FIGURE 89.—Localization of alkaline phosphatase in the mantle of *C. virginica* (longitudinal section). Photomicrograph of a preparation treated by Gomori method.

vations are in full agreement with the results obtained by Bevelander (1952).

SUBLIGAMENTAL RIDGE

A small ridge marking the dorsal edge of the mantle along the fusion of its two lobes is known as the subligamental ridge. Its length in the anteroposterior direction corresponds exactly to that of the ligament, which is secreted by the epithelial cells of the ridge. The base of the ridge is flattened and rests on basic elastic membrane; the body of the ridge is semicylindrical in cross section, its surface slightly undulating, as can be seen from the longitudinal section shown in figure 78.

The histological structure of the ridge has been studied in *Mytilus* (List, 1902; Tullberg, 1881), in *Anodonta* (Moynier de Villepoix, 1895; Rassbach, 1912), and in the Portuguese oyster *Crassostrea* (*Gryphaea*) *angulata* (Leenhardt,1926). Leenhardt and Moynier de Villepoix call the structure "bandelette paleale" (pallial strip) but the term subligamental ridge seems to be more descriptive.

In *C. virginica* the subligamental ridge is always well developed and easily recognizable by its shape and by its coloration, which is usually darker than that of the adjacent part of the mantle. The epithelium (fig. 78, ep.) covering the ridge presents a most striking picture. It consists of a layer of extremely tall and narrow cells arranged in fanlike groups and set on a well-developed basal elastic membrane. The length of the cells varies from 50 to 200 μ depending on the position they occupy within the layer. The cells are very thin, with granular protoplasm and an oval-shaped nucleus. At the distal portion of the ridge the boundaries of the cells become indistinct and their protoplasm darker, presumably due to the concentration there of the organic material which they secrete. The free surface of

the epithelium is not attached to the ligament as was described by Moynier de Villepoix (1895).

At regular intervals the row of epithelial cells is interrupted by oval-shaped pockets which appear to be empty, with the exception of occasional amoebocytes and a few connective tissue cells. The significance of these pockets is not clear. The elastic membrane under the epithelium, thicker here than in the other parts of the mantle, includes many muscle fibers arranged parallel to the length of the ridge (m.). Large oval cells containing yellow-brownish granules (pig. c.) are abundant. The ridge is well supplied with blood through a large blood vessel (bl. v.), around which the connective tissue consists of tightly packed globular and spindle-shaped cells. Directly under the basal membrane of the ridge, however, the connective tissue of the mantle is made up of large vesicular cells.

FUNCTIONS OF THE MANTLE

Ciliary currents along the inner surfaces of the mantle form a definite pattern which may be easily observed. If one valve of the oyster is removed the corresponding mantle rolls up and exposes the gills and the inner surface of the mantle on the opposite side. In such a preparation the intact lobe of the mantle remains fully stretched and the ciliary currents can be observed by sprinkling the surface with small quantities of carmine, colloidal carbon, powdered shell material, carborundum, or other powders insoluble in sea water. It is best to use very fine particles, such as powdered mineral willemite and colloidal carbon. Willemite phosphoresces a brilliant green under ultraviolet light, which makes it possible to locate even the tiniest particles not otherwise recognizable. As a source of ultraviolet light I used a small Mineralight lamp. Hard and heavy particles of this mineral may stimulate the cilia by their weight, but this difficulty is avoided by using colloidal carbon.

As can be seen from the diagram in figure 90, drawn from life, the general direction of the currents is from the base of the mantle to its periphery, with the ciliary motion strongest in the anterodorsal sector. In the large oyster (5 inches in height) used for the drawing, this area extended along the margin of the mantle from the level of the labial palps approximately halfway down the anterior side. The upper part of the mantle was usually completely cleared 2 or 3 minutes after

