CHAPTER V

THE MANTLE

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The inner organs of all mollusks are covered with a soft and fleshy fold of tissue called the mantle or *pallium* (Latin for cloak or coverlet). The structure of the mantle is relatively simple: the organ consists of a sheet of connective tissue containing muscles, blood vessels, and nerves and is covered on both sides by unicellular epithelium. Many blood cells invade and wander throughout the entire thickness of the mantle, infiltrating the spaces (sinuses) in the connective tissue, and crawling through the epithelium to aggregate on the outer surface of the mantle.

Although the principal role of the mantle is the formation of the shell and the secretion of the ligament, the organ plays a major part in several other functions. It receives sensory stimuli and conveys them to the nervous system and assists in the shedding and dispersal of eggs during spawning (see ch. XIV). The mantle also participates in respiration by providing direct exchange of gases between the surface tissues of the oyster and the surrounding water. It stores reserve materials (glycogen and lipids), secretes large quantities of mucus and, finally, aids in excretion by discarding blood cells loaded with waste products.

APPEARANCE

The appearance of the mantle reflects the condition of the oyster. At the time of sexual maturity it is a creamy-yellowish color. In oysters which have accumulated large amounts of glycogen with the onset of the cold season the mantle is white and thick. In oysters of poor quality or in those which have not yet recovered after spawning, the mantle is so transparent that the brown or greenish color of the underlying digestive organ is clearly visible through the thin and watery tissue. Oysters in this condition are particularly suitable for the study of muscles, blood vessels, and nerves which in good quality, "fat" oysters are covered by a thick layer of reserve materials.

Pigment cells are concentrated along the free edge of the mantle and in the tentacles in a band varying in color from light brown to jet black. Also, accumulation of copper in the blood cells may produce a distinct green coloration. Different intensities of pigmentation are often found in oysters of identical origin growing together, and cannot be correlated with geographical location or type of bottom.

ANATOMY

For a detailed study of the mantle the oyster should be fully narcotized by Epsom salt (see p. 65) or by refrigerating it overnight at a temperature of about 2° to 4° C. After the valves are forced apart and the body dissected along the median plane, the two halves of the oyster are left attached to their respective valves and the mantle is preserved in its natural position by having a large quantity of fixing fluid poured over it. Portions of the mantle required for study are cut off, stained, dehydrated, cleared, and mounted. In this way very satisfactory whole mounts can be obtained.

The two lobes of the mantle are joined together at the dorsoposterior margin, and form a cap or hood which covers the mouth and the labial palps (fig. 71). Along the anterior and ventral sides of the body the lobes are free and follow the curvature of the shell. When the oyster opens its shell the mantles separate with the valves to

which they adhere, leaving a narrow opening between the two lobes through which sea water can enter the mantle cavity. The edge of the mantle, may, however, occupy various positions: it may extend parallel and beyond the edge of the valves to leave a wide space between the two opposing lobes, or it may bend inward almost perpendicular to the shell surface (fig. 74) to reduce or completely close the opening between the two lobes and thereby limit the access of water to the mantle cavity. The behavior of the mantle edge as a regulatory mechanism controlling the flow of water through the mollusk will be discussed later (p. 185). In a closed oyster the mantle edge is located about midway between the distal margin of the gills and the edge of the shell. Its position is marked by an impression called the pallial line, which is less pronounced in the oysters than in clams and some other bivalves.

At the ventroposterior end of the body the two opposing lobes of the mantle join the gills to form the delicate outside wall of the cloaca (figs. 72 and 75, cl., f.). On the left side of the body the mantle is joined to the visceral mass; on the right side it is separated from the visceral mass by the promyal chamber. The fusion of the mantle with the visceral mass and with the bases of gill plates forms the wall of the epibranchial chamber, which leads to the cloaca (fig. 75, cl.). The relative position of the epibranchial and promyal chambers can be seen in the cross section of the oyster made through the dorsal part of the body (fig. 73, ep.br.ch.; pr.ch.).

An oblong slit between the two mantle lobes on the dorsoposterior side of the body marks the opening of the promyal chamber. The inside of this chamber can be examined by completely narcotizing the oyster and forcing its valves apart as far as possible without tearing the adductor muscle. Viewed from the posterior side the promyal chamber in a relaxed oyster appears as an oval cavity (fig. 75) to the left of the adductor

