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STRUCTURE AND CHEMICAL COMPOSITION OF THE PERIOSTRACUM OF *PERNA VIRIDUS*

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ABSTRACT

The periostracum of *Perna viridis* at an early growth stage showed the presence of three distinct layers with varying structural and chemical features: outer yellow, middle broad light green with vertical striations and innermost yellow layer. At a later growth stage there is an increase in thickness of the middle layer, with three well-marked regions comprising a central green coloured part (in Mallory takes up red colour) which is vacuolated (containing inclusions) and outer and inner parts which are yellow coloured (refractile to stains).

The outermost layer of the periostracum of an animal in a late growth stage becomes amber coloured and resistant to the action of mineral acids indicative of the sclerotized nature. The chemical characteristics of the middle layer differ markedly from those of the outer and inner layers and shows similarity to that of the epicuticle of arthropods.

INTRODUCTION

THE STRUCTURE and formation of the shell in *Mytilus edulis* have been investigated by Ehrenbaum (1884), List (1902), Kessel (1940), Beedham (1958) and Dunachie (1963). Three distinct layers have been made out in the periostracum of *M. edulis*. The outermost periostracal layer through fuchsinophilic in the early stages becomes refractile in later growth stages suggesting hardening of this layer by tanning.

Dunachie (1963) suggested that the middle layer is distinct in chemical composition from the external layer and take up orange colour with Mallory. Beedham's (1958) observations show that this layer is refractile to stains which may suggest that in later growth stages, this layer undergoes hardening. Kessel (1940) reported that in the earlier growth stages, the vacuoles found in the middle layer, which are filled with, a substance, later gets emptied. Kessel's work (1940) does not throw much light on the composition of the middle layer though he indicated that the layer is refractile to stains even after diaphanol treatment which may suggest that the hardening may not be by tanning. Though occurrence of sulphur containing amino acids in the periostracum has been reported by Degens *et al.* (1967) their location and role in the organisation of the periostracum are not known.

The internal layer is shown to be fuchsinophil. Though Beedham (1958) observed that the internal layer differs in chemical nature from the external and middle layers, its composition is not known clearly.

Dunachie (1963) reported that in a light microscope, the periostracum of *Mytilus edulis* showed vertical striations in the middle layer. They were clearly seen in the newly secreted periostracum and later they are not visible. The work of Kawaguti and Ikemoto (1962) may suggest that during growth phase, it is the middle layer that shows a marked increase in thickness presumably by addition of materials passed on from the epithelial cells to the periostracum. This is suggested by the presence of vertical or oblique lines which according to these authors appear as a bundle of short lines in electron micrographs. The significance of these vertical striations in the early growth

stages and their disappearance in the later growth stages may be that they represent passages for transportation of materials during the formation of the periostracum and later they may get occluded.

In the light of the observations reported above, a study of the structure and composition of the periostracum in *Perna viridis*, at three different growth stages was made.

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Material and Methods

Specimens of *P. viridis* belonging to three size groups of 6 mm, 20 mm and 80 - 100 mm in length (3 mm, 8 mm and 20 mm in breadth respectively) were collected from Madras Harbour, where they occur in abundance. The shells were fixed in 10% neutral buffered formalin for 24 hrs and after washing decalcification of the shell was carried out in 5 % acetic acid or 8 % EDTA (ethylene-diamine-tetra acetic acid, disodium salt) (Simkiss and Tyler, 1957). Celloidin and paraffin sections of the decalcified shell were prepared for histological study. Stains used were Mallory's triple stain, Heidenhaine's haematoxylin and Masson's trichrome. Frozen sections were prepared by gelatin impregnation method and used for the application of histochemical tests.

Results and Discussion

In unstained preparations of the periostracum at an early growth stage (Pl. I A) three distinct layers may be made out. The external layer is very thin measuring 2μ and light yellow. Middle layer is broad, light green and about 10μ to 12μ in thickness, showing close vertical striations. The internal layer is light yellow coloured and is about 4μ thickness. The staining, the histochemical reactions of the layers are summarized in Table 1. In Mallory's triple stain the external layer stained deep red, the middle one blue and internal layer red. With Heidenhain's haematoxylin the regions which were fuchsinophilic were blue black. The middle layer stained a light grey. The external and internal layers showed similarity in their reactions to tests for protein. Both regions were positive to Millon's Hg/nitrite and Xanthoproteic tests, suggestive of the presence of a protein rich in tyrosine (Baker, 1956; Pearse, 1961). In the external and internal layers, Sudan black B, tests for bound lipids was positive. The ferric chloride test for diphenols was positive in the external layer, but negative in the innermost layer. It is suggestive that the outermost layer has some of the components necessary for tanning.

