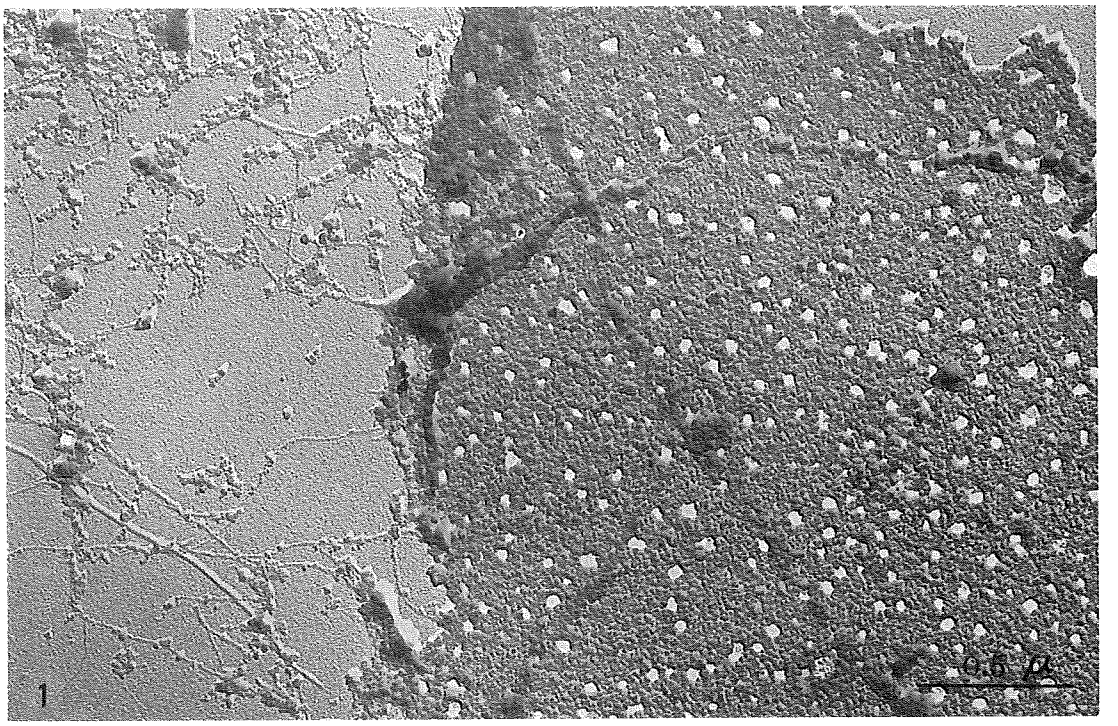

3
0.5 μ

Plate III. EDTA decalcified. Isolated by ultrasonic waves.
Inter-lamellar conchiolin membrane of *Pinctada martensii.*

Fig. 1. Rounded openings (200–500 Å in diameter) are visible sporadically. At the margin of this membrane nacroin fibrils and coating organic materials are loosened from the trabeculae. X 40,000

Fig. 2. Inter-lamellar conchiolin membrane in *Pinna attenuata.*
Openings in the inter-trabecular areas are irregularlly shaped and their size are significantly smaller than in *Pinctada.* X 50,000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1

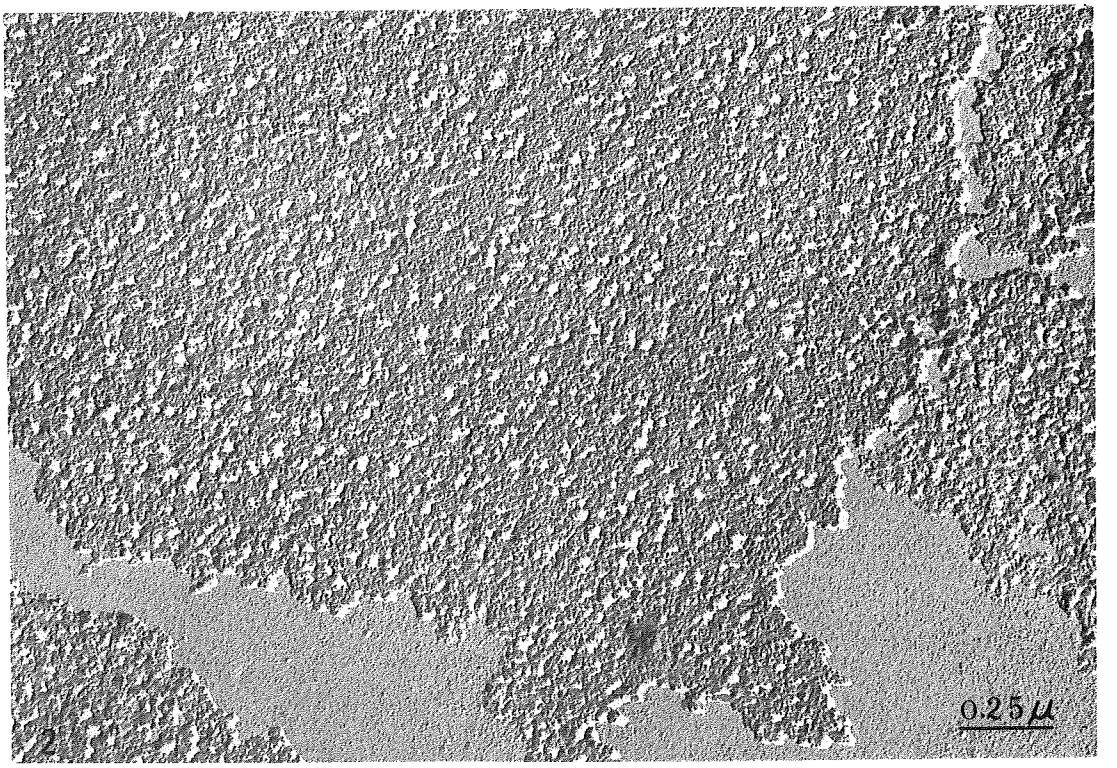
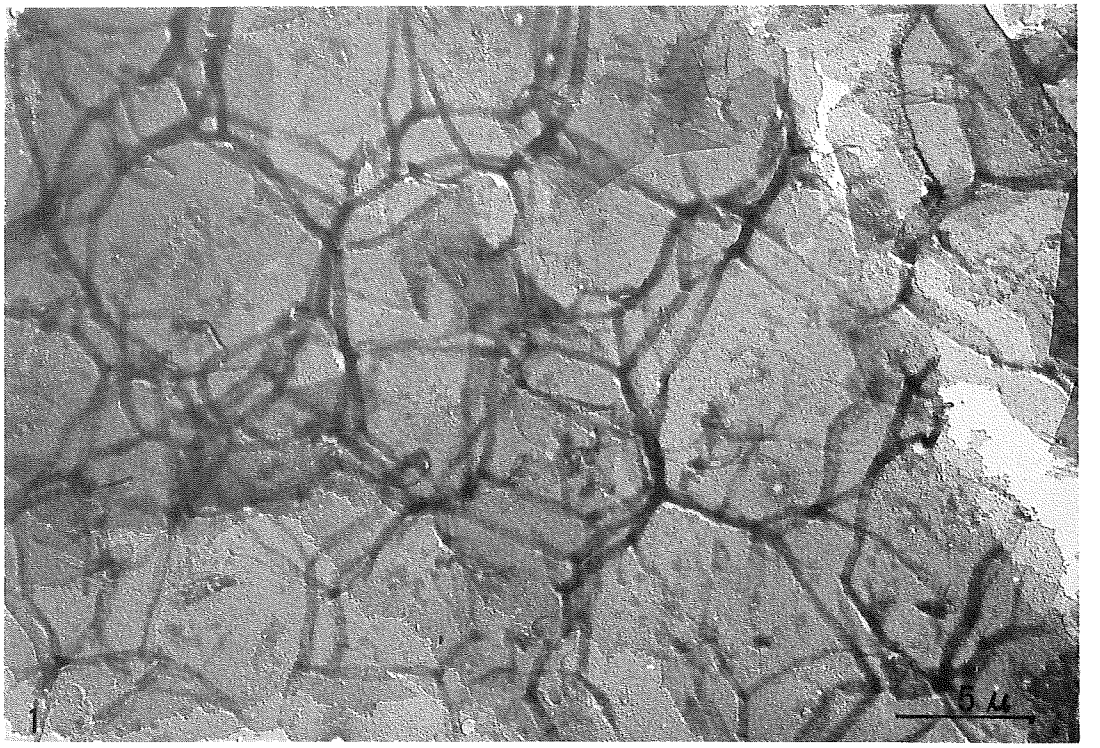

0.25μ
2

Plate IV. EDTA decalcified. Isolated by ultrasonic waves.

