Studies on Shell Formation

VIII. Electron Microscopy of Crystal Growth of the Nacreous Layer of the Oyster *Crassostrea virginica*

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(Received for publication, November 1, 1957)

ABSTRACT

Electron microscope observations have been made by means of the replica method on growth processes of calcite crystals of the nacreous layer of the shell of the oyster, *Crassostrea virginica*. Layer formation is initiated by the secretion of a conchiolin matrix and the deposition of rounded crystal seeds on or in this material. In some areas crystal seeds are elongate and within a given area show a similar orientation, probably due to slower deposition. The seeds appear to increase in size by dendritic growth, and smaller seeds become incorporated into larger ones which come into contact to form a single layer. With further growth, crystals overlap, forming a step-like arrangement. The direction of growth is frequently different in neighboring regions. Crystal seeds deposited on crystal surfaces are usually elongate and oriented. Well-developed crystals have a tabular idiomorphic form and are parallel in their growth. Rounded and irregular crystals were also observed. The crystals show reticular structure with units of the order of 100 Å and striations corresponding with the rhombohedral axes of the crystals. The role of the mantle is discussed in relation to the growth patterns of crystals and shell structure.

Electron microscopy has made possible the study of details of structure of both the organic (Grégoire, Duchâteau, and Florkin (3), Grégoire (4)) and crystalline portions of the mollusk shell (Helmcke (6); Watabe and Wada (13); Tsujii, Sharp, and Wilbur (11)). We have employed this method in the present study to follow details of growth in the nacreous layer of the shell of the oyster *Crassostrea virginica*. Two main structural problems are concerned: the growth and orientation of single calcite crystals and the pattern formed by the association of the crystals. Other aspects of shell growth relate to (1) the contributions of materials by the thin sheet of mantle tissue which covers the inner surface of the shell, and (2) factors both outside and within the oyster which govern mantle processes. The mantle deposits the organic matrix on or in which the crystals are laid down. The presence of this organic material will in itself influence the form of the crystals, as will be described. The mantle is important also in providing the calcium (Jodrey (7)) and the carbonate for calcite formation, and in controlling the environment in which the crystals are formed. The role of the mantle and the influence of the environment upon its activity will be considered in this report only secondarily, as our concern is primarily with the interpretation of shell growth in terms of crystallographic processes.

Materials and Methods

Oysters (Crassostrea virginica) about 6 to 10 cm. in length were collected in March 1957 at Beaufort, North Carolina, from water of temperature 8°C. Some specimens were killed immediately, and the remainder...
were kept from 15 hours to 9 days in aerated sea water in a large wood tank at about 20°C. to induce deposition of new shell. Those kept in the tank showed regular shell movements, and after 9 days new shell growth was present at the margins. The shells were cleaned on the inner surfaces by scrubbing with a toothbrush. After drying, they were cleaned again with ethylene dichloride.

In order to select appropriate areas of the shell for electron microscopy, replicas of the entire inner surfaces were prepared for examination with the light microscope. A relatively thick layer of 3 per cent formvar solution was spread over the surface with a glass rod and left to dry at room temperature. After about 2 hours, the formvar film was easily removed and mounted on a glass plate. Depending upon the size of the shell, from 5 to 20 areas of the surface of each right shell were selected for electron microscope study. In addition to providing a simple means for making a preliminary study of the surface, this technique also removed dust and loose particles.

Two-step replicas of formvar-silicon monoxide or formvar-carbon were prepared for electron microscopy using 3 per cent formvar in ethylene dichloride. Formvar-silicon monoxide replicas shadowed with chromium were prepared according to the method of Hall (5). The method of Bradley (1) was used for formvar-carbon replicas. After the replicas were coated with silicon monoxide or carbon, they were placed in about 20 ml. of ethylene dichloride for 15 to 20 hours to dissolve the formvar, and then mounted on metal grids. More than 700 electron micrographs were taken.

**Observations**

The nacreous region comprises the large central area of the shell exclusive of the area of muscle attachment. It is bounded by a narrow prismatic region just inside the shell margin. The crystals of the nacreous are calcite, and Schmidt (10) has termed the region "Calcitostracum."

The nacreous region of the oyster shell shows a variety of patterns, depending in part upon whether new shell is being deposited. The appearance, as studied by the electron microscope, has been described by Tsuji, Sharp, and Wilbur (11), and is similar to that of shells of oysters discussed below taken during the month of March in sea water at 8°C. On the other hand, when shell deposition was induced, by transferring oysters living at 8°C. to sea water at about 20°C, the pattern of the nacreous layer was markedly different. Through the examination of a large number of shells from oysters at both temperatures, one can obtain a general picture of the growth processes of individual calcite crystals and of the formation of the nacreous layer.

