Studies on the Mineralization of the Calcified Tissue in Molluscs—XIV.

Modification of the Amino Acid Pattern of Proteins in the Extrapallial Fluid during the Process of Formation and Mineralization of Nacreous Conchiolin in Some Bivalves*

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In molluscs, mineral and organic matrices occur in a specific solution, which was termed "extrapallial fluid" by de WAELE¹⁾, secreted by mantle between the tissue and shell valve. It has been evident that the fluid may contain mucoproteins, mucopolysaccharides, calcium, magnesium, carbonate and other inorganic materials. Since a part or almost all of the organic substances in the extrapallial fluid is presumed to be a precursor of conchiolin, it may be denatured to conchiolin, the organic matrix in molluscan shells, in insoluble form.

According to TANAKA and HATANO²⁾, it is possible that the mucous substances secreted by the mantle are insoluble complexes combined with enormous calcium, which is liberated in the course of denaturation of the mucoproteinous substances to form conchiolin and then precipitated as calcium carbonate. It was confirmed by electron microscopic observations that minerals of shells piled up alternately with the sheets of conchiolin (GRÉGOIRE³⁾, WADA^{4,5)}, WATABE⁶⁾). However, we do not know to explain how the alternate accumulation of conchiolin sheet and mineral lamella will occur in the soluble complexes.

It is important for studies of shell formation to investigate whatever will happen in the extrapallial fluid during the process of formation and mineralization of conchiolin. The present author will make comparison of amino acid composition between nacreous conchiolin and the organic substances in the extrapallial fluid in contact with the nacre in this study.

Materials and Methods

Animals used in this study consisted of *Pinctada martensii* (marine bivalve) and *Hyriopsis schlegeli* (fresh water bivalve). The marine species was taken in the pearl farm near the Tatokujima station of National Pearl Research Laboratory in Ago Bay, Mie Prefecture, in February 1966, and the fresh water species taken in

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large outdoor farm ponds of the Osaka Prefectural Fisheries Experimental Station in Neyagawa City, in June 1966.

First the shell valves of the animals were slightly opened with a shell speculum, and then the extrapallial fluid was carefully collected with an injector inserted between the mantle and nacre at the central area of a shell. Secondly small pieces of the nacre of the *Pinctada* shell were made with a hammer and pincers, and cleaned in distilled water and hydrochloric acid. They were decalcified in 3 M acetic acid solutions. The conchiolin obtained was washed in distilled water and dried at room temperature.

One to 4 ml. of the fresh fluid or about 5 mg. of conchiolin was in a sealed tube containing 2 or 3 ml. of 6 N hydrochloric acid for 22 hours at 110°C. Amino acids in the hydrolyzates were determined by using a KLA-3 type Hitachi Amino Acid Analyser according to the method of SPACKMAN *et al.*⁷⁾.

Results

Table 1 shows amino acid composition of mucous substances in the extrapallial fluid of *Pinctada martensii* and *Hyriopsis schlegeli* as compared with that of conchiolin of their nacre. Hydroxyproline and hydroxylysine were not determined in the fluid as in conchiolin⁸⁾. The fluid of both the species contains proteins consisted of relatively larger amounts of aspartic and glutamic acids. The extrapallial fluid of *Pinctada martensii* is in amino acid pattern quite similar to that of *Hyriopsis schlegeli* except

Table 1. The comparison of amino acid composition between mucous substances in the extrapallial fluid and organic matrix in the nacre in the bivalve molluscs (amino acid residues per 1000 total residues).

Materials Amino acid	<i>Pinctada martensii</i>		<i>Hyriopsis schlegeli</i>	
	Mucous substances	Conchiolin	Mucous substances	Conchiolin*
Cysteic acid	13.8	6.9	+	1.5
Taurine	82.2			
Aspartic acid	127.6	164.7	100.8	116.4
Threonine	60.4	9.5	55.6	22.9
Serine	49.9	61.7	65.6	81.5
Glutamic acid	116.4	34.5	149.9	48.7
Proline	36.7	15.6	55.4	19
Glycine	48	236.5	52.3	170.4
Alanine	39.8	191	44.9	216.6
Cystine			+	
Valine	51.9	19.9	46.4	32.9
Methionine	11.2	+	5.6	4
Isoleucine	42.7	12.1	36.9	25.6
Leucine	56.5	84.7	69.2	68.3
Tyrosine	53.7	34.9	63.2	22.2
Phenylalanine	55.6	33.5	60	55.5
Lysine	59.3	25.7	83.5	42.5
Histidine	33.4	+	50.3	8.3
Arginine	60.9	68.9	60.5	63.6
NH ₃	(9.9)	(0.8)	(1.3)	(0.5)

* WADA⁸⁾.

that the rather large amount of taurine is contained in the former but a trace of the amino acid is not determined in the latter.