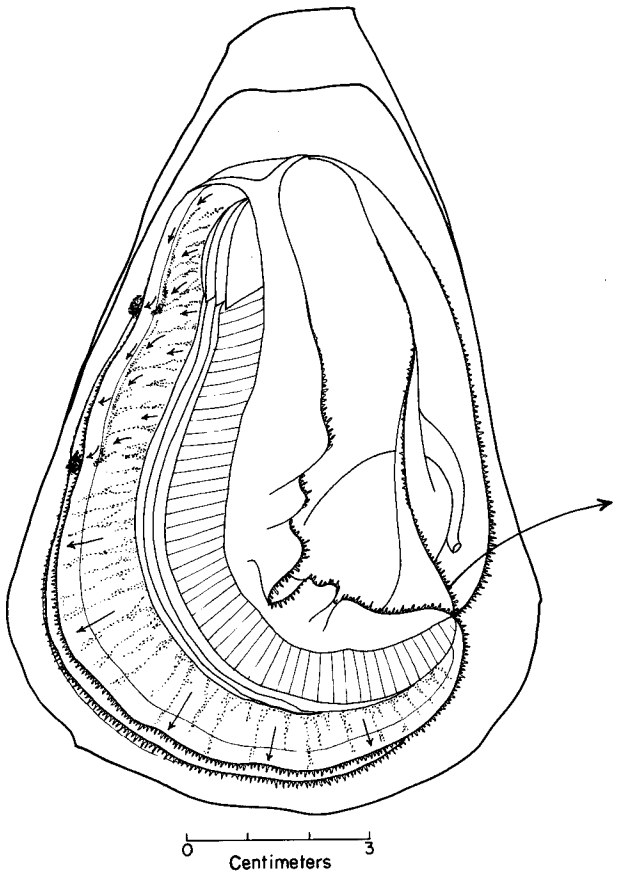

FIGURE 90.—Discharge areas of the mantle. Note the two large lumps of the discarded material at the edge of the mantle which mark the boundaries of the principal discharge area. Small arrows indicate the direction of ciliary currents. The clear path along the periphery of the mantle in the upper left side, indicated by short arrows, marks the ciliary tract located over the circumpallial artery. Long arrow at right indicates the direction of exhalant current of the cloaca. Drawn from life.

it was sprinkled with powder, while in the same specimen 5 to 10 minutes were required to clear the lower (ventral) part. Although the ciliary currents along the posterior side of the mantle in the area adjacent to the cloaca are also directed from the base toward the periphery, this area is swept clear by an exhalant current from the gills (fig. 90, long arrow) which is much stronger than those produced by the mantle epithelium.

The currents along the anterodorsal part of the mantle (upper left of the figure) adjacent to the labial palps are directed at an acute angle to its free margin. There is also a well-defined tract of ciliary movement about 1.5 mm. wide parallel to the edge of the mantle. Upon reaching the level of the lower corners of the labial palps this current

turns sharply about 90 degrees across the mantle edge. This point marks the upper limit of the area throughout which the material settled on the mantle is discarded; in the oysters which received powdered suspensions large lumps of particles entangled in mucus are usually seen here. A similar accumulation of material ready to be discarded marks the anterior boundary of the discharge area which may vary in dimension and location in different oysters.

Along the ventral portion of the oyster body the ciliary currents sweep across the mantle at right angles to its edge and material is discarded along the border. In this respect my observations differ from those of Nelson (1938, p. 24), who thinks that the current in this area is directed along the free margin toward the mouth. Such a current was not present in the large oysters (*C. virginica*) I used in my experiments.

The existence of a special discharge area located in the zone below the labial palps is biologically significant. The so-called pseudofeces, or masses of discarded food particles and of detritus entangled in mucus, which accumulate in this area either slide over the free edge of the mantle and drop off or are forcibly ejected by the snapping of the valves. There is no doubt that the presence of pseudofeces near the edge of the mantle stimulates the strong ejection reaction which constitutes one typical pattern of shell movement in the oyster (see p. 169).

FORMATION AND CALCIFICATION OF SHELL

The principal function of the mantle is the formation of the shell and its calcification. The great structural complexity and intricate pattern of pigmentation found in some species are produced by the mantle. The regulatory mechanisms responsible for this process are not known because the morphogenesis of molluscan shells has never been studied experimentally. From observations on shell growth in some gastropods and lamellibranchs it is clear that the shape of the shell as well as the pattern of pigmentation result from the position assumed by the edge of the mantle during periods of shell secretion and from the rate of deposition of calcium salts and pigments.

It can be easily observed in oysters, scallops, and other bivalves in which the edges of the mantle are not fused together that during periods of growth the mantle extends a considerable distance beyond the border of the shell. In some species it even stretches far out and folds back over the outer surface of the valve. In this way, for instance, the mangrove oysters produce hooks or similar structures by which they attach themselves to branches of trees (fig. 5).

The differential rate of growth along the periphery of the shell as well as the formation of spines, nodes, ridges, and similar sculptural elements are both caused by changes in the rate of deposition of shell material. Two distinct phases may be distinguished in the shell-forming process: (1) the movements of the mantle which stretches and folds itself in order to provide a matrix or mold upon which the shell is formed, and (2) the secretion and deposition of the shell material itself. It is probable that the circumpallial nerve plays a role in the first phase of the process by controlling the muscular activity of the mantle. Our present knowledge of the physiology and biochemistry of shell secretion is inadequate to propose an explanation of the morphogenetic processes involved in shell formation. These processes are not haphazard but follow a definite and predetermined course. This is self-evident from the fact that the final shape of the shell has definite mathematical characteristics (see p. 24) which can be attained only by orderly and regulated deposition of organic framework and mineral salts.

The first step in the formation of the oyster shell is the secretion of conchiolin from the periostracal gland. This process can be easily observed by cutting off a small section of the edge of the upper valve and exposing the intact valve and the underlying mantle of the opposite side. Under a low-power binocular microscope one can see a clear, viscous, and sometimes stringy substance oozing out of the periostracal groove. While secretion is taking place the edge of the mantle appears to be very active, expanding and retracting as successive layers of conchiolin are laid down. Figure 91 shows the position of the mantle at the time of its retraction.

