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Sclerotized Protein in the Shell Matrix of a Bivalve Mollusc

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Abstract

The epsilon-amino groups of lysine and phenolic groups of tyrosine are most heavily concentrated in the newly-deposited organic matrix of the shell of the bivalve *Mercenaria mercenaria*. A phenoloxidase enzyme which oxidizes L-dihydroxyphenylalanine is present only in this "new" area of the shell matrix. Scanning electron micrographs of calcified secretions of the shell show that accretion lines, thought to be layers of organic matrix separating diurnal accretions of calcium carbonate, are not developed until up to 4 d after deposition of shell material. These results suggest that the shell matrix is hardened by some kind of polymerization, and that lysine and tyrosine residues in the matrix are involved in the process. Accretion lines in polished and etched sections become visible only after complete hardening of the polymer occurs.

Introduction

Cross-linked polymerization of protein chains—commonly referred to as tanning or sclerotization—is a distinctive feature of biological materials serving a structural function. Linkage of adjacent protein molecules, by a variety of chemical bonds, is found in hairs, silks, feathers, collagens, and the cuticles and egg-cases of insects and crustaceans.

A variety of cross-linking structures has been described to date, including disulfides, biphenyls, desmosines formed by condensation of lysine derivatives, and aminoquinones, among others, A few of these structures are represented in Fig. 1. In the phylum Mollusca, quinone-tanned proteins are believed to exist in the byssus and periostracum (Brown, 1952; Beedham, 1958; Meenakshi *et al.*, 1969). However, with one exception, tanning has not been investigated for the most substantial structural element of the phylum: the calcified shell.

Over 2 decades ago Beedham (1958) proposed that the organic matrix of bivalve shells was sclerotized. His conclusions were founded on histochemical tests demonstrating an abundance of phenolic groups in shell matrix; he also detected phenoloxidase activity in the ligament, which is partially calcified. Since that time the mechanism of tanning in mineralized molluscan shells has not been explored further.

In this paper, we present evidence that supports and strengthens the conclusions of Beedham (1958). Localized activity of phenoloxidase and tyrosyl hydroxyl groups is found in the area of newly-deposited shell of *Mercenaria mercenaria* (Linné). This activity correlates with physical characteristics of calcified shell sections as seen with a scanning electron microscope. In addition, the major concentration of reactive epsilon-amino groups is demonstrated to exist in the same area. We suggest that polymerization of molluscan shell matrix occurs after the initial depostion of shell, and that the polymerization process involves the reaction of tyrosyl and lysyl groups in the matrix.

Materials and Methods

Scanning Electron Microscopy

Shells removed from live *Mercenaria mercenaria* freshly collected in lower Delaware Bay, USA were cleaned by scrubbing adherent tissues from the shell interior. Radial sections 3 mm thick were cut from each valve with a diamond saw, and subsequently ground with a series of silicon carbide papers (from 220 to 600 grit) on a metallographic polisher. Grinding was followed by polishing with aluminum oxide slurries on Buehler Microcloth, and the final polish carried out with 0.3 micron alumina powder. Sections were cleaned in trichloroethylene, acetone and distilled water, and etched for 30 to 60 s in 0.5% HCL at room temperature (ca 21° C). The polished and etched sections were washed

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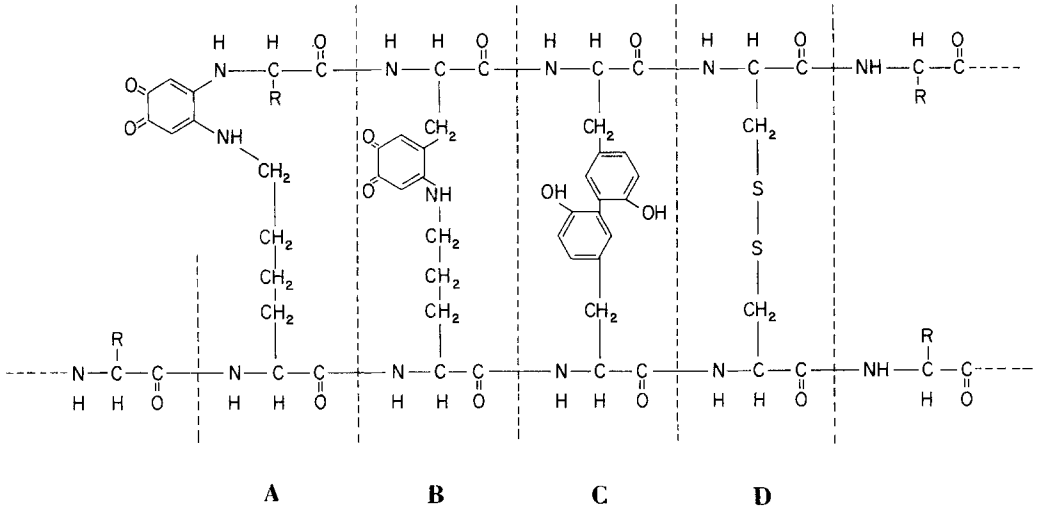

Fig. 1. Representative cross-links between protein molecules. (A) A free o-diquinone reacts with a lysyl amino group from one chain and a terminal amino from another to form an amino-quinone; (B) a quinone derivative of a peptide-bound tyrosyl residue forms an amino-quinone with a neighboring lysyl amino; (C) a pair of tyrosyl residues coupled through a biphenyl linkage; (D) a cystine cross-link. Modified after Brunet (1967)

thoroughly in distilled water, rinsed briefly in ethanol, mounted with silver paint on aluminum scanning electron microscopy (SEM) stubs, and dried for several hours at 65° C. Specimens were coated with approximately 500 Å of pure gold in a Polaron E5000 Sputter-Coating Apparatus, and taken directly for SEM observation or stored in a desiccator until used. Photographs in the magnification range of 50 to 7000X were made with a Cambridge Steroscan microscope.

