STUDIES ON THE MINERALIZATION OF THE CALCIFIED TISSUE
IN MOLLUSCS — V. RADIOAUTOGRAPHIC INVESTIGATIONS
ON THE PATTERNS OF LAYER FORMATION*

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It is probable that calcium carbonate of molluscan shells is crystalized in a so-called extra-pallel fluid¹⁸⁾, mother fluid¹⁹⁾, which is a solution containing protein, carbon dioxide and calcium, excreted between mantle and periostracum through outer epithelium of the mantle. In the mineralization of shells, the embryos of mineral crystals may usually develop in certain fragments of the organic matrix, which was first separated from the specific mother fluid, by heterogeneous nucleation, as a possible hypothesis proposed in bone, teeth and other mineralized tissues of organisms⁴⁾⁷⁾⁸⁾. Recently, ABOLINS-KROGIS (1963) has suggested from his experiments on the regeneration of damaged shell of *Helix* that the organic crystals, their main components being proteins and acid mucopolysaccharides, may act as the fragments of the nucleating matrix. By means of radioautography, WADA (1964 c) ascertained that labeled calcium may associate with certain acid mucopolysaccharides in the organic matrix and deposit concentrically in the metachromatic organic crystals in which calcium spherulites develop subsequently, and has supposed that the acid mucopolysaccharides combined with certain amino acid side groups in the conchiolin proteins may provide specific sites for crystal nucleation. In either case, the subject on crystal nucleation in mineralizing tissues of molluscs may be probably understood in relation with stereochemical structure and chemical components of the organic matrix at a molecular level.

As to crystal growth, many authors had suggested that mineralization and crystallization of molluscan shells may be considered as a special example of crystal grown from a solution and undergone in physico-chemical processes. In his biomineralogical observations on seasonal changes of crystal growth of the aragonite on the inner nacreous surface of the *Pinctada* shell, WADA (1961) has demonstrated that a remarkable correlation exists among the amount of calcium ions in the mother fluid, the rate of deposition of calcium carbonate and crystal size and form, where the amount of calcium carbonate deposition vary apparently in direct proportion to the activity of carbonic anhydrase in the mantle tissue and other organs of *Pinctada martensii* shown by KAWAI (1954). Further, it has been well known that the enzyme inhibitors interrupt calcification in molluscs (WILBUR *et al.* 1955, ABOLINS-KROGIS 1958).

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In regard to the patterns of layer formation in nacre, WATANABE (1950) stated that the alternated stratification of conchiolin membrane and mineral lamella may occur in Liesegang phenomenon, and WATABE (1950) assumed that the growth patterns emerged by growth fronts of stratified lamellae exposed on the nacreous surface are provided by Liesegang phenomenon. While on the basis of anatomical and crystalline structures of the nacre obtained with electron microscopy and X-ray analysis, WADA (1958, 1961, 1962) has proposed the possible idea that as a rule, mineral crystals may grow epitaxially with the protein membrane in alternately stratified accumulation, and that the mechanism of spiral growth of the nacre in which aragonite crystals orient parallel can be probably comprehended by development or modification of the screw dislocation theory defined in the growth of single crystals. Recently, the possibility of epitaxial growth of mineral and protein sheet in the *Nautilus* shells was supported by GRÉGOIRE (1962). On the other hand, WATABE *et al.* (1961) have attempted to explain concerning the layer formation of calcitostracum in the *Crasostrea* shell by the theories on typical tree-like dendritic growth in the third dimension of space and on edge dislocation. The electron microscopic and diffraction investigations on the crystalline structure of pyramidal growth hills in the calcitic shells, *Ostrea gigas* and *Anomia lischkei*, were carried out by WADA (1963 a, b, 1964 a), who has indicated considerable differences in comparison with that of hills in the nacre that foliated calcite crystals emitted radially from the top of growth hills grow as irregular dendritic spherulites, and stated that the mechanism of formation of growth hills between nacre and calcitostracum must be interpreted on rather essentially different ideas.

But, we have still insufficient discussions on the last topic for understand a significant difference between spiral growth hills of the nacre and calcitostracum. To exploring on the mechanism of layer formation is therefore of interest for us. The present author should try to establish the problem with radioautographic stripping method in this paper.