Fig. 1. Leaflets of nacreous conchiolin in *Mytilus coruscus.* X 6,000

Fig. 2. Enlarged electron micrograph of the inter-lamellar conchiolin membrane in Fig. 1. Tight pelecypod pattern characterized by minute openings less than 100 Å in diameter. X 50,000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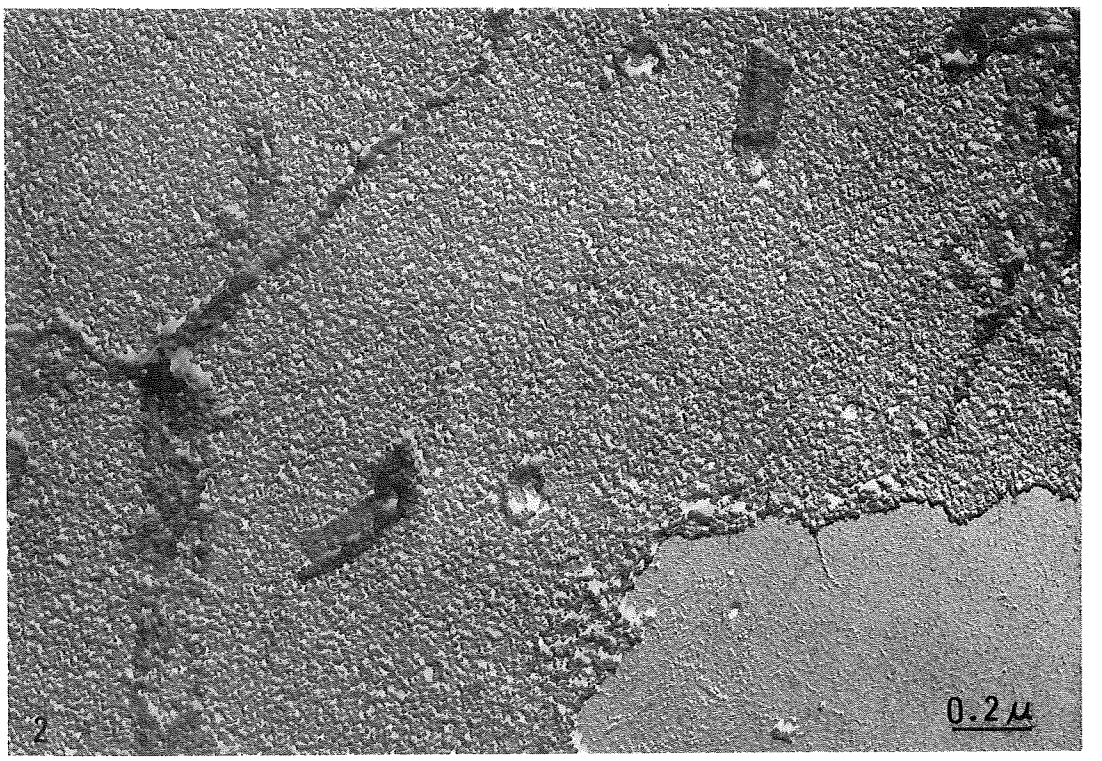
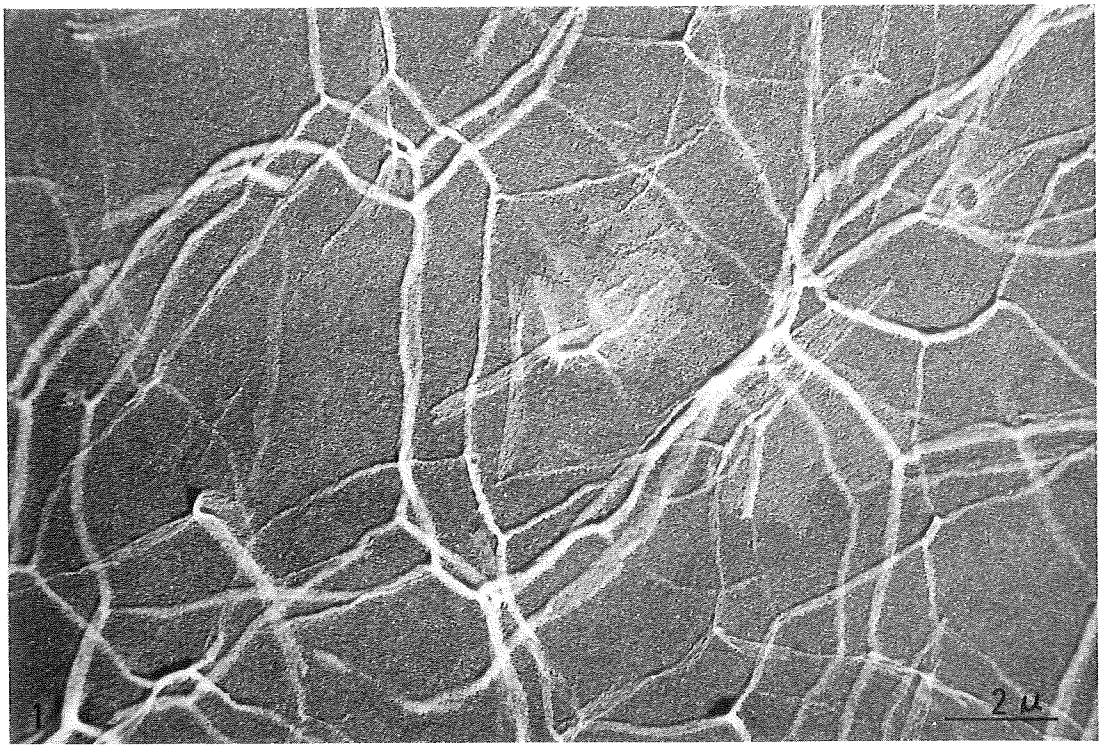

2
0.2 μ

Plate V. EDTA decalcified. Isolated by ultrasonic waves.
Fig. 1,2. Overlapped leaflets of the nacreous conchiolin in *Neotrigonia margaritacea*. Fig. 1. Bright field image X 7,500 Fig. 2. Dark field image X 7,500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2 μ

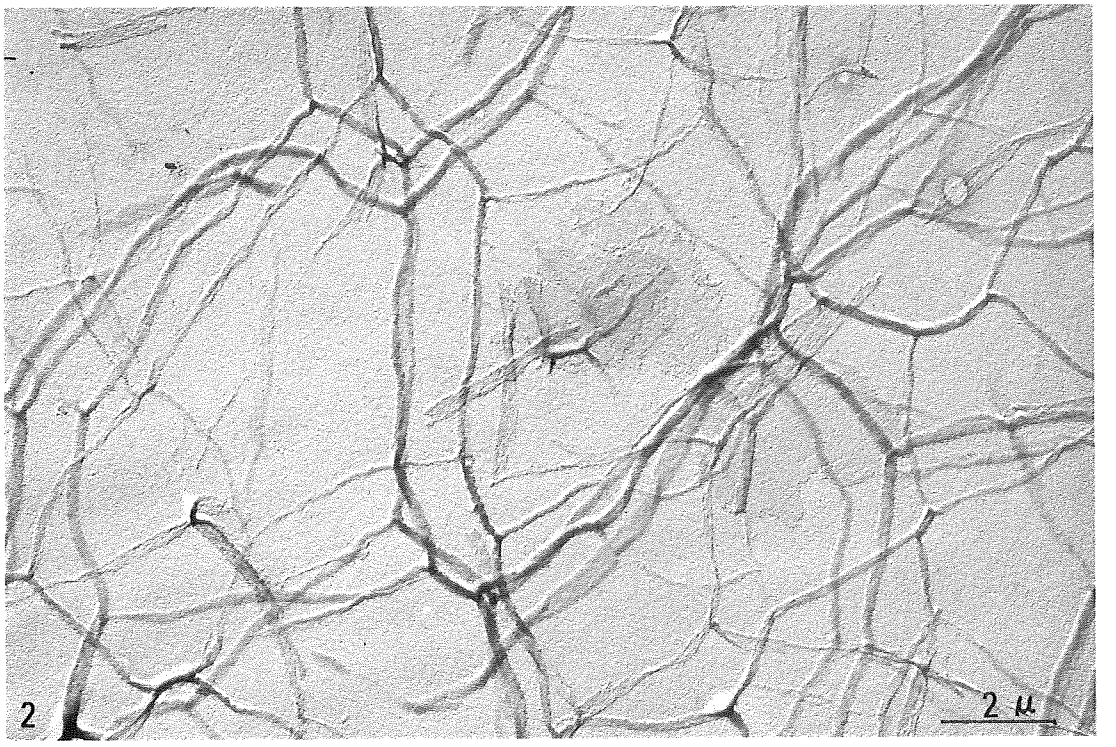

2
2 μ

Plate VI. EDTA decalcified. Fig. 1. Isolated by ultrasonic waves.
Fig. 2. Isolated by hand tearing method.
Inter-lamellar conchiolin membrane in *Neotrigonia margaritacea*. Minute openings (100–200 Å in diameter) of irregular shapes studded sporadically. Sizes of the openings are not greatly different in both methods.

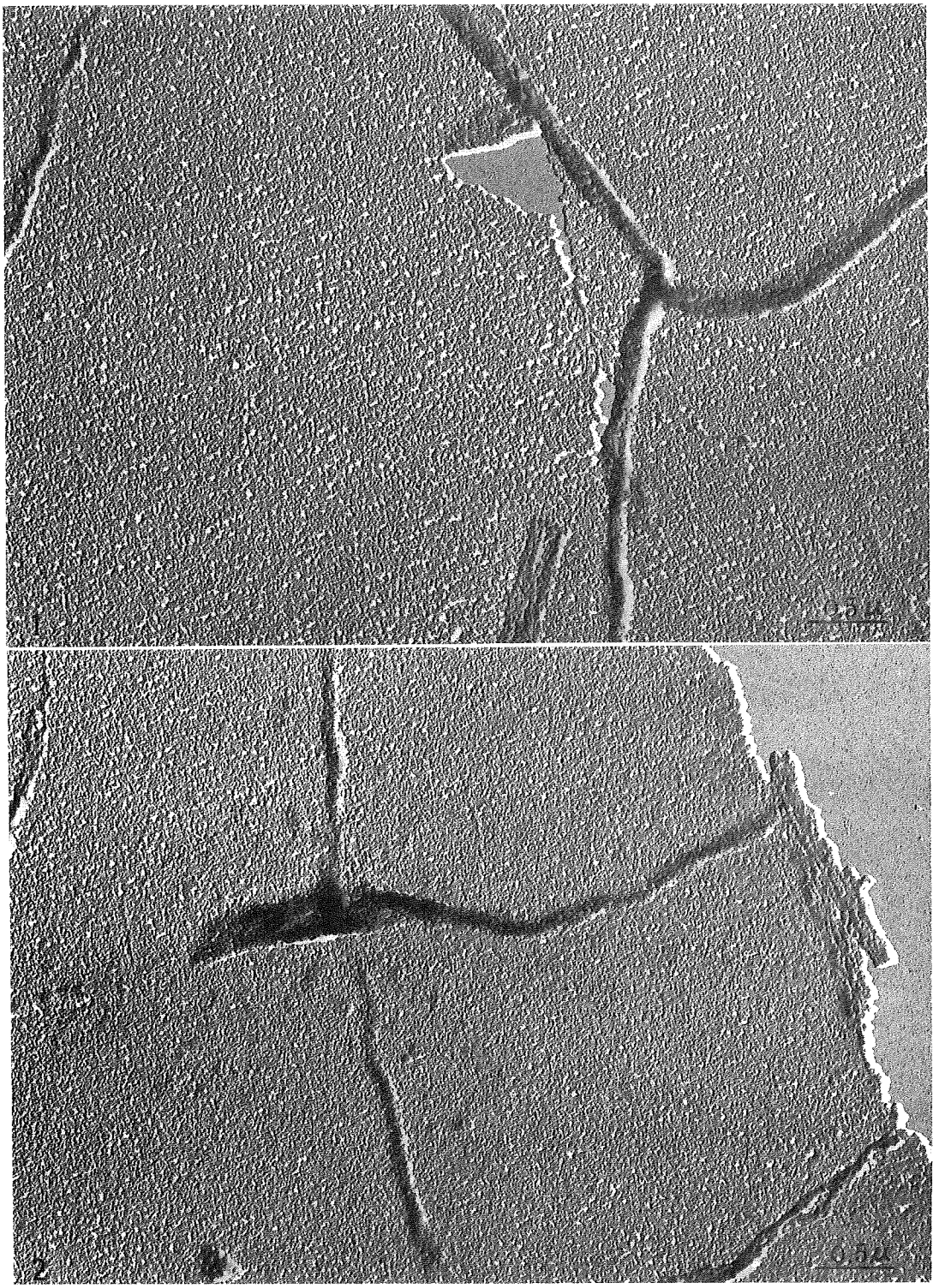
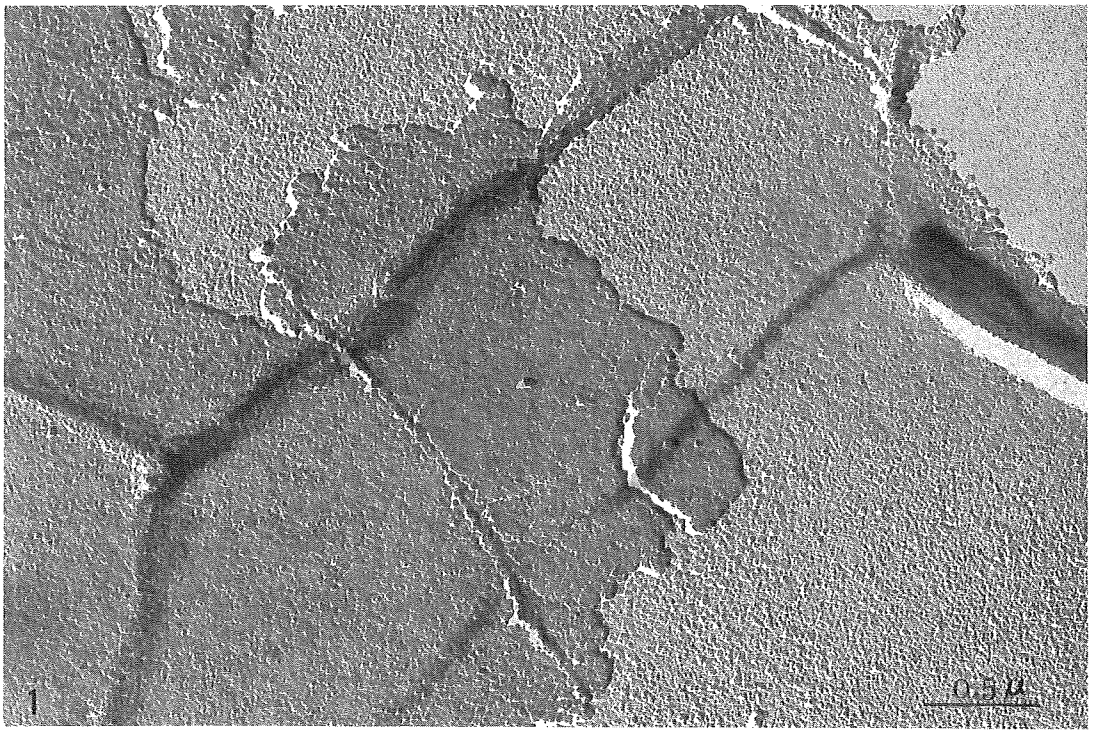

1
2

Plate VII. Chromium sulphate decalcified. Isolated by hand tearing method.