Histochemistry

Both "old" shells (kept dry in the laboratory for a year after removal) and valves from live animals were used. Body tissue and periostracum were removed with a wire brush. However, some specimens were not cleaned on the exterior surface to permit comparison of the reactions of shell matrix and periostracum. Shell pieces were cut or broken to leave at least 2 cm of undamaged margin, then decalcified in a solution of 10% neutral-buffered formalin saturated with sodium ethylenediaminetetra-acetate. At room temperature, 4 to 8 wk were required to completely decalcify shell pieces having an area of 3 to 4 cm^2 on one side. Decalcification was followed by thorough washing in cold-running tap water, dehydration in dioxane (Thompson, 1966), and infiltration and embedding in Tissueprep at 57° C Five micron sections were cut with a rotary microtome and mounted on glass slides.

A variety of stains was used to reveal structural and chemical characteristics of different parts of the matrix sections, including aqueous Methylene Blue, Toluidine Blue, and Mallory's Trichrome Stain. Van Gieson's Picrofuschin was routinely used as a counterstain in conjunction with histochemical tests. Congo Red was applied for identification of glycoprotein (Thompson, 1966) and the Ninhydrin-Schiff reaction for protein distribution (Yasuma and Ichikawa, 1953).

Sulfhydryl and disulfide groups were identified with a modification of the procedure of Danielli (1950). For sulfhydryls, diazonaniline was used as a blocking reagent for tyrosine, tryptophan, and histidine, while amino grops were modified to hydroxyls with acidified sodium nitrite solution. For disulfides, benzoyl chloride served as a general block, and the disulfide linkages were broken with hydrocyanic acid treatment. In both cases, the resulting or remaining sulfhydryl groups were reacted with dinitrofluorobenzene (DNFB) and then with betanaphthol to develop a color reaction.

General reducing ability of matrix sections was identified with the Argentaffin reaction (Lison, 1960), using diammine silver solution as the substrate (Thompson, 1966). Two methods were tried for tyrosine distribution. The first was Baker's (1956) modification of the Millon reaction except that thin sections were incubated at 37° C for 24 h to avoid disruption of the matrix by the boiling step recommended in the original procedure. The second was another variant of the Danielli (1950) method; amino groups are modified in acidified sodium nitrite and sulfhydryl groups are oxidized with hydrogen perioxide. The dinitrophenyl derivative of tyrosine was reduced with 5% sodium hydrosulfite to yield a more satisfactory color reaction (Lillie, 1965).

Arginine was identified by the Sakaguchi reaction (Baker, 1947). Lysine distribution was determined by incubating sections in an ethanol solution of 5-dimethylamino-1-napthalenesulfonyl chloride (dansyl-chloride) at room temperature for 3 h. According to Rosselet and Ruch (1968) this reaction is specific for the epsilon-amino group of lysine; their data further

show that preserving sections in formalin does not affect the accuracy of the test. Dansylfluorescence was observed by exciting the reacted sections with light in the 350 to 400 nm wavelength range and photographing emission at approximately 530 nm; lysine dansylfluorescence peaks at around 540 nm (Rosselet and Ruch, 1968).

Phenol oxidase activity in matrix sections was detected by incubation with tyrosine (Stevenson and Adomako, 1967), catechol (Smyth, 1954) and dihydroxyphenylalanine (DOPA) (Thompson, 1966). In each case a control section was run with 0.005 M KCN included in the incubation medium.

Results

Scanning Electron Microscopy

Etching with HCl was satisfactory for revealing the patterns of growth lines in the shell of *Mercanaria mercenaria* (Fig. 2a, b). Additional etching in 1N KOH revealed more of the fine structure at the level of the individual crystals in the prismatic region. However, this treatment dissolved enough of the organic matrix to render the growth lines less distinct than otherwise (Fig. 2c).

The pattern of growth lines displayed by shell sections resembled the daily and bi-daily frequencies observed by Pannella and MacClintock (1968) in *Mercenaria mercenaria*. Assuming that their interpretation of accretion line frequency is correct, then some evidence of sub-daily activity was also seen in our specimens (Gordon and Carriker, 1978). The distinctness of the growth lines was greatest in the older portions of the shell (Fig. 3a), decreasing near the growing edge (Fig. 3a, b, c). At higher magnifications, the lines seemed to disappear entirely at a distance corresponding to about 2 to 4 days' growth at the edge of the shell; however, a definite gradation of distinctness was observed in specimens extending inward to about 15 to 20 days' growth. At first we assumed that this phenomenon simply represented an artifact of specimen preparation, the most likely cause being insufficient contact with the polishing medium at the edges. However, a number of sections, embedded in and thoroughly supported by a matrix of polyester resin during the polishing step, displayed similar features, while growth lines at the fractured edges of mature shell were uniformly distinct (Fig. 3d).

The occurrence of localized physical differences in growth line patterns led to the tentative conclusion that differences of a chemical nature exist in the shell, either in the mineralized portion or in the organic matrix itself. Accordingly, histochemical experiments were designed primarily to examine the latter possibility. Attention was focused on the existence of cross-linked polymers in shell matrix.

Histochemistry of Shell Matrix

Conspicuous metachromasia was not apparent in any of the sections stained with Methylene Blue or Toluidine Blue. With Mallory's Trichrome, only the periostracum (in those specimens where it had been preserved) stained red, while the shell matrix *per se* was uniformly blue throughout.