Materials and Methods

The films placed on the inner shell surface of the same specimens used in the preceding experiment¹⁶⁾ were observed in detail with a compound microscope in incident light using the vertical illuminator, and afterwards the film which was removed and mounted on a slide glass, in transmitted light. The films were fixed by 25% aquatic solution of sodium thiosulfate alone.

Further, the shells of molluscs used were respectively composed of various structural elements as follows:—(Inserted in the next page)

Observation

The first experiment which was done at the beginning of May corresponded to

species	mineral component	outer layer	inner layer
<i>Chlamys nobilis</i>	calcite	calcitotrachum*	calcitotrachum
<i>Ostrea gigas</i>	calcite	prismatic struc.	calcitotrachum, chalky deposit
<i>Pinctada martensii</i>	calcite, aragonite	prismatic struc.	nacreous struc.
<i>Mytilus edulis</i>	calcite, aragonite	prismatic struc.	nacreous struc.
<i>Anomia lischkei</i>	calcite	{ calcitotrachum (left valve) prismatic struc. (right valve)	{ calcitotrachum (left valve) crossed lamellar struc. (right valve)
<i>Barbatia obtusoides</i>	aragonite	crossed lamellar struc.	complex struc.

* Calcitotrachum is the calcitic layer consisting of foliated structure defined by BøGGILD (1930).

the times soon after hibernation showed that labeled calcium concentrate partially as circular spots here and there on underlying old lamellae, especially on the exposed edge of stratified lamellae. However, a great important difference in the mode of calcium deposition occurs among nacre and other layers at the stages of layer formation, as illustrated in Figs. 1 and 3. That is, in the nacre calcium come to deposit concentrically on the growth fronts of stratified lamellae but lack relatively on the terrace, in which aragonite crystals cemented by intercrystalline membrane of conchiolin were closely packed, occupied between a step and adjacent one, and consequently, the pattern of layer formation is evidently recorded by alternating of black and white zones (Fig. 1). While, in the calcitotrachum the concentration of calcium deposition occurs apparently in no relation with the pattern of layer formation, in some regions is representative of almost parallel streaks (Fig. 3) and in others circular patterns rather uniformly aggregated (Fig. 4). In the layers of prismatic, crossed lamellar and complex structures, calcium deposition images exhibit the circular patterns similar to those in calcitotrachum, though somewhat differ among them, and in the prismatic layer of the *Pinctada* shell, sometimes, concentric patterns similar to those in the nacre. In all cases, calcium appears to deposit specifically in circular patterns as indicated clearly in Figs. 2, 3 and 4.

Observing in detail calcium fixation, it is evident that labeled calcium are located in the free edges and the centre of basal plane of crystals but sometimes absent on the centre and sometimes on the edges, as seen in Figs. 2 and 4.

Discussion

In his considerations based on detailed crystallographic investigations about the spiral growth of the nacre in the genus *Pinctada* shell, WADA (1961) has pointed out that the development of the spiral growth of the layer appears to be similar to that of spiral created on certain planes of single crystals grown by screw dislocations, and

Fig. 1. Radioautograph showing localization of ^{45}Ca in the growth hills of the nacre in *Pinctada martensii*. Note the pattern of ^{45}Ca deposition indicated as blackened zones. $\times 40$

Fig. 2. Distribution of ^{45}Ca in individual crystals grown on the nacre of the *Pinctada* shell. $\times 550$

Fig. 3. Radioautograph of concentration of ^{45}Ca in the ventral marginal region of the calcit-ostracum of the *Ostrea* shell. Note blackened streaks running along the horizontal growth direction of the layer. $\times 40$

