Studies on Shell Formation

VII. The Submicroscopic Structure of the Shell of the Oyster *Crassostrea virginica**

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PLATES 148 TO 151

ABSTRACT

The submicroscopic structure of the growing surface of the shell of the oyster, *Crassostrea virginica*, was studied by means of shadowed replicas. The outer edge of the prismatic region consists of a fine grained matrix enclosing crystals, the surfaces of which show a finely pebbled structure. Crystal size varies continously from $0.01\ \mu$ to $8\ \mu$. The matrix surface shows no evidence of fibrous structure. The outer portions of the prismatic region exhibit a tile-like arrangement of large crystals separated by granular matrix 0.02 to $0.08\ \mu$ in thickness. The exposed crystal surfaces have indentations of varying form which appear as roughly parallel grooves spaced at intervals of approximately $0.3\ \mu$. The final form of this region is believed to result from the random distribution of crystal seeds, which grow without orientation and through coalescence and growth come into contact, producing polygonal areas. The crystal arrangement of the nacreous region is one of overlapping rows of crystals in side to side contact, and with one end of each crystal free, permitting continued increase in length. Crystal angles and plane indices are presented.

The structure of mollusk shell has been comprehensively studied with respect to its microscopic appearance (3, 2, 4, 16, 17, 22, 23), crystallography (3, 21, 24–26), and chemical composition (2, 5); and experimental studies, old and recent, have dealt with growth and repair (1, 2, 13, 15, 18, 27, 28, 32, 33). However, the mechanisms of normal shell growth still remain to be clarified. The information that electron microscopy is providing on the structure of shell (8, 9, 11, 31) and pearl (29, 30) strongly indicates that our understanding of growth mechanisms will be advanced by the further application of this technique to shell studies. The

excellent electron microscope studies of Grégoire, Duchâteau, and Florkin (8) and Grégoire (9) on decalcified shell have shown differences between a large number of species with respect to the pattern of the organic matrix. Watabe (29, 30) has utilized the replica technique of electron microscopy in interesting studies of the crystallography of the nacreous material of pearl.

The investigations reported here concern the submicroscopic structure of the crystalline and matrix material of normal growing shell, as studied by means of replicas made from the inner surface of the shell of the oyster *Crassostrea virginica*. A general description of the main areas of the shell surface is presented, and the succeeding paper gives special attention to crystal structure and growth processes in the nacreous region. The study of normal shell should be followed by studies of the alteration of the submicroscopic structure produced by experimental means. The oyster provides favorable material for such studies in that in-

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formation is accumulating with respect to methods for altering shell deposition under controlled conditions (12, 14, 19, 33).

Materials and Methods

Oysters (*Crassostrea virginica*, formerly *Ostrea virginica*) were collected near Beaufort, North Carolina, during the period of March to June, 1955. During March, oysters were maintained for 13 days in aerated sea water in large wood tanks. The sea water, which was supplied through hard rubber lines, was changed daily, and *Chlamydomonas moewusii* was supplied as food each day. The temperature range was 19°–21°C., and the pH was 7.5–7.9. Other oysters were taken directly from natural waters. No differences were observable between the shell structure of the latter and those maintained in tanks. All specimens used in the studies showed new shell growth on the posterior margin.

The inner shell surface was prepared for replicas by removing all tissue, scrubbing the surface with a toothbrush, and after drying, washing with ethylene dichloride or amyl acetate. The right shell was used throughout. Replicas were prepared with 2 per cent to 3 per cent formvar in ethylene dichloride, and 5 per cent collodion in amyl acetate. The formvar solutions were applied to a portion of the inner shell surface with a single stroke with the side of a glass rod, and the shell was rapidly moved back and forth to hasten drying. Collodion was dropped on the surface, which was then held vertically to permit draining. For stripping the replicas from the shell surface, cellulose tape was applied, the replica was moistened by breathing upon it, and the tape was then pulled free from the shell. The tape with the attached replica could be mounted directly on a glass slide for light microscope observation. In mounting a replica for electron microscopy, a wire grid was placed on the replica while still on the shell. The grid was covered with a tiny piece of moistened lens paper to prevent sticking of the cellulose tape which was applied over the grid and replica.