The mucous substances in the extrapallial fluid differ markedly in amino acid pattern from conchiolin in the nacre which appears to be in contact with the fluid. That is, aspartic acid, serine, glycine, alanine and arginine are less in the fluid than in the corresponding conchiolin, especially the number of glycine and alanine residues in the fluid being 48 and 39.8 in *Pinctada martensii*, and 52.3 and 44.9 in *Hyriopsis schlegeli* per 1000 total residues, respectively. As found in Fig. 1, the each number

Fig. 1. The ratio of amino acid residues in conchiolin (C) to those in the extrapallial fluid (E).

is equal to about one-fifth of glycine and of alanine contained in conchiolin for *Pinctada martensii*, and to about one-third of glycine and about one-fifth of alanine of conchiolin for *Hyriopsis schlegeli*. On the other hand, very higher concentrations of threonine, glutamic acid, proline, lysine and histidine are contained in the fluid of both the species. This amino acid analysis also indicates that taurine residue found in the fluid of the marine species does not be determined in conchiolin, and that rather larger amounts of valine, methionine and isoleucine residues are contained in the fluid of *Pinctada martensii* compared with the nacreous conchiolin and the content of tyrosine residue in the fluid of *Hyriopsis schlegeli* is about three times as much as that in the nacreous conchiolin. We must pay an important attention to the result that changes occurred in the number of each residue between the mucous substances and conchiolin tend to be similar each other in *Pinctada martensii* and *Hyriopsis schlegeli* in spite of different species, except to cysteic acid and leucine (Fig. 1).

Discussion

The electrophoretic pattern of mucous substances in the extrapallial fluid indicated that the fluid of molluscs with aragonite shell such as *Pinctada martensii*²⁾ and other species⁹⁾ contains three or more components of proteinous substances. TANAKA

and HATANO²⁾ have reported that mucoprotein in the fluid may be denatured to form an insoluble proteid which is termed "mucoprotein II" by them and about four-fifth of sulfate of the mucoprotein may lose in the course of formation of the mucoprotein II. That conchiolin of the nacre which is assumed to be the calcified mucoprotein II consists of three fractions of nacrine, nacrosclerotine and nacroïne was pointed out by GRÉGOIRE *et al.*¹⁰⁾. NAKAHARA¹¹⁾ stated that mucous substances secreted by mantle between the tissue and shell show a fine lamellar structure and then appear to be incorporated in the organic matrix of the nacre.

Judging from the present result that amino acid composition shows a rather marked difference between the proteins in the extrapallial fluid and those in the nacre adjoined the fluid, it is probable that some changes occur in the proteinous substances in the process of formation and mineralization of organic matrix of the nacre; presumably, (1) protein fractions constituting conchiolin and mucous substances in the extrapallial fluid may be same in kind, respectively, but differ in content of each fraction between each other, (2) the kind of protein fractions may be same between each other, but some of amino acids of protein fractions in the fluid may increase or decrease in the course of formation of the nacre, and (3) if some of protein fractions contained in the fluid be not directly involved in formation of the nacre, the member of protein fractions may decrease or disappear in conchiolin. When modification of amino acid pattern found in this study is in detail compared with the report by GRÉGOIRE *et al.*¹⁰⁾ that the essential reticular structure of the nacreous conchiolin is made by nacrosclerotine and nacroïne which are about 70% of the conchiolin and have very large amounts of alanine and glycine residues, one could cast a light on the hypotheses mentioned above. That is, it is evident that alanine and glycine which are the main amino acids of nacrosclerotine and nacroïne increase considerably in the course of formation and mineralization of organic matrix in the nacre, but threonine, glutamic acid, proline, tyrosine, lysine and histidine residues which are very larger in nacrine than in nacrosclerotine and nacroïne tend to decrease at a high rate. The consideration suggests that the protein fractions in the extrapallial fluid which correspond to nacrosclerotine and nacroïne may be incorporated into the organic matrix of the nacre at a high rate. Further the disappearance of taurine residue in conchiolin may support the third hypothesis. Some of amino acids of remains in the fluid may decrease or lose during denaturing to become the member of the organic matrix in the nacre, as shown by increasing or decreasing of amino acids in no connection with amino acid pattern of nacrine.

Summary

1. Amino acid composition of mucous substances in the extrapallial fluid forming the nacre of *Pinctada martensii* and *Hyriopsis schlegeli* were compared with that of conchiolin of the nacre, respectively.

2. Some of protein fractions or amino acid residues of the mucous substances may decrease or lose during denaturing to form the organic matrix of the nacre and so mineralizing.

3. Alanine and glycine residues in the nacreous conchiolin were five times as much as those in the fluid. This result may suggest that the protein fractions in the fluid corresponding to nacroscerotine and nacroïne are incorporated in conchiolin at a very high rate.

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