The newly deposited shell (n.sh.) extends outward along the plane of the valve; the edge of the mantle (mn.e.) rolls upward; its outer lobe (o.mn.l.) is parallel to the plane of the valve, while the middle and inner lobe (m.l.) face the observer. The tentacles of the inner lobe extend down; those of the middle lobe are slightly contracted. The outer lobe underlies the sheet of

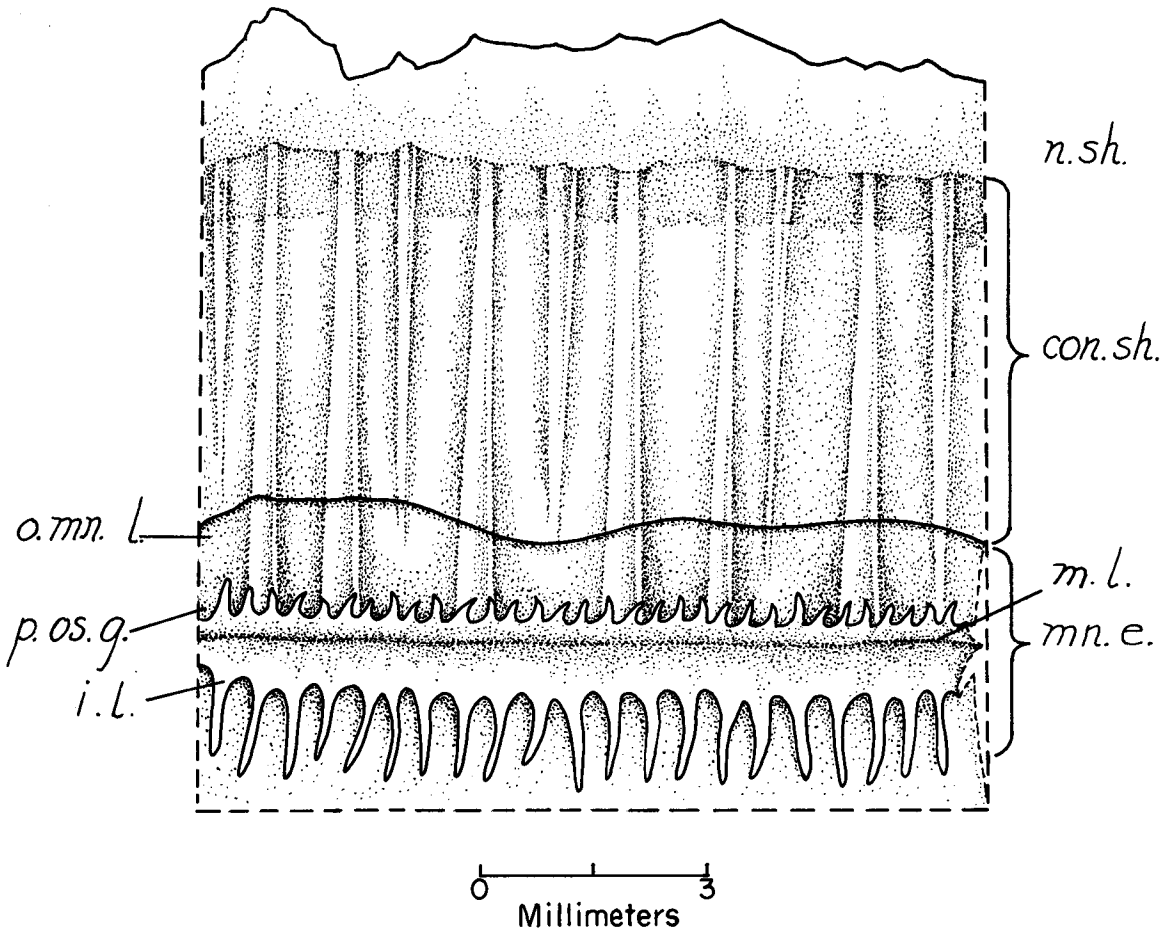

FIGURE 91.—Small area of the mantle edge with the adjacent part of the newly secreted shell viewed from above. The mantle was exposed by cutting off a piece of the opposite valve, and the oyster was placed in sea water under a binocular microscope. con.sh.—conchiolin sheet; mn.e.—edge of the mantle; i.l.—inner lobe; n.sh.—new shell; o.mn.l.—outer lobe of the mantle, contracted; p.os.g.—periostracal groove. Drawn from life. The position of structures at the edge of the mantle in relation to one another: the new shell area (n.sh.) marked on three sides by a broken line is in the plane of the drawing, next to it is the outer lobe (o.mn.l.), then the conchiolin sheet (con.sh.), the middle lobe (m.l.), and the inner lobe (i.l.) is at the top, nearest to the observer.

viscous conchiolin (conch.sh.) which oozes out from the periostracal groove (p.os.g.) between the outer and middle lobes. The distal edge of the conchiolin sheet (end of stippled area) indicates the previous maximal extension of the outer lobe before the withdrawal of the mantle edge. The entire group rests on the newly formed and already solidified shell (n.sh.).