At the shell margin, stripping of the replica was very often unsuccessful. Replicas thick enough to strip without tearing from these rough surfaces were usually too thick to give good quality images in the electron microscope. With these, therefore, the silicon monoxide technique of Hall (10) was used. Briefly, after chromium shadowcasting (15° angle), about 5 mg. of SiO was evaporated normal to the surface from a distance of 14 cm. The grids were then transferred to a flat bottomed vessel, whose bottom was partly covered with solvent (amyl acetate for collodion, or ethylene dichloride for formvar). Gentle tipping brought the solvent into contact with the grid, and usually the collodion or formvar was dissolved without dislodging the SiO film from the grid.

Approximately 300 replicas were prepared for electron microscopy and light microscopy. The electron microscope used was the RCA model EMU-2d.

OBSERVATIONS

The growth of shell results from the deposition of crystalline material within an organic matrix of conchiolin by a thin sheet of tissue called the mantle, which lines the inner surface of the shell. Different parts of the mantle produce shell of characteristic pattern. In the oyster, *Crassostrea virginica*, two regions are distinguished: prismatic and nacreous regions. The outer portion of the prismatic region is a narrow border of variable width of the order of 1 mm. at the edge of the shell, with the remainder of the prismatic region forming a second border of similar width immediately central to it. The remainder, and the greater portion of the shell, is the nacreous region. These regions are well known from microscopic studies on various mollusks (see, for example, Schmidt (22)); and Hirata (12) has given a description of them in *Crassostrea virginica*.

The microscopic structure of the regions of the shell surface as reproduced by unshadowed formvar replicas photographed by the light microscope is shown in Figs. 1 to 3. The photographs are presented for orientation with respect to the electron micrographs which follow, and as illustrative of the usefulness of the replica technique for the microscopic study of the surfaces of opaque biological structures.

Prismatic Region:

Two features characterize the outer portion of the prismatic region: (1) crystals of various sizes, and (2) a large amount of matrix material (Figs. 1, 4 and 5). The crystals are birefringent under polarized light and disappear on treatment with dilute HCl (Hirata (12)). The crystals show a continous range of sizes from approximately 0.01 μ to 8 μ (Fig. 4), and, as they appear in replicas, have rounded borders. Schmidt (21) also reported small rounded crystals in the prismatic layer in *Malleus*. Their true shape is probably obscured by matrix covering the edges. Because of matrix at the borders, their size is probably larger than measurements of replicas indicate. At the lower size limit, crystalline material cannot be distinguished from irregularities in the matrix. The larger crystals undoubtedly result from growth (Fig. 4) and coalescence of individual smaller crystals. Crystal

coalescence is also strongly indicated in electron micrographs of material deposited by the oyster on nitrocellulose films (Maroney and Wilbur (19)). With a continuing increase in size, the crystals would make contact with other crystals and assume a polygonal shape. The crystal surfaces are very finely pebbled (pitted in replica) and show delicate indistinct elongate depressions (Fig. 5). The surface of the matrix is granular without evidence of fibrous structure (Fig. 5).

The inner portion of the prismatic region has a tile-like arrangement of large polygonal crystals separated by a thin layer of matrix (Figs. 2 and 6). A vertical section through the shell would show the crystals to be columnar (Coker *et al.* (6), Schmidt (22)). The surface area of the crystals increases with increasing distance from the periphery as in other pelecypods (Coker *et al.* (6), Schmidt (22)). Measurements of the mean maximal crystal width of two shells gave a value of 9.1 μ near the outer margins, and 44.6 μ at the central border. The variation in crystal size in any one area of the prismatic region is generally large (Fig. 2).

The exposed crystal surface is rugose, and increasingly so in crystals nearer the center of the shell. The surface indentations vary in form (Fig. 6), but may appear as roughly parallel grooves, which in measurements on 3 shells were spaced at mean intervals of 0.28 μ. (The replicas are, of course, reversed from the condition on the shell surface. Raised areas in the replicas in Fig. 6 appear light.) The matrix separating the crystal areas is about 0.16 μ to 0.75 μ wide, and is higher in the center with grooves at its edge. This is the form to be expected if approaching crystal edges squeeze the matrix.

It is apparent that some mechanism exists for maintaining the height of the matrix and the crystal surface at approximately the same level. Movements of the mantle probably accomplish this by distributing secreted matrix. If the crystal faces become higher than the matrix boundaries through crystal growth, the mantle movements would fill in the depressions between crystals with matrix. Excess material would be moved across the crystal faces. Such material is probably related to the grooves and delicate indentations in the crystal surface. The reason for the regularity of grooves is, however, not clear.

