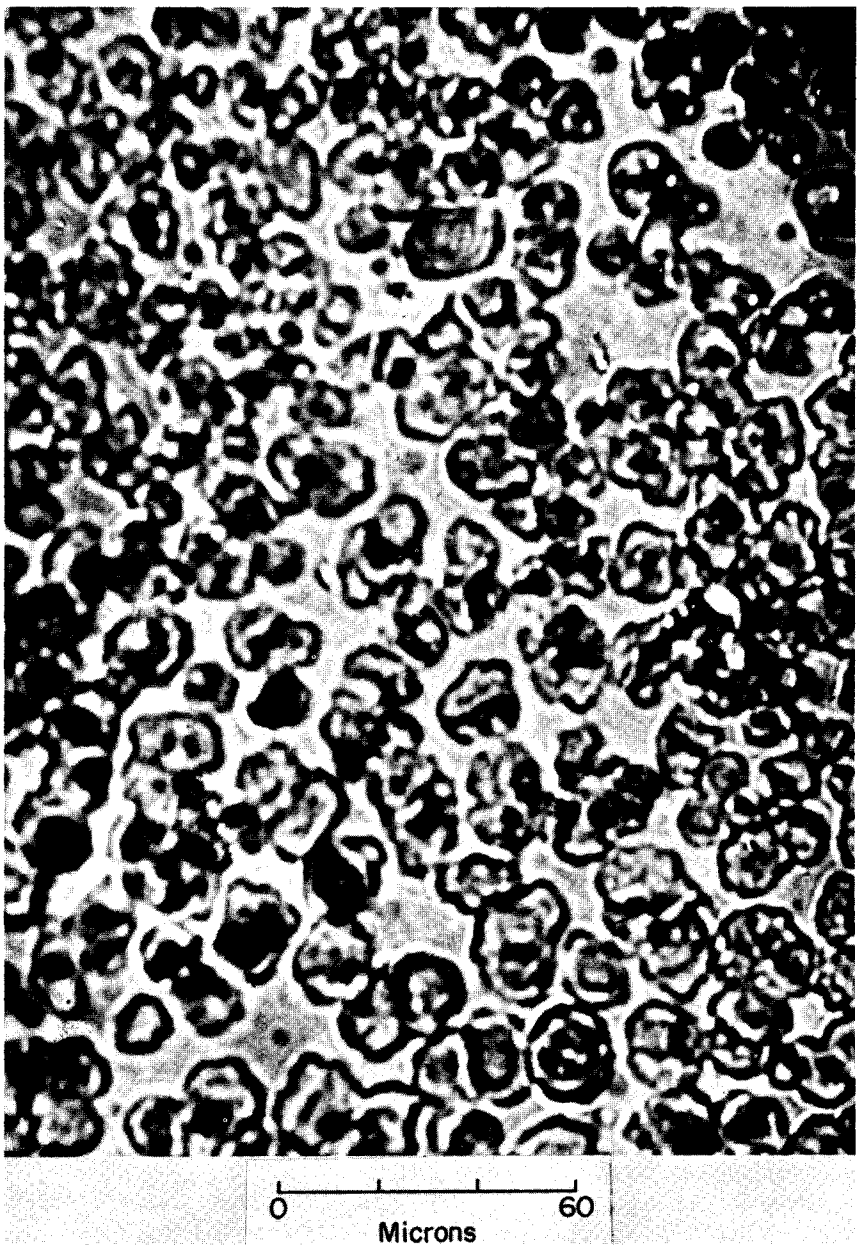

FIGURE 97.—Calcite crystals deposited on a piece of conchiolin. Photomicrograph taken 38 hours after the secretion of conchiolin has started.

contribute to this process by transferring phosphate to some substrate and removing the phosphate ions. This tentative explanation suggests a number of biochemical studies that should be made to obtain a better understanding of the process of calcification.

An important factor in the process of shell calcification is the enzyme phosphatase, which is generally present in the ossifying cartilages of young animals and in other tissues and organs in which calcium is deposited. The action of the enzyme consists of hydrolysis of hexosemonophosphoric ester and glycerophosphoric ester and consequent liberation of inorganic phosphate. The role of phosphatase in the shell formation of mollusks was established by Manigault (1939), who found a direct correlation between phosphatase activity in the digestive diverticula, mantle, and blood and precipitation of calcium in the shell. He concluded that phosphatase is