Fig. 1,2. Inter-lamellar conchiolin membrane in *Neotrigonia margaritacea*. Any openings can not be observed in the inter-trabecular areas of the inter-lamellar conchiolin membrane. Instead of the openings minute protuberances (mostly 100 Å in diameter) are seen on the whole parts of this sheet.

Bundaries of the trabeculae and the inter-trabecular areas difficult to be distinguished. Besides these protuberances aggregations of granules with high electron density are sometimes observed. (Fig. 2) Fig. 1,2 X 30,000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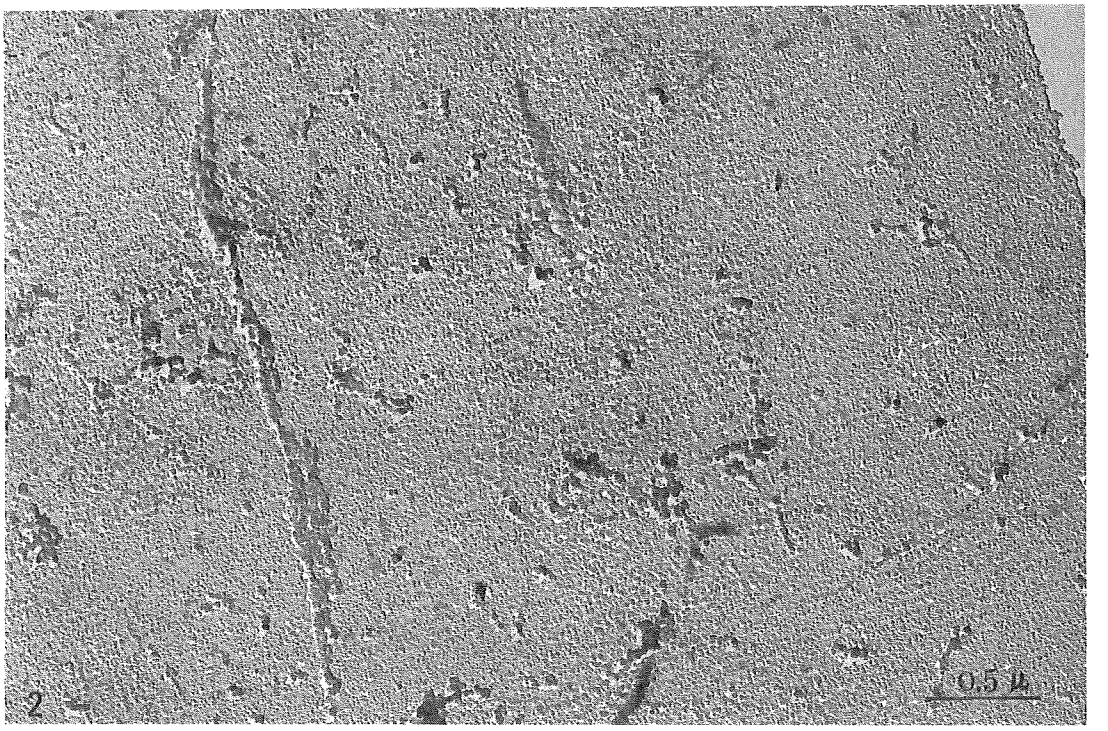
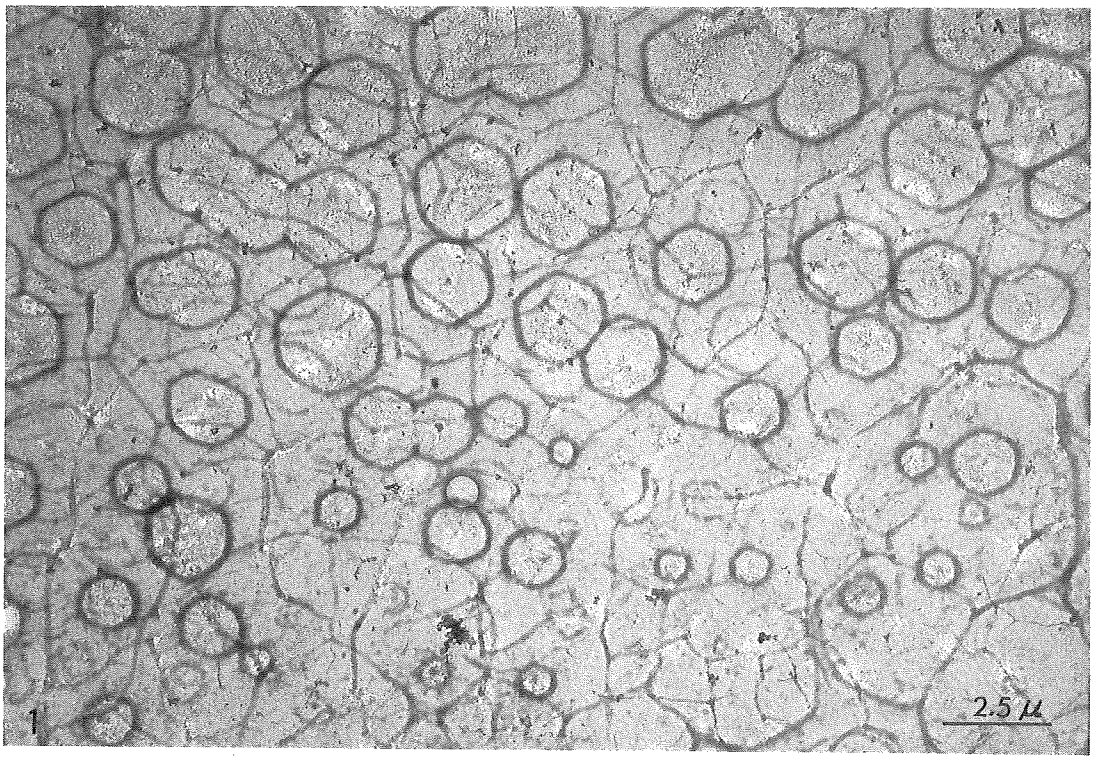

2
0.5 μ

Plate VIII. Chromium sulphate decalcified. Isolated by hand tearing method.

Fig. 1,2. Overlapped leaflets of several pieces of the inter-lamellar conchiolin membrane in *Pinctada martensii.*

Hexagonal or rounded outlines of high electron density might reflect ghost image of aragonite crystals just before perfect demineralization. Minute openings can often be observed in the inter-lamellar conchiolin membrane, but their sizes are much smaller (mostly less than 100 Å in diameter) than the conchiolin obtained after decalcification with EDTA. (See Plate III. Fig. 1) And in some places elaborate region without openings can also be observed. Fig. 1. X 6,000 Fig. 2. 15,000


1
2.5 μ

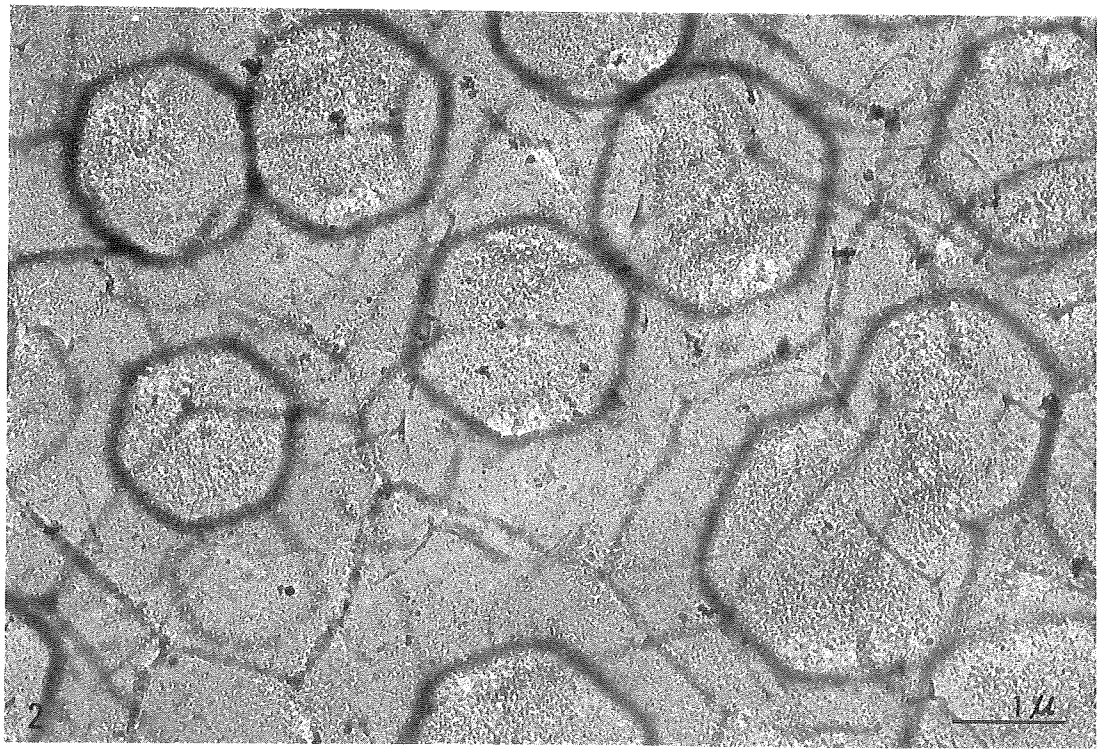
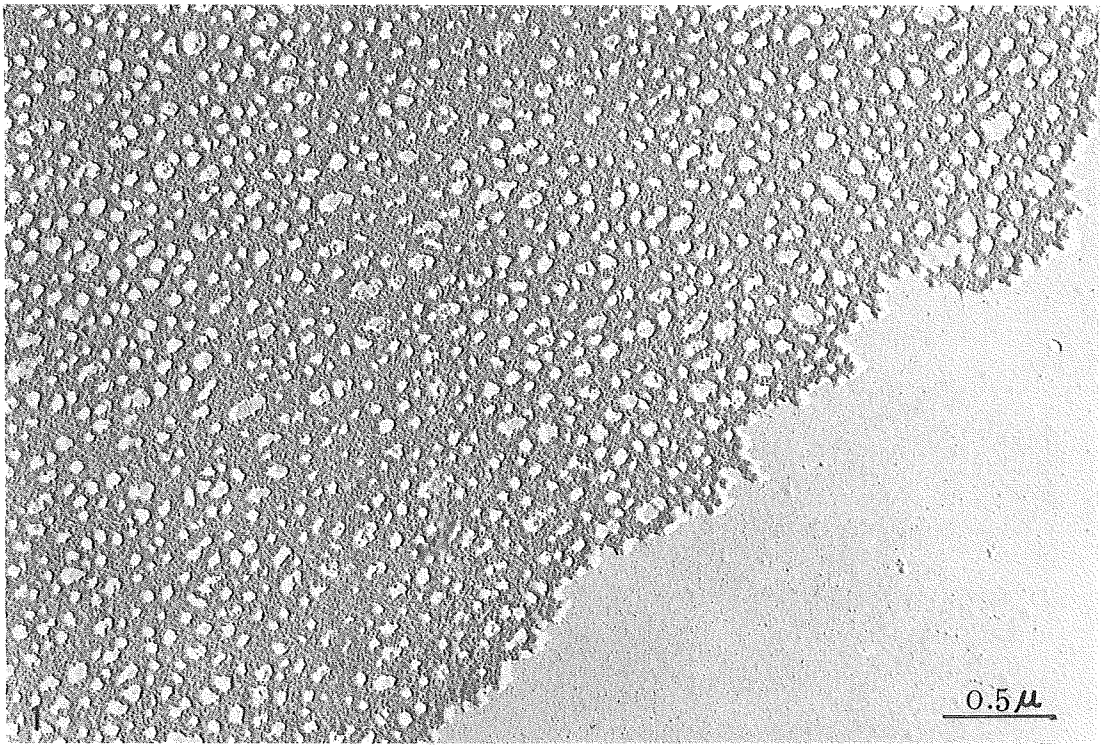

