STRUCTURAL AND BIOCHEMICAL CHARACTERISTICS IN ADULT SCALLOP SHELLS

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ABSTRACT

This paper presents results of studies carried out on the microstructure, composition of organic matter and amino acids in abnormal shells of adult *Pecten maximus*. Results indicate that abnormal *P. maximus* shells can have two types of brown material on the inner layer of the valves. Brown calcified areas can be present and they have abnormal crystals on the internal foliated layer of the shell. An index (MIBC) was developed to describe this type of abnormality that depended on the extent of the brown areas. The other type abnormality had brown material consisting of uncalcified membranes that were embedded in mud tubes made by *Polydora* sp. worms. The amino acid composition of these two materials was completely different. When compared to white (normal) calcified shell, the brown calcified areas were characterized by decreases in aspartic acid and the proportion of serine, and the presence of a brown pigment that was resistant to decalcification and hydrolysis in 6N HCl. Biochemical analysis showed the brown uncalcified areas caused by *Polydora* contained chlorophyll, carotene and fucoxanthin pigments. The crystal abnormalities were considered to result from natural ecological disturbances. Crystal abnormalities have been noticed when scallop broodstock have been submitted to unfavourable rearing conditions in the laboratory.

INTRODUCTION

The presence of two different types of brown areas on the internal surface of bivalve shells has been reported in the literature. Brown membranes were observed to be associated with parasites (Blake and Evans 1973) or bacteria (Goulletquer et al. 1989; Paillard and Maes, in press), or were the result of stressful rearing conditions (Mori 1975; Marin and Dauphin 1992). In other work, brown calcified material was observed in *Venerupis pullastra* by Johannessen (1973). In the latter situation the shells were deformed. The brown coloration seemed to be a biological response to pathological or stressful conditions.

IFREMER staff in France recently observed during seeding surveys that in some areas natural mortalities of three year old scallops, *P. maximus*, were associated with intense brown coloration on the internal surface of the shells (Dao, in press). Devauchelle (Pers. Comm.) observed that microstructural modification can be induced in the internal layer of the valves when scallops are reared under poor conditions in the laboratory. An investigation was begun to determine the cause of the brown discoloration. The aim of this study was to determine if the brown coloration was caused by disturbances to adult *P. maximus*, and whether this coloration could be used as a criteria for selecting good quality broodstock for breeding.

Results of initial work describing the brown coloration, scanning microscope observations and amino acid composition of the organic matter of the internal foliated layer of scallop shells are presented.

MATERIALS AND METHODS

DESCRIPTION OF THE BROWN COLORATION

The scallops were dredged in one area in the Bay of St. Brieuc and four areas of the Bay of Brest (Rade de Brest): Roscanvel, Saint Pierre, Caro and Pen a Lan. Thirty scallops from two to six years of age were collected from each area to establish the variation in extent and intensity of the brown coloration.

MICROSTRUCTURE OBSERVATIONS

Ten shells of wild scallops with completely white inner shell surfaces and 20 others that showed some brown coloration on the internal surface were compared. The brown coloured shells were sampled from scallops dredged in one site in the Rade de Brest (Tinduff), the white shells were from scallops dredged in the Bay of the Seine River. All shells were from three or four year old scallops and were collected during the autumn. Shells from five scallops reared without algal food in the laboratory at 9°C were also studied. After freshwater and ultrasonic rinses, dry fragments or sections were metallized with gold palladium for microstructure examination with a scanning electronic microscope (SEM 505 Phillips).