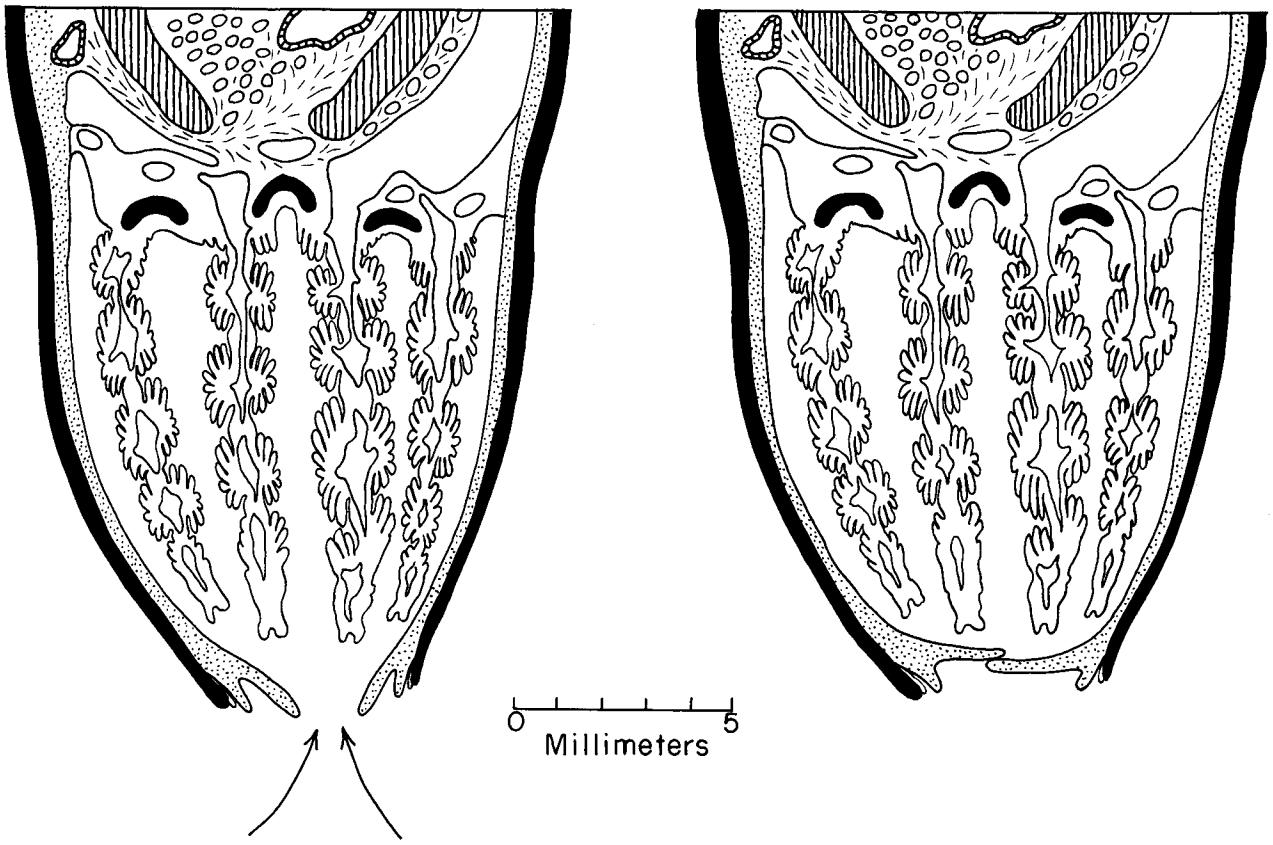

FIGURE 74.—Cross sections of the valves, mantle, gills, and adjacent portion of the visceral mass of *C. virginica*. In both diagrams the valves are open; the open pallial curtain (at left) permits free access of water to the mantle cavity; the closed pallial curtain (at right) prevents water from entering the mantle cavity. The outer lobe adheres closely to the valve and is not visible. Drawn from the photomicrographs of cross section of adult oyster. Bouin, hematoxylin-eosin.

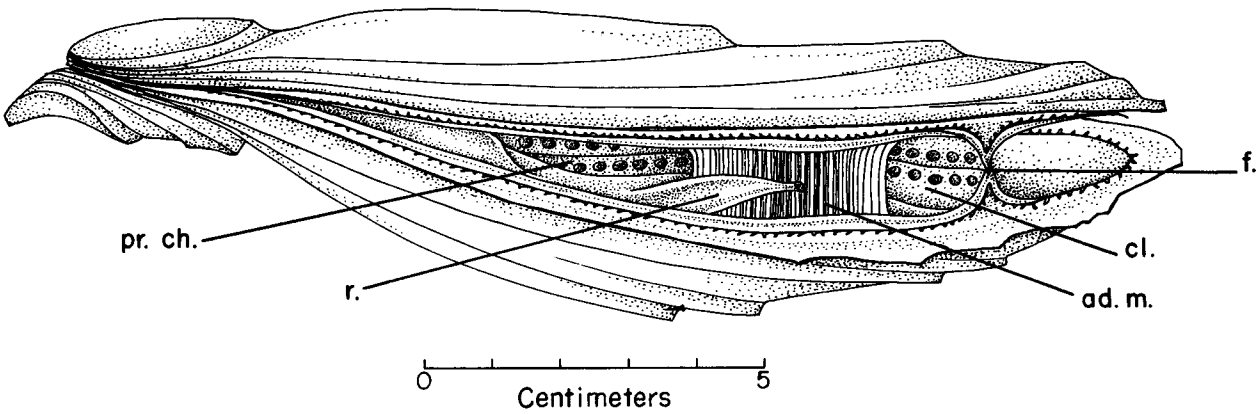

FIGURE 75.—Promyal chamber (at left) and cloaca (at right) viewed from the posterior side of a large oyster (*C. virginica*) completely relaxed by narcosis. Note the fusion of the two opposing lobes of the mantle, and the adductor muscle (in the middle). Drawn from life. Actual size. ad.m.—adductor muscle; cl.—cloaca; f.—fusion of mantle lobes and gills; pr.ch.—promyal chamber; r.—rectum.

muscle. The large round openings of the water tubes of the gills can be seen on the inner wall of the chamber. The rectum extends along the edge of the chamber, ending with a round anus adhering to the side of the adductor muscle; the opening of the cloaca lies to the right of the muscle. The water tubes emptying into the cloaca and the fusion of the mantle with the gill lamellae are also clearly visible.

The most conspicuous components of the mantle are the radial muscles, the blood vessels, and the nerves (fig. 76). All these structures can be identified in a piece of fresh tissue stretched over a glass slide and examined under strong illumination with a low-power microscope. For more detailed study, it is necessary to prepare whole mounts or to section the preserved tissues.

The radial muscles extend from the place of their attachment to the visceral mass to the edge of the mantle. At about two-thirds of their length from their base they begin a fanlike expansion toward the periphery before terminating in the base of the tentacles. The majority of the muscles are accompanied along their length by nerves, blood vessels, and blood sinuses. Much more slender than the radial muscles are the concentric muscular bands which parallel the free edge of the mantle (not shown in fig. 76) and are more abundant at its thickened distal edge.

Because of its strongly developed musculature, the mantle is highly contractile. It may stretch a considerable distance beyond the edge of the valve, or withdraw inside the shell, and even roll up into a tube. Contraction of the radial muscles will throw the inner surface of the mantle into ridges which serve as temporary channels for discarding mucus and foreign particles accumulated on it. These movements may involve either the entire surface of the mantle or only a small portion of it, depending on the intensity of stimulation received by the tentacles.

The wide circumpallial artery (fig. 76, cp.a.) follows the entire periphery of the mantle. At low magnification it is usually visible as a wide tubular structure with many branching vessels which communicate with the irregular spaces (blood sinuses) within the connective tissue. A large pulsating blood vessel, called the accessory heart (ch. XI, fig. 236), is located in the anteroventral part in each lobe of the mantle. The structure and the function of this vessel are discussed in chapter XI, p. 254.

Just outward from the circumpallial artery runs the circumpallial nerve, which also extends along the entire margin of the mantle. In whole mount preparations seen under low power, the circumpallial nerve appears as a compact unbranching band. Examination under high power, however, reveals a fine network of small nerves connecting the circumpallial nerve with nerves and with the visceral and cerebral ganglia. Since nerve fibers on the surface of the mantle and in the tentacles lead to the circumpallial nerve, stimuli received by the neuroreceptors of these areas are transmitted through the circumpallial nerve to the radial nerves and reach either the visceral or the cerebral ganglia.