2
1 μ

Plate IX. EDTA decalcified. Isolated by ultrasonic waves.
Inter-lamellar conchiolin membrane in *Omphalius rusticus.*
Typical gastropod pattern characterized by round openings in the inter-trabecular areas (mostly 300–500 Å in diameter) can be observed. Thickness of this membarene is slightly larger than in pelecypods. Fig. 1. Bright field image. X 24,000 Fig. Dark field image. X 30,000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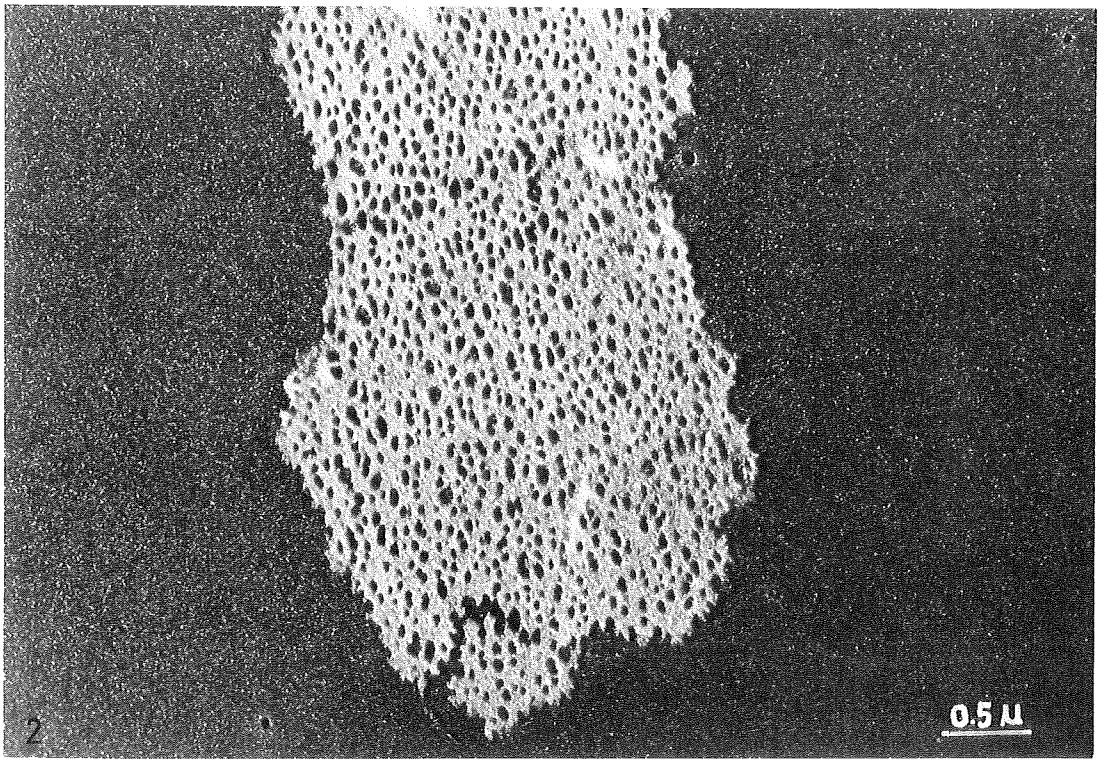

2
0.5 μ

Plate X. Chromium sulphate decalcified. Isolated by hand tearing method.

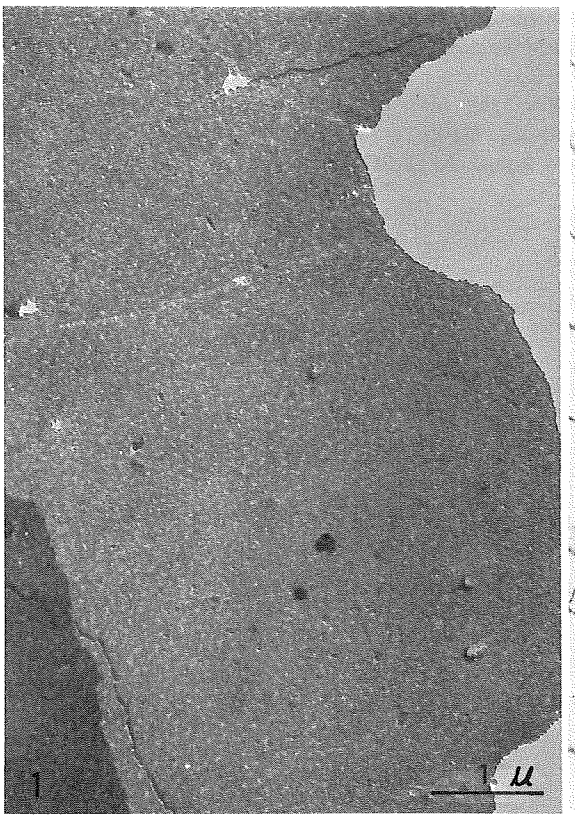
Fig. 1,3. Inter-lamellar conchiolin membarene in *Omphalius rusticus.*

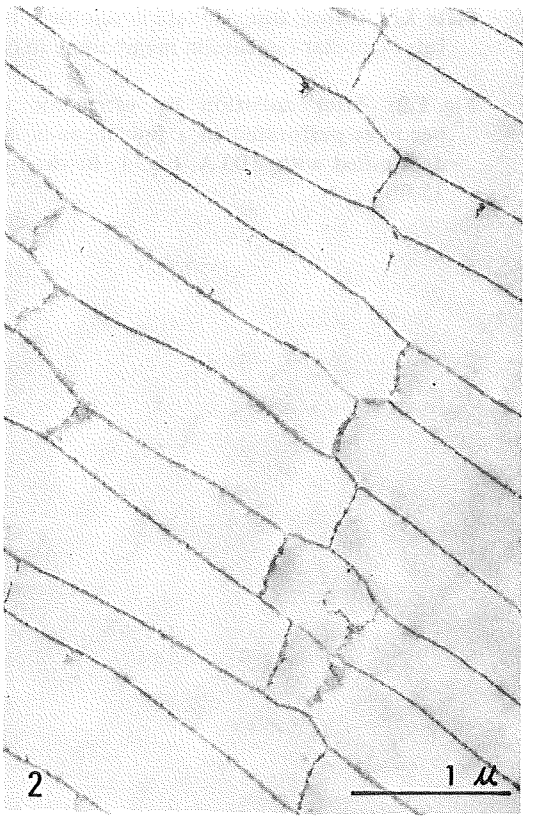
In this conchiolin membarne no openings can be observed. Round protuberances are seen on this membarene, and in other regions small patches of relatively low electron density can be found sporadically.

Fig. 2. Ultrathin section of the nacreous conchiolin in *Omphalius.*

Chromium sulphate decalcified.

Openings are not discernible in the inter-lamellar conchiolin membrane. Fig.1. X 15,000 Fig.2 X 21,000 Fig.3 X 45,000


