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HISTOCHEMISTRY OF THE PERIOSTRACUM OF AN ESTUARINE BIVALVE *MERETRIX CASTA*

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ABSTRACT

Histochemical studies of the periostracum of the bivalve *Meretrix casta* during the early and late growth stages were carried out.

The periostracum is composed of an outer part formed of lipoproteins, the middle layer showing the presence of a sulphur containing protein and the inner-most similar to that of the outer layer.

The periostracum of *M. casta* shows in the early stages of growth, minute transverse canals traversing its entire width. In later stages, the canals appear to enlarge into large vesicles in the middle layer. But the canals are occluded by material which is indistinguishable from the surrounding region. The canals are exposed after treatment with detanning agents like alkaline stannite solution suggesting that the materiial in them may be tanned protein.

[Changes taking place in the chemical composition of the periostracum during growth involve the occurrence of quinone tanning in the outer-most layer.

The middle layer of the periostracum which undergoes hardening by sulphur bonding in the later growth stages recall strongly the -S-S- bonding reported in some arthropod cuticle.

INTRODUCTION

PREVIOUS works on the periostracum of eulamellibranch bivalves belonging to the order Heterodonta is confined to some aspects of *Tellina tenius, Mercenarria mercenaria, Cardium corbis, Pitar morrhuna* and *Tagelus divisus.* Truemen (1949) reported in the course of his studies on the ligament of *Tellina tenius* that the periostracum was fuchsinophil and gave indication of the presence of a protein containing tyrosine and inferred from the positive reaction to Millon's test. He did not distinguish the constituent layers of the periostracum. In *Mercenarria mercenaria,* a more detailed account of the chemical composition of the periostracum has been given by Hillman (1961). He noted that the periostracum contains a protein which undergoes quinone tanning and also noted the presence of an oxide involved in tanning.

Degens and Spencer (1966) and Degens *et al.* (1967) analysed chromatographically the amino acids in the protein of the entire periostracum of *Pitar morrhuana* and *Tagelus divisus.*

The amino acid composition suggests that more than one protein fraction may be present. But the nature of the protein fractions and chemical composition of the constituent layers of the periostracum have not been fully investigated. The occurrence of cystine and cysteic acid in the protein hydrolysate of the periostracum of *Pitar morrhuana* and *Tagelus divisus* may suggest the occurrence of a keratinous protein. The nature and role of such a protein, if present, is not known. In the light of the previous observations, a study of the structure and chemical composition of the periostracum of *Meretrix casta,* a closely allied species was made.

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Material and Methods

Two size groups of *M. casta* representing two different growth phases were collected from the brackishwater areas near Madras. An earlier growth phase (A) showed a length of 10 - 15 mm and 8 - 10 mm in breadth and the other, Late growth stage (B) 60 - 65 mm in length and 50 mm in breadth.

The fixation of shells were carried out in 5% formalin or 10% neutral buffered formalin for a minimum period of 24 hrs. They were washed and decalcified in 5% acetic acid or 8% EDTA (ethylene diamine tetra acetic acid, disodium salt). Paraffin and celloidin sections (5 - 8 μ) of the decalcified shell and periostracum were prepared for histological study.

Mallory's triple stain, Heidenhain's haematoxylin and Masson's trichrome stains were used. Frozen sections were prepared by gelatin impregnation method and used for the application of histochemical tests.

Chitosan test (Campbell, 1929), formic acid test (Rudall, 1955), PAS test (Runham, 1961), the modified chitosan method (Clark and Smith, 1936) and Schultz test (Lillie, 1954) were applied for the detection of chitin.

The product resulting from chitosan test was analysed for sugar constituents by circular paper chromatography and unidimensational ascending chromatography (Giri and Nigam, 1954). The solvent used were n-butanol : pyridine : water (6 : 4 : 3), n-butanol : acetic acid : water (4 : 1 : 5) and acetic acid : butanol : water (7 : 2 : 1).

Results and Discussion

Sections passing transversely across periostracum showed a layered structure. In an early growth phase of the periostracum it is seen, in unstained preparation, the outermost layer measuring 2 to 3 μ in thickness, was light yellow in colour and underlying it was a region which measured 4 to 5 μ in thickness; this region showed a spongy appearance. The middle region about 5 to 7 μ in thickness was colourless and traversed by canals. The inner-most layer 2 to 3 μ was differentiated from the middle layer by its light yellow colour (Pl. I A). The condition of the periostracum appears to undergo a change in later growth stages. The general features are similar except that the middle region is widened and differentiated into a well marked outer (10 to 12 μ thickness) and inner part (25 to 30 μ) (Pl. I B). When stained with mallory the periostracum in an early growth phase shows the outer-most layer blue in colour and the spongy layer below it was very lightly red stained (Table 1). In the middle region an outer part was funchsinophil, while the inner part was unstained. The inner-most yellowish layer stainred red (Pl. I A). With Masson's trichrome stain, the outermost layer stained green while the layer underlying it was less intensely stained. In the middle region, the outer part which was fuchsinophil in Mallory, stained with xylidine ponceau while the inner part was feebly stained red. The inner-most layer took up a red colour. With Heidenhains haematoxylin, the outer-most layer was unstained, while the underlying layer was lightly stained blue. In the middle region, the outer part took up a deep blue colour while the part below it was refractile to the stain. The inner-most layer took up a blue colour.