The middle layer showed distinctive features different from those shown by the outer and inner layers. While the Xanthoproteic and Millon's tests were negative in this region, it was positive to biuret test, indicative of the presence of a simple protein. A feature of interest is that this region also gives evidence of the Presence of organic sulphur, probably contained in the protein present in this region. This is borne out by the results of reactions obtained with nitroprusside test (Pearse, 1961) as well as with ferric ferricyanide and blue tetrazolium tests (Chēvremont and Frederic, 1943; Barnett and Seligman, 1954). Although a protein containing tyrosine is not found in this region, a feature which may be of interest is the presence of phenolic substances indicated by a positive reaction to ferric chloride and argentaffin tests (Lison, 1936).

Plate I B shows a section passing through the periostracum of an animal measuring 20 mm in length and 8 mm in breadth representing a later growth stage. A prominent feature is the increase in thickness of the middle layer which is now distinguishable into three well marked regions comprising a central green coloured part which is vacuolated and outer and inner parts which are yellow coloured.

The staining reactions and results of histochemical tests of the layers of the periostracum are summarized in Table 1. The middle layer comprising three distinct sub-layers shows different staining reactions compared to those in the early growth stage. With Mallory, the central part takes up a red colour and the outer and inner parts are refractile to stains. The innermost layer is more intensely coloured red with Mallory than the outermost layer. The external and internal layers stained blue black in Heidenhain's haematoxylin while the central part of the middle layer stained blue black and the outer and inner parts remained refractile to stains.

The results of histochemical tests performed on the constituent layers of the periostracum show some difference compared to those reported in an earlier growth stage. The layers which are fuchsinophil are positive to Millon's, Hg/nitrite and Xanthoproteic tests as in the corresponding layers in the earlier growth stage. Similarly the external layer is positive to argentaffin and ferric chloride tests, suggesting the presence of reducing substances as has been found in the earlier growth stage. Free lipid disappears in the external layer of the periostracum in the later growth stage. A similar absence of free lipids has also been noted in the middle layer with the exception of the middle part. The internal layer still gave evidence of the presence of free lipid (Table 1). Where free lipids are not indicated, the layers in question except the internal layer showed the presence of bound lipids. The significance of the reaction is that the disappearance of the free lipids may be due to the partial hardening undergone by those layers involving the lipids which tends to become bound with proteins.

The external and internal layers may be formed of lipoproteins recalling the chemical composition of the unhardened epicuticle of arthropods, such as *Periplanata* (Dennell and Malex, 1955).

Plate I C shows a section passing through the periostracum of an animal in a late growth stage measuring 80 mm to 100 mm length and 20 mm breadth. The outermost layer is amber coloured and is 4μ to 6μ thick. The middle layer measured about 50μ to 80μ thick and appears yellow when viewed through a light microscope. A central vacuolated region is prominently seen. The vacuoles appear almost empty. The inner layer is light brown and measure 6μ to 8μ thick. The results of standing and histochemical tests on the periostracum of this stage are summarized in Table 1. It is seen that the outermost layer during subsequent stages of growth undergoes further changes as revealed by the results of staining and histochemical tests. Unlike the innermost layer, it becomes amber coloured and resistant to action of mineral acids (Table 1). At this stage, the outermost layer when treated with Millon's, Hg/nitrite and Xanthoproteic tests, does not show a positive reaction. The transformation of the outermost layer from a fuchsinophil condition to a condition in which it is chemically resistant and mechanically rigid has already been reported in *M. edulis* (Beedham, 1958). These changes that take place in the outermost layer appear to be due to a process recalling tanning reported to occur in Arthropod cuticles. As in the latter, there occurs in this layer a tyrosine containing protein together with a lipid content which possibly undergo condensation forming a lipoprotein complex. The presence of a lipoprotein complex is inferred from the positive, Millon and Xanthoproteic tests as well as positive reaction obtained for bound lipids. In the earlier growth stage, both ferric chloride and argentaffin tests were positive, but in the later growth stage the ferric chloride test was negative. It may be suggested that phenols which occur in the earlier phase, may undergo oxidation yielding quinones. This view is supported by the observation, that the external layer in the later growth stages gives a positive reaction to chromaffin test for quinones. The above