1
1 μ


2
1 μ

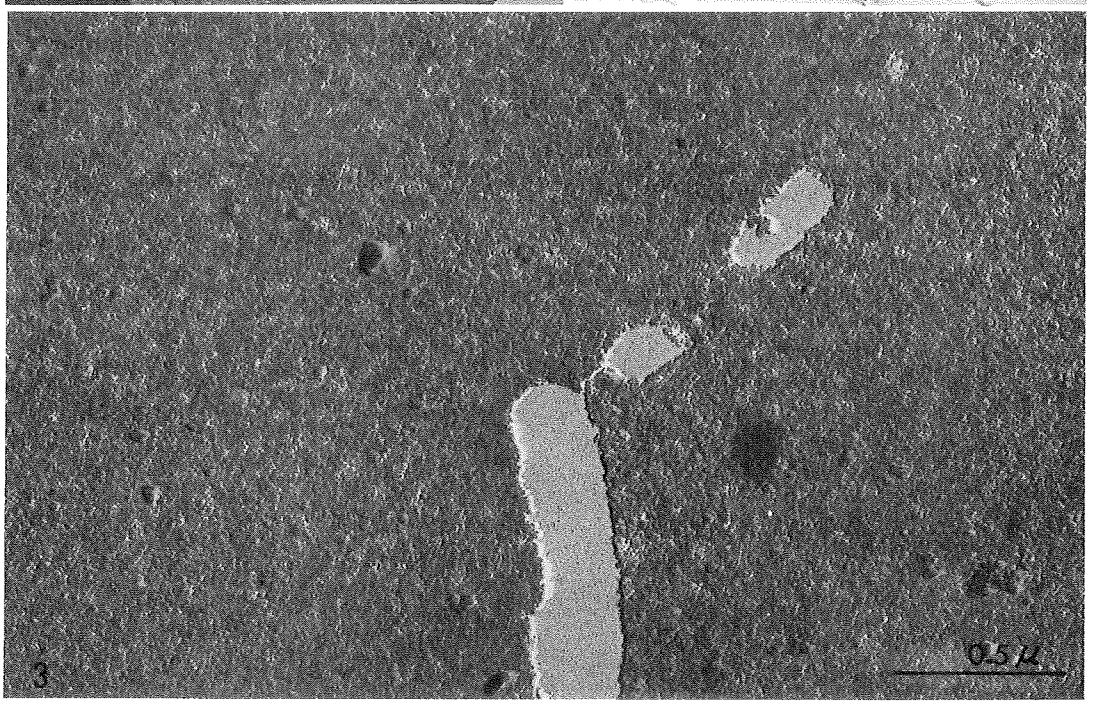
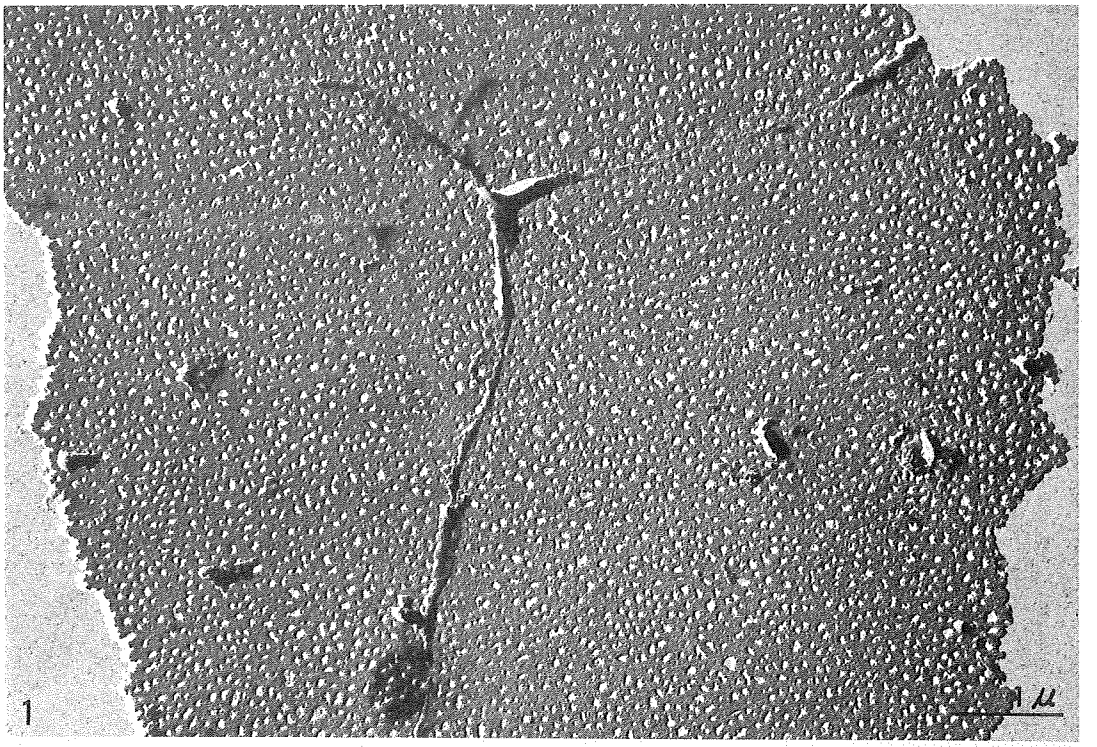

3
0.5 μ

Plate XI. Chromium sulphate decalcified. Isolated by ultrasonic waves.
Inter-lamellar conchiolin membarene in *Omphalius rusticus.*

Fig. 1,2. After radiation by ultrasonic waves the inter-lamellar conchiolin membrane becomes perforated in a few seconds, and this ultrastructure is very similar to the one decalcified with EDTA. Fig. 1. Bright field image. X 15,000 Fig. 2. Dark field image. X 15,000


1
1 μ

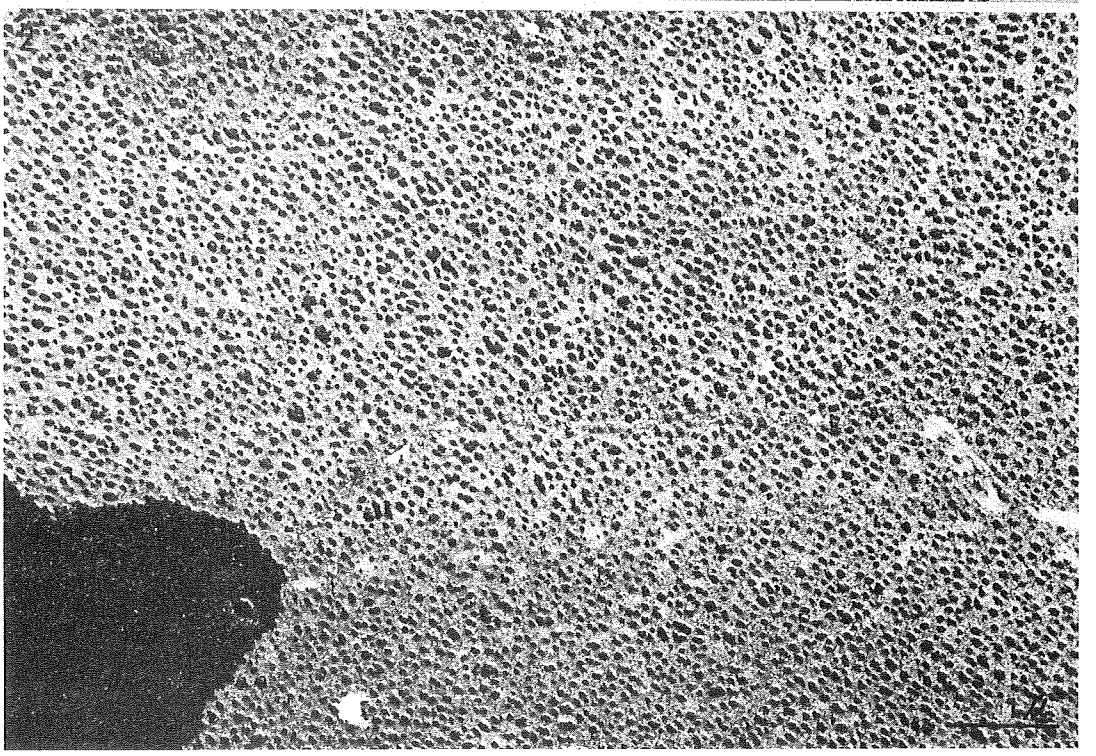
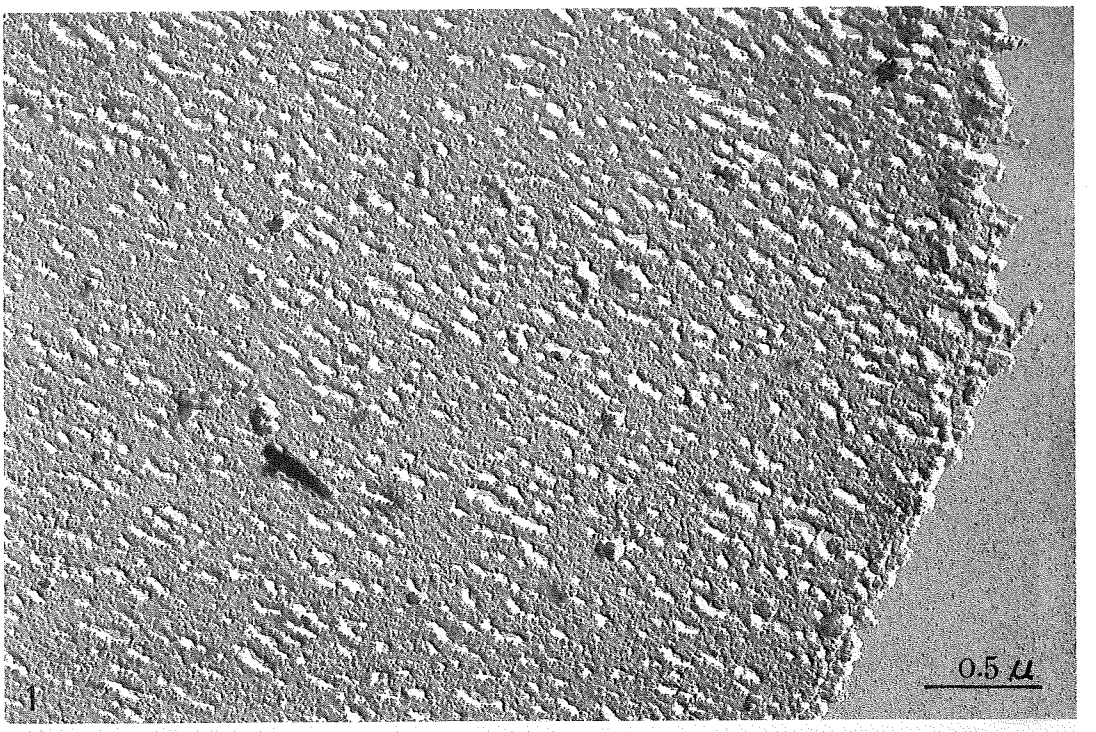

2

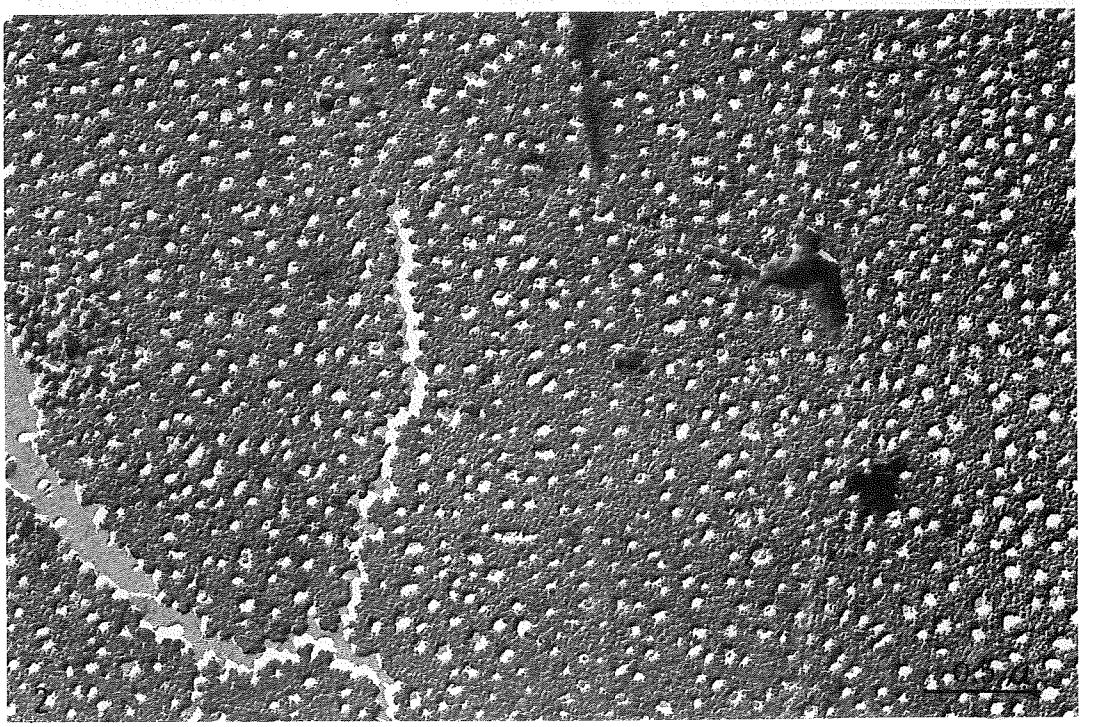
Plate XII.