A transition zone (Schmidt (22)) lies between the prismatic and nacreous regions and, while clearly different from either region, shows certain features of both (Fig. 7). Prismatic-type matrix is shown in Fig. 7 as a band in the upper left corner. Matrix in the transitional zone is abundant. The dark shadowed areas in Fig. 7 are probably crystals and the remainder is matrix.
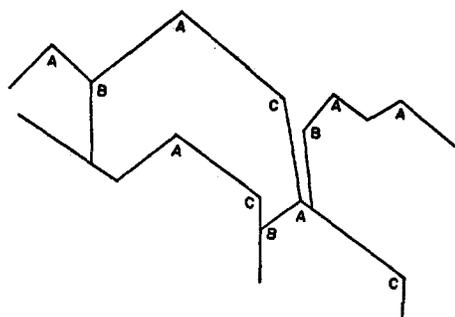
Nacreous Region:

The crystal form and arrangement of the nacreous region are strikingly different from the prismatic regions. The pattern in any local area resembles that of a shingled roof with the individual crystals arranged in overlapping rows (Figs. 3, 8, and 9. See also Schmidt (22)). Over a larger area a skewing of rows is often found, with neighboring areas showing rows of crystals running at very different angles (Fig. 3). Within the nacreous region, differences in detail between large areas are clearly evident in electron micrographs (Figs. 8 and 9). The nacreous region near the transitional zone is a region of abundant deposition of matrix. This material coats the crystals, and in vertical section would appear as organic septa between the crystal layers. At the free ends of the crystals it becomes piled up, apparently as a result of crystal growth. The matrix appears granular (Fig. 9).

The crystal form stands out with great clarity in the central parts of the nacreous (Fig. 9). This is due to the relative scarcity of matrix material. However, the white areas at the crystal edges undoubtedly represent matrix. The crystal faces are relatively smooth (Figs. 8 and 9), though small elevations and rather deep depressions (Fig. 9) can be seen. Crystals are always in side to side contact. Variation in width of crystals can be seen in Figs. 3, 8, and 9.

The calcium carbonate of the oyster shell is calcite (Bøggild, 3). Measurements have been made from electron micrographs on 168 angles appearing on basal planes of calcite. The values fall between 81° and 155°, with increased frequencies of angles of 96° to 100° for angle A (Text-fig. 1), and 131° to 135° for angles B and C (Table I). The frequency distribution is similar for angles B and C. In a separate study,[1] the most

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[1] The following data and discussion of crystal angles have been kindly contributed by Mr. N. Watabe from studies reported in the accompanying paper. We are also indebted to Mr. Watabe for his aid in the preparation of the manuscript.

Text-Fig. 1. Illustrations of angles reported in Table I.

TABLE I

Distribution of Angles of Calcite Crystals of the Nacreous Region

Angle A		Angle B		Angle C	
Degrees	No. of cases	Degrees	No. of cases	Degrees	No. of cases
81–85	4	111–115	1	111–115	0
86–90	6	116–120	4	116–120	4
91–95	8	121–125	6	121–125	8
96–100	24	126–130	7	126–130	6
101–105	12	131–135	20	131–135	10
106–110	12	136–140	3	136–140	5
111–115	11	141–145	4	141–145	4
116–120	5	146–150	0	146–150	2
121–125	1	151–155	0	151–155	1
	83		45		40

frequent values were approximately the same as those in Table I, and in addition an angle of 120° (115° to 125°) was also frequent. In some replicas the angles were 90°, 120°, and 150°. The range of angles for each of these three values may be explained by inclination of each crystal with the shell surface. That is, the angle measured in a replica will appear to be different from the true angle if the crystal was not precisely at right angles to the outline of the crystal projected by the electron microscope. For example, a crystal whose angles are 120°, 90°, and 150° may at a certain inclination of the crystal appear to have angles of 118°, 105°, and 137°. The angles 90°, 120°, and 150° are those found between $\{10\bar{1}0\}$ and $\{11\bar{2}0\}$ of calcite, e.g. 90° for $(\bar{1}\bar{1}20) \wedge (1\bar{1}00)$, 120° for $(1\bar{1}00) \wedge (10\bar{1}0)$, and 150° for $(10\bar{1}0) \wedge 11\bar{2}0)$.

