STUDIES ON THE MINERALIZATION OF THE CALCIFIED TISSUE IN
MOLLUSCS — III. LOCALIZATION AND DISTRIBUTION OF ^{45}Ca IN
THE MANTLE TISSUE AND ON THE GROWING SHELL SURFACE
IN SEVERAL MARINE BIVALVES BY RADIOAUTOGRAPHY*.

Koji WADA**

(Received February 17, 1964)

Biochemical studies¹⁾⁵⁻⁹⁾¹¹⁾¹⁸⁻²⁰⁾²⁵⁻²⁸⁾ on calcium metabolism in marine and fresh water bivalves using ^{45}Ca by many authors have discussed the possible picture of accumulation, transference and excretion of ^{45}Ca in various organs of them and showed that the great part of the absorbed ^{45}Ca is accumulated in both the organs of gill and mantle, the latter being the most active in the ^{45}Ca excretion. HORIGUCHI (1957, 1958, 1959) stated on the base of his experimentally obtained results that ^{45}Ca responsible for the shell formation in *Hyriopsis schlegeli* and *Pinctada martensii* is absorbed through the digestive system via mouth and through the tissue surface directly and then the absorbed ^{45}Ca is transferred from one organ to another by blood stream. It is probable that almost all of ^{45}Ca excreted through a mantle epithelium is incorporated into calcium carbonate crystals in shells (JODREY 1953, HORIGUCHI 1959), in addition WILBUR *et. al.* (1959) had pointed out the various rates of ^{45}Ca deposition in different areas of the *Crassostrea* shell.

In his histochemical studies on calcification in molluscs by radioautography, BEVELANDER (1951, 1952) showed several good micrographs of localization of ^{45}Ca and ^{32}P in the mantle of marine and fresh-water molluscs and elucidated that the excreted ^{45}Ca and ^{32}P are located in crystals and periostracum, respectively. TUJII *et. al.* (1954) obtained the same results that in *Pinctada martensii* the absorbed ^{32}P is abundant in regions of the mantle below the inner surface epithelium as indicated by BEVELANDER⁹⁾. While NAKAHARA (1962 a) reported that ^{45}Ca injected into the large posterior adductor muscle appears in the mucus contacted with the central areas of the outer surface epithelium of the mantle of *Pinctada martensii* at half an hour and subsequently in marginal one.

For exploring the mechanism of mineralization of molluscan shells, the present author observed the local patterns of ^{45}Ca deposition on the growing shell surface and the distribution of ^{45}Ca in the mantle tissue in several marine bivalves by radioautography and would report the obtained results in this paper.

Materials and Methods

Two experiments were carried with the following marine molluscs collected from

* Contribution No. 112 from National Pearl Res. Lab.

** National Pearl Res. Lab. (和田浩爾 国立真珠研究所)

Tatokujima water in Ago Bay in May and October, 1962.

Order	Pseudolamellibranchia	shell length*
	<i>Chlamys nobilis</i> (REEVE)	6~6.5 cm.
	<i>Ostrea gigas</i> (THUNBERG)	5~7 cm.
	<i>Pinctada martensii</i> (DUNKER)	6~7 cm.
Order	Filibranchia	
	<i>Mytilus edulis</i> (LINNÉ)	3.5~4 cm.
	<i>Anomia lischkei</i> DAUTZENBERG et BAVAY	4.5~5 cm.
	<i>Barbatia obtusoides</i> (NYST)	3~4 cm.

Shell valves of these animals were cleaned free of sessile organisms and mud, and they were placed in a large bottle for some time, before the injection of ^{45}Ca . Animals were respectively injected into their adductor muscle with 0.1 ml. of NaCl solution containing the activity of 0.13 microcuries of ^{45}Ca at each time, and were afterwards reared at the water-depth of 1.5 meters in the water around Tatokujima station of our laboratory. ^{45}Ca was used as CaCl_2 in HCl solution. The pH of the injection was neutralized by the calculated amount of sodium bicarbonate. The water temperature was about 17.6°C in the first experiment and about 22.1°C in the second.