Positive tests were indicated with the periodic acid-Schiff, ninhydrin-Schiff and Congo Red strains. Color was uniformly distributed in each of these tests. Neither proteins nor carbohydrates showed localized concentration differences.

In stained decalcified sections, it was possible to distinguish regions of the matrix corresponding to those seen in sections of calcified shell. A characteristic structure was apparent for both the prismatic and inner homogeneous (Pannella and MacClintock, 1968) layers. Homogeneous matrix showed many fine lamellae arranged parallel to the inner surface of the original shell (Fig. 4). These layers were continuous into the prismatic area, except that they bent at the prismatic-homogeneous junction to become perpendicular to the inner surface and parallel to the curved growing edge at the margin (Fig. 4). Indistinct lamellae were also observed in the homogeneous region of calcified shell sections viewed with the SEM (Fig. 5). In addition to these lines, which correspond to accretion lines seen in SEM photographs, there was a series of lines perpendicular to the marginal growth edge of the prismatic region. The spacing between these lines and their orientation, which continues directly out to the margin, suggests that these are shell prism boundaries (Fig. 4). The combination of prism boundaries and accretion layers presents a reticular appearance reminiscent of SEM photographs of radial sections of clam shells.

The use of specific histochemical tests was adopted with the following point of view: if, as scanning electron micrographs suggested, there was a chemical change occurring in the organic matrix as the shell matured, it ought to be possible to identify a nonuniform distribution of materials participating in the hypothesized polymerization. Any protein side-groups implicated in cross-linking should show a greater abundance in the matrix closest to the growing margin, and a corresponding absence in the matrix of the fully developed shell.

Although sulfhydryl groups were not expected to survive the prolonged decalcification procedure, a positive test was obtained for sulfhydryls throughout the matrix section. The same was true for disulfides. No localized differences in the distribution of these groups was apparent (Fig. 6).

Incubation with diammine silver identified a strong reducing potential which was restricted to the margin of prismatic matrix and to the newly-deposited inner homogeneous matrix. Because of the shrinkage and distortion inherent in decalcification and embedding procedures, it is not possible to state with any accuracy

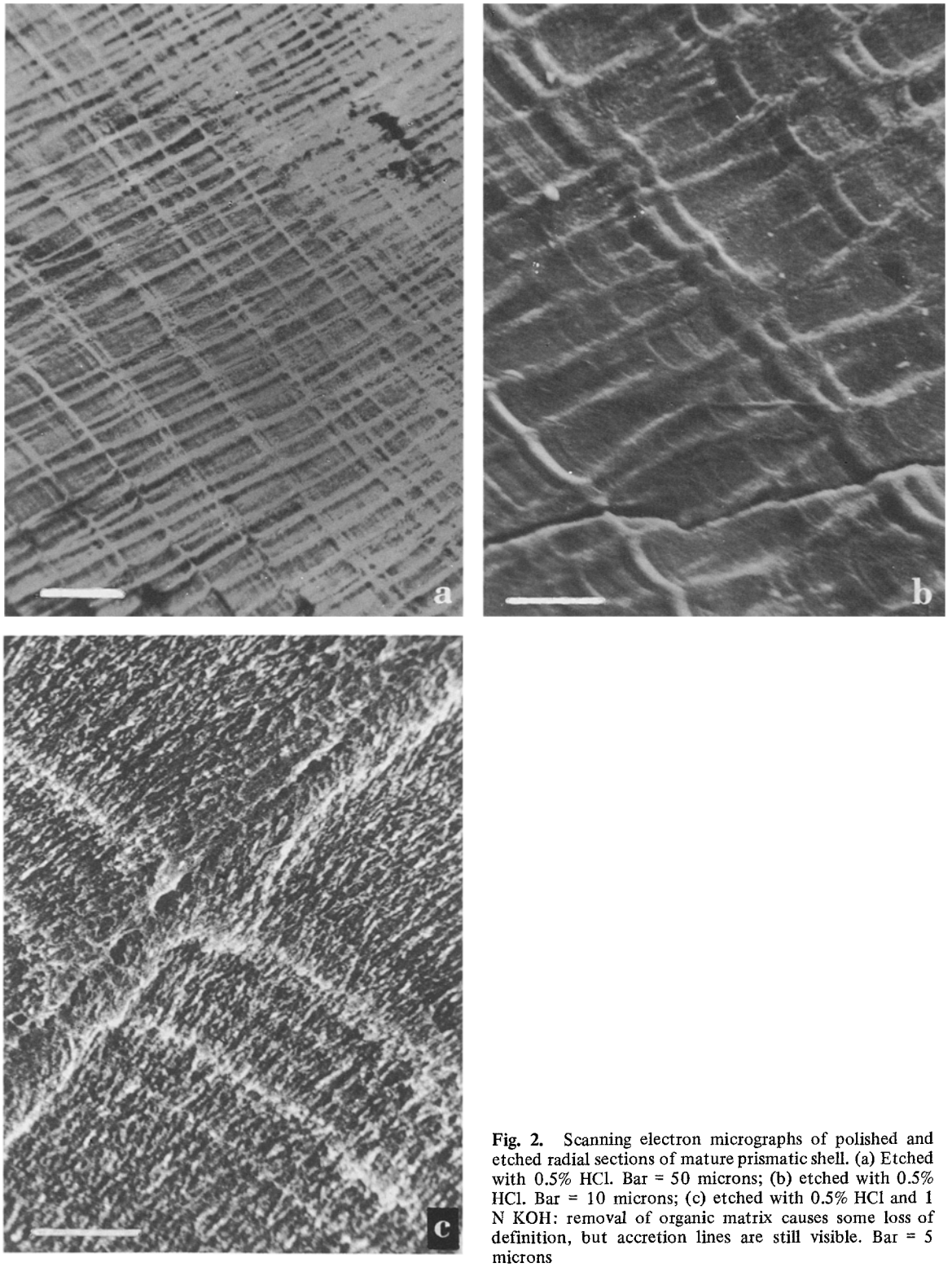

Fig. 2. Scanning electron micrographs of polished and etched radial sections of mature prismatic shell. (a) Etched with 0.5% HCl. Bar = 50 microns; (b) etched with 0.5% HCl. Bar = 10 microns; (c) etched with 0.5% HCl and 1 N KOH: removal of organic matrix causes some loss of definition, but accretion lines are still visible. Bar = 5 microns

the depth to which such reducing capacity penetrated the original shell material (Fig. 7).