observations together with the presence of a polyphenol oxidase as seen from a positive Nadi reaction (Table 1) may suggest that tanning of the protein in the outermost layer may take place. The mechanism of tanning may be similar to what has been suggested to occur in arthropod cuticles. In support of such a view, it has been noted that when the amber coloured resistant outer layer of the periostracum was treated with diaphanol or sodium hypochlorite, the amber colour was lost and the layer was rendered soft. After such pretreatment the layer in question resumed its staining properties taking up a red colour with Mallory. It is known that diaphanol and sodium hypochlorite are detanning agents which bring about a reversal of the tanning process restoring the protein to its original condition (Kennaugh, 1957). The results reported above provide evidence for the occurrence of sclerotization in the outermost layer of the periostracum. It is suggestive that as in the epicuticle of arthropods, its function may be protective. It appears reasonable to suggest that the chemical similarity of the periostracum to the arthropod epicuticle may be suggestive of functional similarity.

The fully hardened periostracum when subjected to diaphanol treatment shows the vacuoles to be enlargements of vertical canals which traverse the periostracum from the internal layer to the external layer (Pl. I D). Similar results were obtained by treatment with sodium hypochlorite (Pl. I E). The exposure of the canals after treatment with detanning agents may suggest that the contents of the canals may be of the nature of tanned protein. In the normal condition of the fully grown periostracum, these canals are not distinguishable from the surrounding periostracal substance except in the region of the central part of the middle layer. It was noted that in the earlier growth stages the transverse canals are seen prominently, but later they are not visible except in the central part of the middle layer where they appear to be vacuoles in nature. The transverse canals stain pink with Mallory and react positively to argentaffin test. They also showed positive reaction to Millon's tests unlike the surrounding regions of the middle layer, which were positive to Biuret test. Apparently these canals are continuous from the internal fuchsinophil layer to the outermost surface of the external layer and serve as channels for transport of materials. Although the occurrence of tanning in a constituent layer of the periostracum and the presence of transverse canals, may be analogy recall a similar condition reported in the cuticle of arthropods, particularly insects, it is not known how far the functional role of the canals suggested to occur in periostracum is similar to those of the pore canal of arthropod cuticles. Kawaguti and Ikemoto (1962) refers to the existence of microvilli in the epithelial cells which appear to be in contact with the periostracum. In Gastropods like *Berthelinia limax*, the outer layer of the periostracum is unaffected while the middle layer increases in thickness as it approaches the mantle edge and is in association with microvilli.

Several structural continuities were recorded between the periostracum and the other layers of the shell based on the occurrence of the so called tubules across the shell layers penetrating even the periostracum (Hudson, 1969; Tayler *et al.*, 1969). However evidences regarding the existence of the structures establishing a continuity between the periostracum and the epithelium are still unsettled. It is considered that there is no evidence for any addition to the periostracum once it is formed at mantle edge. (Fretter and Graham, 1962; Hyman, 1967). But some authors considered that periostracum after it is formed may show polymerization of some of the constituents contained in it (Taylor and Kennedy, 1959; William, 1969). The question is how far chemical changes if any, taking place in the periostracal layers would contribute to the increase in thickness of the layers concerned. In the present study, it has been noted that the periostracum of an

older shell, as indicated by size is thicker than that of an younger one.

The chemical characteristics of the middle layer differ markedly from those of the outer and inner layers. Unlike the outer and inner layers, it is negative to tests for a tyrosine containing protein. In addition to the presence of a protein positive to Biuret test, this region gives evidences for the presence of organic sulphur. In later growth stages, the region gets differentiated into three parts, an outer and inner part refractile to stains, separated by a central green coloured fuchsinophilic one having vacuoles, which appear to contain inclusions. The central region is positive to tests for SH groups like intraprusside, ferric ferricyanide tests (Table 1). The outer and inner regions did not react positively to these tests, but to lead acetate test and blue tetrazolium test (Table 1), indicating the presence of S-S groups. The presence of sulphur in the periostracal layer was confirmed by additional tests of Hawk Oser and Summerson, 1954. The chemical reactions are summarised in Table 1. The reactivity of the entire middle layer in an earlier growth stage to nitroprusside and ferric ferricyanide tests and the restriction of such reaction in the later stages of growth to the central part together with the observation that the outer and inner regions referred to above, react positively to lead acetate test may suggest that in these regions SH groups may be transformed to S-S bonds contributing to stabilization of protein. Such a condition recalls the process of Keratinization involving formation of disulphide links conferring rigidity and resistant properties on the protein. The suggestive evidence referred to above has been tested by application of other tests. The alkaline sodium sulphide splits the S-S bonds and convert it to SH which bring about a reversal of the process of Keratinization. The middle layer was disrupted and separated at various regions. It was rendered untanned and fuchsinophil (Pl. I F). The external amber coloured layers and internal fuchsinophil layers of the periostracum are not affected. In chemical composition, the middle layer of the periostracum recalls the epicuticle of arthropod like *Palamneus swammerdami*.