During the secretion of conchiolin the edge of the mantle frequently extends out and then withdraws to the position recorded in the drawing. At the time of expansion the outer lobe temporarily supports the semiliquid conchiolin and by moving in and out spreads it over the shell. Because of this action the proximal part of the newly formed valve receives a larger amount of conchiolin and becomes thicker than the distal portion. When secretion is interrupted, the conchiolin layers become incorporated into the shell substance and the conchiolin sheet as shown in figure 91 is no longer visible.

The rate of secretion of the new shell varies at different parts of the mantle edge. Quantitative data are lacking, but observations made during the periods of more rapid growth in *C. virginica* (May to June and October to November in New England waters) show that the area of newly formed shell is always largest at the ventral side of the valves near the principal axis of growth (fig. 92).

The organic matrix of the shell can be produced by the pallial epithelium at any place along the

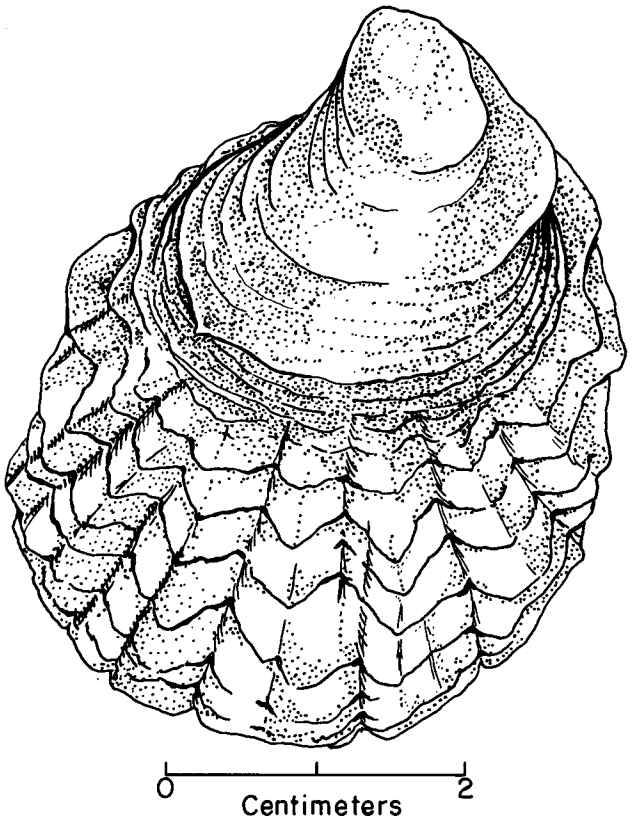

FIGURE 92.—New shell growth formed during 1 year along the periphery of the valve of an adult oyster from Long Island Sound planted in the Oyster River, Chatham, Mass. The newly formed shell is recognizable by zigzag lines of the material; its width is greatest along the ventral edge.

entire outer surface of the mantle and is not restricted to the periostracal groove. Such secretion, first observed in pearl oysters (Bøggild, 1930), can be experimentally demonstrated in *C. virginica*. Oysters with one valve removed and the edges of the mantle cut off above the periostracal groove secreted a new conchiolin layer over the entire surface of the exposed mantle within 5 days. Although the operated specimens remained alive in the laboratory tanks at Woods Hole over 3 weeks this conchiolin membrane remained uncalcified. In another experiment three adult oysters were removed from their shells and kept alive in sea water for 3 weeks. They formed rather thick coats of periostracum which was very lightly calcified. The repair of holes made in oyster shells by boring snails and sponges also shows that conchiolin is secreted by the entire surface of the mantle. The damaged area is rapidly covered by a layer of organic material which later becomes calcified.

Soon after being secreted, the conchiolin becomes calcified. Progressive stages of this process can be observed on the growing edge of the shell, or by inserting pieces of plastic or small glass cover slips between the edge of the mantle and the valve and removing them at regular intervals for inspection. The earliest stage of calcification is recognized by the appearance of minute granules of calcium salts, which become visible in polarized light as brightly sparkling dots (fig. 93). At this early stage the distribution of the granules (calcospherites) does not show any definite pattern or arrangement. In a living oyster they can be found entangled in strands of mucus left on the conchiolin sheet by the back and forth movements of the mantle edge. Within the next 24 to 48 hours typical hexagonal crystals of calcite can be seen (fig. 94, black crosses). They gradually increase in size and present a picture of great brilliance and beauty in polarized light (fig. 95).