Although a positive reaction was obtained with our modification of Baker's (1956) tyrosine test, the color reaction was too weak to be unequivocal. However, the DNFB reaction proved positive in every section tested. Figs. 8a and b show that the availability of tyrosyl hydroxyl groups was greatest at the growing margins of the shell. The reducing capacity of these areas can probably be attributed to phenolic groups.

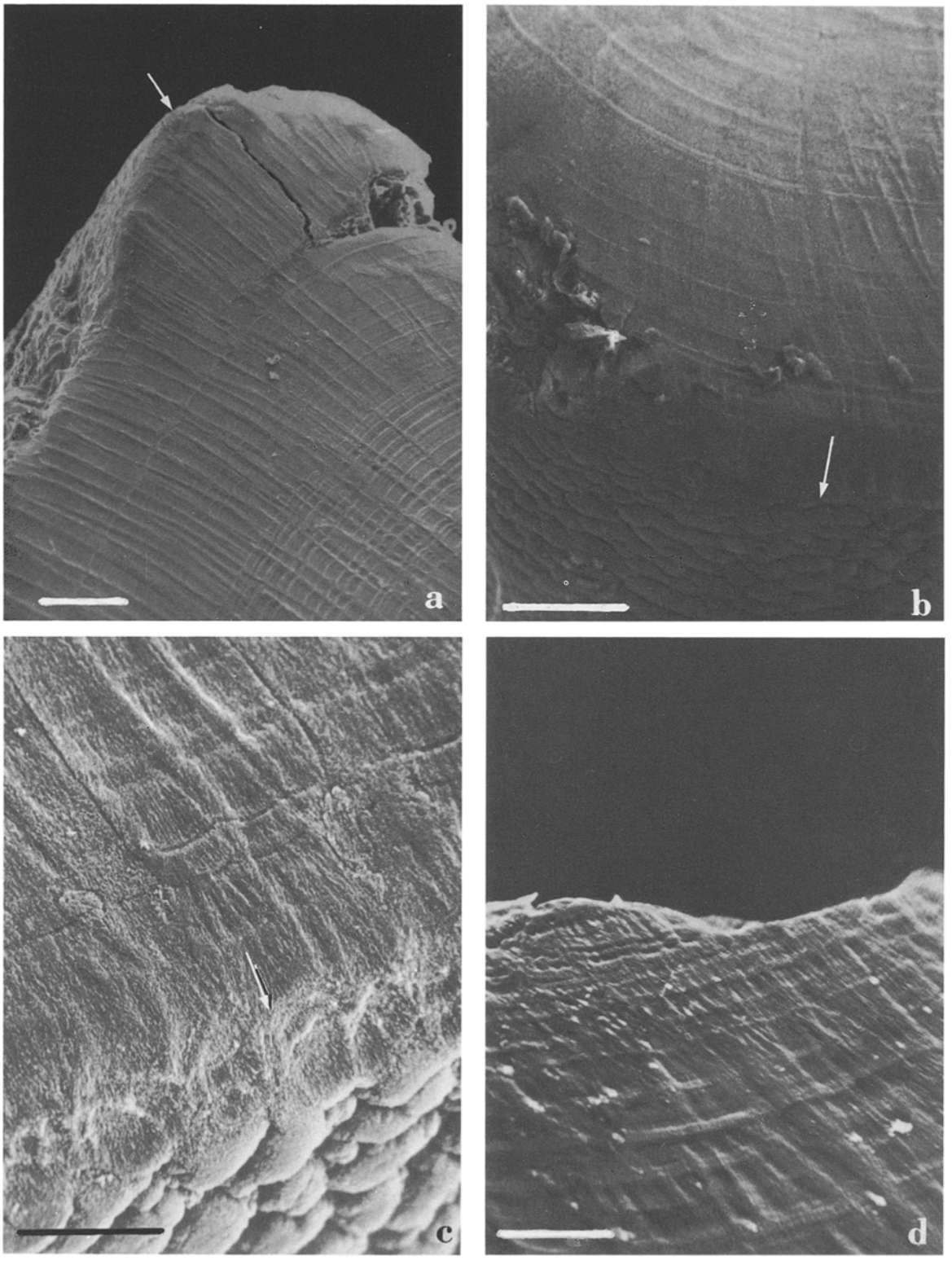

Fig. 3. Scanning electron micrographs of polished and etched radial sections of prismatic shell. Arrows indicate newly formed edge of shell. (a) Accretion lines are distinct in older portion of shell, but fade out toward margin. Bar = 100 microns; (b) and (c) quite close to newly-deposited edge the accretion lines can no longer be visualized. Bars are 100 and 20 microns, respectively; (d) polished and etched radial section of an "artificial margin" created by fracturing the section prior to polishing. Accretion lines are distinct to the very edge of the specimen. Bar = 50 microns