Fig. 1. HCl decalcified. Isolated by hand tearing method.
Inter-lamellar conchiolin membrane in *Nautilus pompilius*.
Reticulated nautiolid pattern is similar to the one decalcifed with EDTA. But thickness of this membrane and protuberances on them decreased. X 30,000

Fig. 2. Chromium sulphate decalcified. Ultrasonic dispersed.
Enlarged electron micrograph of the inter-lamellar conchiolin in *Omphalius rusticus*. X 30,000



1



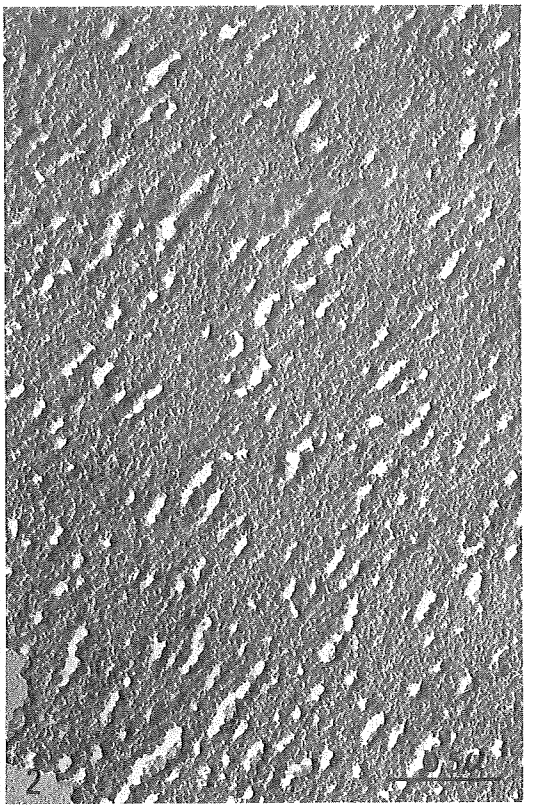
2

Plate XIII. EDTA decalcified. Nacreous conchiolin in *Nautilus pompilius.*

Fig. 1. Ultrathin section cut obliquely against shell layer of the body whorl.
Lace-like reticulate nautiloid pattern can be observed in this profile. (Compare with Plate XVIII, XIX) X 15,000

Fig. 2,3. Inter-lamellar conchiolin membrane isolated by ultrasonic waves.
Elongated openings of ovoid shapes in the inter-trabecular areas are distinguished from gastropods and pelecypods. Fig. 2. X 30,000 Fig. 3. X 24,000


1
1 μ


2
0.5 μ

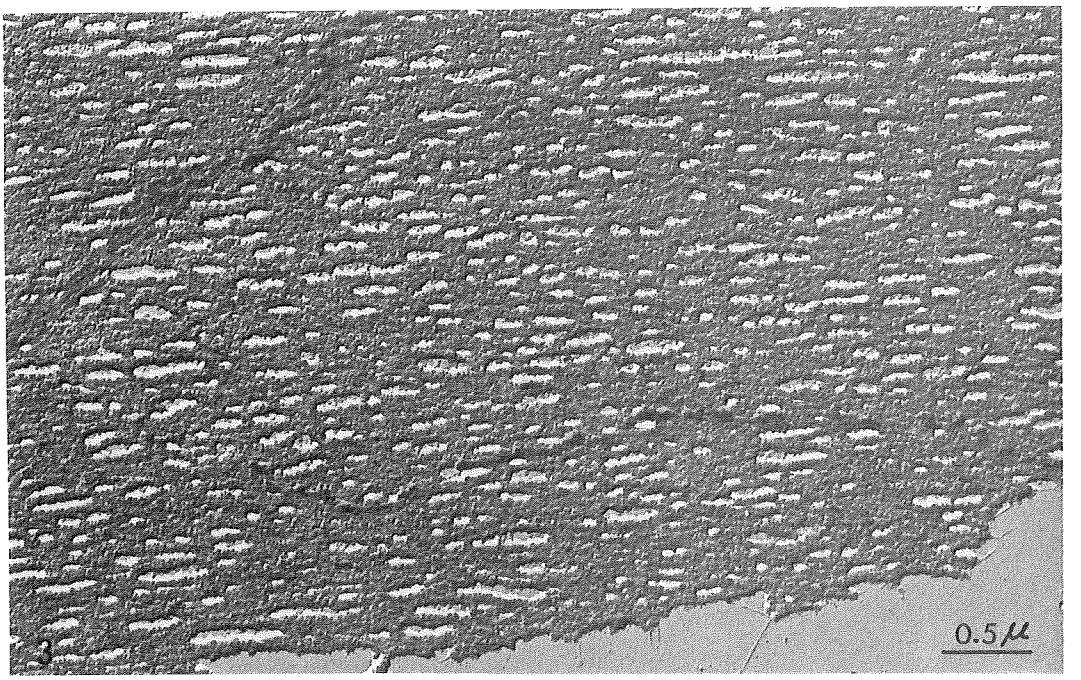
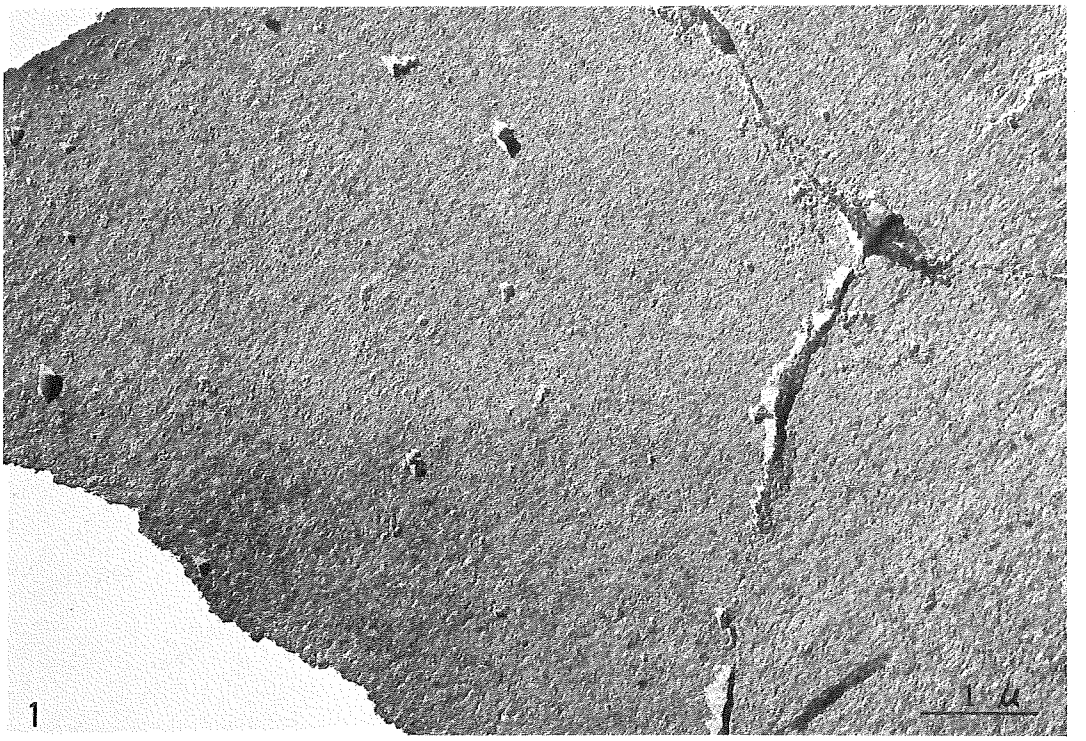

3
0.5 μ

Plate XIV. Chromium sulphate decalcified. Isolated by hand tearing method.
Inter-lamellar conchiolin membrane in *Nautilus pompilius*.
Elongated openings characteristic in the EDTA decalcified conchiolin are not utterly observed, and boundaries between the trabeculae and the inter-trabecular areas can not be easily discernible. Granular protuberances (mostly 100 Å in diameter) are adhering on this membrane. Aggregations of dense granules are also found sporadically. Fig. 1. X 15,000 Fig. 2. X 30,000


1
1 μ

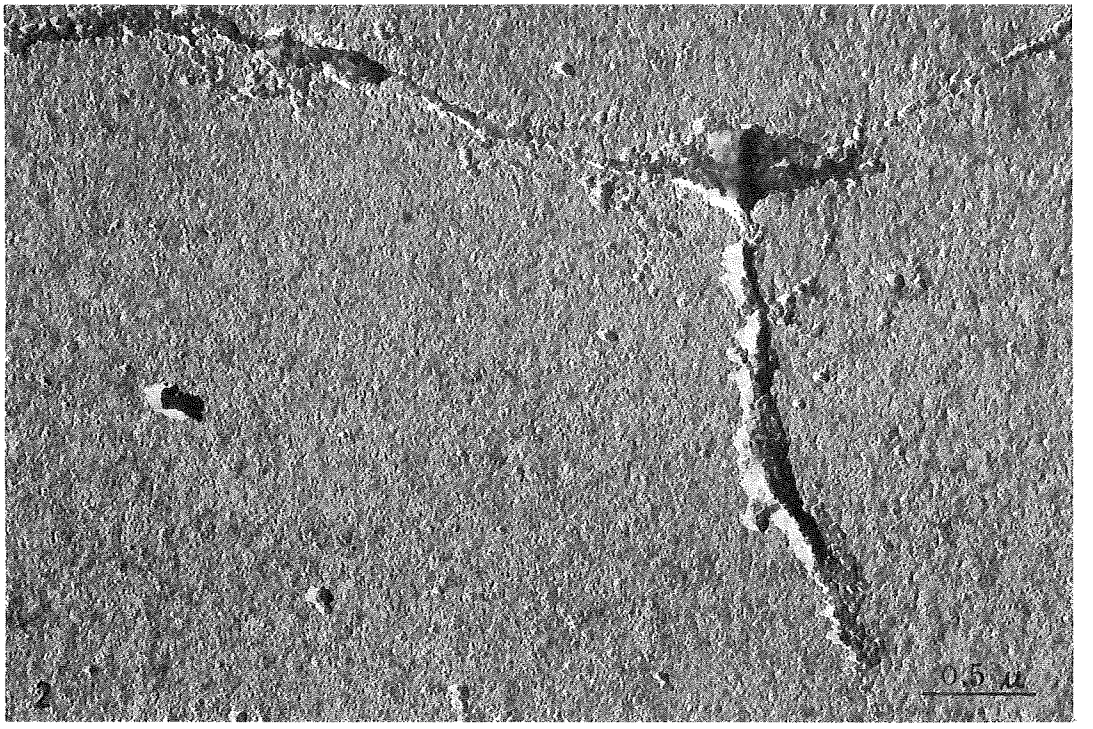
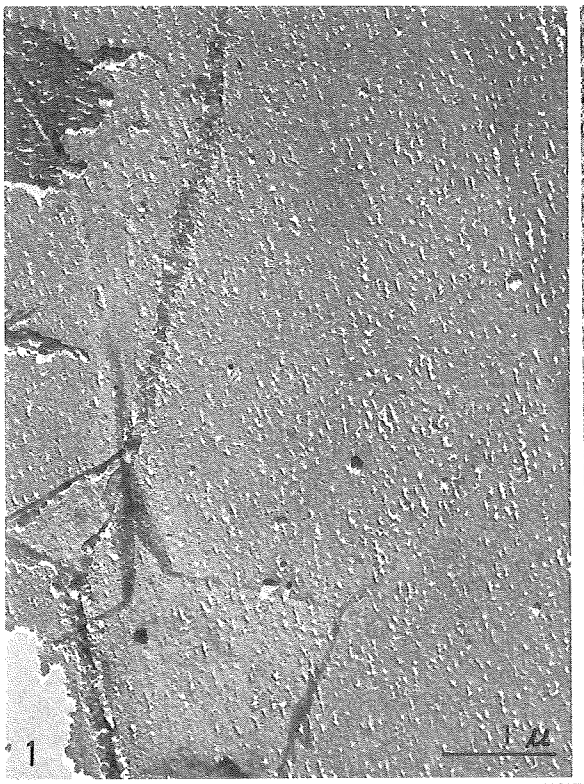
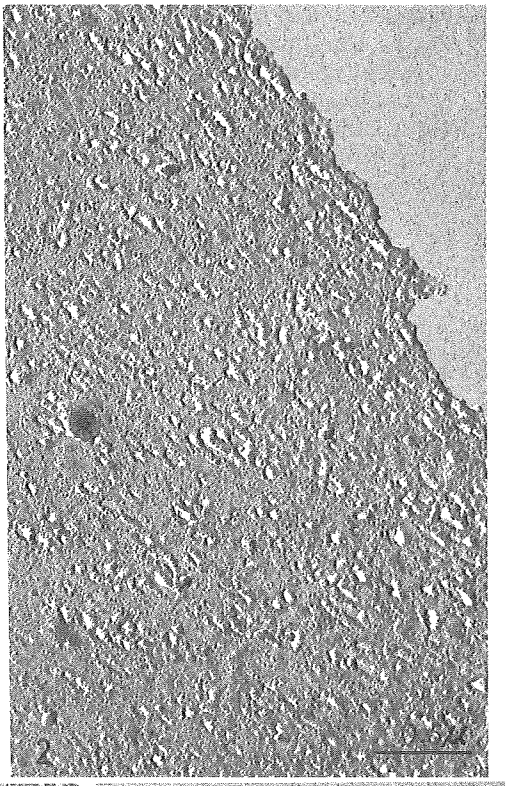