The thick and muscular border of the mantle is divided into three lobes (fig. 77) which have been described in the literature as "folds" (Awati

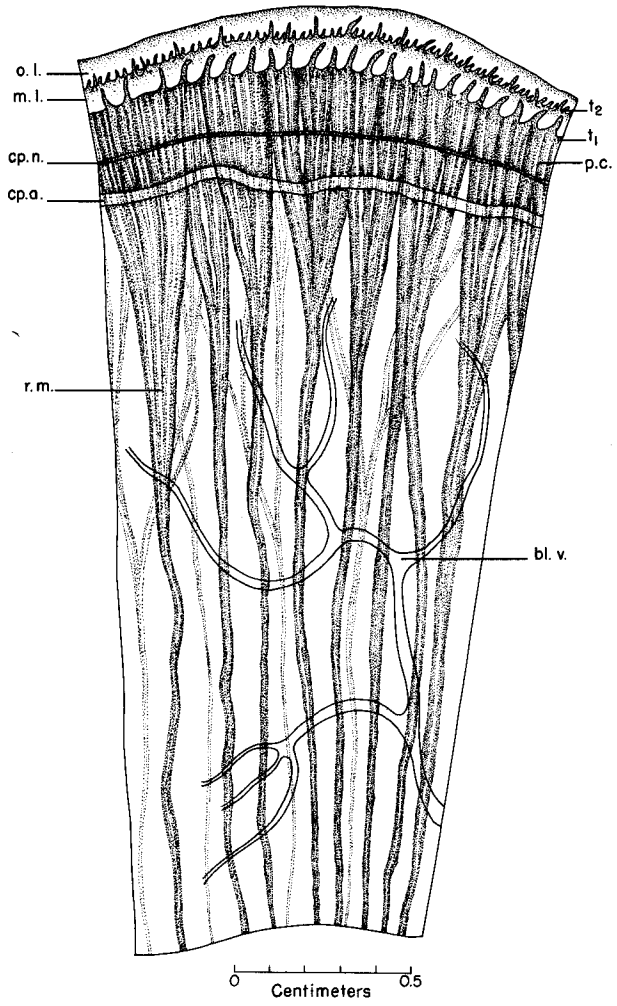

FIGURE 76.—Whole mount of a piece of mantle. Major portion of connective tissue was removed by maceration. Safranin stain. Magnified about 10 times. bl.v.—blood vessels; cp.a.—circumpallial artery; cp.n.— circumpallial nerve; m.l.—middle lobe; p.c.—pallial curtain (inner lobe); r.m.—radial muscle; o.l.—outer lobe or shell lobe; t_1—tentacles of inner lobe; t_2—tentacles of middle lobe. Radial nerves surrounded by radial muscles are not visible. Formalin 5 percent, hematoxylin.

and Rai, 1931), "reduplications" (Nelson, 1938; Pelseneer, 1906), "lamellae" (Hopkins, 1933), "lames" in French (Leenhardt, 1926), and "Klappe", in German (Rawitz, 1888). The term "reduplication" is misleading because the lobes are not formed by the duplication of the mantle tissue, being comparable rather to a fringe or flounce at the margin of a soft material. To avoid confusion the term marginal lobes is retained in this text.

The mantle border of all the species of oysters studied, namely, *C. virginica, C. angulata, C. gigas, O. edulis,* and *O. lurida* is divided into three projecting lobes, the outer or shell lobe (sh.l.), the middle lobe (m.l.), and the inner lobe or pallial curtain (p.c.). Hopkins' statement (1933, p. 483) that "The border of the mantle (of *C. gigas*) divides into two lamellae, each bearing a row of tentacles" is an obvious inaccuracy of description.

The outer or shell lobe (sh.l.) is narrow and devoid of tentacles. It lies in contact with the margin of the shell and may be seen protruding beyond the edge of the valve during periods of rapid growth. The middle and the inner lobes each bear a row of sensitive and highly contractile tentacles.

The inner lobe or pallial curtain (fig. 77, p.c.) is especially broad and turned inward. In describing this structure in scallops Pelseneer named it the "velum" (1906). Although that term has been used by several investigators (Awati and Rai, 1931; Dakin, 1909b) Nelson (1938) pointed out that the term "velum" is better known as the swimming organ of the pelecypod larvae and proposed to call the inner lobes of the mantle the "pallial curtains". This term seems to be appropriate, but is used in this book in the singular since there appears to be no advantage in the plural recommended by Nelson.

The inner lobe may be projected into the mantle cavity (fig. 74). Depending on the degree of contraction of various sets of muscles the inner lobe assumes different angles in relation to the mantle as a whole. In a fully relaxed mollusk the lobe of each side extends outward in the general plane of the mantle and shell. In a contracted state the lobes on both sides project inward almost at right angles to the surface of the mantle; in this position the mantle borders touch and the tentacles of the two sides interlock, effectively sealing the entrance to the mantle cavity. This function of the inner lobe was first described by Rawitz in 1888 and was redescribed in 1933 by Hopkins. As will be shown later (p. 304) the pallial curtain also plays an important role during the spawning of female oysters.

The deep furrow between the shell lobe and the middle lobe is called the periostracal groove (fig. 77, per.gr.), the name referring to the secretion site of organic shell material by glandular cells concentrated in the deepest portion of the groove and collectively known as the periostracal or conchiolin gland (c.gl). During the shell-