The results of staining of the constituent layers of the periostracum may indicate nonhomogenity in chemical features. With a view to assess the chemical nature of the different

TABLE 1. *Results of staining reactions and histochemical tests obtained with the periostracum of* Meretrix casta

Stains and tests	(A) Early growth stage 10-15 mm length and 8-10 mm breadth					(B) Late Growth stage 60-65 mm length, and 50 mm breadth				
			Middle layer					Middle layer		
	Outer layer	Spongy layer	Outer part	Inner part	Inner layer	Outer layer	Spongy layer	Outer part	Inner part	Inner layer
Mallory's triple stain (Mallory, 1938)	Blue	Light red	Red	Unstained	Light red	Blue	Unstained	Red	Unstained	Red
Masson's trichrome (Pantin, 1948)	Green	Light red	Red	-do-	-do-	Green	-do-	-do-	-do-	-do-
Heidenhain's haematoxylin (Lillie, 1954)	Unstained	Light Blue	Blue	-do-	Light Blue	Unstained	-do-	Blue	-do-	Blue
Linder Thomas Phosphomolybdic acid (Lillie, 1954)	+	+	—	—	—	+	—	—	—	—
Weigert acid Iron chloride (Lillie, 1954)	+	+	—	—	—	+	—	—	—	—
Biuret test (Fearon, 1946)	+	+	—	—	—	+	—	—	—	—
Millon's test (Bensley & Gersh, 1933)	—	+ +	—	—	+	—	+	—	—	+
Hg/nitrite test (Baker, 1956)	—	+ +	—	—	+	—	+	—	—	+
Xanthoproteic test (Pearse, 1961)	—	+ +	—	—	+	—	+	—	—	+
Sudan black-B (Lison, 1936)	+	—	+	—	+	+	—	+	—	+
Sudan black-B (Bereubaum, 1958) in hot acetone	+	—	+	—	+	+	—	+	—	+
Argentaffin test (Lison, 1936)	+	+	+	+	+	—	+	+	+	+
Sodium hypochlorite (Brown, 1950)	—	+	—	—	—	—	+	—	—	+
Diaphanol (Kennaugh, 1957)	—	+	—	—	—	—	+	—	—	+
Alkaline Stannite Solution (Trim, 1941)	—	+	—	—	—	—	+	—	—	+
Azide iodide reagent (Feigl, 1954)	—	—	+	+	—	—	—	+	—	—
Nitroprusside test (Pearse, 1961)	—	—	+	+	—	—	—	+	—	—
Ferric-ferrycyanide test (Chevremont and Frederic, 1943)	—	—	+	+	—	—	—	+	—	—
Blue tetrazolium test (Barnett and Seligman, 1954)	—	—	—	+	—	—	—	+	+ +	—
Lead acetate test (Pearse, 1961)	—	—	+	+	—	—	—	+	+ +	—
Alkalin sodium sulphide (Brown 1950)	—	—	—	+	—	—	—	—	+ +	—
Thioglycollate (Pearse, 1961)	—	—	—	+	—	O	O	O	O	O
Con. mineral acids (Solubility in)	O	O	O	O	O	+	—	—	—	—

+ Positive reaction; — Negative reaction; O Not carried out.