DISCUSSION

By means of the shadowed replica method, the submicroscopic surface structure of the major

regions of the growing shell of the oyster has been described. Specifically, information has been obtained on the form and arrangement of calcite crystals in different regions and on the organic matrix material and its relation to the crystals.

There are marked differences between the outer (prismatic) and the central (nacreous) areas of the shell in addition to the well known difference in crystal form. Whereas the crystals of the prismatic region show no orientation, the crystals of the nacreous region are definitely oriented. Also, the matrix material is commonly more abundant in the prismatic, as compared with the central nacreous region. The central area of the shell is also a region in which the rate of calcium deposition, as measured with radioactive calcium, is relatively low as compared with the area near (but not at) the shell edge (Wilbur and Jodrey, 32). The more rapid growth in the peripheral region and the slower growth of the central portions of the shell have the effect of preventing a disproportionate thickening of the older regions, as the shell increases in size.

The two factors of abundant conchiolin and a high rate of calcium deposition would favor unoriented crystal growth in the prismatic region. The formation of a new layer of shell is probably initiated by the secretion of the conchiolin matrix. The next step is the deposition of crystal seeds in random distribution. The conchiolin, apparently lacking an organized structure, would not provide a substrate which would orient the crystals; and with rapid crystal growth, orientation would not occur. With growth and coalescence of small crystals, large crystals would be formed. With continued enlargement, crystals would come into contact to form contiguous polygons (Biedermann (2)), which is the characteristic form of the prismatic regions. The polygonal crystals further from the edge of the shell were of larger size. They are probably older, and may enlarge beyond their original boundaries as they become thicker. Vertical sections through the shell should clarify this point.

The nacreous region is in reality a mosaic of areas differing slightly or strikingly from each other. The basic pattern of overlapping rows of oriented crystals is seen throughout, but the details of crystal form, the orientation of the crystal axes, and the presence or absence of an overlying layer of secreted conchiolin differ from area to area. The growth of the nacreous region is also best considered in terms of semiautonomous areas, as we shall describe in the following paper.

Grégoire, Duchâteau, and Florkin (8) and Grégoire (9), in their extensive studies on decalcified shell, found that the matrix was generally amorphous or granular. However, certain of their electron micrographs suggest a fibrillar character (See reference 8, plate 23, Figs. 1 to 4). The matrix, as it appears on the uneven surface of the oyster shell, is finely granular without evidence of fibrils. An electron microscopic study of partially decalcified shell may reveal further details of matrix structure, but from the evidence at hand it appears that shell matrix differs greatly from the oriented matrix of bone (20, 7) along which the crystals are arranged.

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EXPLANATION OF PLATES

All electron micrographs were taken at a magnification of 2,600 to 7,500. The enlarged prints were made from reversed negatives.

PLATE 148

FIG. 1. Photomicrograph of replica of outer prismatic region. Crystals appear as black dots. Compare with Figs. 4 and 5. The replica was made with 2 per cent formvar and is unshadowed. $\times$ 1,100.

FIG. 2. Prismatic region. Photomicrograph of unshadowed formvar (2 per cent) replica of surface. See also Fig. 6. $\times$ 1,100.