ORGANIC MATRIX ANALYSIS

Ten shells with brown coloration were collected from the Tinduff area and 10 white shells were collected from the Seine River area. All were dredged in the autumn and were five years old. Samples of the internal foliated shell layer were isolated from between the pallial line and the edge of the shell in the region of the 4^{th} annual ring. The external shell layer was removed with a saw in order to select the brown colored areas. Shell samples were rinsed with dilute sodium hypochlorite and distilled water, then dried and crushed. Decalcification of 2.5 g of powdered shell was carried out with acetic acid at pH 4 for 24 hours. After a low speed centrifugation (30 min, 3600 cycles per min) acid soluble and acid insoluble organic fractions were separated and collected. The insoluble fraction was rinsed five times with distilled water and centrifuged, then lyophilized before storage at room temperature. The soluble fraction was desalted with low pressure chromatography on a Sephadex G50 gel (Pharmacia) followed by ultrafiltration (Filtron cells, 3K) and lyophilized. For amino acid analysis, soluble and insoluble fractions were hydrolyzed with 6N HCl at 110°C for 24 hours. After PITC (phenylisothiocyanate) derivatization, amino acids were separated with a reverse phase HPLC system following an acetonitrile gradient. Brown membranes embedded in *Polydora* muds were subjected to the same treatment as described above. Three insoluble fractions from membranes of three scallops were analyzed.

DETECTION OF ALGAE AND PIGMENTS

Scallops sampled during the spring had green coloration under and around the muscle. Analysis for algae was made on brown and green coloured calcified fragments that were decalcified with acetic acid and observed under a light microscope. For the pigment study, the brown calcified areas were scratched and treated with a solution of acetone, ethanol, methanol, and ether

in the following proportions: 5:2:2:1. Preparations were incubated for 30 min at 4°C in the dark and centrifuged for 10 min at 4°C and 12,690 G. Supernatants were isolated and concentrated by nitrogen bubbling and then added to 1 ml of acetone. The samples were analyzed by absorption spectrum between 400 and 700 nm to detect the presence of chorophyll.

RESULTS

DESCRIPTION OF THE BROWN COLORATION

Six developmental stages were determined for the brown coloration (Table 1, Fig. 1). Values of 1 to 6 were assigned to these stages and an index system was developed to describe them (MIBC, Mean Index of the Brown Colour). Similar MIBC values were observed at all sampling sites in the Rade de Brest and Bay of St Brieuc except at the Pen a Lan site in the Rade de Brest. This site was situated in shallow water (2 m) and scallops there were strongly affected by brown coloration of the shells (Fig. 2). Results show that MIBC increases with age of the scallop (Fig. 3). The brown colour was not found in one year old scallops.

MICROSTRUCTURE OBSERVATIONS

General Observations on Calcified Shells

The distal part of the mantle epithelium produces a finely perforated layer that is strongly curved towards the umbo. Successive laminae are clearly visible on the opercular valve and production of daily layers has been reported which permits precise aging of the shells (Fig. 4).

The foliated layer begins in the more internal areas of the shell, the basic unit being a long and narrow crystal. In the first zone (about 250 to 400 μ in thickness), the foliated units are slightly divergent, the external part being more or less continuous with the perforated layer. On the inner surface they appear to be organized in a fan-like arrangement, sometimes strongly divergent with respect to the direction of growth.

The major component of *Pecten* shells is a thick and homogenous layer of foliated crystals. In well calcified systems, these foliated units are closely packed and form large fascicles with straight cut edges in which growth is parallel (Fig. 5(1)).

Observations on the Crystals of the Internal Foliated Calcified Layer

White areas from completely white shells or from shells with some brown coloration from animals collected in the wild had a similar structure to those described in Figure 5(1), regardless of age. The crystals were very regular. Shell parts of animals held in the laboratory were white (Fig. 6(1)), however, the crystal structure was completely disorganized (Fig. 6(2)). They were aggregated and very small compared to those observed in white shells of scallops from wild populations.

Crystals in the brown calcified parts were more or less disorganized (Fig. 5(2,3,4)). The level of disorganization was not correlated with the MIBC. When the shells had a value of 5 or 6 MIBC and were dark brown, they were largely perforated with parasites (Fig. 5(5,6)).