After sampling of various times for 24 hours, the shell valves were removed from animals, their inner surface was well washed with a large stream of fresh water and then dried at a room temperature. On the other hand, a fragment of the mantle was taken off of animals which were fixed in buffered formalin (pH 7.0) for 72 hours and processed for sectioning in the ordinary paraffin method. Radioautographic film for the stripping method was subsequently stuck on the section of the mantle tissue and on several different regions of the inner shell surface, was exposed for 28-34 days and then developed with the developer D-72. The mantle tissue was stained with 1/400,000 toluidine blue solution for a short time after the fixation of film, and control specimen of mantle deparaffinized in the same procedure with Mallory's triple-stain for the comparative investigation.

Observation

A part of ^{45}Ca injected into an adductor muscle may be immediately transferred to a mantle tissue. The localization of calcium appeared in the mantle of any species at half an hour after the injection seems to reveal no considerable difference followed with increase of time through this experiment, though varies slightly among different species, and is essentially similar in the species mentioned above, in the longitudinal section. An example of radioautograph on the localization of ^{45}Ca in the mantle of *Pinctada martensii* is shown in Fig. 2 in comparison with control preparation (Fig. 1), illustrating that an abundance of calcium is found in the periphery of the mantle (Fig.

* The shell length is here defined by the longest length crossed at a right angle the shell height drawn from a umbo to the furthest ventral edge.

2 a') and that calcium is concentrated in the epithelium itself and sub-epithelium, where, in general, calcium is more abundant in inner surface (Fig. 2 b') than in outer surface (Fig. 2 c') in the pallial regions but contrariwise more in the latter than in the former in the central regions (Fig. 2). On the outer surface of the mantle (Fig. 2), the highest concentration occurs within the epithelium and sub-epithelium in the

Fig. 1. Control preparation of longitudinal section of mantle of *Pinctada martensii* ($\times 11$). a, b and c are respectively high power of the inner surface of the middle fold, the inner and outer surface of the mantle marked in Fig. 1. ($\times 70$).

Fig. 2. Radioautograph of longitudinal section of mantle corresponding to that shown in Fig. 1. ($\times 11$), illustrating localization of ^{45}Ca shown by blackened dots. a', b' and c' are marked in Fig. 2. ($\times 70$).

marginal regions and the lowest concentration within those near or in the pallial and adductor muscles. Further, ^{45}Ca is located in the connective tissue, muscle- and nerve-fibres in the mantle and in mucus contacted with the outer surface epithelium. But the concentration of calcium in a mantle appears to be varied with increase of time and by sections of a specimen and each species.

The most of calcium compounds excreted through the outer epithelium of a mantle may be incorporated as calcium carbonate into shell valves, growth rate of which differs individually among not only species but also various regions of a shell of single species. Accordingly, when ^{45}Ca deposition on the inner shell surface is examined with radioautographic stripping film, it can be expected that mineral crystals containing ^{45}Ca are concentrated in specific pattern in different regions of a shell and in species. Radioautographs of growing shell surface of several marine molluscs indicate that amount and feature of calcium deposition are rather individuated in each species and moreover represented in specific local patterns among different areas of a shell of single species. That is, in nacre of the *Pinctada* shell, although the concentration of calcium deposition differs somewhat among various parts, the whole surface is

Fig. 3. Radioautographs of the growing shell surface of marine molluscs, *Pinctada martensii* (A), *Ostrea gigas* (B), and *Anomia lischkei* (C), which were killed at 6, 2 and 4 hours after the injection, respectively. Localization of ^{45}Ca is shown by blackened patterns.