2
0,5 μ

Plate XV. Chromium sulphate decalcified. Radiated by ultrasonic waves.

Fig. 1-3. Inter-lamellar conchiolin membrane in *Nautilus pompilius*.

After radiation by ultrasonic waves elongeted openings are clearly visible. (Fig. 3) In the initial stage of radiation half-perforated portions can be observed. (Fig. 1,2) Fig. 1. X 15,000 Fig. 2. X 25,000 Fig. 3. X 30,000


1
1 μ


2
1 μ

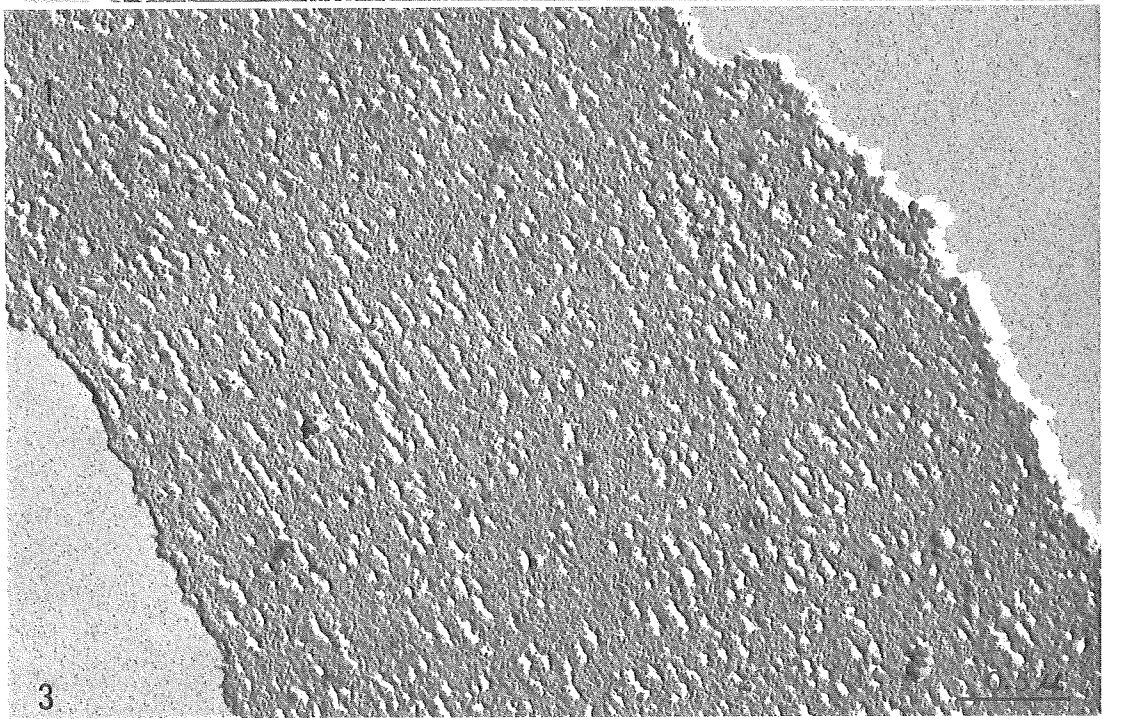

3
1 μ

Plate XVI. EDTA decalcified.

Fig. 1. Ultrathin section of the nacreous conchiolin in *Mytilus coruscus,* cut obliquely against the shell layer.
Minute openings in the inter-lamellar conchiolin membrane are vaguely visible. X 15,000

Fig. 2. Ultrathin section of the nacreous conchiolin in *Neotrigonia margaritacea.*
Inter-lamellar conchiolin are observed as notched membrane owing to the existence of minute openings in the inter-trabecular areas. (See in Plate VI) X 12,000


1
1 μ

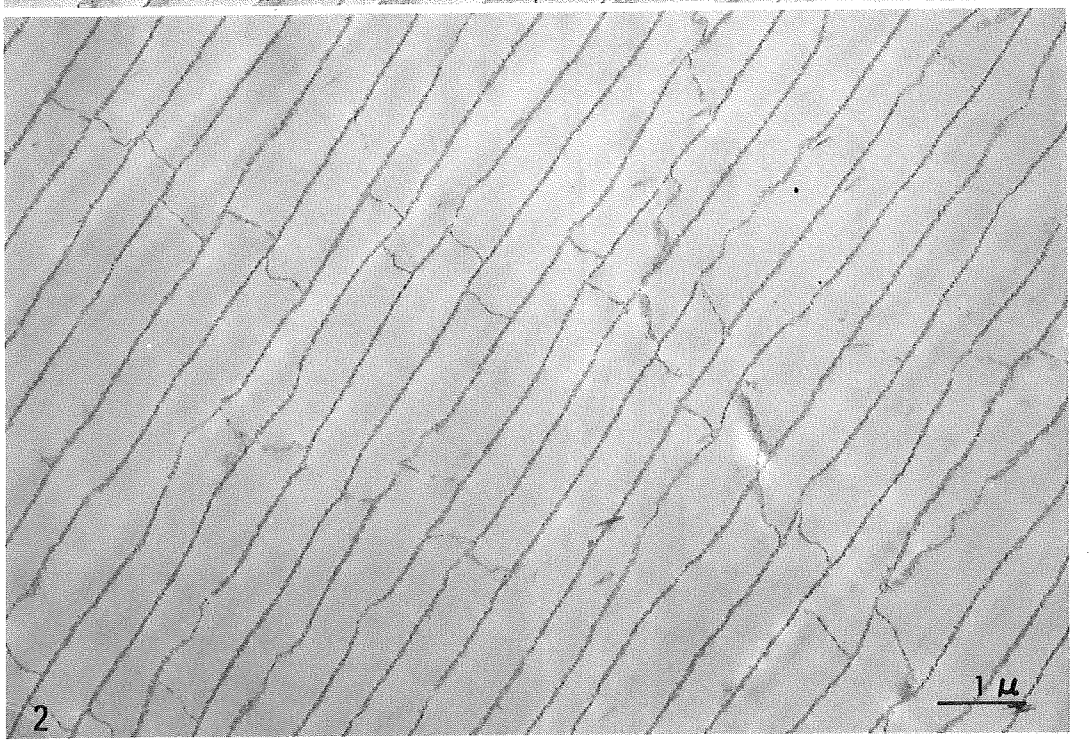
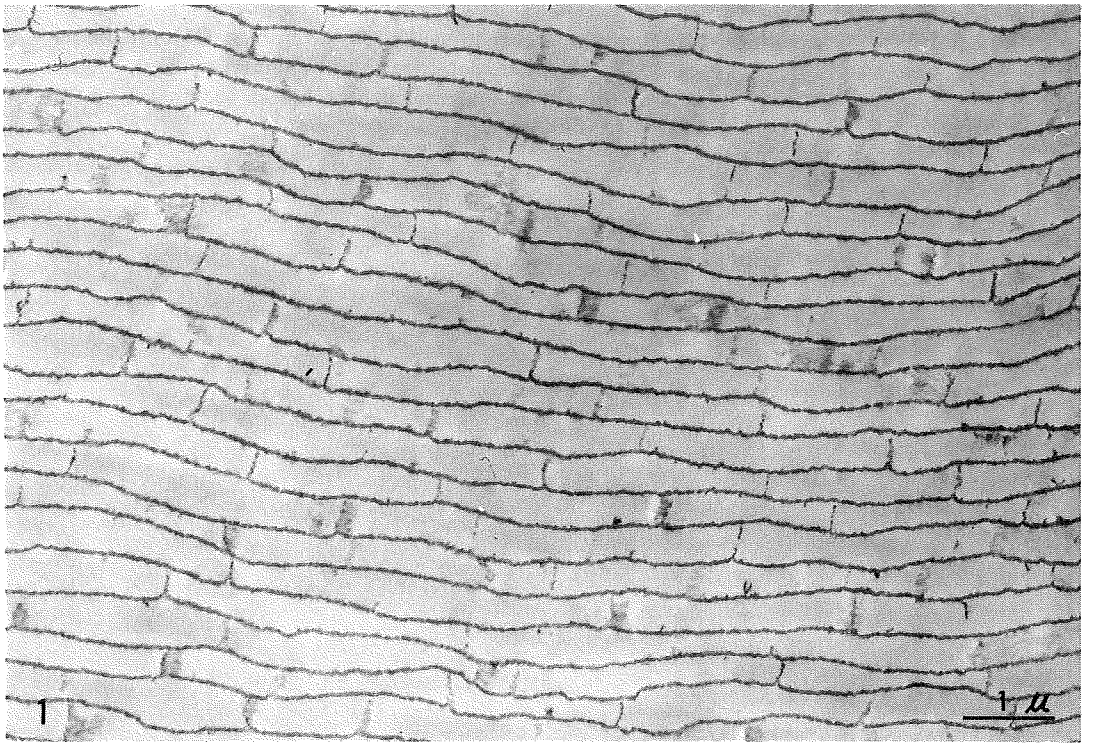

2
1 μ

Plate XVII. Chromium sulphate decalcified.