AMINO ACID ANALYSIS

Composition of the Calcified Parts

Organic content of the dry material was 1.36 ± 0.01%. Four main amino acids were found in the soluble and insoluble fractions: aspartic acid, serine, glycine and glutamic acid (Table 2). They represent more than 75% of the total amino acids found in the brown and white parts of shells. In all cases dispersion was remarkably high. The soluble and insoluble fractions had a similar composition except for aspartic acid and lysine. The insoluble fraction contained significantly lower amounts of aspartic acid and higher amounts of lysine ($P=0.01$) compared to the soluble fraction. This led to variable values for the ratio of acidic amino acids/basic amino acids. The ratio was more than twice as high in the soluble fraction as in the insoluble fraction.

The difference in amino acids in the white and brown calcified parts of the shell was not significant except for aspartic acid in the insoluble fraction and serine in the soluble fraction. Both decreased in the brown calcified parts compared to the white calcified parts ($P=0.05$). Further, a deep brown residue remained after hydrolysis of the brown insoluble fraction that was isolated from the brown calcified parts.

Composition of the Brown Polydora Chamber Membranes

The main amino acids found in the brown *Polydora* chamber membranes were aspartic acid, glutamic acid, valine, and serine (Table 3). The content of threonine, glycine, arginine and alanine were lower but similar. The ratio of acidic amino acids:basic amino acids was approximately 2:1.

DETECTION OF ALGAE AND PIGMENTS

Observation under the light microscope showed the presence the boring green alga, *Ostreochromis* sp., especially under the muscle. When green algae are present the muscle does not adhere to the shell. Absorption spectra are shown in Figure 7. The reference was obtained using only the solvent (Fig. 7(1)). Samples from the white calcified parts (Fig. 7(2)) produced a similar spectrum to the reference. Observations of the brown calcified parts (Fig. 7(3)) and green calcified parts (Fig. 7(4)) showed three peaks: one at 664 nm, one at 467 nm and one at 437 nm. The peak at 664 nm shows the presence of chorophyll *a*. The two other peaks indicate the presence of carotene, fucoxanthin and perhaps chlorophyll *c*.

DISCUSSION

Shell deformities in Pectinids have been reported in the literature (Mann and Taylor 1981; Palmer 1980; Epifanio 1976; Mori 1975). Similar structural abnormalities caused by calcified brown areas was reported by Johannessen (1973) on *Venerupis pullastra*. The presence of brown membranes in Pectinids was reported by Mori (1975), Blake and Evans (1973) and in clams by Goulletquer et al. (1989) and Paillard and Maes (in press). It was generally concluded that the presence of brown membranes resulted from pathogenic or parasite attack (Paillard 1992; Getchell 1991). Moreover, Paillard (1992) stated that the brown pigment in non-calcified membranes was melanin. Results presented here show that *P. maximus* shells have both symptoms and they seem to be linked to adverse environmental conditions, but the mechanisms that control production of brown membranes or brown calcified parts are probably different because their amino acid composition is different.

However, results of laboratory experiments show that environmental disturbances quickly produce crystal deformation even when no shell deformation or brown coloration of calcified parts is observed. Crystal distortions were also observed in the brown calcified parts. The Tinduff sampling site, where the occurrence of brown shells was high, is well known for its environmental instability. Probably the environment has a direct influence on the development of brown calcified shells. It is possible that environmental disturbances can affect the calcified areas of the shell prior to or independent of brown coloration. Hence younger scallops, which are always white, could have crystal disturbances depending on rearing sites, but this hypothesis must be confirmed.

The aspartic acid composition of the insoluble fraction was slightly modified in brown areas compared to white areas. Aspartic acid is generally considered as the main ligant for calcium and is directly involved in the calcification process (Krampitz et al. 1983; Alzieu et al. 1982). Decreases in aspartic acid content could be related to problems in the calcification process in scallops with brown coloured areas.

It is necessary to conduct controlled experiments to establish a relationship between causes and shell abnormalities. This will be undertaken soon in our laboratory. Another objective of this research will be to find criteria that permit selection of healthy animals. Initial results concerning this particular point are presented by Larvor et al. (in press).