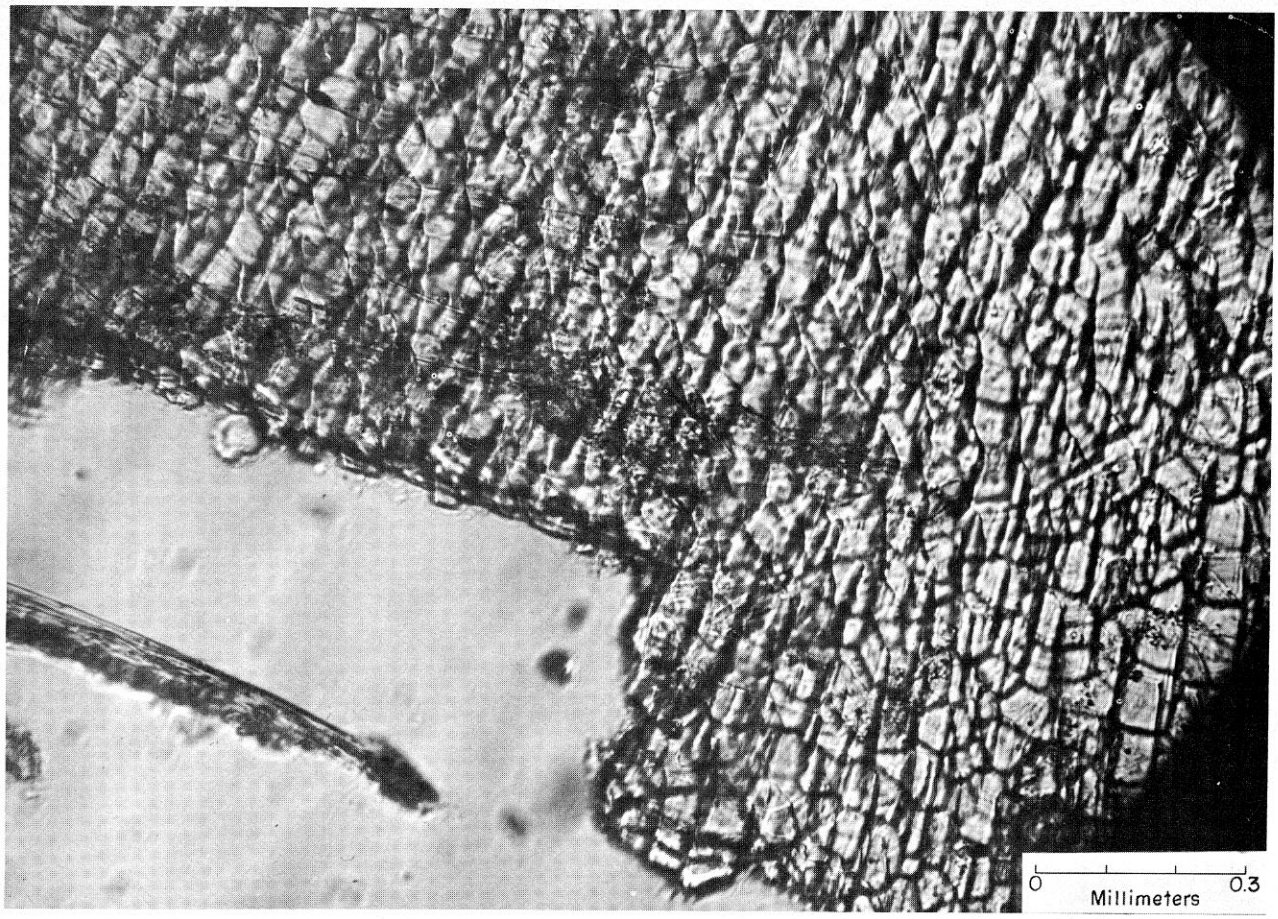

FIGURE 98.—Prismatic layer of the new growth of shell at the edge of the mantle. Four to five days old. Photomicrograph of an unstained whole mount.

probably a transfer agent involved in the mobilization of calcium. The localization of this enzyme along the border of the mantle and in the surface epithelium of the oyster, shown by the Gomori technique (fig. 80), confirms the opinion of Manigault and of Bevelander that the phosphatase plays an active role in the calcification of oyster shells.

During the last decade considerable advance was made in studies of the metabolic aspects of shell formation. Hammen and Wilbur (1959) paid particular attention to carbon dioxide conversion to shell carbonate and to the secretion of conchiolin matrix in which the calcium carbonate crystals are deposited by the oyster (*C. virginica*). The work of Jodrey and Wilbur (1955) on high activity of the enzyme oxalocetic decarbohylase in the mantle tissue of this species suggested that the deposition of carbonate may be related to decarboxylation reactions of the mantle. Experimental work conducted by Hammen and Wilbur at the Duke University Marine Laboratory at Beaufort, N.C., corroborated this hypothesis. Living oysters and isolated shells were placed for 12 hours in sea water containing 240 microcuries of $NaHC^{14}O_3$ per liter. The radioactivity of the shell surface was determined near the posterior margin of the right valve and corresponding correction was made for self absorption on the surface. By incubating pieces of oyster tissues in $NaHC^{14}O_3$ it was found that C^{14} is incorporated into organic acids of the mantle. More than 90 percent of the radioactivity occurs in succinic and smaller amounts in fumaric and malic acids. The initial step in the process is the fixation of carbon dioxide by propionic acid resulting in the formation of succinic acid. Both acids were found in relatively high concentrations in the shell forming tissues of the oyster. The fact that in these experiments labeled amino acids were found in the radioactive