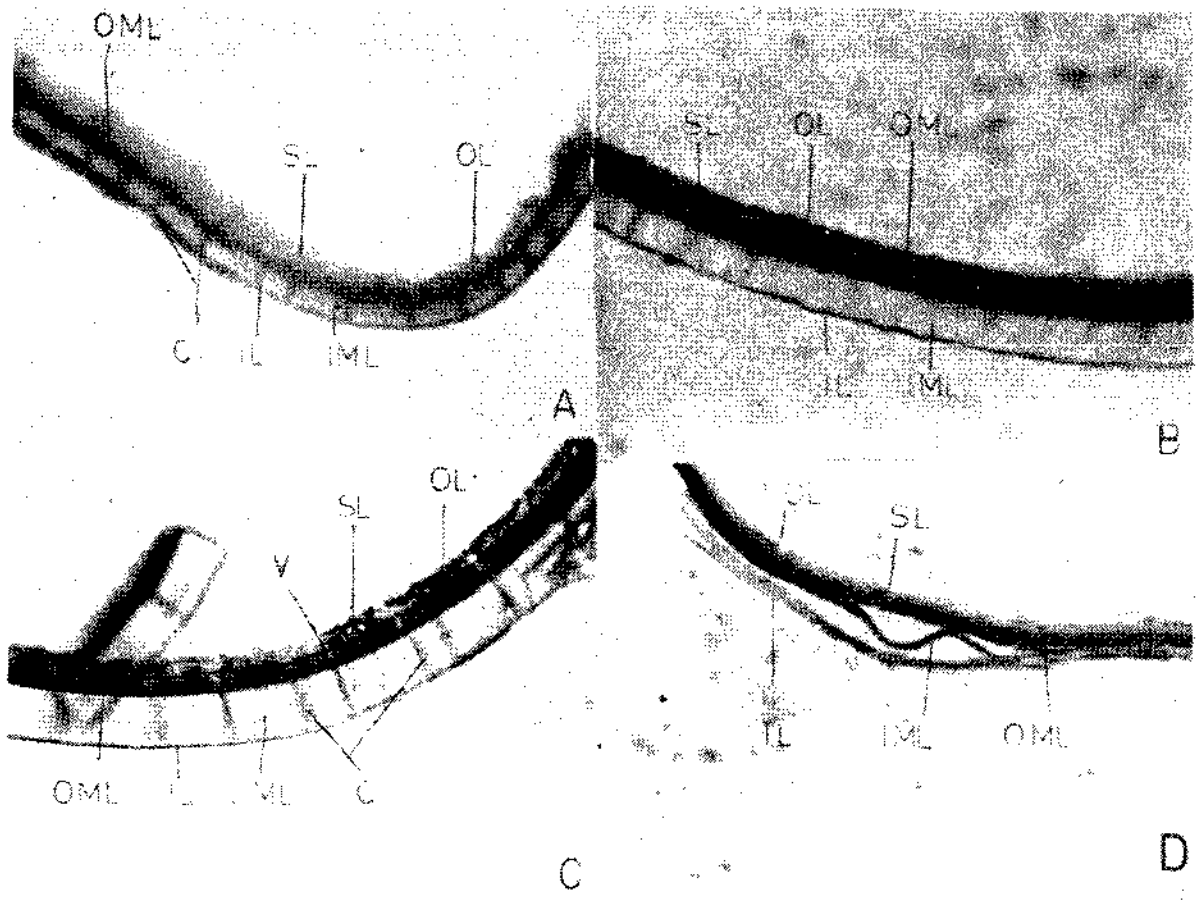

PLATE I A. Transverse section through the periostracum at an early growth phase x 1000 Stained in Mallory's triple stain; B. Transverse section through the periostracum at a late growth phase x 1000 Stained in Mallory's triple stain; C. Transverse section through the fully hardened periostracum treated with sodium hypochlorite x 1000 Stained in Mallory's triple stain and D. Transverse section through the fully hardened periostracum x 1000.

After treatment with alkaline sodium sulphide Stained in Mallory's triple stain (C – Canal; IL – Inner layer; IML – Inner part of the middle layer; OL – Outer layer; OML – Outer part of the middle layer; SL – Spongy layer and V – Vacuole).

layers, histochemical tests were performed on the periostracum in two different growth stages A and B referred to above, the results are recorded in Table 1.

Tests for proteins, show that the outer-most blue staining layer was positive to biuret test and only faintly positive to Millon, Hg/nitrite and Xanthoproteic tests. But the layer underlying it, was more intensely positive to Millon and Xanthoproteic tests and also showed positivity to biuret test. In the later growth stages, the reactions of the second layer were different. It now did not react to biuret, but retained the Millon's positivity. These observations may suggest that in this layer, there may be present more than one protein fraction, one of them containing phenyl groups. The absence of a more intense reaction to Millon and Xanthoproteic tests in the periostracal layer of the later growth stage and the assumption of refractility to stains may suggest that during growth, this layer undergoes chemical changes resulting in hardening and refractility. Beedham (1958) noted that in *Mytilus edulis* the outer-most layer of the periostracum at its origin stained red in Mallory, but this reaction faded later.

In the present study, sections of the periostracum were prepared from animals of two different sizes representing an early and later growth stages (A & B). Sections compared were those cut from identical regions of the shell of the animal in the two stages of growth referred to above. This region lies inside the pallial line and close to it. By analogy with the condition reported in the periostracum of *Mytilus viridis*, the changes in chemical composition may involve a process of tanning. This is suggested by the presence of a protein containing phenyl groups, of lipids and of argentaffin positive substances. To verify if tanning does occur in the region, frozen sections of the periostracum were subjected to treatment with diaphanol which has been widely used as a detanning agent (Kennaugh, 1957). After such treatment the refractility to stains in this region was lost and such regions stained blue with Mallory. Very similar results were obtained after treatment with alkaline stennite solution and sodium hypochlorite (Brown, 1950) (Pl. I C). Both these results are known to produce effects very similar to those of diaphanol on hardened proteins restoring them to an original unhardened condition. It is suggestive that spongy and the outer most layer may have similar chemical composition, but the inner part may undergo a process of tanning resulting in the effects referred to above. That tanning results in chemical resistence of this layer is indicated by the results of treatment with mineral acids (Table 1).