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Table 1. Criteria used to determine the level of the MIBC (Mean Index of the Brown Colour).

Stage	Localization of Brown colour on the inside of the upper shell	Colour under the muscle
1	small marks	white
2	along the margin and hinge	white
3	continue strip maximum 1 cm wide from the margin	white
4	continuous strip approximately 2 cm wide from the margin	white
5	covers the whole shell except the part from 0 to 2 years old	while
6	as in 5	green (algae)

Table 2. Amino acid composition of white and brown calcified parts of the internal layer of *Pecten maximus* shells. IM = Insoluble matrix. SM = Soluble matrix.

		White Shell IM n = 10		Brown Shell IM n = 10		White Shell SM n = 9		Brown Shell SM n = 10	
Amino Acids		X	SD	X	SD	X	SD	X	SD
1	ASP	32.84	4.75	28.11	4.66	40.74	5.62	37.19	4.02
2	GLU	6.17	0.69	6.34	0.61	6.74	1.21	7.39	0.74
3	SER	25.56	4.07	24.15	2.34	25.56	2.20	22.94	2.27
4	GLY	15.75	4.70	17.32	3.21	14.53	3.81	15.16	2.78
5	THR	2.55	1.59	5.89	3.63	1.42	1.20	3.17	1.40
6	ALA	4.61	0.65	5.23	0.75	4.36	0.66	5.64	1.17
7	PRO	1.39	1.54	1.22	0.48	1.36	2.19	1.10	0.96
8	HIS	0.89	0.58	2.16	1.20	0.33	0.31	1.16	0.82
9	ARG	1.96	1.19	1.68	0.98	1.37	1.67	1.22	0.68
10	TYR	0.23	0.08	0.43	0.15	0.17	0.20	0.50	0.56
11	VAL	1.15	0.12	1.80	0.32	0.57	0.16	1.01	0.28
12	ILE	0.70	0.09	1.18	0.18	0.32	0.11	0.80	0.29
13	LEU	1.02	0.15	1.77	0.37	0.61	0.21	1.36	0.47
14	PHE	0.26	0.10	0.56	0.25	0.13	0.10	0.40	0.37
15	LYS	5.93	1.43	3.96	1.66	2.38	1.15	2.30	0.97
Acidic AA/Basic AA			4.70		4.62		11.64		10.14
1 to 4			80.32		75.92		87.58		82.68
5 to 15			19.68		24.08		12.42		17.32

Table 3. Amino acid composition of brown uncalcified membranes found in mud created by *Polydora* worms on the inside *Pecten maximus* shells.

Membrane IM Amino Acids		Samples 1	2	3
1	ASP	18.00	18.50	18.50
2	GLU	11.80	12.10	12.60
3	SER	8.30	8.40	8.30
4	GLY	6.50	6.30	6.00
5	THR	7.00	6.80	6.00
6	ALA	5.70	5.40	5.80
7	PRO	0.80	0.90	0.90
8	HIS	3.80	4.10	4.00
9	ARG	7.20	7.20	6.80
10	TYR	3.10	3.10	2.40
11	VAL	9.90	9.60	9.70
12	ILE	5.20	5.10	5.20
13	LEU	5.40	5.40	6.10
14	PHE	3.90	3.70	4.00
15	LYS	3.40	3.40	3.70
Acidic AA/Basic AA		2.07	2.08	2.14
1 to 4		44.60	45.30	45.40
5 to 15		61.90	61.00	60.60