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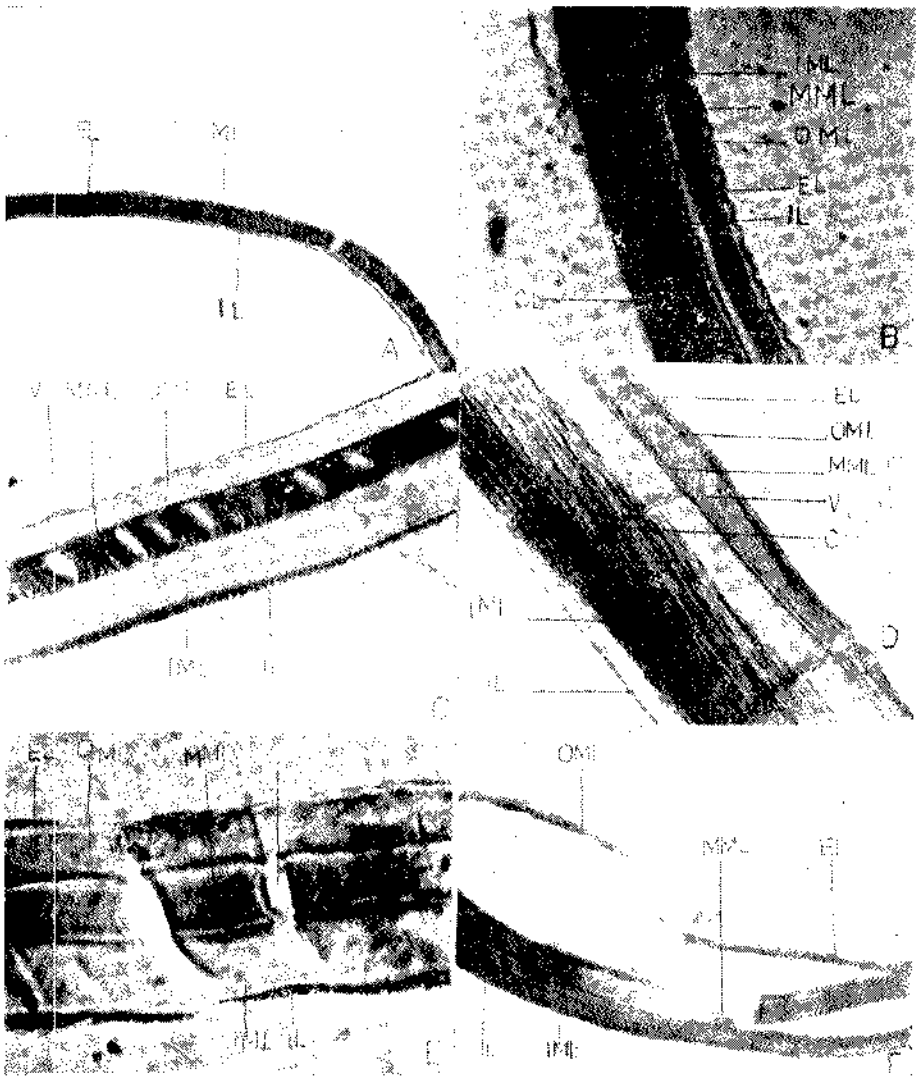

PLATE 1 A. Transverse section (TS) through the periostracum at an early growth stage, B. TS through the decalcified shell at a later growth stage to show the differentiation of the middle layer of the periostracum, C. TS through the periostracum at a late stage, D. TS through the fully hardened periostracum treated with Diaphanol, E. TS through the fully hardened periostracum treated with sodium hypochlorite and F. TS through the fully hardened periostracum treated alkaline sodium sulphide (All x 1000, Stained in Mallory's triple stain). C - Canal; CL - Calcareous layer; EL - External layer; IL - Internal layer; IML - Inner part of the middle layer; ML - Middle layer; MML - Middle part of the middle layer; OML - Outer part of the middle layer and V - Vacuole.

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