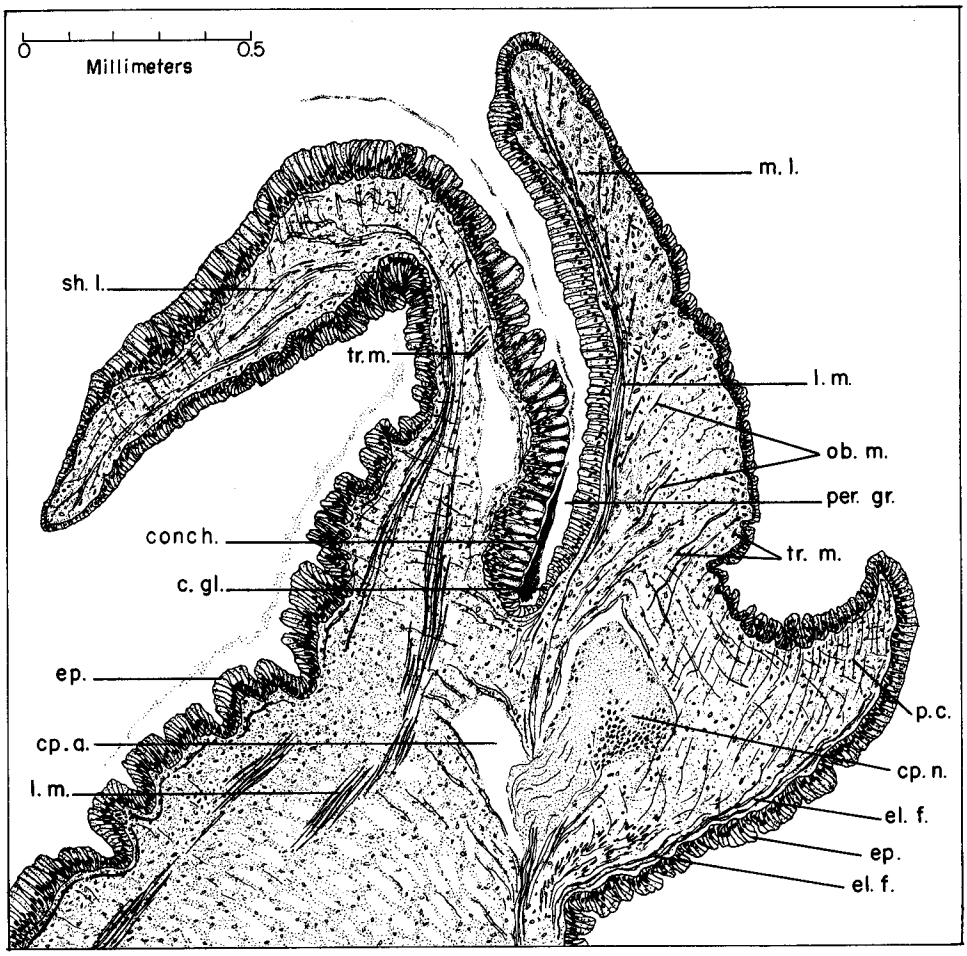

FIGURE 77.—Transverse section of the edge of the mantle of adult *C. virginica*. Bouin 3, hematoxylin-eosin. The outer or shell lobe at left faces the valve (not shown) and is bent as a result of fixation. The section passes between the tentacles of the inner lobe (pallial curtain); only the tentacle of the middle lobe (m.l.) is seen. c.gl.—conchiolin (or periostracal) gland; conch.—sheet of conchiolin spread over the shell lobe; cp.a.—circumpallial artery; cp.n.—circumpallial nerve; el.f.—elastic fibers; ep.—epithelium; l.m.—longitudinal muscles of tentacles; m.l.—middle lobe; ob.m.—oblique muscles; p.c.—pallial curtain (inner lobe); per. gr.—periostracal groove; sh.l.—shell or outer lobe; tr.m.—transverse muscles.

growing season, viscous yellowish material (fig. 77, conch.) accumulates in the groove and gradually oozes out to the periphery of the outer mantle lobe, where it solidifies into the periostracum. The groove between the middle lobe (m.l.) and the pallial curtain secretes mucus, which is gradually moved by ciliary currents to the outer margin of the mantle and there discarded.

It has already been noted that the edges of the middle and the inner lobe each bear a row of highly extensible, tapering tentacles; however, their arrangement and size in the two lobes are different. Two types are clearly visible along the edge of the middle lobe: numerous short and slender tentacles, and less abundant long and stout ones (fig. 76). The order of the tentacles follows a certain pattern, namely, each long tentacle is succeeded by a group of four to six small ones (t_2). The stout tentacles frequently occupy a position slightly out of line with the small ones, being a little nearer to the inner fold. The inner lobe bears only the long and stout tentacles (t_1).

There is great variation in the size of all the tentacles and in their pigmentation. Since they are highly sensitive to touch and other stimuli and retract at the slightest disturbance, their relative size can be observed only when they are completely relaxed. In fully narcotized adult oysters the ratio between the numbers of tentacles on the

inner and middle lobes was found to vary from 10:18 to 10:32.

It has not yet been definitely established whether the two types of tentacles contain different receptors and therefore respond to different stimuli. According to Elsey (1935) the large tentacles of *C. gigas* are more sensitive to hydrochloric acid than the small ones. Hopkins (1932) does not specify which row of tentacles was under observation in his work on sensory stimulation of *C. virginica*. In my experiments (see p. 293) observations were made exclusively on the long tentacles of the inner lobe.

A narrow and slightly pigmented cylindrical structure along the dorsal edge of the mantle (fig. 78) marks the position of the subligamental ridge, the organ which secretes the ligament. The ridge consists of a layer of specialized epithelium underlined by connective tissue. Large blood vessels are found close to the base of the ridge. Microscopic structure of the ridge is given on p. 83.

RUDIMENTARY MUSCLE OF THE MANTLE

A small and sometimes hardly visible muscle is located on the dorsal part of the mantle. Its location is sometimes marked by light violet pigmentation and by a shallow depression in the corresponding part of the valve to which the muscle adheres. The attachment is weak, and in the majority of oysters the muscle separates from the valve when the valve is lifted. Leenhardt (1926) states, however, that in some *O. edulis* the muscles were so strongly attached to the shell that they could not be separated without rupturing the mantle tissue. Examination of sections of the mantle of *C. virginica* from the Woods Hole area convinced me that muscle fibers do not extend from one side to the other, but end in the connective tissue of the mantle. The muscle is apparently nonfunctional and morphologically is not analogous to the anterior adductor of bivalves. Leenhardt (1926) considers the rudimentary muscle of the mantle as a vestige of the larval foot retractor which disappears during metamorphosis. Stenzel (1963) states that this muscle is present in all the Ostreidae and calls it Quenstedt's muscle in honor of its discoverer (Quenstedt, 1867).

HISTOLOGY

The mantle consists of connective tissue which envelops the muscles, blood vessels, and nerves and is covered on both sides with the epithelium.

CONNECTIVE TISSUE

The most conspicuous structural element of the connective tissue is the vesicular cell, characterized by large globular or oval body and relatively small nucleus without nucleoli. In zoological literature these cells appear under a variety of names and were even incorrectly considered as lacunae (Leenhardt, 1926) and mucus cells (List, 1902). Well-developed membranes outline cell boundaries sharply; the protoplasm within forms a delicate network of fine granules. In preparations dehydrated with alcohol the inside of the vesicular cells appears almost empty, but in tissues treated with osmic acid and in frozen sections stained with Sudan II and other fat stains large globules of lipids are seen to fill the inside of the cells (figs. 79 and 80). Less abundant are the smaller round cells with more compact protoplasm. They often occur near small arteries (fig. 81, r.c.). The fusiform cells (f.c.) with small bodies and oval nuclei form long branching processes which anastomose and touch each other.

Examination of frozen sections of connective tissue treated with toluidine blue or other metachromatic stains shows clearly the presence of a cytoplasmic ground substance with a very fine reticulum supporting various inclusions. After the removal of glycogen this substance can be stained very deeply with periodic acid fuchsin (McMannus reagent)or with Hale stain which is used to test for acid polysaccharides of the hyaluronic acid type (Hale, 1946). The results of such staining reactions have been interpreted in the literature as indicating the presence of mucopolysaccharides or mucoproteins. Histological methods are not entirely dependable (Meyer, 1957), but so far no chemical analyses of the connective tissue of the mantle have been made. It is known, however, that acid mucopolysaccharides are among the components of the ground substances in mammalian tissues. It is very likely that they are also present in the connective tissue of the oyster.

Elastic fibrils are scattered throughout the connective tissue of the entire thickness of the mantle but appear to be more abundant at the free edge and in the layers underlying the surface epithelium (fig. 77, el.f.). Muscle fibers are also very abundant and will be discussed in detail later.