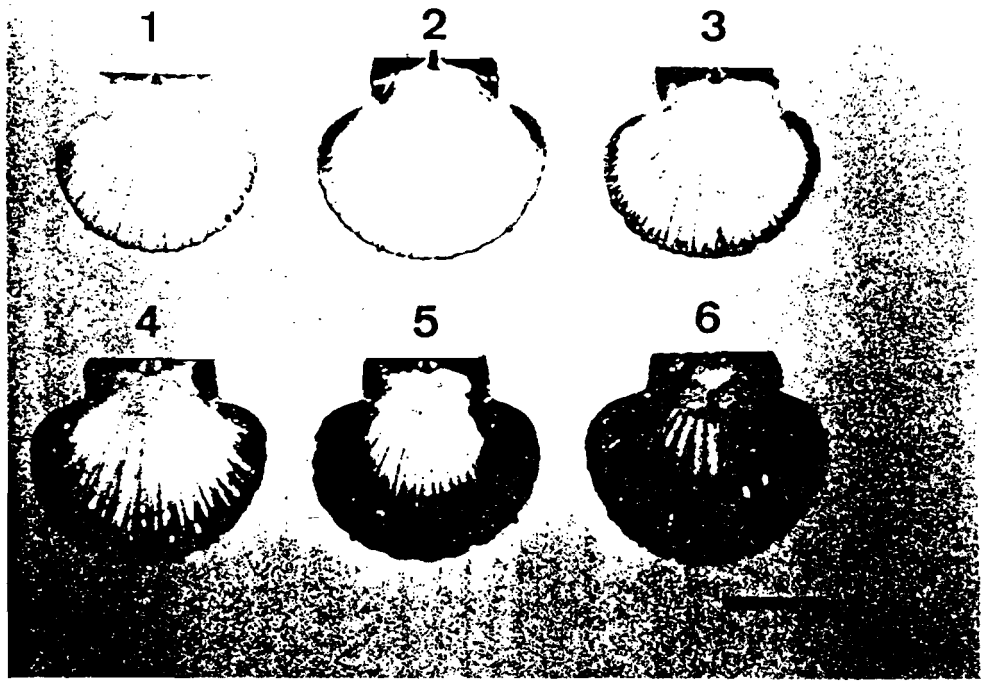


Fig. 1. Photograph of *Pecten maximus* shells representing typical MIBC levels, 1 to 6.

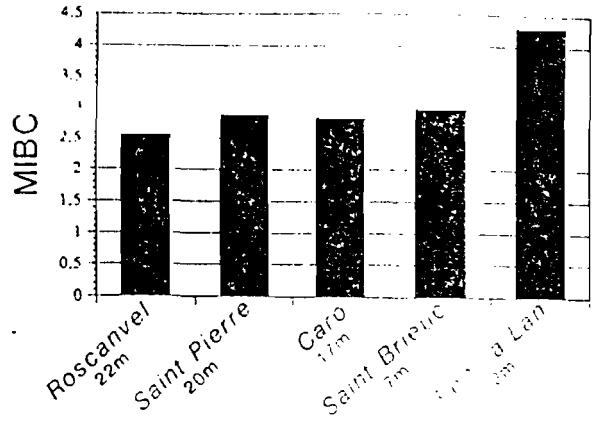


Fig. 2. MIBC level of *Pecten maximus* shells from different areas of the Bay of Brest (Rade de Brest) and in the Bay of St Brieuc.

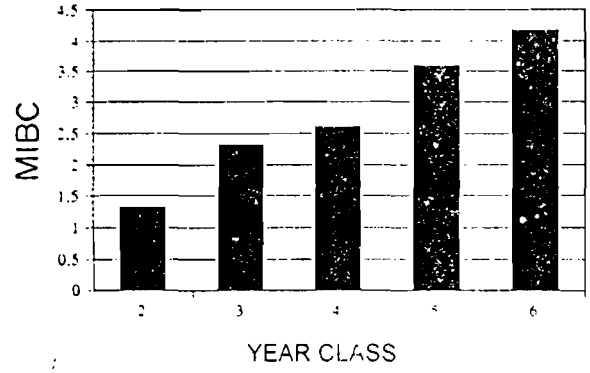


Fig. 3. Relationship between MIBC levels in *Pecten maximus* shells and age of scallops.