Distribution of calcospherites at the stage of their transformation into small calcite crystals on the surface of the newly secreted shell (fig. 96) does not show any distinct orientation in relation to the growth axis of the shell. Some of the calcospherites are scattered over the entire field of vision, while others are packed tightly between the larger crystals (see large group of crystals at the lower part of figure 96). Within the next 48 hours the calcite crystals increase in size (fig. 97). In the final stage of shell formation the calcite crystals become arranged in a distinct pattern to form the prismatic layer in which each unit is a prism oriented with its long axis at about a 90° angle to the edge of the shell (fig. 98). The form of the individual prisms varies greatly, some of them are even wedge-shaped and slightly curved. This can be observed after boiling a piece of shell in a strong sodium hydroxide solution to separate the prisms (Schmidt, 1931).

Each calcite prism is surrounded by a capsule of conchiolin. By dissolving the mineral in weak hydrochloric acid it is possible to obtain intact the organic meshwork of the conchiolin layer. The walls of each capsule, as can be seen in figure 99, are very thin and slightly iridescent. Since in the earliest stages of shell formation the conchiolin sheet appears to be amorphous under the light microscope, it is reasonable to assume that the organic capsules of the calcite prisms are formed

733-851 O—64——7

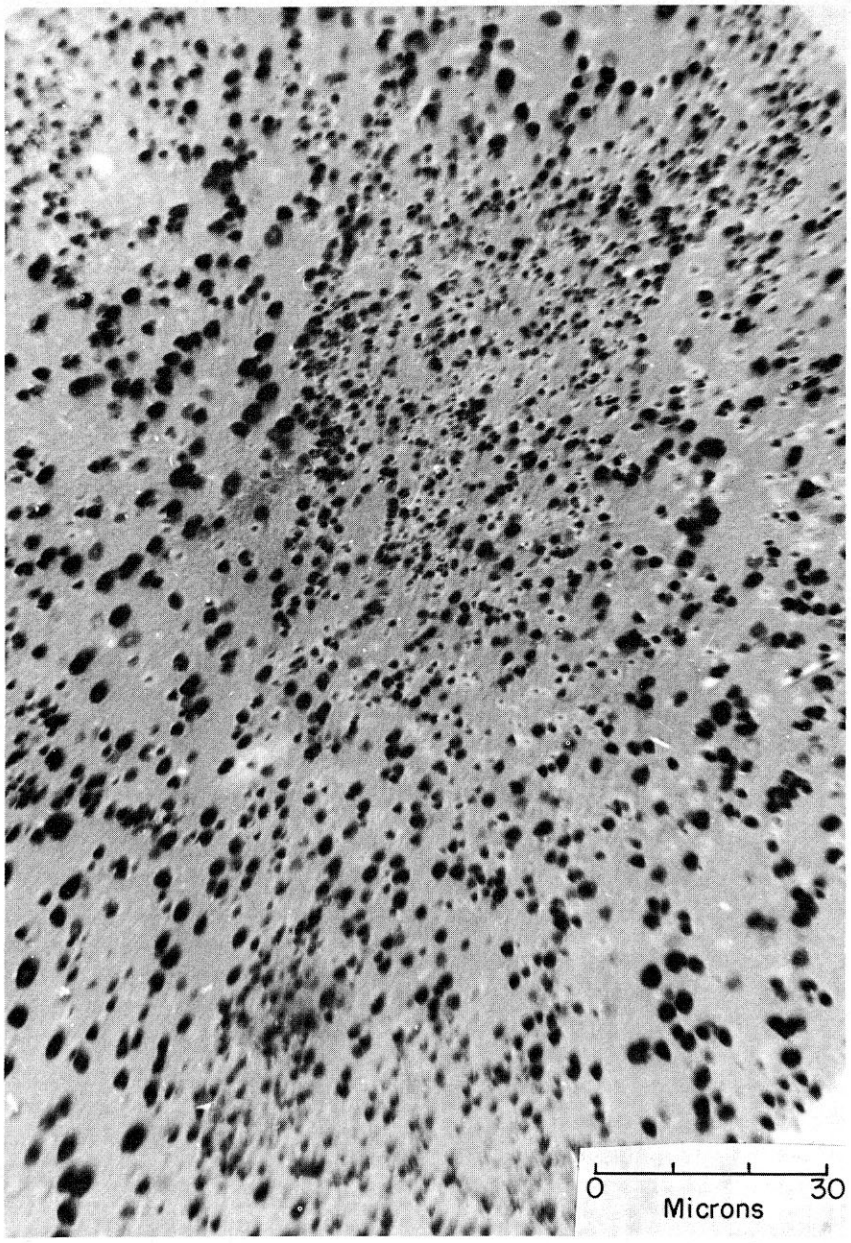

FIGURE 93.—Small granules (calcospherites) in conchiolin shortly after its secretion by the mantle. Black and white enlargement of a Kodachrome photograph taken with polarized light.

by later deposition of conchiolin, the secretion of which continues during calcification. The details of this prosess have not yet been described.