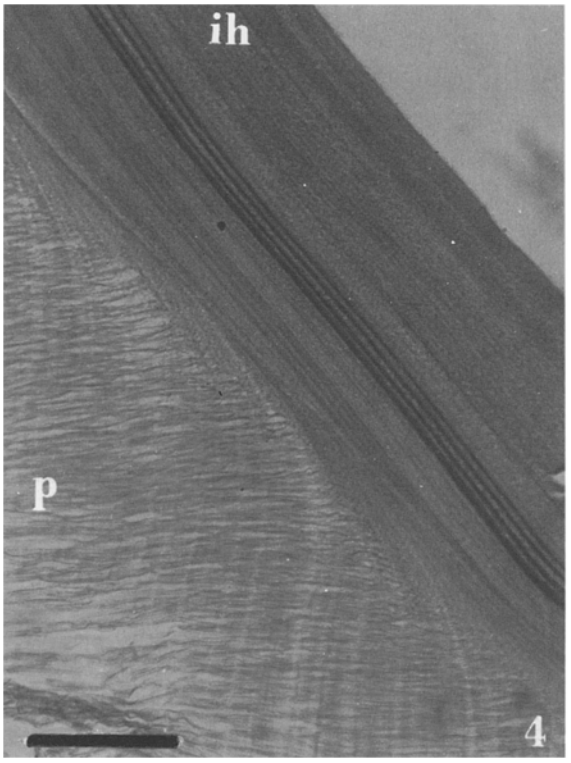

Fig. 4. Light micrograph. Thin section of decalcified matrix of mature shell stained with Mallory's Trichrome. Inner homogeneous region (ih) displays growth lines parallel to inner edge of original shell. Prismatic region (p) has a reticulate appearance due to the intersection of prism boundaries with accretion lines. Growth lines are continuous from one region to the other. Bar = 0.1 mm

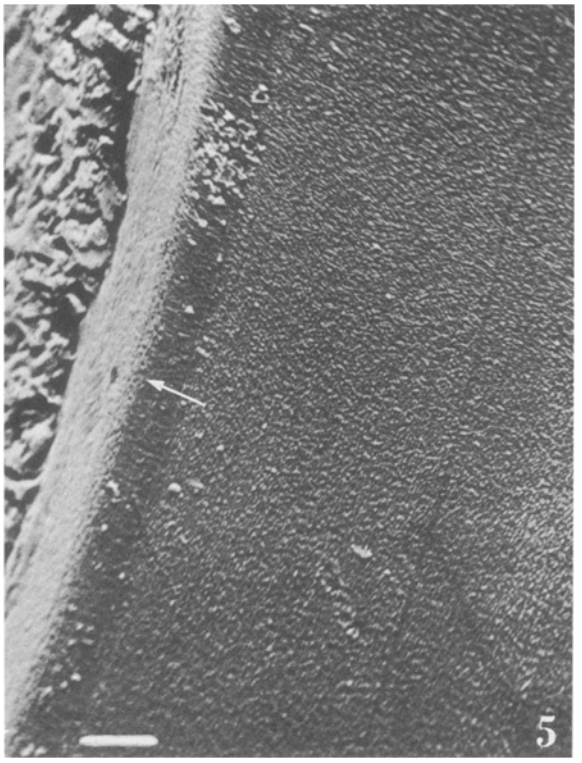

Fig. 5. Scanning electron micrograph. Polished and etched section of inner homogeneous material, showing accretion lines running parallel to inner edge of calcified shell (arrow). Bar = 25 microns

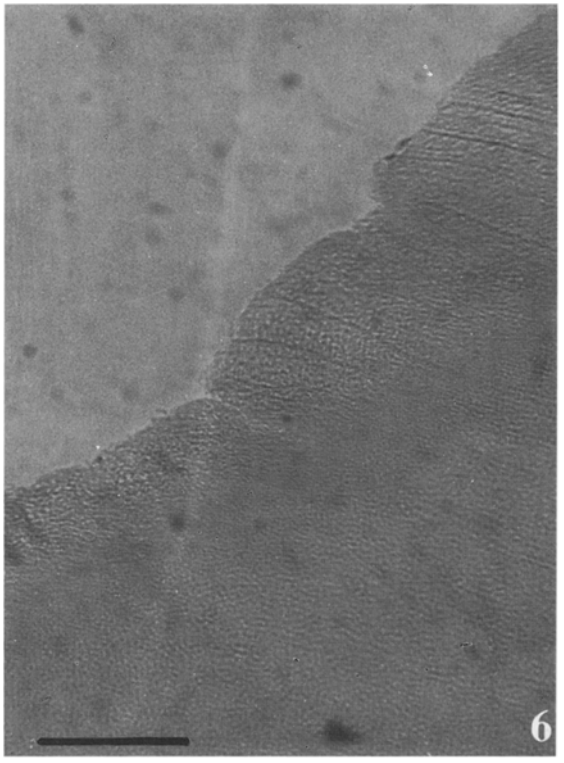

Fig. 6. Optical microscope. Thin section of decalcified prismatic matrix stained with dinitrofluorobenzene (DNFB) and beta-naphthol to demonstrate sulfhydryl groups. Color is uniform throughout section. Bar = 0.05 mm