FIG. 3. Nacreous region. Photomicrograph of unshadowed formvar (2 per cent) replica of surface. Compare with Figs. 8 and 9. $\times$ 1,100.
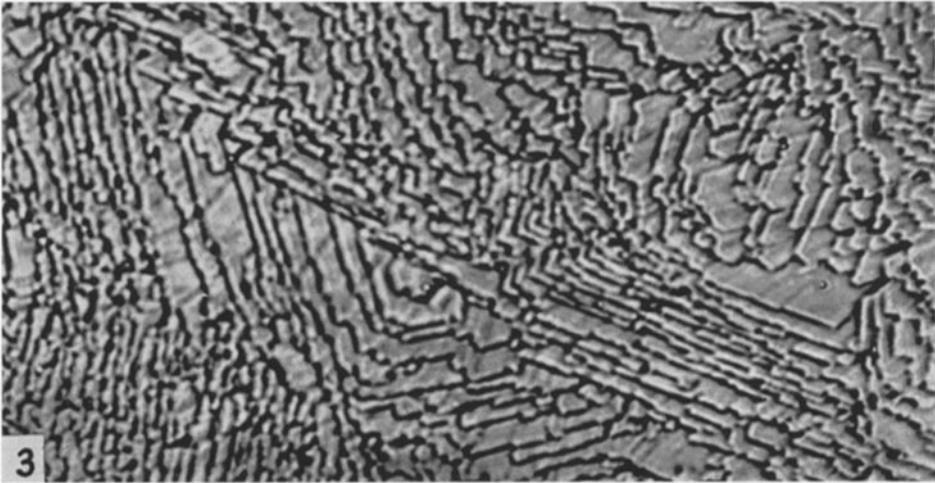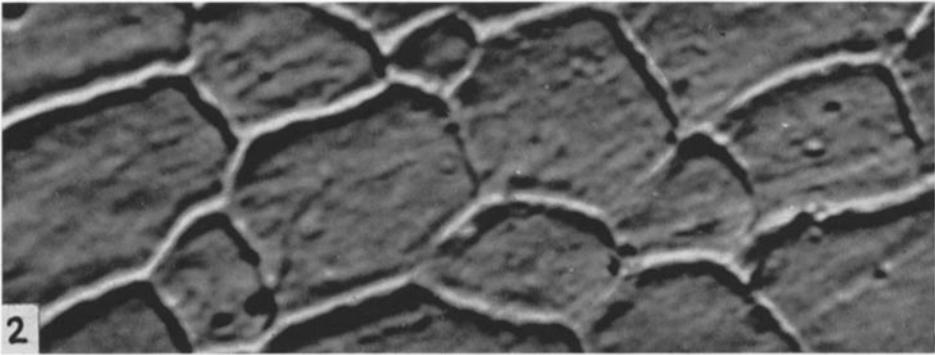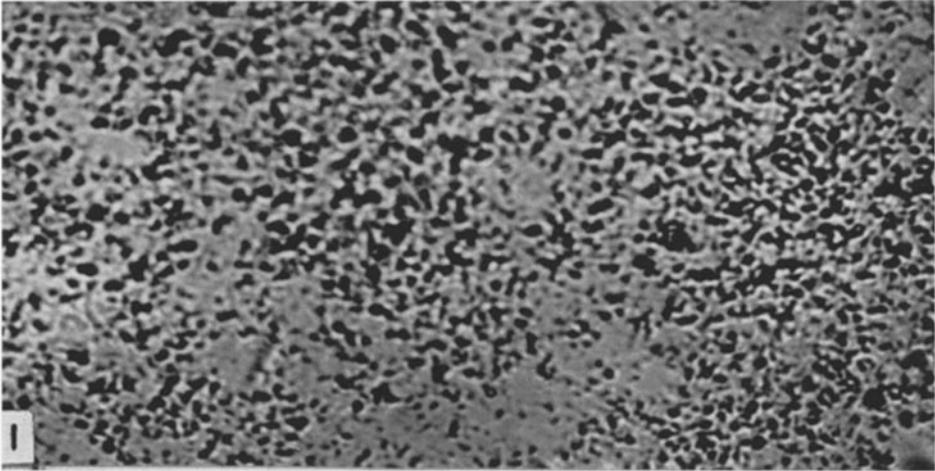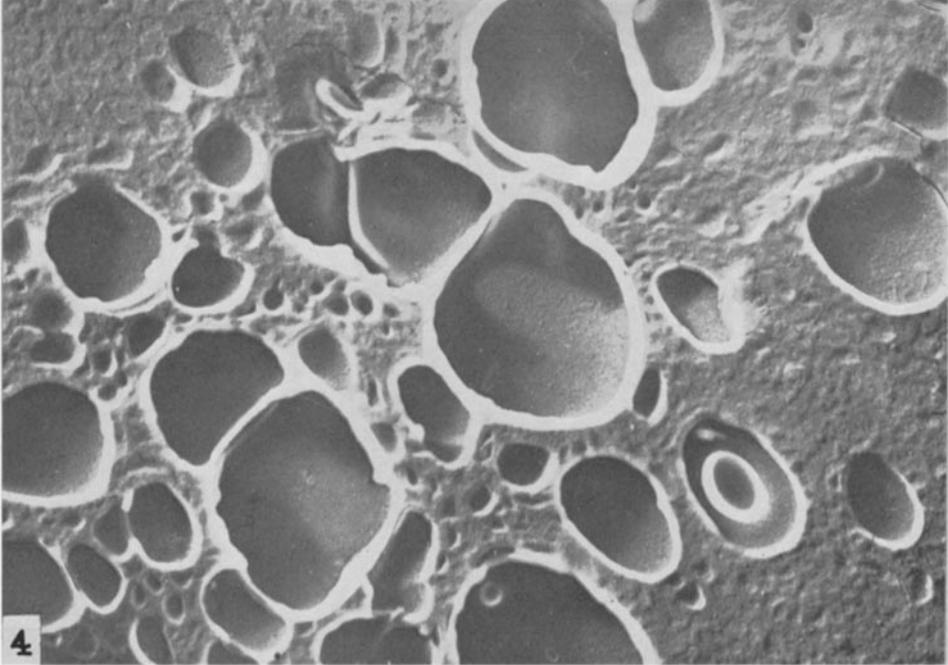
(Tsujii *et al.*: Shell structure)