In some specimens the mantle may be thin and transparent whereas in others it is thick and

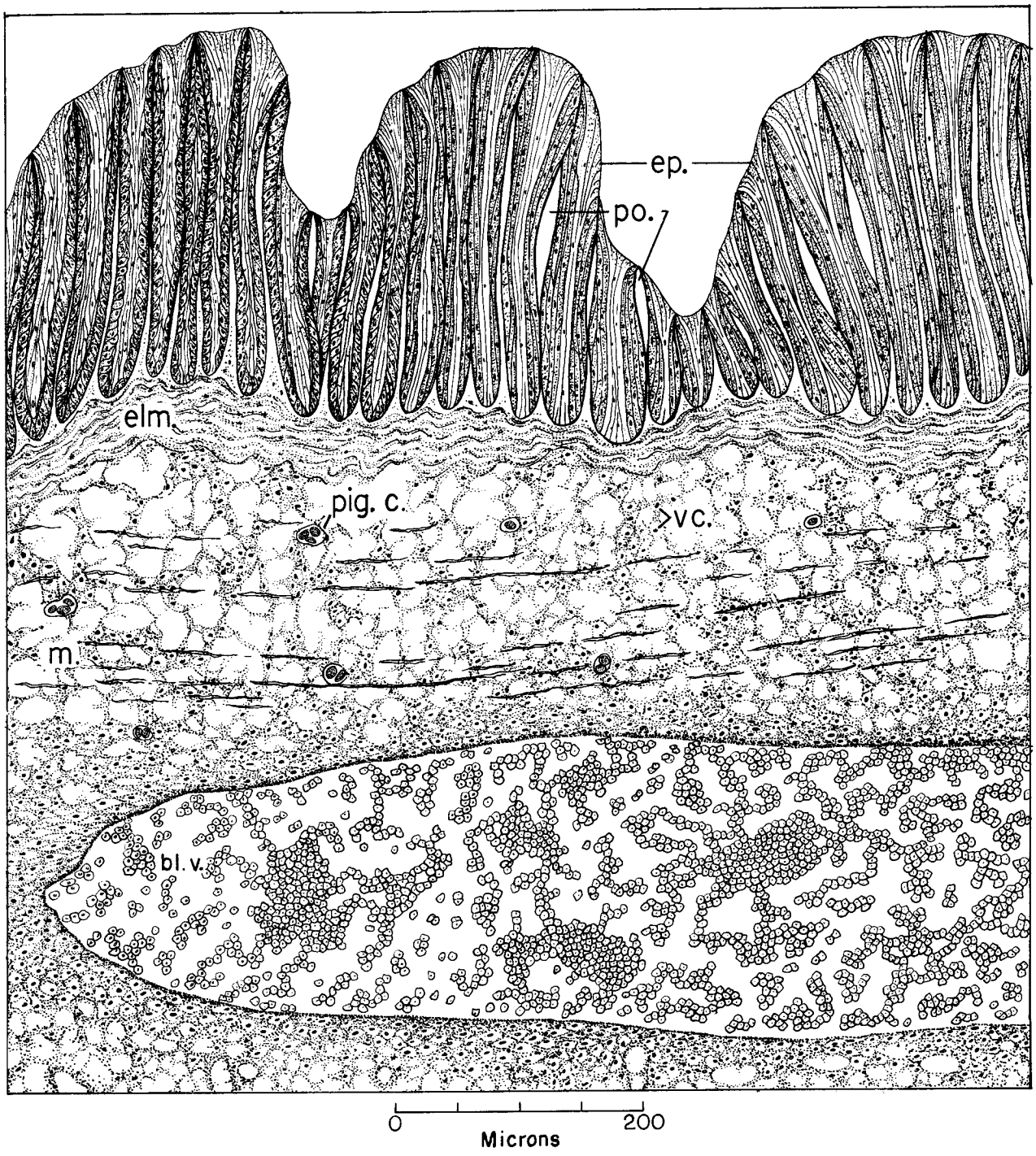

FIGURE 78.—Longitudinal section of the subligamental ridge made at right angles to its dorsal surface. Bouin 3, hematoxylin-eosin. bl.v.—large blood vessel; el.m.—basal elastic membrane; ep.—epithelium; m.—muscle fibers; pig.c.—pigment cells; po.—pockets between the epithelial cells; v.c.—vesicular cells.

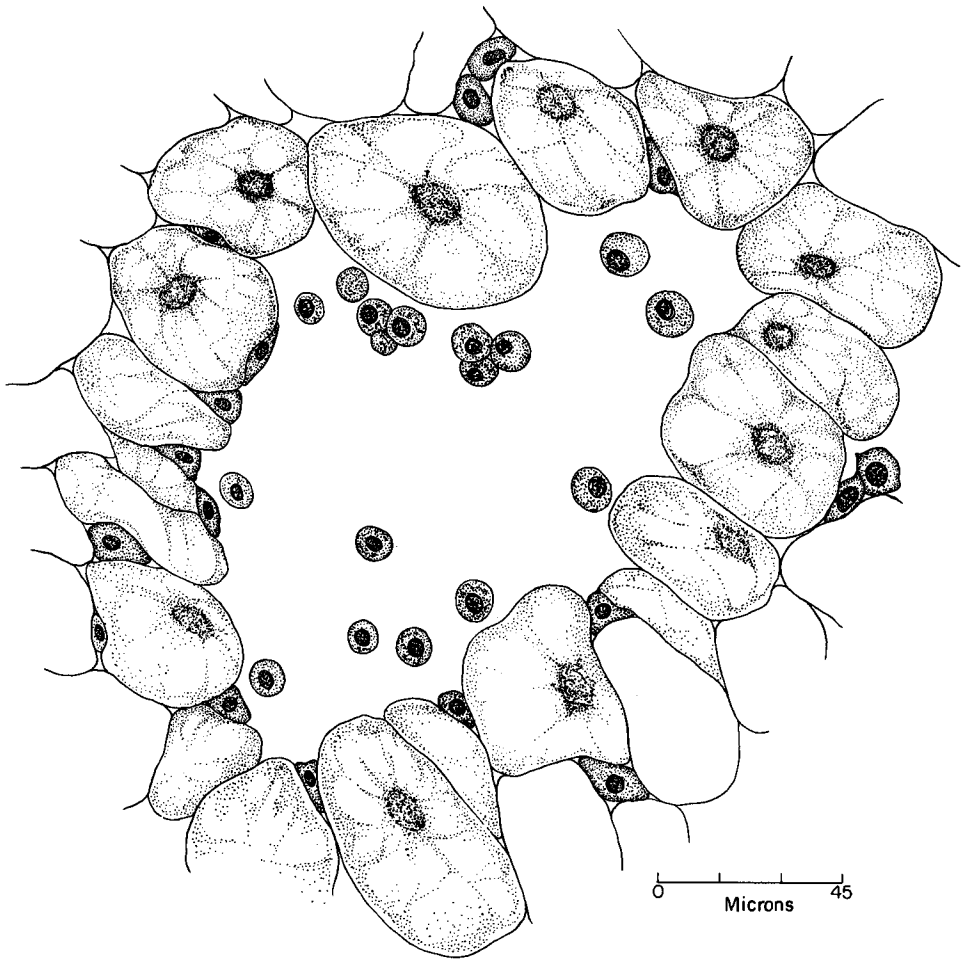

FIGURE 79.—Vesicular cells of connective tissue from the mantle of an adult *C. virginica* surrounding the blood sinus. Blood cells crawl between the cells of connective tissue and penetrate into the sinus. Bouin 3, hematoxylin-eosin.