Fig. 1,2. Ultrathin section of the nacreous conchiolin in *Pinctada martensii.* Vertical profile. Brick wall construction of the conchiolin matrices is remarkable. Inter-lamellar conchiolin membrane are slightly undulated, but openings are invisible in any portion. Fig. 1. X 12,600 Fig. 2. X 18,000


1
1 μ

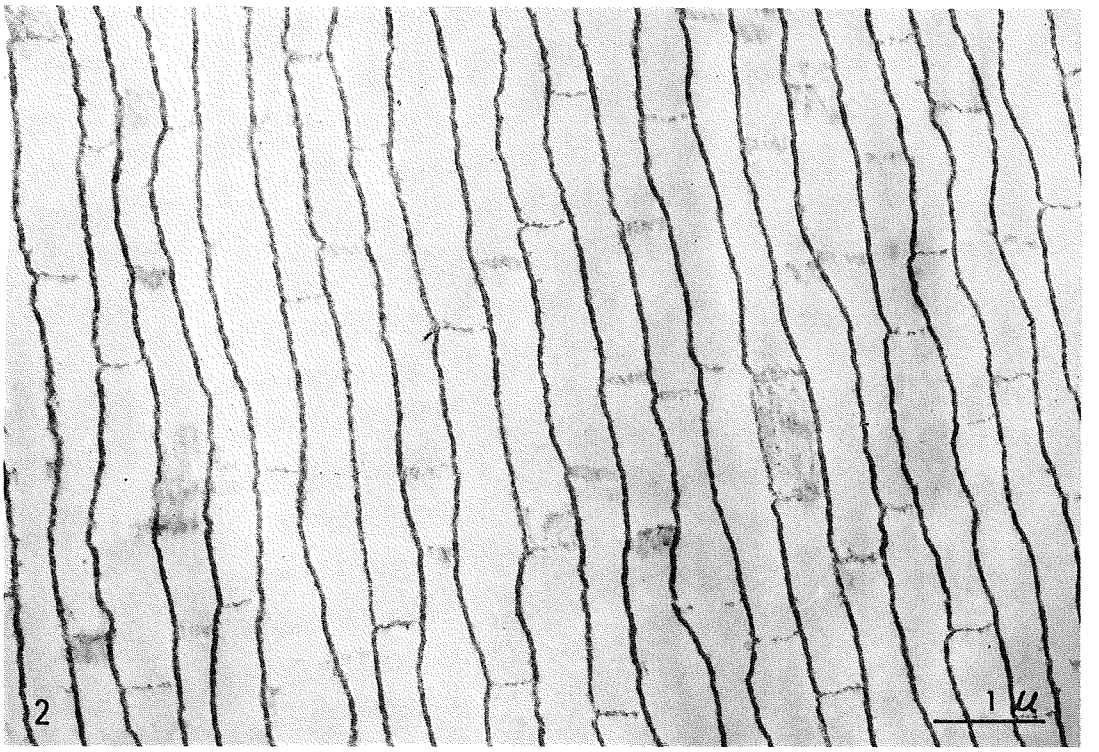
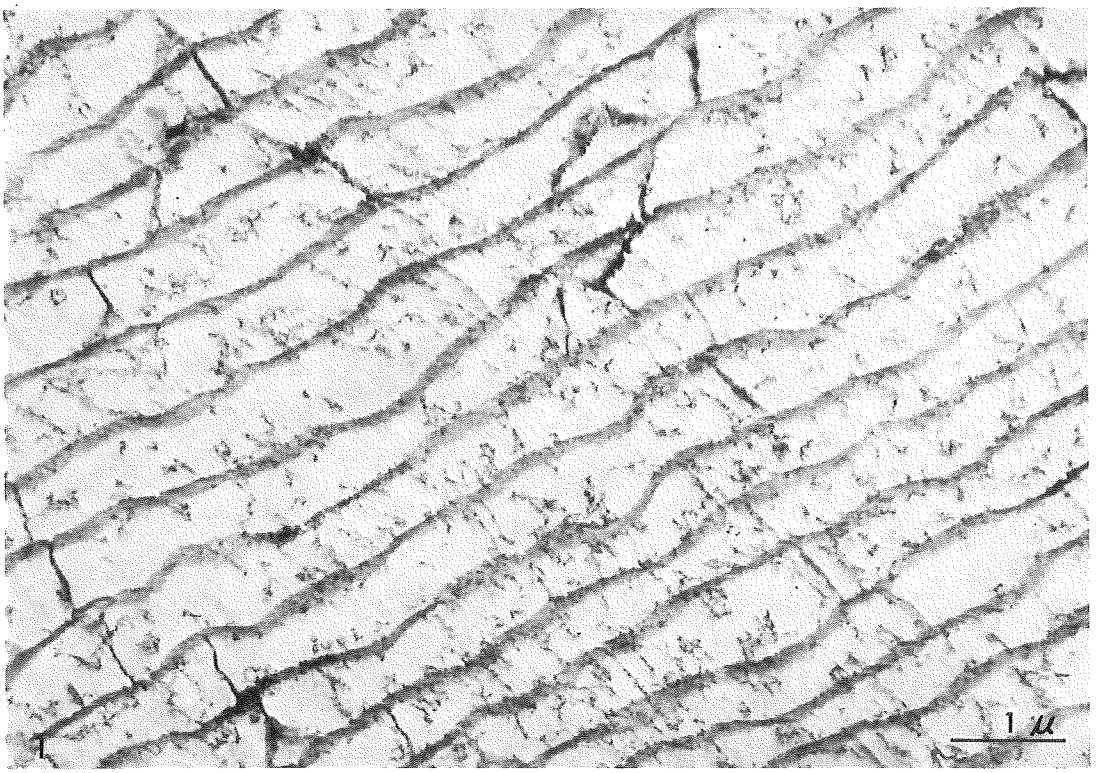

2
1 μ

Plate XVIII. Chromium sulphate decalcified.

Fig. 1,2. Ultrathin section of the nacreous conchiolin in *Nautilus pompilius*. Oblique profile. Openings can not be observed in any inter-lamellar conchiolin membranes. Thin organic membrane adhered and hung from the inter-lamellar conchiolin seems to be the intra-crystalline matrices. Fig. 1. X 15,000 Fig. 2. X 21,000

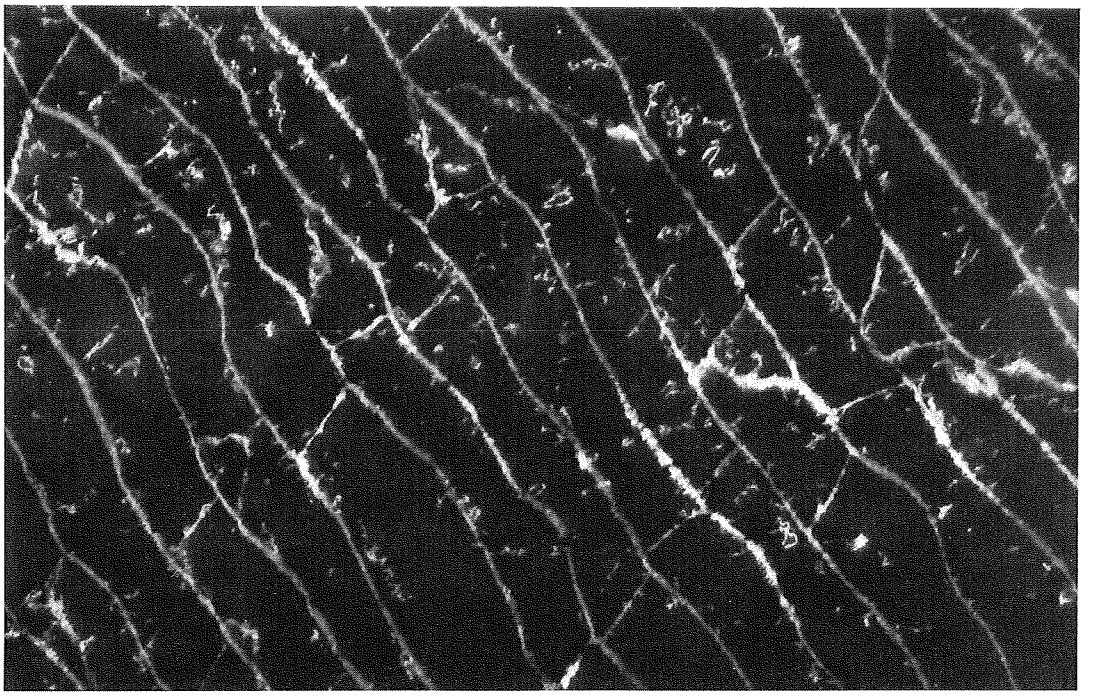

1 μ

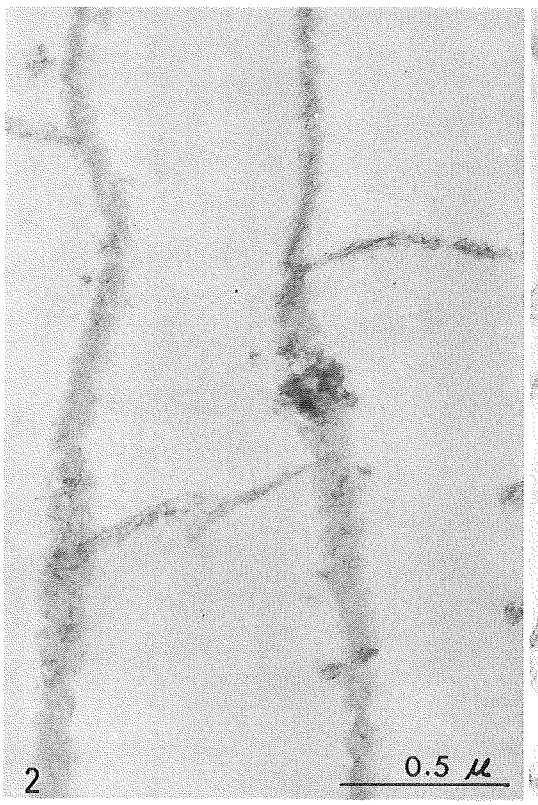

2
μ

Plate XIX. Chromium sulphate decalcified. Ultrathin section of the nacreous conchiolin in *Nautilus pompilius*.

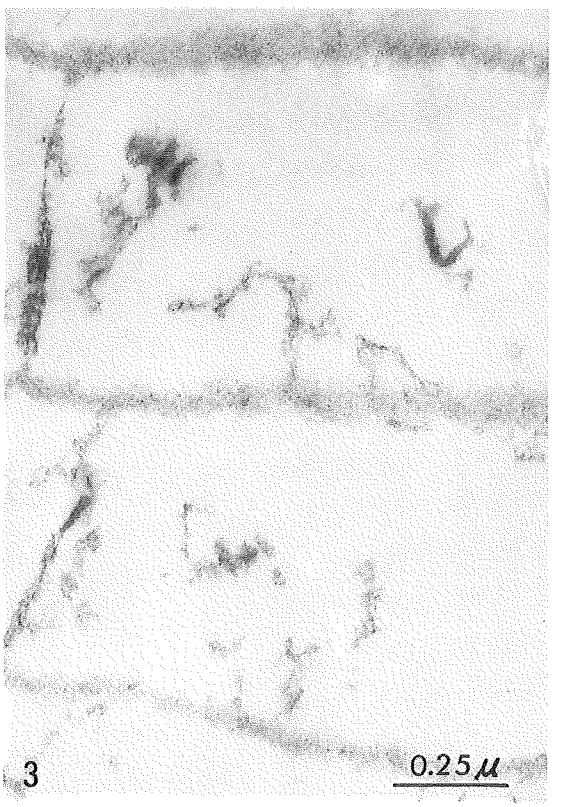
Fig. 1. Dark field image of the vertical profile of the conchiolin matrices.

Fig. 2,3. Enlarged pictures of the inter-lamellar conchiolin membrane.
Slightly oblique profile. Openings are invisible at a high magnification. Compare with Plate XIII. Fig. 1. X 12,000 Fig. 2. X 45,000 Fig. 3. X 60,000





2



3