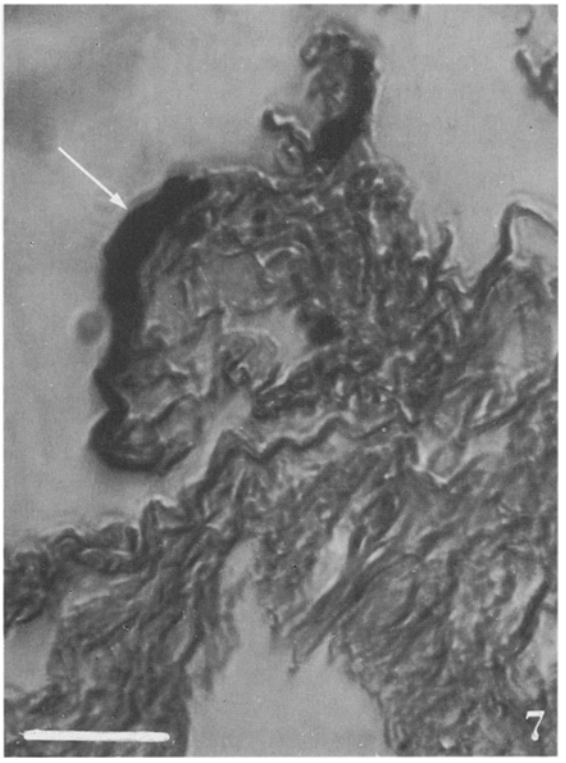

Fig. 7. Light micrograph. Arrow indicates sites of reducing activity in thin section of prismatic portion of decalcified matrix. Staining with diammine silver occurs only at periphery of the matrix. Bar = 0.025 mm

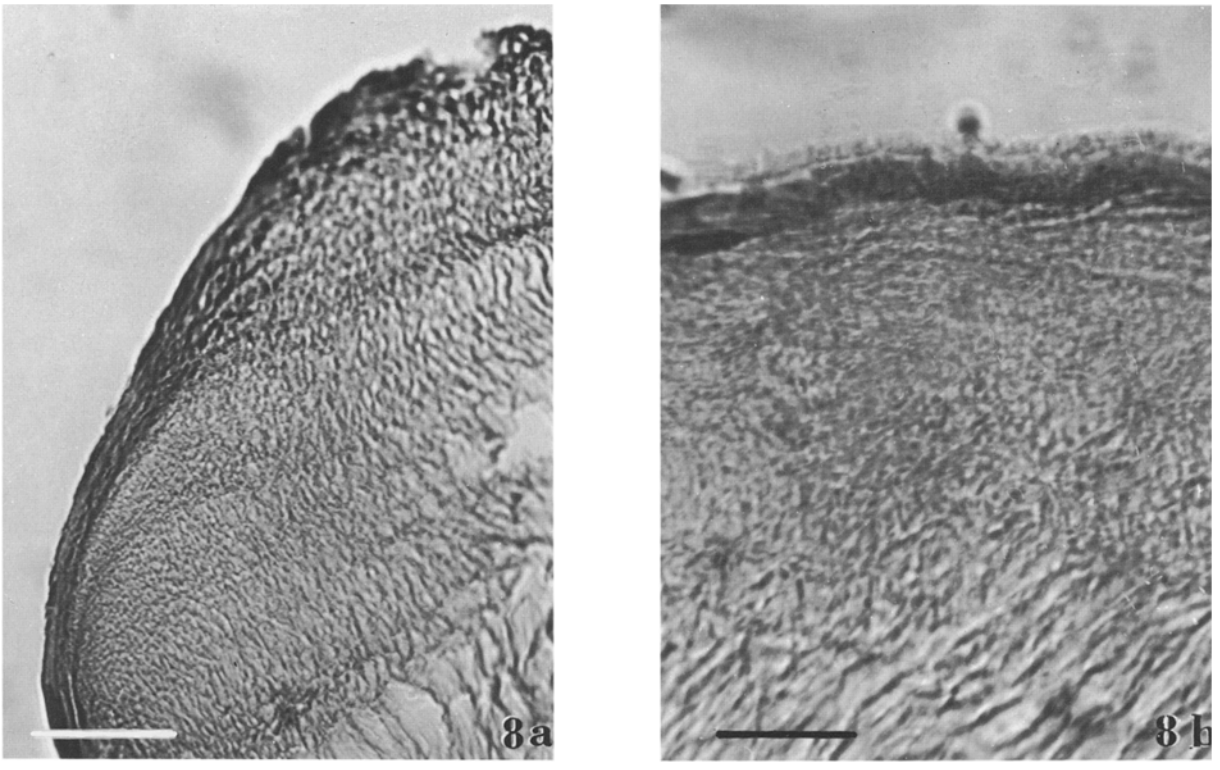

Fig. 8. Light micrographs. Thin sections of decalcified matrix near shell margin stained to demonstrate tyrosine. Color distribution indicates greatest abundance of tyrosyl residues at the extreme margin of shell. (a) Prismatic material. Bar = 0.025 mm. (b) Inner homogeneous material, close to pallial line. Bar = 0.025 mm

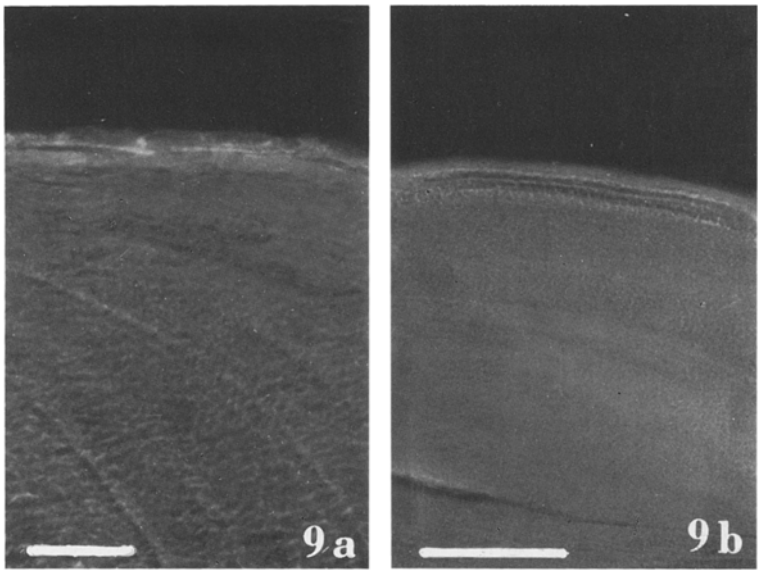

Fig. 9. Fluorescence micrographs. Dansyfluorescence of lysyl amino groups in thin sections of decalcified matrix. (a) Prismatic material near margin: bar = 0.025 mm; (b) inner homogeneous material, close to pallial line. Bar = 0.05 mm