THEORIES OF CALCIFICATION

Studies of shell calcification fall into two major categories. One type of work places the emphasis on the identification of calcium-secreting cells or organs; the other approaches the problem from the biochemical point of view. It has been generally accepted that calcium carbonate, separated from blood, is secreted as colloidal gel by certain cells at the edge of the mantle and that crystallization takes place outside the cells (Crofts, 1929; Dakin, 1912; Kuyper, 1938) between the conchiolin sheet and the mantle. Separation of calcium is not, however, confined to the surface cells of the mantle. The calcium-secreting cells may be subepithelial, as in *Patella* (Davis and Fleure, 1903). In the calcification of the epi-

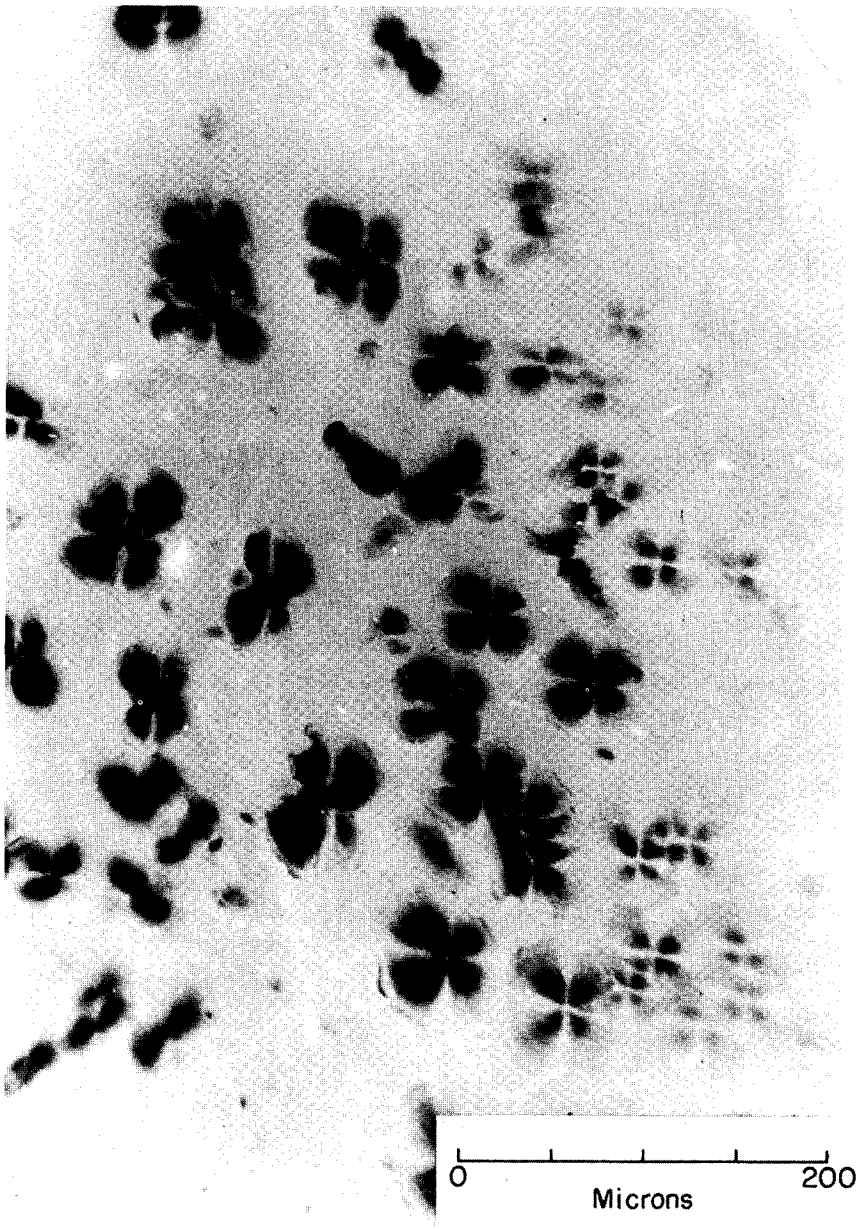

FIGURE 94.—Calcite crystals of new oyster shell about 24 to 36 hours after its formation. Black and white enlargement of a Kodachrome photograph taken with polarized light.

phragm of *Helix pomatia* (Prenant, 1924, 1928), the calcium is liberated by the leucocytes in the connective tissue of the mantle. In the case of pearl formation, Boutan (1923) has shown that calcareous deposits are formed by amoeboid cells which crawl through the mantle epithelium, while the latter secretes the concentric layers of the organic matrix (conchiolin).