**Layer Formation:**

The first step in the formation of a layer is the deposition of conchiolin by the overlying mantle tissue. This is followed by the formation of crystal seeds on or in the conchiolin. Watabe (12) reported that thick conchiolin was deposited on the surface of pearls as the first layer formed after hibernation, and that crystal seeds were deposited on this material. In the oyster, conchiolin deposition was stimulated by increasing the temperature from 8°C. to 20°C. Fig. 1 is an electron micrograph of the nacreous region, showing conchiolin and small crystal seeds. The diameter of the crystal seeds varied from 200 A to 3000 A at this stage. The incorporation of small crystal seeds into large ones was often evident. In Fig. 1 the crystal seeds are rounded in form and are distributed randomly, whereas in other regions (not shown), some seeds are of rather polygonal and elongated form and have similar orientation. The difference in form and orientation is probably due to slower deposition of the crystals in the latter case. The outlines of large crystals underneath the conchiolin can be seen in Fig. 1.

With the growth of crystal seeds, the surface becomes covered by larger crystals with small seeds scattered among them (Fig. 2). In Fig. 2 the crystals are seen to be composed of many branches. The larger crystals were about 1 micron in their greatest dimension. This configuration seems to indicate dendritic growth and incorporation of seeds into the larger crystals. Other areas not shown exhibited rhombohedral crystals about 4 microns in length, all having similar orientation.

In a more advanced stage of crystal growth, crystals come into contact to form a single layer. Fusion of crystals may not take place in all areas, probably due to conchiolin or impurities. Growth up to and including this stage is largely two-dimensional and corresponds with that found by Watabe (12) in the growth of pearls.

It is interesting that the surface patterns described above were usually limited to dorsal and anterior parts of the shell. In other regions, a great number of well developed crystals were observed.

The next stage is the formation of a step-like arrangement of the crystals resembling the
shingles of a roof (Fig. 3). Here a crystal grows over one or more adjacent crystals.

These crystals will now be discussed.

Form, Arrangement, and Growth of Crystals:

The form of the calcite crystals was typically tabular and idiomorphic even though there was variation in form.

The corresponding axes of the crystals of a single area were parallel, showing that the crystals developed with parallel growth (Fig. 3). The measured angles between corresponding edges were somewhat different in different parts of the shell, but as discussed in the previous paper (Tsujii, Sharp, and Wilbur (11)), angles of 120°, 90°, and 150° are probably the true angles. Accordingly, the side planes are considered to be \{1010\} and \{1120\}. Therefore, one of the rhombohedral axes lies at an angle of 60° with one of the crystal edges (Fig. 3, arrows).

The growth rate of the side planes (hexagonal prisms) of adjacent crystals is usually the same, as evidenced by a similar length of the free edges (Fig. 3). An irregularity of growth rate of side planes is shown in Fig. 4, in which the free crystal edges running diagonally in the field from upper left to lower right are longer and more regular, due to slower growth of the corresponding side planes. The rounded crystals in Fig. 5 exhibit a further variation which may be due to impurities. Differences in crystal form are often found in a limited area, crystals in an upper layer being different from those in a lower layer.

The direction of growth of individual crystals and the crystal layer may be governed by several factors. The important factors are probably local concentration differences of calcium carbonate, adsorption of impurities, and the presence of neighboring crystals. If the mantle can bring about local differences in availability of calcium carbonate, this would cause more rapid growth where the concentration is higher and would, in turn, govern the predominant direction of growth. Such differences would thus account for the observed differences in direction of growth in different areas. Two different growth directions in a limited area near the adductor muscle are seen in Fig. 6. One runs from the right middle toward the lower left, the other curving from the upper middle to the lower left, where it meets the former.

The Surface Structure of Calcite Crystals:

The surface of the crystals showed two characteristic features, reticular structure and striations. The size of the units in the reticular structure (Fig. 8) is of the order of 100 Å, as measured by the average distance between rows. This dimension corresponds to the length of about 20 unit cells of calcite. Finer details could not be resolved, but elongation of the units is clearly evident. Striations are present in both idiomorphic and irregular crystals. In a limited area of a crystal the striations are parallel. Over the whole surface of the crystal three directions were sometimes observed, the angle between any two being roughly 120°. The direction of the striations corresponds with the rhombohedral axes of the crystal.

Crystal seeds were commonly observed on the upper surfaces of well developed crystals (Figs. 5 and 7). Their form was elongate, and the direction of elongation was the same for all seeds on one crystal. The direction of elongation would correspond to one of the rhombohedral axes of the large crystal. In any one area of shell the crystal seeds take a similar orientation, demonstrating that the large crystals on which they form have a similar direction of growth. The angles of the large crystals also provided evidence of parallel growth, as mentioned above.

DISCUSSION

The formation of a layer of calcite crystals in the nacreous region begins with the deposition of crystal seeds either on or in conchiolin secreted by the mantle or on calcite crystals. The crystal seeds increase in size, make contact with neighboring crystals, and fuse with them. In this manner larger crystals are formed, and with continued growth a layer is completed. Crystals continue to increase in size and overlap adjacent crystals. A new layer will be initiated by the secretion of a new layer of conchiolin or an interruption in the supply of calcium carbonate which, on renewal, will bring about the formation of seed crystals.