The middle layer of the periostracum appears to be different chemically from the outer two sublayers. Here the results obtained are that of Millon's Hg/nitrite and Xanthoproteic tests are negative, but it reacted positive to tests for organic sulphur. In this respect, the outer part of the middle layer differed from the inner part. A distinction may be seen between the outer part which was positive to nitroprusside (Pearse, 1961) ferric ferricyanide (Chevremont and Frederic, 1943) and blue tetrazolium (Barnett and Seligman, 1954) tests and the inner part which was negative to nitroprusside and ferric ferricyanide test, but positive to blue tetrazolium and lead acetate tests. The distinction between the outer and inner part of the middle region is more prominently seen in later growth stages. Here the region reacting to lead acetate test was wider and it may also be inferred that the outer part reacting to nitroprusside and ferric ferricyanide test may be gradually transformed into one reacting to lead acetate and blue tetrazolium tests. These differences are reflected in the results of staining reactions of the two regions. The outer region still stained red with Mallory and Masson's trich-

rome stain and blue with Heidenhain's haematoxylin whereas the wider inner part was refractile to these stains. It is suggestive from the above observations that in the outer part sulphur is present in the form of SH groups and in the inner part such may be transformed into -S-S- bonds, contributing to hardening.

most layer remains fuchsinophil in all the growth stages studied, suggesting that there is no alternation in its chemical composition during growth stages. The presence of a protein containing phenyl groups together with lipid, both in free as well as bound state may suggest that precursors of tanning though present, it remains permanently in unhardened

TABLE 2. *Summary of the results of histochemical tests performed on the periostracum of* Meretrix casta *for the detection of chitin at a late growth stage*

Stain and tests	Outer layer	*Middle layer* Spongy layer	Outer part	Inner part	Inner layer
Chitosen test (Campbell, 1929)	—	—	—	—	—
Modified Chitosen test (Clark and Smith, 1936)	+	+	+	+	—
Schultze test (Lillie, 1954)	+	+	+	+	—
Formic acid (Rudall, 1955)	+	+	+	+	—
PAS test (Pearse, 1961)	++	++	+	+	—
PAS after acetylation (Pearse, 1961)	—	—	—	—	—

\+ Positive reaction; — Negative reaction

++ Intensely positive reaction.

Support for this view was found from the results of treatment of these two layers with alkaline sodium sulphide and thioglycollate reagent. From the results summarized in Table 1, it may be inferred that the inner colourless part of the middle region reacting positively to lead acetate is susceptible to the action of alkaline sodium sulphide and thioglycollate reagents whereas the outer part apparently containing SH groups was not reactive to these reagents (Pl. I D). The inner-

condition. The outer-most part is either phenolically tanned or contains a protein precursor of tanning while the middle layer shows the presence of a sulphur containing protein which is hardened in the inner region by formation of -S-S- bonds. The inner-most layer remains incompletely tanned. The transverse canals which enlarge into vacuoles may serve for transport of material which may include those involved in the tanning of the outer layers of the periostracum.

Trueman (1949) reported absence of chitin in the periostracum of *T. tenius* based on a negative reaction with schultze test. But the presence of hexosamine has been reported by Degens *et al.* (1967). It is suggestive that such amino sugars may indicate the presence of chitin. In the present study, tests were performed on the periostracum of *M. casta* for the detection of chitin.

It was found that the periostracum does not survive the chitosan test (Campbell, 1929), but the modified chitosan test and PAS test (Ranham, 1961), yielded positive results in the periostracum by giving a violet colour (Table 2). Supporting evidences for the presence of chitin were obtained by the application of formic acid test (Rudall, 1955). The results are recorded in Table 2. It is seen that chitin is indicated in the external and middle layers, but not in the innermost layer.

The chemical nature of the chitin-like substance of the periostracum was tested by chromatographic analysis of the sugars present in a hydrolysate of the chitinous material. It has been suggested that the chitin drawn from different sugars may vary and that glucose need not be the only sugar present in it. The work of Degens and Spencer (1966) and Degens *et al.* (1967) may indicate that both galactosamine and glucosamine occur in the periostracum of *P. morrhuana* and *T. divisus.*

The relationship between these amino sugars and chitin in *M. casta* is not clear. The sugars in the acid hydrolysate of the periostracum were analysed chromatographically. The results showed the presence of glucose, galactose and rhamnose.

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