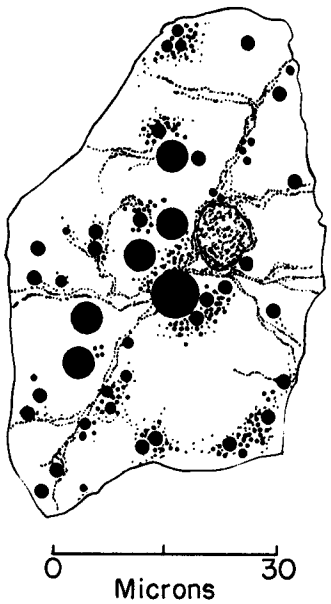

FIGURE 80.—Vesicular cell of connective tissue with fat globules. Frozen section. Sudan II.

opaque. These changes in appearance usually coincide with seasonal cycles in the glycogen content of the connective tissue and with the progressive stages of gonad development.

The presence of glycogen can be easily demonstrated by treating the tissue with Lugol solution (1 percent iodine in 2 percent potassium iodide in water). Specific reagents used for the identification of glycogen, such as Best's carmine and Bensley's modification of Bauer-Feulgen reagent (which stains glycogen granules red-violet), also give good results.

In the live oyster glycogen can be seen as small colloidal granules which ooze from the tissue under slight pressure. In preserved and stained material it appears in the form of granules or rods (fig. 82). The total amount of glycogen in the connective tissue may be so great that the blood vessels and nerves of the mantle are completely hidden under it and cannot be traced by

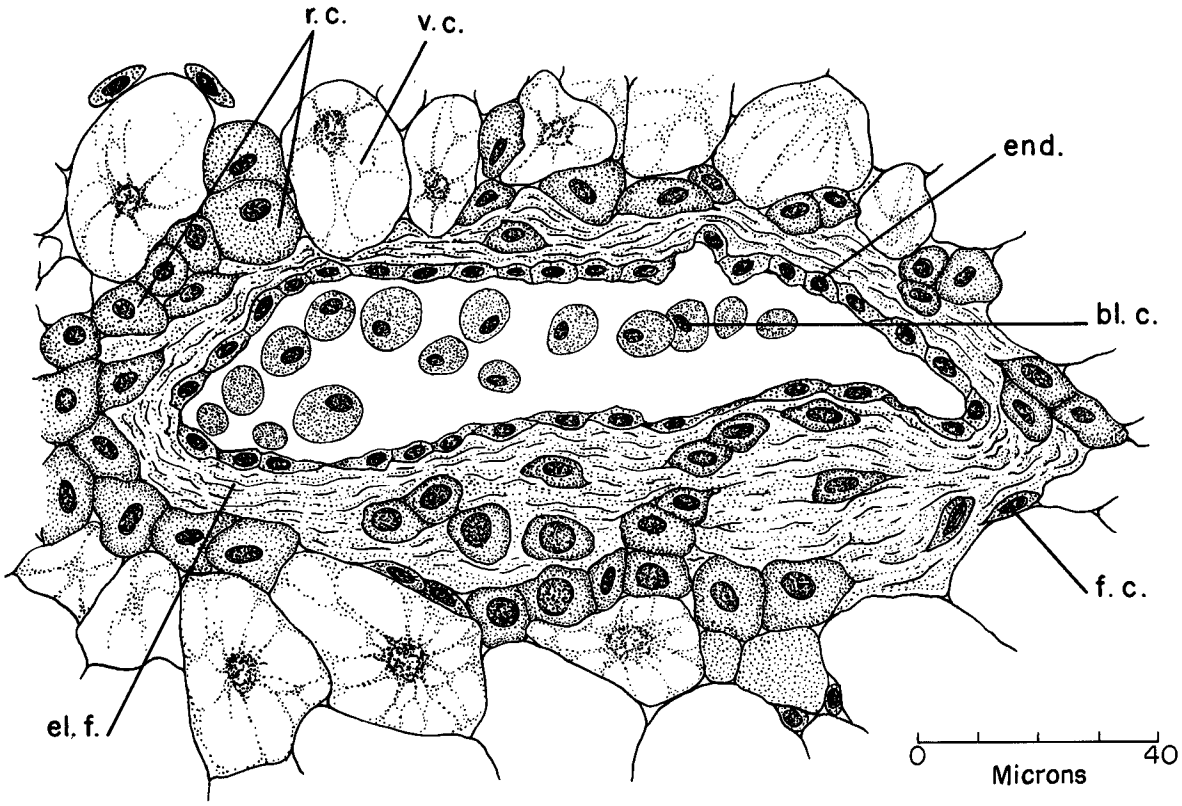

FIGURE 81.—Cross section of a small artery of the mantle. bl.c.—blood cells; e.lf.—elastic fibrils; end.—endothelium; f.c.—fusiform cells; r.c.—round cells; v.c.—vesicular cells. Kahle, hematoxylin-eosin.

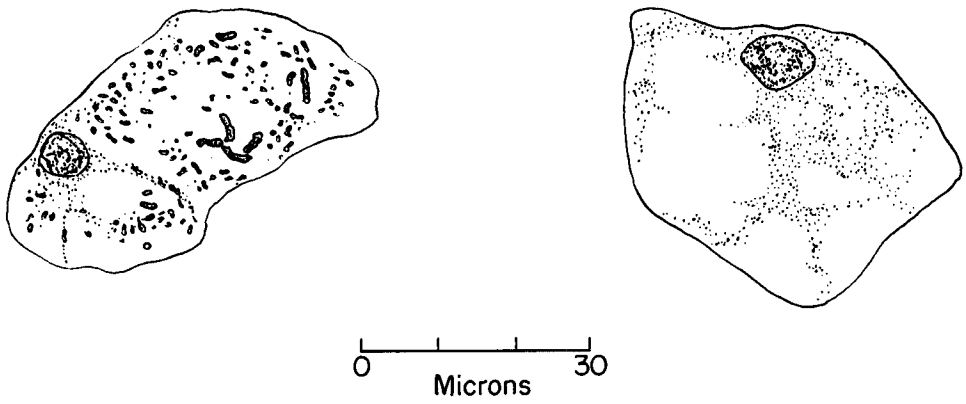

FIGURE 82.—Two vesicular cells from the mantel of an adult *C. virginica*. Left—the cell contains glycogen stained with Best's carmine; fat globules were dissolved in processing. Right—similar cell after fixation with Bouin 3; note complete absence of glycogen and fat, both dissolved during fixation and dehydration.

dissection. Such abundance of reserve material led one of the earlier investigators (Creighton, 1896, 1899) to conclude that its storage in the connective tissue of lamellibranchs is a special adaptation comparable to the storage of fat in the connective tissues of vertebrates.