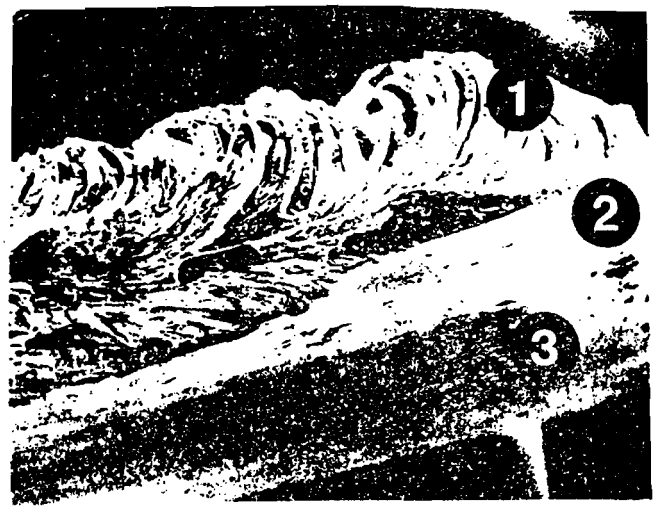
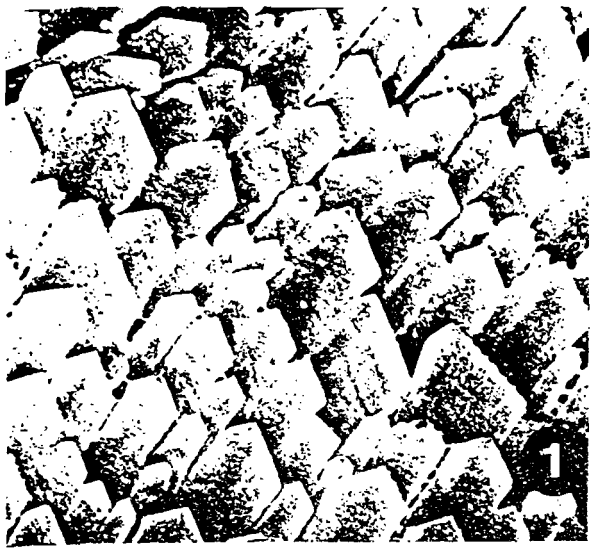


Fig. 4. View of the shell organization at the growing edge, 43x. (1) Perforated layers. (2) External foliated layer. (3) Internal foliated layer.