De Waele (1929) approached the calcification problem from the physiochemical point of view. Working with *Anodonta cygnea* he has shown that the extrapallial fluid between the mantle and the shell is chemically identical with blood. Exposure of this fluid to air causes the formation of a precipitate, which consists of a suspension of calcium spherules in protein solution. He therefore assumed the existence in the pallial fluid of a hypothetical compound consisting of protein, carbon dioxide, and calcium carbonate. The escape of carbon dioxide would then cause the

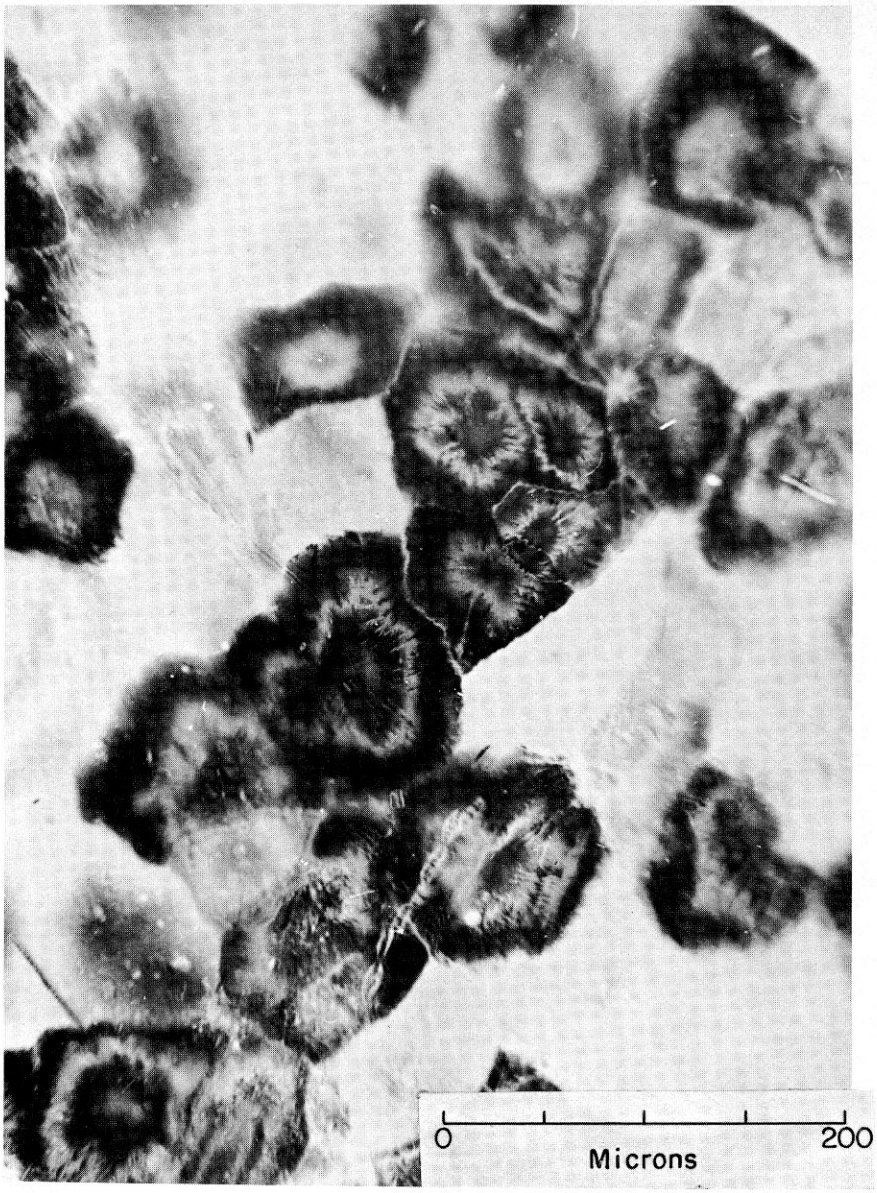

FIGURE 95.—Early stage of the formation of prismatic layer. Photographed with polarized light.

precipitation of calcium carbonate. Dotterweich and Elssner (1935) found, however, that calcium carbonate crystals are formed in the extrapallial fluid of *Anodonta* only in an atmosphere containing less than 1.5 percent carbon dioxide. In *Helix*, regeneration of the shell will take place in an atmosphere containing up to 15 percent of carbon dioxide, according to Manigault (1933). Although the latter accepted De Waele's theory, his own results seem to prove its inadequacy; and Robertson (1941) remarks that De Waele's hypothetical protein compound is without a real chemical basis. Furthermore there are other discrepancies in De Waele's results which invalidate his theory. The calcospherites and the protein precipitated from blood and from extrapallial fluid contained 50 percent organic matter, whereas the new shell contained only 4 percent of it. To reconcile these facts it would be necessary to assume that a great proportion of the organic matter in the new shell must be reabsorbed. The entire process as outlined by De Waele appears to be highly improbable.

