Mayerson submits "guestimates" of the barley crop of the Bedouin, and is not astonished that we reaped 125 kg of barley per dunam in a drought year of 40 mm rainfall. However, the Bedouins of the area *were* astonished, since they had a complete drought year and did not reap 1 kg per dunam. The purpose of our experimental farm is to replace these popular "guestimates" with scientific data.

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31 May 1961

Formation of the Periostracum in Mercenaria mercenaria

Abstract. A series of histochemical tests performed on the mantle of the northern quahog *Mercenaria mercenaria* L. suggest that the epithelium of the inner surface of the first fold and the underlying secretory cells function together in the formation of the periostracum in this mollusk. The secretory cells supply a phenolic substrate which, when oxidized, provides quinones capable of tanning the periostracum.

A row of columnar epithelial cells along the inner surface of the outer—that is, first—fold of the mantle of bivalve mollusks is regarded as being functional in the secretion of the periostracum (*1–4*). Our studies indicate that a similar group of epithelial cells in the bivalve, *Mercenaria mercenaria* L., also functions in the formation of the periostracum (*5*), although a conflicting report states otherwise (*6*). The periostracum of bivalve mollusks consists largely of a quinone-tanned protein, but the nature of the tanning process is obscure. Our histochemical tests on the mantle of *Mercenaria mercenaria* suggest that material formed in secretory cells underlying the inner epithelium of the first fold is a precursor of the tanning agent in this organism. A summary of the tests and procedures follows.

Quahogs were dug from an intertidal flat in Delaware Bay, off Cape Henlopen, Delaware, fixed in Bouin's solution within an hour after collection, and tested with Millon's reaction (*7*) and the argentaffin reaction (*8, 9*). Millon's reagent reacts with proteins containing large amounts of phenolic amino acids, principally tyrosine, while the argentaffin test has been used extensively to detect polyphenols involved in the tanning of proteins (*4*).

Paraffin sections cut transversely from the mantle edge were deparaffinized and treated with Millon's reagent at room temperature until maximal color developed (approximately 6 hours). Similar sections were treated with ammoniacal silver nitrate (Fontana's solution) (*8*) for 18 hours at 37°C and counterstained in 1-percent aqueous neutral red for 30 seconds.

A test for a polyphenol oxidase (*10*) was carried out on whole mantles from quahogs taken off Slaughter Beach, Delaware, a few miles north of Cape Henlopen. The clams were refrigerated (5°C) overnight and then removed from the shells while they were still alive. Whole mantles were treated with 10-percent neutral formalin for 1 hour at 22°C to harden the tissues. They were then incubated for 13 hours in 0.0056*M* 3,4-dihydroxyphenylalanine (DOPA) in 0.1*M* phosphate buffer at *p*H 7.4. The DOPA solution was changed after the first hour of incubation.

After the mantles were incubated in the DOPA solution, pieces were cut from the mantle edge (see Fig. 1), fixed for 24 hours in Bouin's solution, and imbedded in paraffin. Sections were counterstained in 1-percent aqueous neutral red solution. Control mantles were treated in the same manner except that they were incubated only in phosphate buffer.

The inner epithelium of the first fold, as seen in Table 1, although not reacting with either Millon's reagent or ammoniacal silver nitrate of the argentaffin reaction, did give a positive DOPA oxidase reaction, particularly near the opening of the periostracal groove. The underlying secretory cells did not react with DOPA, but their behavior in Millon's reagent and Fontana's solution showed that they contain a protein substance that has large amounts of phenolic groups.

The secretory cells have been demonstrated by several histochemical and histological techniques (*5*) and do not appear to be artifacts of fixation or staining. Ducts or extensions of these cells feed through the inner epithelium of the first fold to the periostracal groove.

Table 1. Summary of reactions observed in histochemical tests on *Mercenaria* mantle.