Figure 5 (opposite). Crystals from the internal layer of *Pecten maximus* shells. (1) Normal crystals, 3325x. (2) to (4) Abnormal crystals: (2) 5368x, (3) 2452x, (4) 4911x. (5) and (6) Parasite tunnels: (5) 320x, (6) 380x.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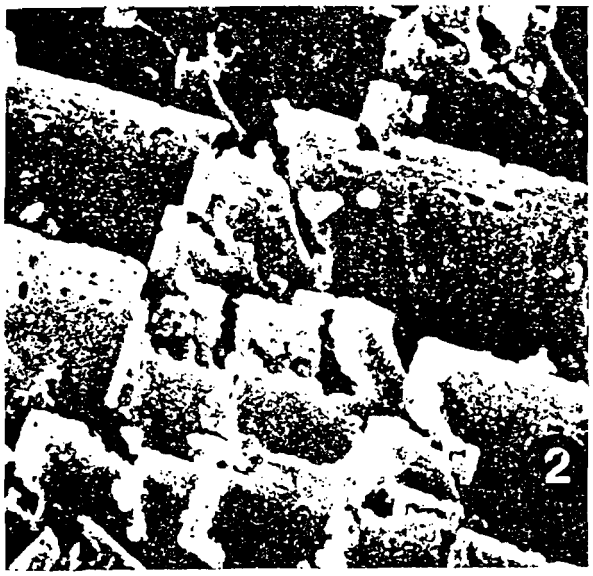
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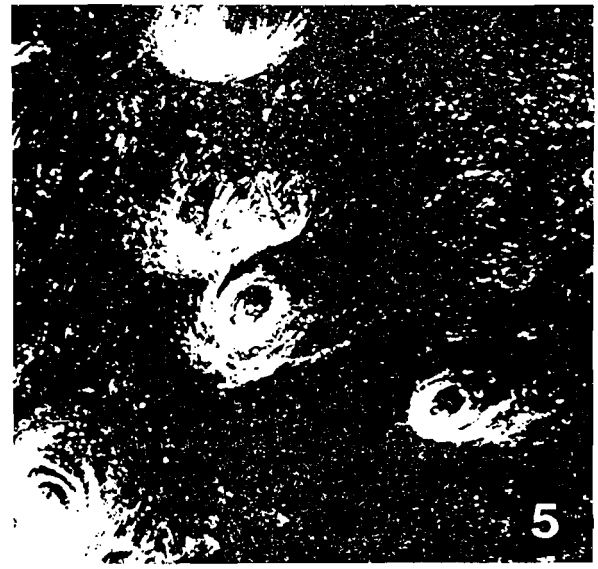
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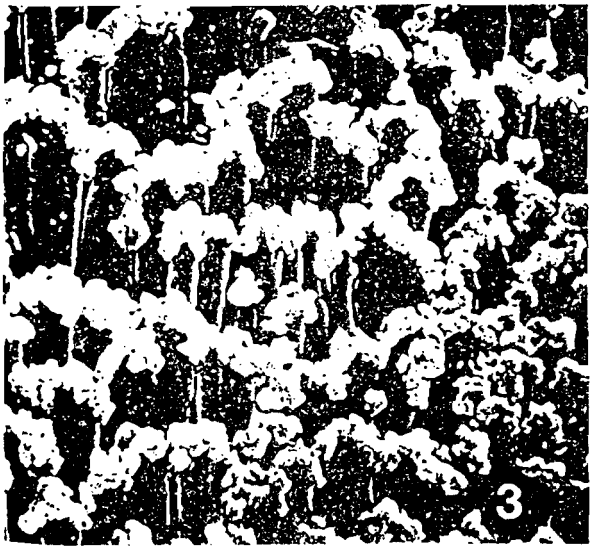
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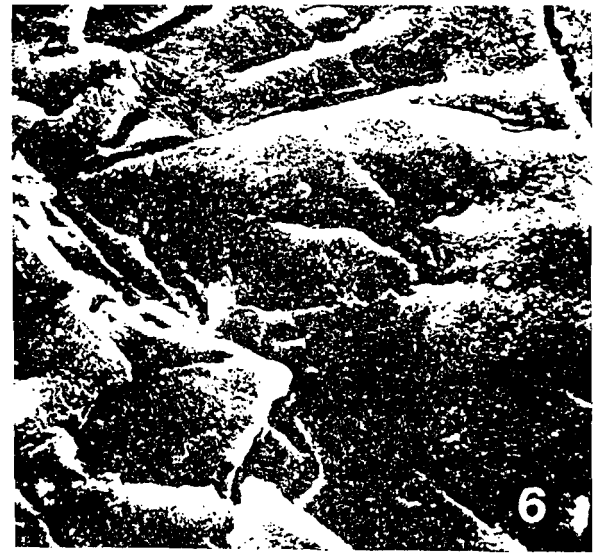
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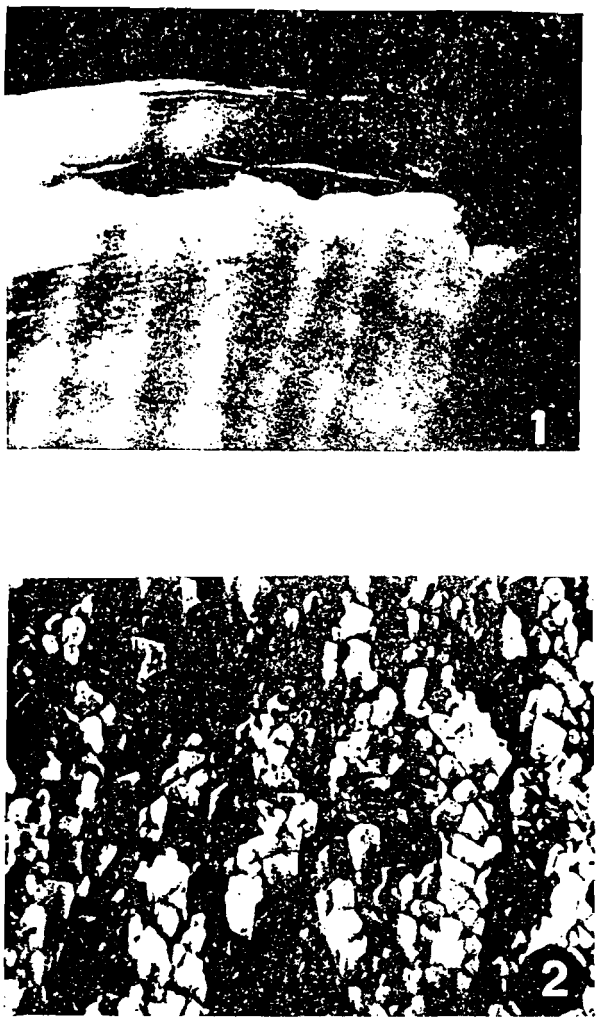