Steinhardt (1946) assumed that calcification of the oyster shell is associated with the formation of citrate, probably the tricalcium-citrate

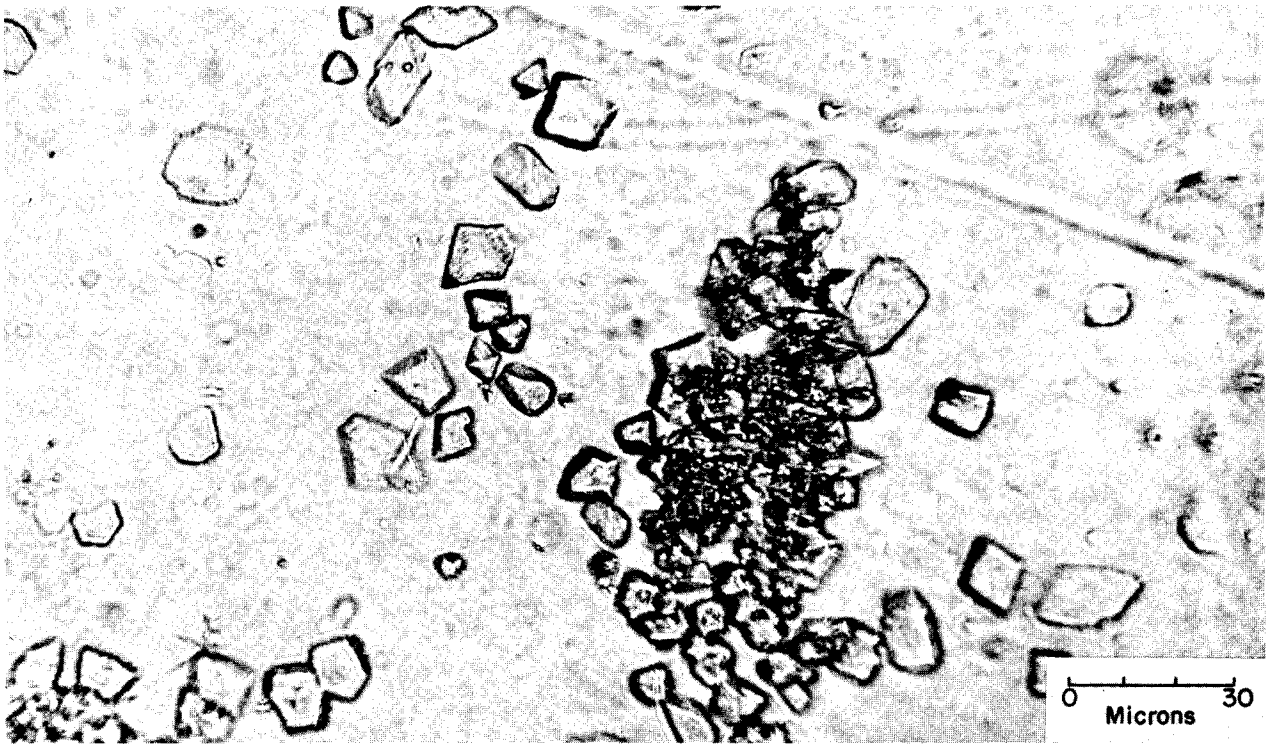

FIGURE 96.—Photomicrograph of a piece of new shell of *Crassostrea virginica* taken 28 hours after the beginning of calcification. Small calcite crystals are randomly distributed, and calcospherites scattered over the entire field of view are in places densely packed between the larger crystals.

$(C_6H_5O_7)_2Ca_3+4H_2O$. The observation that citric acid is formed in connection with carbohydrate metabolism, and that citrate is qualitatively precipitated from a solution which also contains phosphate and calcium ions in a suitable concentration (Kuyper, 1938, 1945a, 1945b), forms the basis of his conclusion. The citrate in the precipitate is found not as calcium citrate but in a somewhat more complex form in which calcium is combined with both phosphoric and citric acids. This is verified by the results of the analyses shown in table 11, in which the oyster shell was presumably *O. edulis*. It is rather difficult to arrive at a definite conclusion regarding the role of citric acid in the calcification of oyster shells, but Steinhardt's observations establish the presence of calcium phosphate in the oyster shell, which was supposed to consist primarily of carbonates; and an abundance of calcium phosphate in the mantle was demonstrated by Biedermann (1914).

During recent years (Bevelander, 1952; Bevelander and Benzer, 1948; Bevelander and Martin, 1949; Hirata, 1953; Jodrey, 1953) considerable advances in the study of the processes of calcification have been made. It had been generally assumed that the small granules appearing on the surface of the conchiolin consisted of calcium carbonate, but Bevelander and Benzer found that they are made of calcium phosphate. It is not at all clear how the calcium phosphate of the granules is converted into calcium carbonate, which is the final product of calcification in the oyster shell. It is doubtful that the conversion is accomplished by direct reaction between the calcium phosphate and the carbonate, because such a process would require very high concentrations of carbonate. The explanation proposed by Bevelander and Benzer implies that calcium phosphate may be dissolved by the action of organic ions which in some manner bind calcium. Phosphatase may

TABLE 11.—*Analyses to calcified materials according to Steinhardt*

[All figures are in percentages]

Material	Citric acid	Phosphorus	Calcium
Concretions from crayfish stomach	1.56	9.0	25.6
Chicken egg shell	0.15	0.154	33.3
White coral	0.013–0.024	0.007	35.2
Oyster shell	0.017	0.019	32.8