Tissue	Millon's	Argentaffin	DOPA oxidase
Inner epithelium of first fold	−	−	+
Underlying gland cells	+	+	−
Periostracum	+	+	+?
Outer epithelium of second fold	−	−	−

The periostracum gives intense Millon and argentaffin reactions, but there were no discernible differences between the DOPA oxidase-treated periostracum and the untreated control portions. Since the periostracum is a tanned protein, it is difficult to tell whether a positive DOPA oxidase reaction is due to the enzyme or the already-tanned periostracum.

The epithelial cells themselves do not seem to contain appreciable amounts of phenolic amino acids. Apparently the only available material capable of sup-

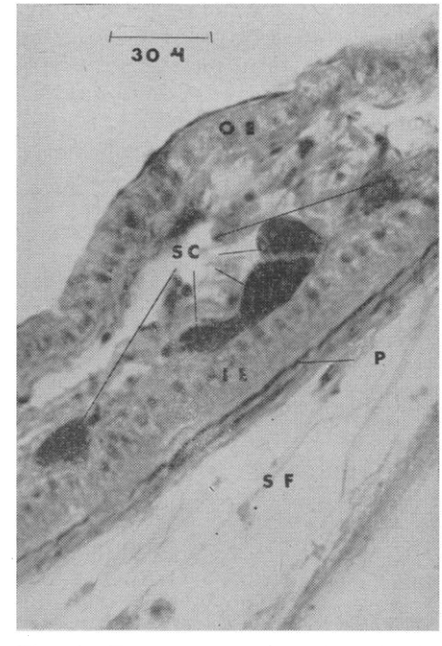

Fig. 1. Transverse section through the mantle edge of *M. mercenaria* showing the dark-stained response of five secretory cells to Millon's reagent. In this photograph the periostracum is adpressed to the epithelium of the inner surface of the first fold by a flap of tissue which arises from the outer surface of the second fold. *OE*, Outer epithelium of first fold; *IE*, inner epithelium; *SC*, secretory cells; *P*, periostracum; *SF*, flap from second fold.

plying the necessary tanning agent is the product of the underlying secretory cells. A positive argentaffin reaction plus the identification of a protein substrate and a phenol oxidase provide support for the existence of a complete quinone tanning system (*11*). On the basis of the present research it can be postulated that the hardening of the periostracum of *M. mercenaria* may be brought about by the action of an orthoquinone produced by the oxidation of the side chains of the phenolic compound secreted by the underlying cells of the first fold. The combination of this oxidation product with either the main chains of the polypeptides, or the side chains, results in the tanning reaction. Brown (*2*) has suggested a self-tanning, that is, one in which the phenolic protein acts as both a substrate and a tanning agent, for the byssus of *Mytilus edulis*. Whether the product of the secretory cells undergoes self-tanning or, in an oxidized state, aids in tanning another protein, perhaps the product of the epithelial cells themselves, is not clear.

It is interesting that, except for the sulfide link found in keratinized proteins, the quinone bonding mentioned above is the only other covalent link found so far in skeletal proteins (*12*), and might possibly be responsible for the sclerotization of such proteins throughout the animal kingdom (*11; 13*).

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13. I express my appreciation to Drs. J. H. McClendon, R. R. Ronkin, and G. F. Somers for their critical reading of the manuscript. This problem was suggested to me by Dr. C. N. Shuster, Jr., and the work was sponsored by grant G-6154 to Dr. Shuster from the National Science Foundation. This paper is contribution No. 22, University of Delaware Marine Laboratories.

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Luminescence Potency of the Cypridina System

Abstract. Visible light is emitted on oxidation of 0.00001 μg/ml ($2 \times 10^{-11}M$) of *Cypridina* luciferin with 0.01 mg/ml of luciferase protein, or with 1 μg/ml of luciferin with 0.0000001 μg/ml ($2 \times 10^{-15}M$) of luciferase. Data on yields indicate the average content of luciferin and luciferase to be, at best, about 1 μg of each in a single living organism.