PLATE 149

FIG. 4. Outer prismatic region. Electron micrograph of replica of surface, showing crystals of various sizes in the matrix. Silicon monoxide replica, chromium shadowed. × 27,800.

FIG. 5. Outer prismatic region. Replica showing portion of large crystal (lower half of photograph) and matrix. The bubble-like structure apparent here is probably an artifact. × 38,300.
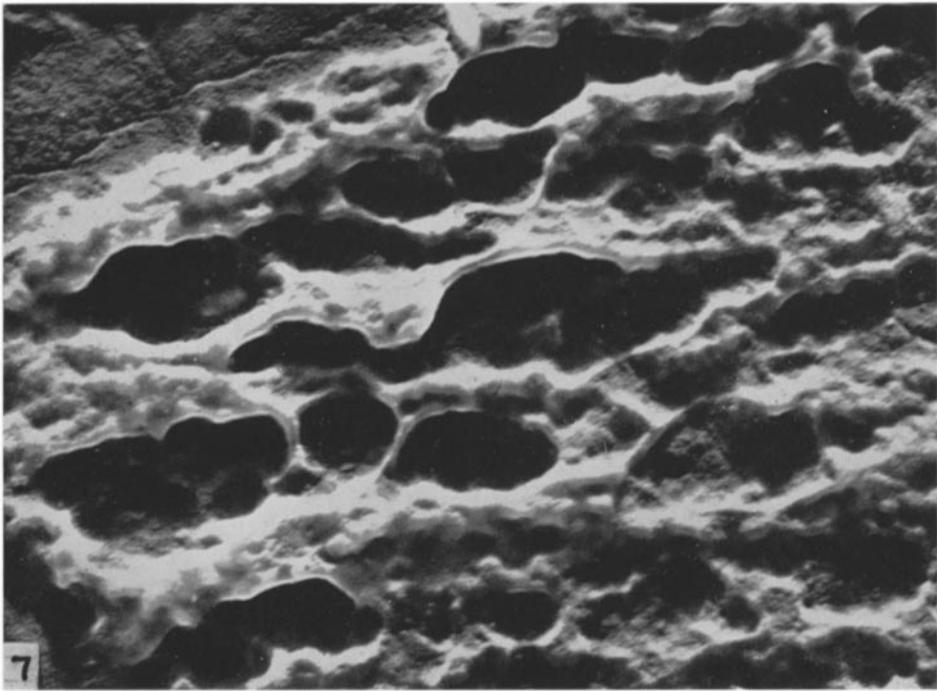
(Tsujii *et al.*: Shell structure)

PLATE 150

FIG. 6. Prismatic region. Replica showing prismatic crystals separated by thin bands of matrix. The surfaces of the crystals show irregular grooves which are roughly parallel. 2 per cent formvar replica, chromium shadowed. × 11,800.