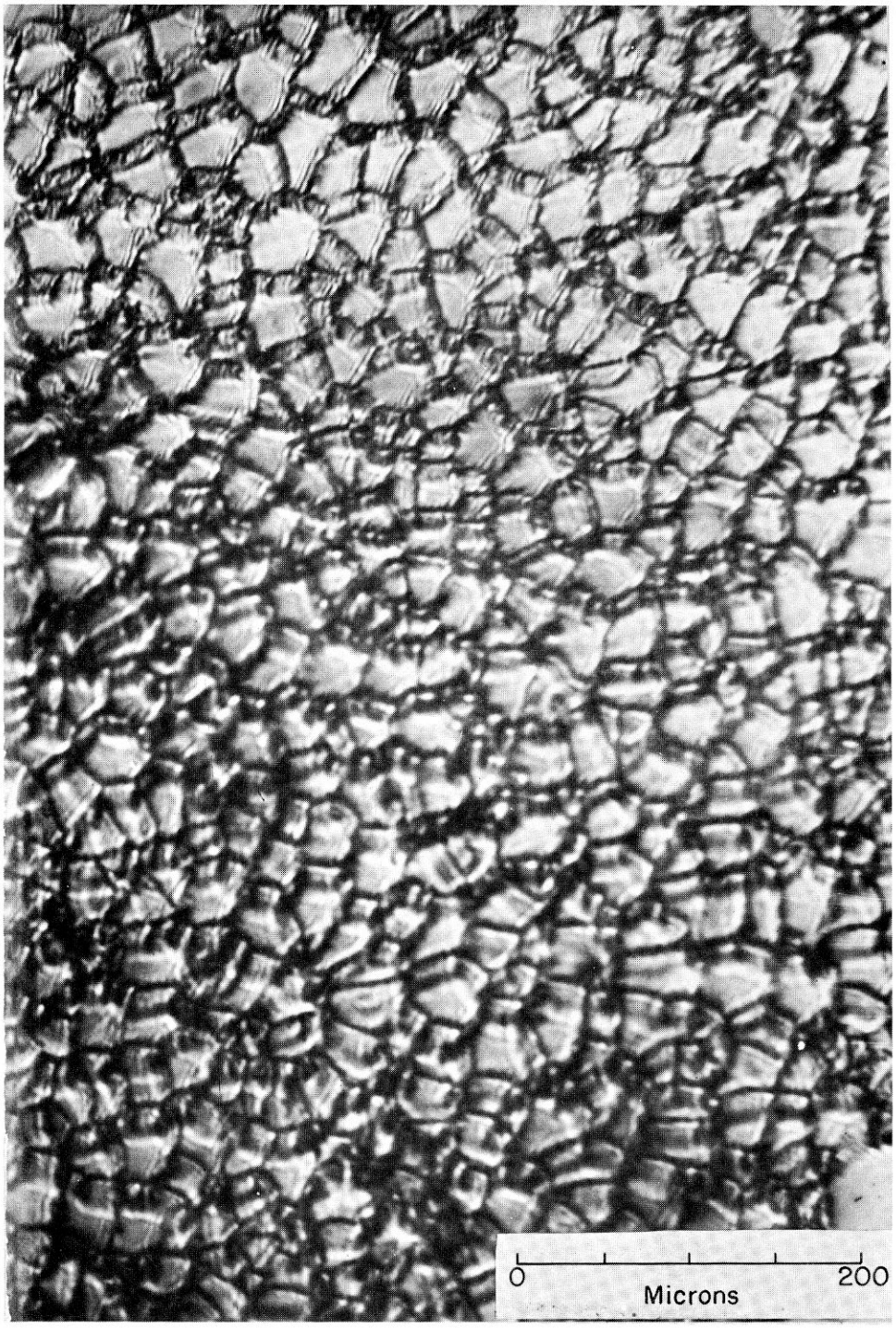

FIGURE 99.—Photomicrograph of organic meshwork of prismatic layer of shell after decalcification in weak hydrochloric acid. Note that the outlines of the capsules retain the shape of the mineral prisms.

conchiolin of the shell indicate that carbon dioxide fixation also contributes to the syntheses of the organic matrix of the shell.