The form of the crystals in the nacreous layer of the oyster shell is typical of calcite. Further, crystal growth occurs in a manner similar to that of an inorganic system. Very small seed crystals have an appearance and scatter such as to suggest their origin from a highly supersaturated solution.
CRYSTAL GROWTH OF SHELL

The mantle plays an important role in the growth and arrangement of the calcite crystals by bringing about localized differences in calcium carbonate concentration. These differences are seemingly made possible by the close apposition of the mantle to the inner shell surface. The relation of the mantle to the shell also furnishes an explanation for the particular type of crystal growth observed. It is well known that when crystals grow in constantly agitated solutions (metastable), uniform growth in all directions will result. On the other hand, when crystal growth takes place in thin layers of solution in which circulation is only very limited, the crystals assume a tabular form (Buckley, (2)). Such conditions may be presumed to obtain between mantle and shell.

The early growth of crystals on or in the conchiolin is dendritic, and this form of growth would be favored by a thin layer of solution. Further, the dendrites have a definite orientation, as shown by the orientation of crystal seeds on the surface of the larger crystals, which themselves have developed through dendritic growth. The difference in direction of growth in different areas of the shell might result from local differences in concentration.

The striations observed on the surface of the crystals might be explained in several ways: by discontinuity of crystal growth, by incorporation of impurities, and by mosaic structure formation. The reticular structure shown in Fig. 8 might represent the mosaic structure, since the dimensions of the units of structure are of the order of 100 Å, and correspond to that of "Zwicky" cracks (14, 15). Pfefferkorn (9) reported lamellae of 100 Å thickness in the etched cleavage surface of calcite. As he pointed out, the nature of this structure can scarcely be discussed in terms of its form alone. For the interpretation of these surface structures, electron diffraction analysis is planned. This method is also expected to provide further information on the form and arrangement of the calcite crystals.

The present paper has given only a general outline of crystal and layer growth of the nacreous region. We have left out of this discussion a detailed consideration of the relations between the conchiolin and the crystal layers, the etching of the growing surface which appears to be a common rather than a rare event, and other processes associated with growth. These problems will be reported on later.

Physiological differences between the mantle edge and its central region are indicated by differences in the amount of conchiolin secreted, by a difference in the respiratory rate (Jodrey and Wilbur, (8)), and by a difference in the turnover rate of calcium (Jodrey, (7)). The study of the fine structure of the nacreous region provides evidence of localized differences in the central area of the mantle as well. The differences in crystal form and growth patterns in localized areas result from localized physicochemical conditions. Such conditions would undoubtedly be brought about by the mantle, with portions of the mantle overlying these areas functioning as physiological entities.

We wish to express our sincere thanks to Dr. Sterling B. Hendricks, United States Department of Agriculture, Soil and Water Conservation Research Branch, Beltsville, Maryland, and Dr. Carl A. Zapffe, Baltimore, Maryland, for their helpful discussions.

BIBLIOGRAPHY

EXPLANATION OF PLATES

All the electron micrographs shown were taken of formvar–silicon monoxide replicas shadowed with chromium. The prints were enlarged from original negatives. Shadows in the figures are white.
FIG. 1. The first stage of nacreous layer formation, showing deposition of rounded crystal seeds, about 200 A to 3000 A in diameter, in or on thick conchiolin which covers crystals. Edges of the underlying crystals are seen in the upper right and lower middle of the figure. The seeds are randomly distributed. X 25,000.

FIG. 2. The second stage of layer formation, showing dendritic growth of seed crystals. X 24,000.

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Fig. 1. The first stage of nacreous layer formation, showing deposition of rounded crystal seeds, about 200 A to 3000 A in diameter, in or on thick conchiolin which covers crystals. Edges of the underlying crystals are seen in the upper right and lower middle of the figure. The seeds are randomly distributed. X 25,000.

Fig. 2. The second stage of layer formation, showing dendritic growth of seed crystals. X 24,000.
(Watabe et al.: Crystal growth of shell)
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Fig. 3. Well developed calcite crystals of tabular idiomorphic form. The corresponding free edges of the crystals are parallel. The directions of the rhombohedral axes are shown by arrows. The corresponding axes run in parallel with one another. X 22,500.
(Watabe et al.: Crystal growth of shell)
PLATE 154

Fig. 4. Crystals of semimorphic form. The edges running diagonally from the upper left to lower right are longer and more regular than others, due to slower growth rate of the corresponding side planes. × 16,500.

Fig. 5. Rounded crystals. The form may be due to impurities. Crystal seeds are deposited on the surface. They are elongate and have similar orientation, which corresponds to one of the rhombohedral axes of the large crystals. × 25,000.
(Watabe et al.: Crystal growth of shell)
PLATE 155

FIG. 6. Difference of growth direction of crystals. One direction is from the right middle to the lower left of the figure, and the other curves from the upper middle downward to the lower left. X 16,000.

FIG. 7. Irregular crystals. Seeds of the same orientation are deposited on the large crystals, showing that the large crystals developed in parallel growth. X 16,000.
(Watabe et al.: Crystal growth of shell)
PLATE 156

Fig. 8. Reticular structure of crystals. The size of the units in the structure is of the order of 100 Å, which corresponds to the dimension of about 20 unit cells of calcite. The direction of rows in the structure would correspond to rhombohedral axes of the crystal. × 38,000.
(Watabe et al.: Crystal growth of shell)