Fig. 4. Localization of ^{45}Ca in the growth hills developed in the central area of the calcit-ostracum in *Ostrea gigas*. $\times 125$

that some calcium in mineral crystals may bind with the suitable parts of the organic matrix on which they seem to grow epitaxially. Further, it was demonstrated that the occurrence of the spiral growth may correlate with concentration of calcium ions in the mother fluid and its pH, and that the steps on the nacreous surface are very often by the spiral growth of micro-crystals. From several proofs, he has supposed that the mechanism of spiral growth of the nacre is reasonably interpreted by developing the theory of the dislocation mechanism of crystal growth. As to crystal nucleation, it is suggested that certain acid mucopolysaccharides bound with specific regions of amino acid side groups in the conchiolin protein may probably offer appropriate sites for the nucleation of mineral crystals (WADA 1964c). But, in the nacre the step may be supplied only at definite parts of the layer which do not differ from others in superficial structure, and thus the growth fronts of the characteristic growth spiral developed by rotating of the end of the step as clockwise or counter-clockwise appear to advance by addition and deposition of calcium carbonate¹⁰⁾. From the series of WADA's observations mentioned above, it is possible that the formation of spiral steps is confined in certain specific plane in spite of more great chances for the development of embryos of mineral crystal may exist in specific regions distributed in the organic matrix. The present results indicated the possibility of the above-mentioned considerations, and seem to suggest that in the nacre, the spiral steps advance actively on the growth fronts of them and the occurrence of crystal embryos may be undergone rather independently of the formation of the spiral step. Therefore, the present author proposes again here the fundamental idea that the mechanism of formation of the spiral steps themselves in the nacre, which may be considered as a kind of mosaic crystals, can be reasonably consistently explained by the theory of screw dislocation, taking account of the crystallographic structure of the layer, independent of deposition of crystals which assumed to grow epitaxially with the conchiolin protein.

In 1961, WATABE *et al.* have stated that formation of the overlapping rows of oriented, elongated crystals which suggest the presence of strong concentration gradients of calcium carbonate in a mother fluid may take place in the three processes of two-dimensional tree-like and three-dimensional dendritic growth and dislocation of crystal surface, in the calcitostracum of shell of the American oyster *Crassostrea virginica*. Characteristic growth hills in the calcitostracum of the *Ostrea* shell pointed out by WADA (1963 a, c, 1964 a) suggested the irregular dendritic spherulite consisting of radially oriented crystals elongated corresponding to a strong concentration gradients of calcium carbonate towards the all peripheral regions of a hill from its central part. Further it is evident that the dendrite of calcite in the hills appears to occur in an extremely high concentration of calcium carbonate in the mother fluid surrounding them in comparison with those of the nacre, as has been suggested from the amount of calcium deposition¹⁶⁾. In the results obtained in the present experiment,

one can observe that labeled calcium fix rather uniformly overall the growth hill, diffusing around its top. Judging collectively these facts, the growth hills in the calcitostracum of shell of *Ostrea gigas* must be able to be interpreted on the available idea based on the theory about the mechanism of dendritic growth, considering on diffusion and concentration gradients of solutes in the mother fluid. Accordingly, the mechanism of formation of the growth hills may be theorized on the essentially different idea from the mechanism of formation of the spiral steps in the nacre. The growth hills in the *Anomia* shell will be also explained by identity or modification of the idea proposing for the *Ostrea* shell in the present paper.

It is illustrated with radioautography that individual mineral crystals appear to grow only on the free side planes, only on the basal planes, or on both planes, in the progressive processes of layer formation. Sometimes, the growth of individuals completely packed in a terrace appears to be discontinued on all planes of them.

Summary

1) The investigations on the mechanism of layer formation in several marine bivalves were carried out with radioautographic stripping films.

2) The distribution and concentration of labeled calcium may indicate the mode of layer formation.

3) The circular deposition of labeled calcium on the inner shell surface appeared to be essentially similar to the selective fixation of calcium in the organic matrix and among various layers.

4) The mechanism of formation of the spiral steps and growth hills must be theorized on possible ideas independent of the position of development of embryos of mineral crystals which seems to be determined in certain specific fragments of the conchiolin protein.

5) The mechanism of development of growth hills in the nacre and the calcitostracum seems to be respectively understood on essentially different ideas. That is, the mechanism of formation of spiral growth steps in the nacre can be consistently explained by the theory of screw dislocation, while the growth hills in the calcitostracum by the theory of dendritic growth.

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