Arginine was uniformly distributed in matrix sections. However, a strikingly different result was found for lysine. Free lysyl amino groups were more heavily concentrated in the same areas as were the phenolic groups (Figs 9a, b). Furthermore, in sections where the periostracum was preserved, the highest concentration of epsilon-amino groups was seen in the periostracum (Fig. 10). The results obtained with all histochemical tests reported so far were qualitatively the same whether "old" or "new" shells were used in the experiments.

Negative results were found for all sections incubated with either tyrosine or catechol. The latter test (Smyth,

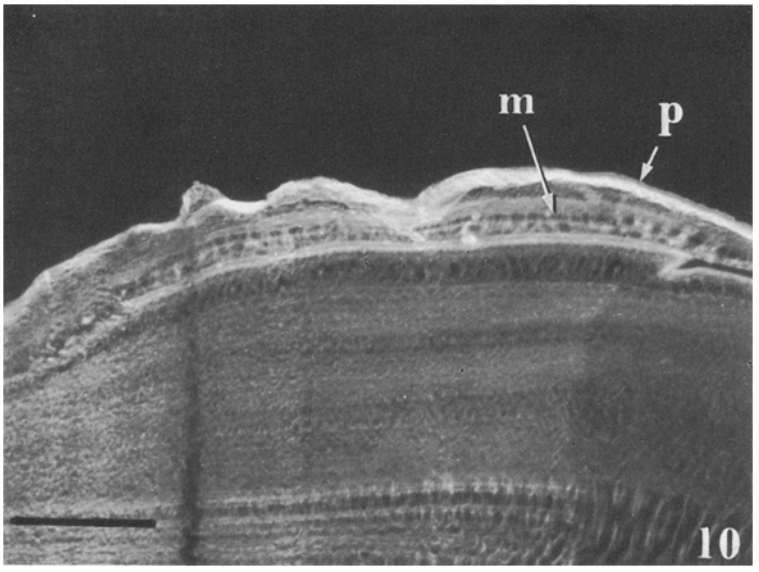

Fig. 10. Fluorescence micrographs of thin section of decalcified matrix close to pallial line. Dansyfluorescence of the periostracum (p) is considerably brighter than that of the underlying conchiolin matrix (m). Bar = 0.05 mm

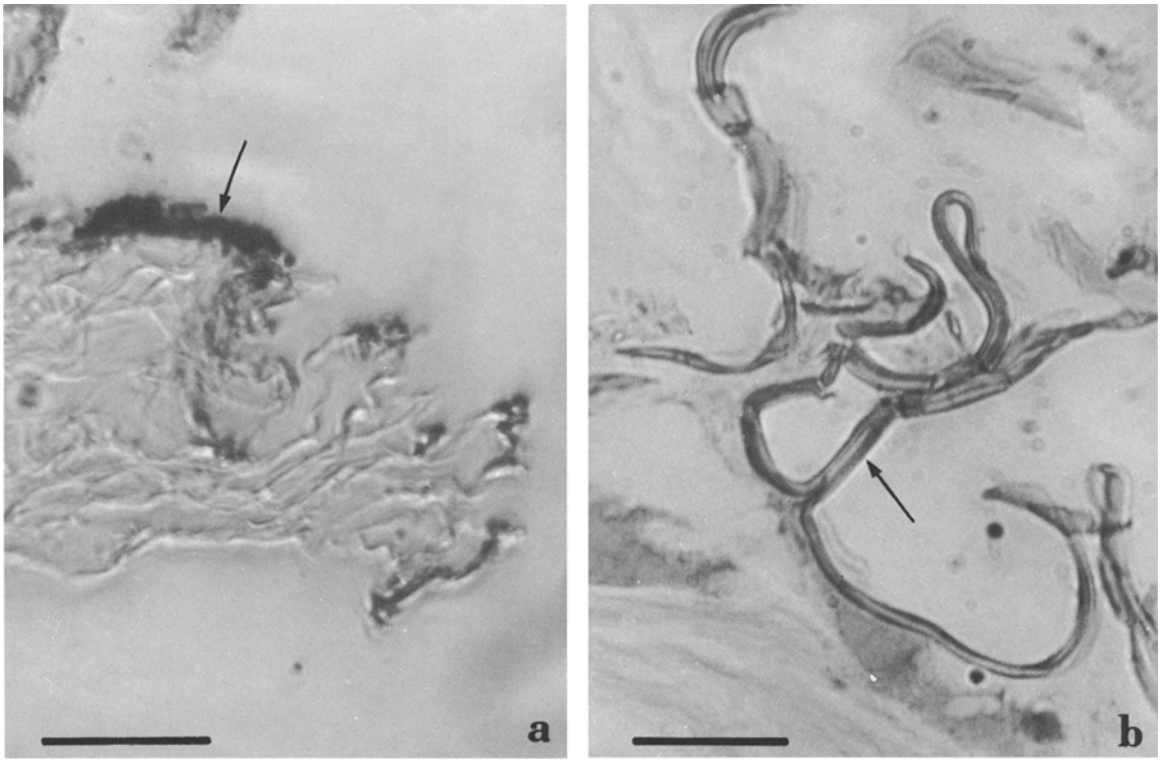

Fig. 11. Light micrographs. Thin sections of decalcified matrix which has been incubated with L-DOPA. (a) Shows presence of phenoloxidase activity only at periphery of matrix. Bar = 25 microns: (b) fragments of periostracum, indicated by arrow, remain clear after incubation with L-DOPA. Bar = 10 microns