Fig. 6. (1) Morphology of the growing edge of scallop shells from animals held in the laboratory and not supplied with algal food. (2) Microstructure of the newly reconstructed edge, 4400x. Grained crystals are produced without any definite organization which is probably related to modification of the external envelopes.

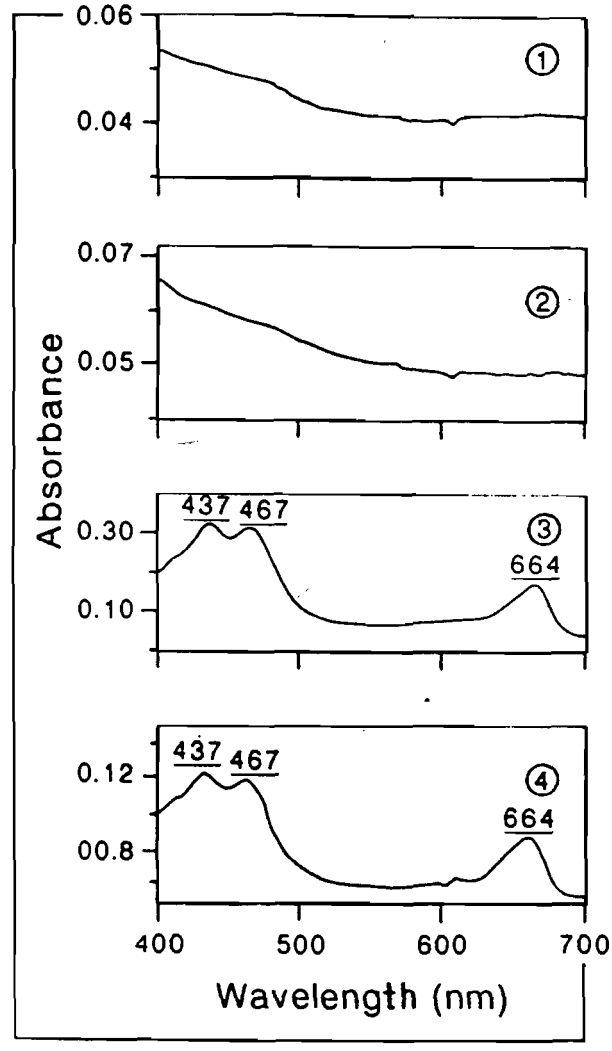

Fig. 7. Absorption spectra. (1) Solvent only. (2) Solvent with white calcified parts. (3) Solvent with brown calcified parts. (4) Solvent with green calcified parts.