FIG. 7. Transitional zone between prismatic and nacreous regions, including an area near the edge of a large prismatic crystal. Note intercrystal matrix in upper left. × 11,100.
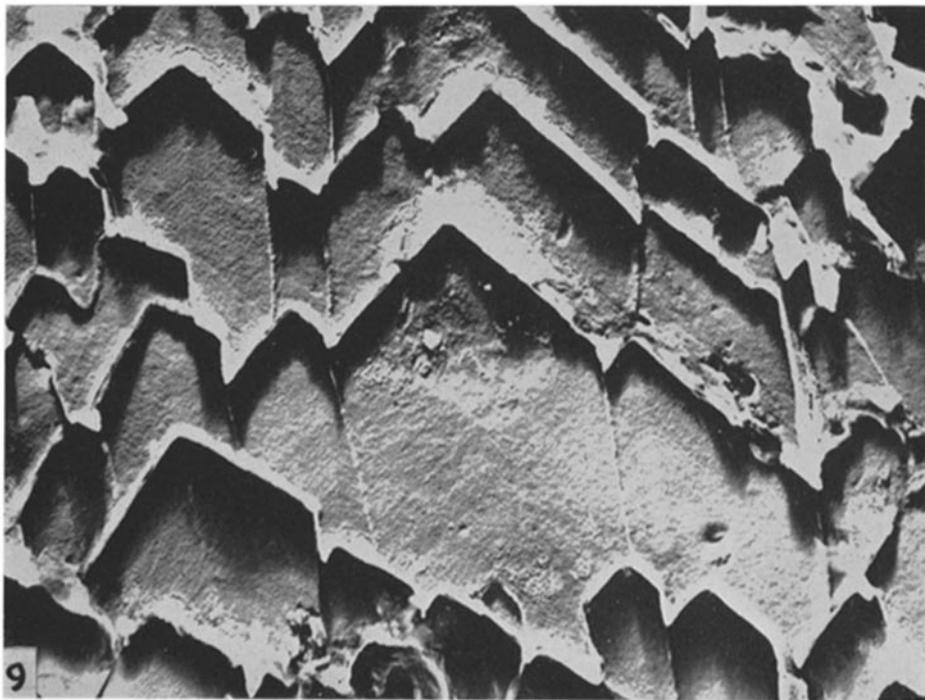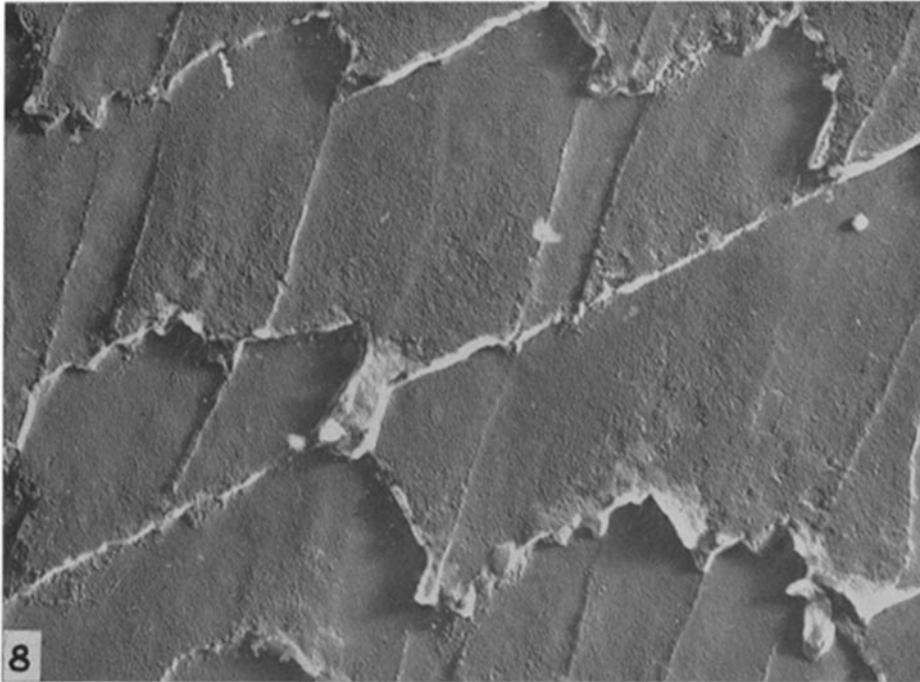
PLATE 150
VOL. 4

(Tsujii *et al.*: Shell structure)

PLATE 151

FIG. 8. Nacreous region in center of shell. Note that edges of crystals are not always sharp. Compare with Fig. 9. Silicon monoxide replica, chromium shadowed. × 20,000.

FIG. 9. Nacreous region near center of shell. Edges of crystals are more clearly defined than in Fig. 8. Silicon monoxide replica, chromium shadowed. × 11,100.

(Tsujii *et al.*: Shell structure)