1954) is considered to be specific for a tyrosinase enzyme involved in the production of melinan in insect cuticle. Neither was any melanin found by Lillie's (1965) ferric iron uptake method. On the other hand, incubation of matrix sections with L-DOPA showed a strong reaction when the sections were prepared from new shell (Fig. 11a), and an equivocally positive reaction with old shell. In both cases the reaction was limited in extent to the extreme periphery of the prismatic margin. The reaction was completely blocked by inclusion of cyanide in the incubation medium. Close examination of fragments of periostracum revealed an absence of DOPA-oxidase activity (Fig. 11b).

Discussion

In his extensive biochemical and histochemical analysis of the organic matrix from the shells of 3 lamellibranchs, Beedham (1958) adduced evidence for a tanning reaction in both the periostracum and the conchiolin of the valves. He used relatively nonspecific terms ("outer" and "inner" layers) to describe the origins of the non-calcareous matrix relative to the shell, and it is not clear in his histochemical report whether "outer" refers to the prismatic region of the shell, the unbounded shell surface in general, or the actively growing marginal regions. Clear-cut structural differences in regions of the shells of all the animals he worked with have since been reported (Taylor *et al.*, 1969), and these differences should have been observed in sections of decalcified matrix as they were in the present work.

Beedham inferred from the "presence" of phenolic groups in the conchiolin that the matrix was tanned. Curiously enough, we have reached a similar conclusion from an opposite viewpoint. The evidence presented here shows that tyrosyl phenolic groups are indeed present in the shell matrix of *Mercenaria mercenaria*. We deduce that phenols are involved in the cross-linked polymerizations of matrix from the fact that some of these groups are rendered non-reactive as the shell matures. Beedham's (1958) histochemical evidence is partially corroborated by amino acid analysis of different parts of the valves, although it does not clarify the locality of chemical groups in specific shell regions. In our work and that of Beedham the soluble matrix, a significant portion of the total conchiolin, was lost due to the decalcification procedures (Hare, 1963; Crenshaw, 1972; Grégoire, 1972).

In *Mercenaria mercenaria*, as in all other bivalves analyzed, lysine is an ubiquitous minor constituent of shell matrix (Hare, 1963; Degens *et al.*, 1967; Saleuddin and Hare, 1970; Crenshaw, 1972; Weiner and Hood, 1975). The evidence presented here shows that free amino groups of lysine, like the phenolic groups of tyrosine, are more reactive in the marginal, newly-deposited regions of the shell matrix. While the localization of lysine epsilon amino groups has not been reported before in molluscan shell, an analogous situation does exist for insect cuticles. Lysine, and indeed all amino groups, disappear during the hardening of the ootheca of the cockroach (Pryor, 1940), and Hackman (1953b) could not detect amino groups in the insoluble portion of the cuticle in the coleopteran *Aphodius Howitti*.

Our results also suggest the presence of a phenoloxidase enzyme at the shell margin. When this enzyme acts upon a DOPA substrate the product is a black "melanoid" substance which is not further identified. DOPA has been found in the shell matrix of a number of gastropods and bivalves, but not in *Mercenaria mercenaria* (Degens *et al.*, 1967). Lerner *et al.* (1949) reported that a mammalian DOPA oxidase (or tyrosinase; the terms are used interchangeably by some authors, cf. Thompson, 1966) catalyzes both the conversion of tyrosine to DOPA and the oxidation of DOPA to a melanin intermediate which is probably a quinone. The phenoloxidase in the shell of *Mercenaria mercenaria* is clearly different, since we found no colour reaction to matrix sections incubated with tyrosine. Neither was any reaction noted with catechol, an important distinguishing characteristic from insect phenoloxidase which converts catechol to quinone derivatives (Hackman and Todd, 1953).

The localized concentration of tyrosine and lysine in the area of recently deposited shell may be considered circumstantial evidence for a polymerization involving these amino acids. The fact that the sections were thoroughly washed with water during preparation eliminates the possibility that these were free amino acids; they must be still embedded in a proteinaceous chain. The presence of a phenol oxidase near the shell margin suggests that tyrosine may be a precursor for either free or protein-bound quinones formed by enzymatic activity (Sizer and Wagley, 1951; Hackman, 1953a; Sizer, 1963; Dabbous, 1966; Kawasaki *et al.* 1974). At the moment this idea is entirely speculative, however, as we have not demonstrated either the quinones themselves or any intermediate products.

The concept of active chemical changes occurring in the marginal area of the shell, probably polymerization, is supported by the distinctive physical character of calcified shell at the ultrastructural level. We are able to observe growth lines in calcified shell sections because layers of organic matrix are less soluble in HCL solution than calcium carbonate. Accretion lines are left in relief after the etching step. A similar difference in solubility explains why accretion lines become less distinct towards the growing edge of the shell. "New" shell matrix has not had time to harden chemically and dissolves at the same rate or faster than calcified material. A gradient in distinctness from the margin to the older part of the shell indicates that polymerization is a relatively slow process. Using the model of Pannella and MacClintock (1968) and assigning 1 d of growth to each major accretion line in a section, we observe that up to 4 d may be required before the matrix is sufficiently hardened to permit the appearance of distinct growth lines. "Setting" of the organic matrix may not be complete for several weeks after deposition (Fig. 3a).

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