Calcium enters the mantle directly from sea water, as was demonstrated by Jodrey (1953) using mantle-shell preparation and radioactive Ca^{45}, and can be taken up through other parts of the mollusk and transported to the mantle. The

enzyme carbonic anhydrase which is present in various mollusks may be expected to accelerate deposition of calcium carbonate, and the rate of deposition is retarded by carbonic anhydrase inhibitors.

Complex metabolic cycles involved in shell formation have been reviewed by Wilbur (1960), and probable relations of carbon dioxide to shell conchiolin and carbonate deposition are shown by him in a summary diagram (fig. C, p. 25 of Wilbur's paper).

CYTOLOGICAL IDENTIFICATION OF CALCIUM

Several methods for the identification and localization of calcium salts in the oyster tissues are available, but none are completely reliable. Gomori (1939) suggests that soluble calcium could be demonstrated by treating the frozen sections with ammonium oxalate, the insoluble octahedral crystals of calcium oxalate being easily recognized. The use of a fixative consisting of formalin and ammonium oxalate was also proposed (Rahl, quoted from Gomori). Both methods tried in my laboratory on sections of oyster mantle gave unsatisfactory results. The difficulty is the dislodging of calcium-bearing granules and mucus during sectioning, since the granules are easily carried out by the knife's edge from their original location inside the cells to the outside of the epithelium. This difficulty can be avoided to a certain extent by double embedding the tissue in colloidin-paraffin.

Indirect methods of Ca^{++} identification are based on the use of heavy metals (silver, cobalt, copper, and iron). Because almost all insoluble calcium compounds in the tissues are either phosphate or carbonate, any procedure which would demonstrate the presence of these anions may be considered specific for calcium. When the sections are immersed in a solution of one of the heavy metals the corresponding metallic salt is formed at the sites of phosphate or carbonate. The reduction may be effected by exposing to light if silver nitrate is used, or by immersing in appropriate reducing reagents (ammonium sulfide, acidified potassium ferricyanide). Identification by staining of calcium is based on the formation of insoluble lacs with several hydroxyanthraquinine dyes (alizarin sulfonic acid, purpurin, anthrapurpurin). Calcium deposited in the process of shell formation may, however, contain substances which interfere with the lac-forming reaction of alizarin. Also, the dye frequently fails to stain old deposits and its color is affected by the presence of iron. Although these complications limit the usefulness of alizarin as a reagent for the determination of calcium, I found that a 1 percent water solution of alizarin S (sodium alizarin sulphonate) is probably the best histochemical reagent for identification of calcium in the oyster mantle. It readily reacts with new deposits of calcium carbonate or calcium phosphate and forms compounds resistant to both acids and alkalies.

To study the cytology of calcium secretion, the deposition of conchiolin and its calcification was stimulated by cutting off small pieces of shell along the posterior margin of the oyster. Laboratory experience shows that such injury made during the warm season is rapidly repaired. Small pieces of the mantle border with the adhering and partly calcified conchiolin were excised and 3 days later preserved in neutral formalin or absolute ethyl alcohol. Sectioned tissues were stained with alizarin S and other reagents for demonstration of calcium. The preparations showed a large number of alizarin stained globules or granules, about 1.5 μ or less in diameter adhering to the surface of the mantle. Identical granules were found inside the goblet cells of the epithelial layer along both sides of the mantle (fig. 100).

The results of the staining and other histo-

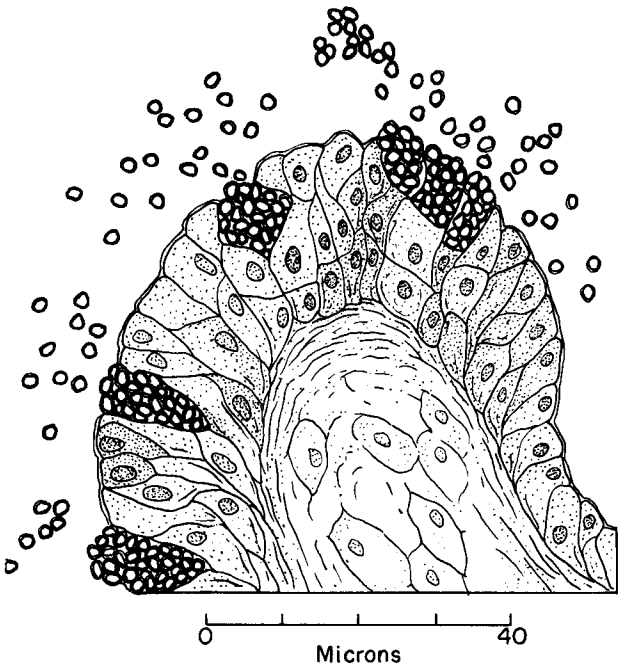

FIGURE 100.—Calcium containing granules discharged by the epithelial cells at the edge of the mantle of *C. virginica*. Neutral formalin 3 percent, alizarin.