The quantity of glycogen stored in connective tissue gradually decreases as the gonads of the oyster increase in bulk. This was first reported for *O. edulis* by Pekelharing (1901) and confirmed by the more recent investigations of Bargeton (1942). Evidence presented in the latter work strongly suggests that the growing sex cells utilize the glycogen stored in the vesicular cells surrounding the gonad tubules, but cytological details of this process are still unknown and the problem

has not yet been studied from a biochemical point of view.

After the disappearance of their contained glycogen the vesicular cells do not shrink or collapse. A hypothesis was therefore advanced (Semichon, 1932) that the glycogen granules are supported by a framework of a special substance which remains intact after the dissolution of glycogen. It is claimed that this framework can be revealed by staining with black anilin inks. The evidence for the existence of such a special substance is not, however, convincing. In cells with a moderate content of glycogen the latter can be seen in close contact with the protoplasmic network typical for vesicular cells. Furthermore, the walls of the vesicular cells are fairly rigid and the cells retain their shape even when they are empty. The shrinkage of connective tissue frequently caused by changes in osmotic pressure when the salinity of the water surrounding the oyster is suddenly increased is not associated with the disappearance of glycogen.

The fat globules in vesicular cells vary greatly in size and number, usually forming distinct vacuoles that are easily dislodged. The relationship between the fat and glycogen content of the oyster and the role of lipids in the physiology of lamellibranchs have not been studied.

Large oval cells containing a brown pigment are scattered throughout the connective tissue of the mantle. The pigment is not soluble either in acids or fat solvents. Its chemical nature and physiological significance are not known.

Wandering blood cells are commonly seen in the mantle. They crawl between the connective tissue cells, aggregate in the vicinity of blood vessels and blood sinuses (fig. 79), and are gradually discarded through the surface of the mantle. As a rule, the oyster continually loses a certain amount of blood by diapedesis or bleeding. Any excess of heavy metals accumulated by blood cells (see p. 390) is also discarded by this normal process.

MUSCLES

The radial muscles consist of large, regularly spaced bands of fibers which extend almost the entire width of the mantle from the line of its fusion with the visceral mass and with the adductor muscle to the free margin. For a study of the anatomy of the muscular system the connective tissue in which the bands are firmly enclosed should be macerated in 1 percent potassium hydroxide for about 24 hours. After being washed in distilled water the loosened tissues are removed with a small stiff brush and fine forceps.

The radial muscle bands are composed of large bundles of unstriated fibers which begin to branch toward the distal edge of the mantle about one-third of the distance from that edge. At this level the muscles appear fanlike and enter into all three lobes, where they terminate.

The central part of a muscle band is usually occupied by one or two radial nerves, although muscles without a central nerve (figs. 83 and 84) do occur.

The contraction of the radial muscles pulls the entire mantle inside and throws its surface into ridges. Such a general reaction usually precedes the contraction of the adductor muscle and the closing of the valves. The contraction may occur spontaneously in response to some internal stimulus or it may develop as a result of external irritation produced by chemicals, mechanical and electrical shock, or sudden change in illumination. In response to a weak outside stimulus only a small sector of the mantle contracts, making a slight V-shaped indentation along its periphery. This response may or may not be followed by contraction of the adductor muscle. Strong stimuli, as a rule, result in complete withdrawal of the mantle, contraction of the adductor muscle, and closing of the valves. Besides the large radial bands there are many smaller bundles of transverse fibers (fig. 77, tr.m.) extending diagonally across the thickness of the mantle, a well-developed system of longitudinal muscles (l.m.), and the oblique muscles (ob.m.) of the tentacles.

The longitudinal or concentric muscles follow the general outlines of the edge. They are more abundant at the thickened distal edge of the mantle but do not exhibit the definite pattern of distribution apparent in the radial muscles. The transverse muscle fibers are more numerous in the pallial curtain (fig. 77, tr.m.) than in the other parts of the mantle. They are so arranged that the position of the curtain may be quickly changed in response to external or internal stimuli.

All the muscle cells are of the smooth, nonstriated type with typical elongated nuclei. In some bivalves the muscle fibers of the mantle appear to show a double oblique striation; this was shown to be an optical effect created by a series of fine fibrillae spiralling around the larger fibers (Fol, 1888; Marceau, 1904). Muscle fibers