rather uniformly blackened except adductor and pallial muscle impressions in lower concentration of ^{45}Ca but there is very often found indistinct radial pattern running towards a whole ventral margins from muscle impressions (Fig. 3 A), calcium deposition in nacre of the *Mytilus* shell is characteristic of distinct concentric pattern intersected the faintly radial streak elongated from the central area to mid-ventral margin (not shown), and in *Barbatia obtusoides* radial pattern occurs towards ventral margin outside and inside near pallial muscle line but not in central areas of the shell (not shown), in which the inner layer is composed of complex structure and the outer layer concentric crossed lamellar one. On the other hand, calcitostracum of the *Ostrea*, *Chlamys*, and *Anomia* shells show differences in amount and feature of calcium deposition among each other: in *Ostrea gigas*, more distinct pattern radially emitted from the root of a posterior adductor muscle impression to pallial line occurs in ventral area but faintly uniformly blackened parts are definitely distributed over the whole surface of the shell (Fig. 3 B), in *Chlamys nobilis* marginal regions are blackened in faintly radial pattern and limited small area near the umbo in irregular pattern (not shown), while in the *Anomia* shell, radial pattern is formed towards a whole ventral margin from muscle impressions confined in mid-dorsal area (Fig. 3 C). Further in the above-mentioned other species, muscle impressions also show the lowest concentration of ^{45}Ca as in *Pinctada martensii*. Local pattern of calcium deposition is essentially similar between a corresponding area of left and right valves of a single animal except *Anomia lischkei* (Fig. 3 C) in which the left valve consists only of calcitostracum and the right one crossed lamellar and prismatic structures.

If it is supposed that the difference of the activity of ^{45}Ca injected per unit weight of an animal does not effect on the activity of calcium metabolism and further experimental conditions, seasonal change of calcium metabolism of species and variance of age of animals used are disregarded in the present experiment, the activity of calcium metabolism might be relatively determined among experimental animals. Now, from an average concentration of ^{45}Ca deposition per unit area of the inner shell surface of animals reared for 2 hours after the injection, the activity of calcium metabolism per unit area of a mantle among species may be roughly defined by the following equation:

$$Ostrea > Anomia, Mytilus > Chlamys, Pinctada > Barbatia$$

Especially, calcium metabolism in *Ostrea gigas* appears to be extremely active in comparison with other species but reveal characteristic differences among local parts of the mantle.

Discussion

From several studies⁴⁾¹⁵⁻¹⁷⁾²²⁾²³⁾ on the crystalline structure of some molluscan shells, it has been assumed that tension and elongation of mantle tissue may affect

directly or indirectly on orientation of mineral crystals in the processes of shell mineralization. In his initial observations on relationships among configuration of crystal, fibre axis of protein membrane and behavior of mantle tissue or of grafted mantle epithelium, WADA (1960, 1961) has discussed the effects of dynamic force of mantle tissue upon orientation of crystal and pointed out the possibility of such ideas that crystals are oriented in parallel by growing epitaxially with protein membrane the fibre axis of which assumes to depend on tension and elongation of a mantle and on movement of cilia on free surface of outer epithelium or that crystals deposit in parallelism along a definite current occurred in the solution, in which calcium carbonate is crystallized, by movement of a mantle and its cilia. He has again ascertained this ideas on bases of his own (1964), NAKAHARA's (1962 b) and GRÉGOIRE's (1962) studies. But our investigations about this subject are still incomplete and we have no sufficient knowledge for understand role of a shell-forming tissue in reference to crystal growth and crystalline structure of a shell. The present results obtained do not give us a concrete evidence but a superior guidance for exploring the mechanism of specific orientation of mineral crystals on growing shell surface. Radioautographs of labeled calcium deposited on inner shell surface show apparently identity and similarity of local pattern of calcium deposition, specific pattern related towards ventral periphery of a shell from a certain part specialized in species, among species used in this experiment. For instance, the feature of the radial pattern in the *Ostrea* shell appears to keep an exact copy of disposition of muscle within the mantle and occurs in definite relation with elongation of calcite crystals and orientation of their rhombohedral axes in the corresponding areas, while the rather faintly uniformly blackened parts appears to correspond to the areas containing the development of many growth hills, as has reported by WADA (1963). The source of blackened streaks is located in the root of the posterior adductor muscle impression, which coincides completely with the orientation of crystallographic axes in the shell (WADA 1963, unpublished). The relations between crystalline structure and local pattern of calcium deposition in the *Pinctada* and the *Anomia* shells appear to occur in the same way as in the *Ostrea* shell, but in the *Pinctada* shell the orthorhombic *b* axis of aragonite may take calcite rhombohedral axes place. The source of radial streaks is laid inside the posterior adductor muscle impression in the *Pinctada* shell and near the umbo or the root of muscle ones in the *Anomia* shell. In all cases, it is evident that mineral crystals may elongate dominantly and arrange regularly their definite crystallographic element along radial blackened pattern which seems to depend closely on movement and tension of a mantle. And crystals in the regions of strong radial pattern may elongate and develop more rapidly along the blackened streak than in faintly radial one.