In early studies on the biochemistry of the light-emitting enzyme-substrate ("luciferase-luciferin") system of the small ostracod crustacean *Cypridina*, Harvey (*1*) estimated that the addition of crude luciferase to a solution containing 1 part of luciferin in from 4 to 40 billion parts of water resulted in the production of light visible to the dark-adapted eye. This estimate was based on the assumption that the raw material, consisting of whole dried organisms, contained between 1 and 10 percent luciferin by weight. Since pure, crystalline luciferin has recently been isolated from dried specimens (*2*) as well as from initially living organisms preserved in Dry Ice (*3*), and a tentative description of its structure has been made (*4*), more definitive estimates can now be made regarding the actual luciferin content of the organism as well as the minimal concentration, in terms of number of molecules (molecular weight 469) required for visible luminescence. The same is true with respect to luciferase; it has very recently been obtained in an essentially pure state, and its major properties have been determined (*5*). Moreover, when both the enzyme and substrate are pure, quantitative errors due to the influence of unknown substances, which might exert inhibitory effects on the activity of luciferase or quenching effects on the total light emission, can be eliminated.

For the experiments reported here (*6*), a methanolic solution of crystalline luciferin was calibrated by optical density at 435 mμ in a Beckman spectrophotometer. Pure luciferin is relatively stable in methanol, although it rapidly autoxidizes in aqueous solution exposed to air. Therefore, dilutions were made in methanol; and in accordance with Harvey's observations (*1*), extreme precautions (using all new glassware, and so forth) were exercised to avoid errors due to adsorption of luciferin on, and its release from, glass surfaces of pipettes and containers. Tests for light emission were made by adding, in total darkness, 4.9 ml of a solution of 0.5 mg protein of practically pure luciferase in 0.1M sodium chloride plus 0.05M sodium phosphate buffer, pH 7.0, to 0.1 ml of a given dilution of luciferin, for simultaneous observation by three or four persons with dark-adapted eyes. A faint, momentary luminescence was clearly seen at a dilution of 1 in 10^{11} ($2 \times 10^{-11}M$ luciferin), but none at 1 in 10^{12}.

The potency of luciferase was similarly tested by adding dilutions of the enzyme in the aforementioned salt solution (glassware and blank solutions were first autoclaved) to 1 μg of luciferin in 0.1 ml of methanol. Light was observed at a final concentration of 1 part of luciferase in 10^{13} parts of solution, though not with certainty at 1 part in 10^{14}. On the basis of 50,000 as the molecular weight (*5*), a luciferase concentration of somewhat less than $10^{-15}M$ ($<10^{-7}$ μg/ml) is thus sufficient to yield visible light.

Analogous experiments with respect to the actual visibility of luminescence at great dilutions of firefly luciferin and luciferase have not been reported, but the quantum efficiency of the firefly system has been measured with the lu-

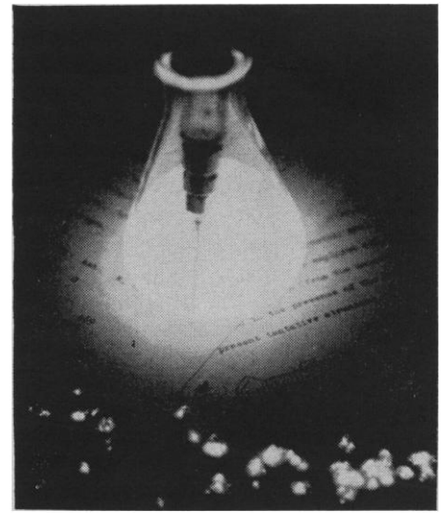

Fig. 1. Photograph taken by the light of the *Cypridina* reaction. The foreground shows individual specimens and small clumps of the organisms luminescing after removal from Dry Ice in which they were stored for about 2 months. The reaction mixture in the flask consisted of 28 ml of buffered salt solution containing 0.002 mg of pure luciferin to which was added, by the hypodermic syringe, 2 ml of salt solution containing 2 mg of practically pure luciferase. Exposure time, 1 minute at $f/2$; Tri-X Pan film.