It has been pointed out that calcium metabolism in *Classostrea virginica* is considerably active in comparison with *Pinctada martensii* and *Hyriopsis schlegeli* (Horiguchi 1957). According to WILBUR *et al.* (1952) and NAKAHARA (1961), histogram of

amount of calcium deposition suggested the remarkable differences of calcium metabolism in local areas of a mantle in *Crassostrea virginica* and *Pinctada martensii*. Further, NAKAHARA (1961) ascertained by the administration of tetracycline that relative amount of calcium deposition through a year is somewhat different among local areas of the *Pinctada* shell. The relative comparison of amount of calcium deposition appears to be possible among not only local areas of a single shell but also species by means of radioautography, as indicated in the present observation. In addition, the mode of calcium deposition in local areas can be identified from the difference of feature of blackened pattern, where we must determined true images taking account of the radiography for uneven specimens as described by JODREY *et al.* (1951). In the present radioautographs, it is also elucidated that calcium metabolism in *Ostrea gigas*²⁶⁾ is extremely active in comparison with experimental specimens and the relative amount of calcium deposition among local areas agrees well with that in the *Crassostrea* shell shown by WILBER *et al.* (1952). But the blackened pattern in the *Ostrea* shell appears to be markedly varied among local areas and also in the *Anomia* shell. On the other hand, the relative amount of calcium deposition among local areas in the *Pinctada* shell takes place in rather indistinct difference during the periods of this experiment but appears to vary in October more than in May, as suggested in NAKAHARA's report¹²⁾. There is hardly found local difference of calcium deposition in the *Mytilus* shell in this experiment. In either case, specific difference occurs in the mode and relative amount of calcium deposition among local areas of an inner shell surface, and there is found an extremely small amount of calcium deposition in muscle impressions of all specimens.

Radioautographs of localization of ^{45}Ca in the mantle of fresh-water and marine molluscs which were reared in the water containing ^{45}Ca were shown by BEVELANDER (1952) who demonstrated that high concentration of calcium localized in all periphery of the mantle and occurred within epithelium and sub-epithelium in which mucous cells distribute. TANAKA *et al.* (1955, 1956) NAKAHARA (1962) detected ^{45}Ca in mucus excreted through outer epithelium, respectively. The results obtained in the present are entirely similar to their observations, and may suggest a certain relation between calcium and organic matters in calcification of mollusc.

Summary

1) The localization of ^{45}Ca in the mantle and on inner shell surface was determined for several marine molluscs with radioautographic stripping film, and discussed calcification of shell in special reference to mineralization and crystalline structure of shell.

2) Specific and local differences of calcium deposition revealed in amount and mode on the inner surface of a shell of experimental specimens and whereby specific patterns might result.

3) Specific pattern suggested the mode of development and formation of shell, while local pattern took place in close connection with crystalline structure in the corresponding areas and appeared to be characterized depending on tension and elongation of mantle tissue.

4) High concentration of ^{45}Ca was localized in epithelium and sub-epithelium of mantle.

5) The presence of ^{45}Ca was detected in mucus excreted through outer epithelium.

6) In all specimens, muscle impressions indicated the smallest amount of calcium deposition in a whole surface of a shell.

The present author expresses his sincere thanks to Drs. Yaichiro OKADA and Tadashi TUJII for their useful advices as well as for kindness in the loan of valuable manuscripts. Thanks are extended to Mrs. Shigeru ŌTA and Katuo TAKAYAMA for their encouragement.

References

- 1) ABE, Y., KADO, Y., SHINAGAWA, M., TAKANAKA, J. and YAZIMA, S.: *Jour. Sci. Hiroshima Univ., Ser. A*, 18, 249 (1954).
- 2) BEVELANDER, G.: *The New York Journal of Dentistry*, 21, 305 (1951).
- 3) ———: *Biol. Bull.*, 102, 9 (1952).
- 4) GRÉGOIRE, Ch.: *Bull. Inst. roy. Sc. natur. Belgique*, 38, 1 (1962).
- 5) HATANO, H., MATUI, Y., SAWADA, Y., NAKAKA, S. and UCHIDA, Y.: *Bull. Nippon Inst. Sci. Res. Pearl*, 3, (44) 1 (1955).
- 6) HORIGUCHI, Y., MIYAKE, M., YOSHII, Y., OKADA, Y., INOUE, Y. and MIYAMURA, M.: *Bull. Jap. Soc. Sci. Fish.*, 20, 101 (1954).
- 7) HORIGUCHI, Y.: *Bull. Jap. Soc. Sci. Fish.*, 22, 747 (1957).
- 8) ———: *ibid.*, 23, 710 (1958).
- 9) ———: *Fac. Fish. Pref. Mie Univ.*, 3, 381 (1959).
- 10) JODREY, L. H. and WILBUR, K. M.: *Proc. Soc. Exp. Biol. and Med.*, 77, 80 (1951).
- 11) JODREY, L. H.: *Biol. Bull.*, 104, 398 (1953).
- 12) NAKAHARA, H.: *Bull. Natl. Pearl Res. Lab.*, 6, 607 (1961).
- 13) ———: *ibid.*, 8, 871 (1962 a).
- 14) ———: *ibid.*, 8, 879 (1962 b).
- 15) RAMA, S.: *Mem. Ind. Ins. Sci.*, 1, 871 (1935).
- 16) ———: *Proc. Ind. Aca. Sci.*, 2, 345 (1935).
- 17) SCHMIDT, W. J.: *Zool. Jahrb.*, 45, 1 (1924).
- 18) TANAKA, S. and HATANO, H.: *Jour. Chem. Soc. Jap.*, 76, 602 (1955).
- 19) ———: *Aisotopu Kenkyu Riyo Souran*, 307 (1956).
- 20) TUJII, T., YOSHII, Y., OKADA, Y., INOUE, Y. and MIYAMURA, M.: *Fac. Fish. Pref. Mie Univ.*, 2, 199 (1956).
- 21) WADA, K.: *Bull. Jap. Soc. Sci. Fish.*, 26, 549 (1960).
- 22) ———: *Bull. Natl. Pearl Res. Lab.*, 7, 703 (1961).
- 23) ———: *Venus*, 22, 281 (1963).
- 24) ———: *Bull. Jap. Soc. Sci. Fish.*, 30, 319 (1964).
- 25) WATABE, N., YOSHII, Y. and OKADA, Y.: *Bull. Jap. Soc. Sci. Fish.*, 23, 139 (1957).
- 26) WILBUR, K. M. and JODREY, L. H.: *Biol. Bull.*, 103, 269 (1952).
- 27) ———: *ibid.*, 108, 359 (1955).
- 28) KADO, Y.: *Jour. Sci. Hiroshima Univ., Ser. B*, 19, 163 (1960).