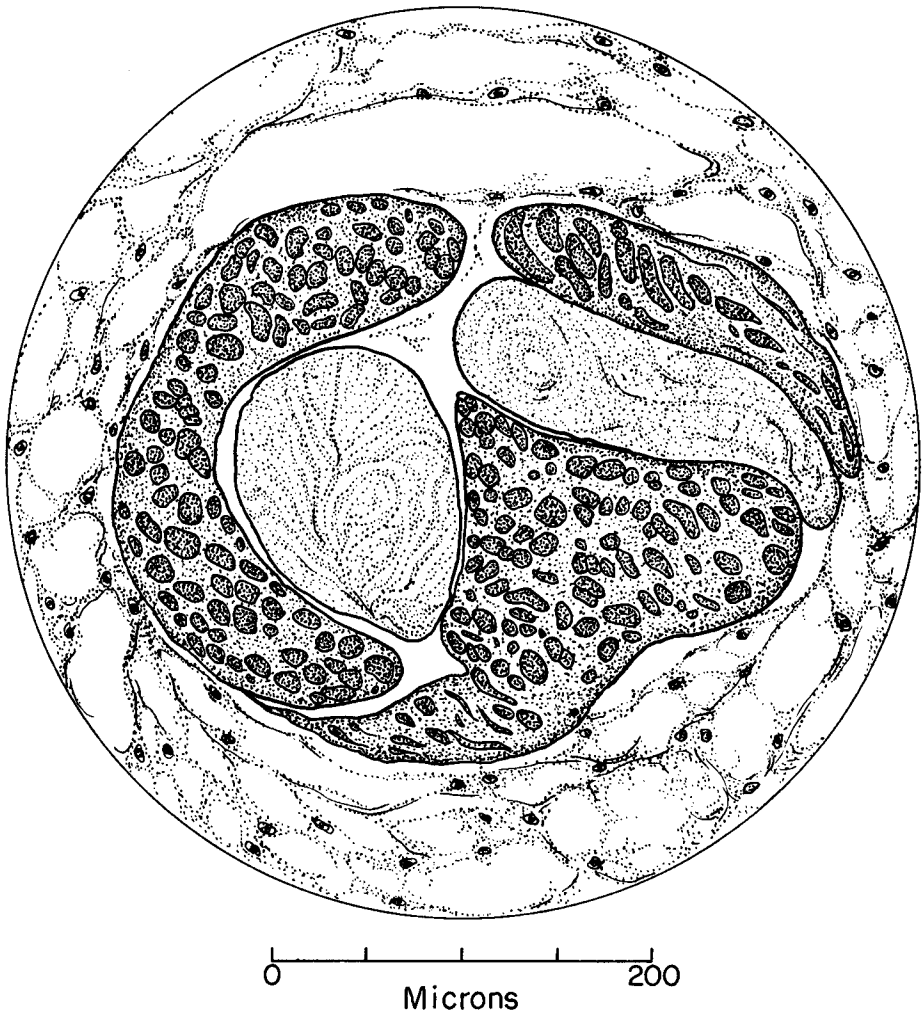

FIGURE 83.—Cross section of the radial muscle of the mantle of an adult *C. virginica*. The muscle completely surrounds two nerves. Bouin 3, hematoxylin-eosin.

with true transverse striation, described in the mantle of *Pecten jacobaeus* and *P. opercularis* (Dakin, 1909a), are not found in the oyster mantle.

BLOOD VESSELS

The principal blood vessels of the mantle (fig. 232 in ch. XI) are the circumpallial artery (cr.p.a.), which runs along its entire periphery and sends out many branches; the common pallial artery (co.p.a.); and a large pulsating vessel in the anteroventral part of the mantle called the accessory heart (fig. 236 in ch. XI). The latter can be observed by dissecting the wall of the epibranchial chamber and spreading the cut tissues apart. The structure and function of these vessels are discussed on page 253.

The small arteries and veins of the mantle can be recognized easily by their histological characteristics. The walls of the arteries have a thick, elastic, muscular layer lined with endothelium (fig. 81, end.). In the veins the elastic layer is much less developed and the endothelium absent (fig. 85). The sinuses (fig. 79) are irregularly shaped spaces in the connective tissue. Since they have no walls of their own they cannot contract. The size of the opening or lumen may be reduced by growth of the surrounding vesicular cells and by accumulation of blood cells.

EPITHELIUM, TENTACLES, AND NERVES

Both sides of the mantle are covered by cylindrical epithelial cells set on an elastic basal membrane (fig. 77). Large goblet cells which secrete mucus and cells containing eosinophile granules are abundant on both sides of the mantle. The cells of the side facing the pallial cavity are long

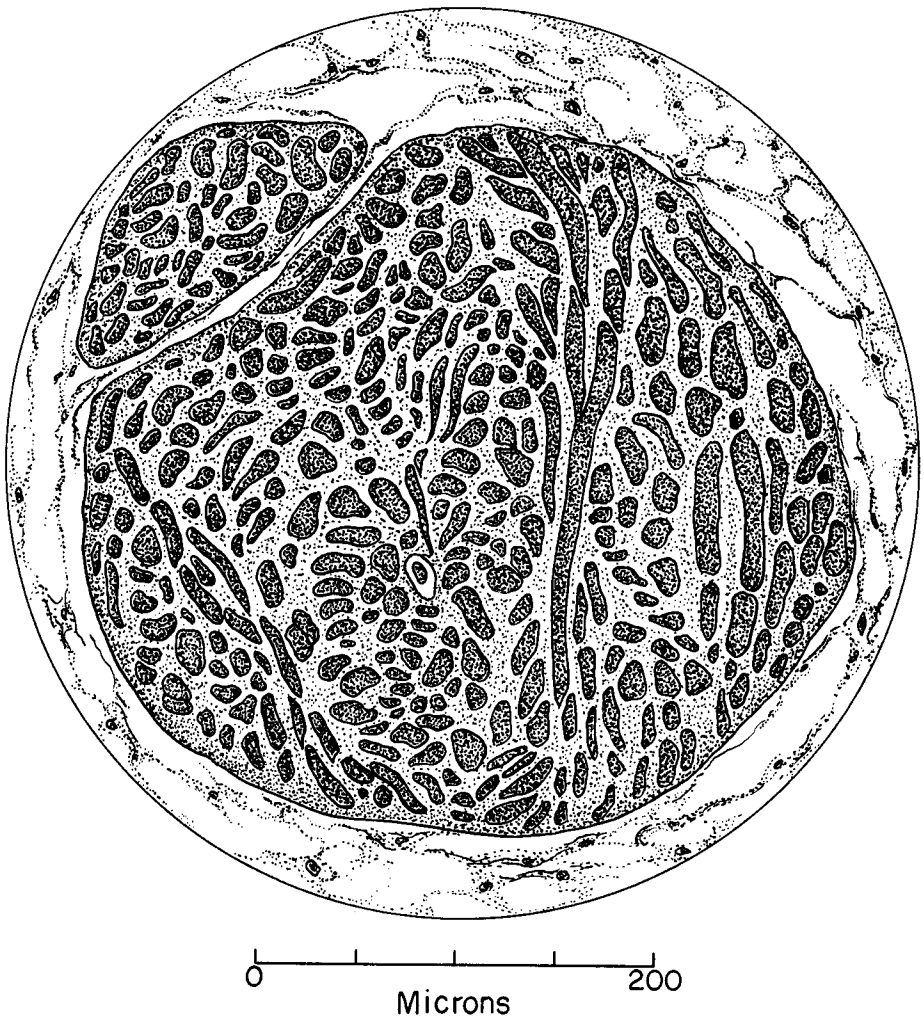

FIGURE 84. Cross section of the radial muscle of an adult *C. virginica*. The muscle is not accompanied by nerve. Bouin 3, hematoxylin-eosin.

and ciliated; those on the outside under the valves bear no cilia and are much shorter, in places almost cubical.

The two sides of the mantle perform different functions. The inner side maintains ciliary currents, which in general move from the base of the mantle to its edge and carry mucus and sediments settled from the water; this material is passed to the margin of the shell to be discharged. The epithelium of the outer side secretes the inner layer of the shell, the so-called calcito-ostracum.

Although the ciliated epithelium of the edge of the mantle contains the same kind and proportion of cellular elements found in other parts of the organ, the cilia at the border of the mantle are especially powerful. The tentacles themselves consist of a core of connective tissue with associated blood vessels, elastic fibrils, and muscle fibers which emerge from branches of the radial muscles. On the outside the tentacles are covered with a single layer of ciliated epithelium to which black or brown pigment imparts a dark color. Special sense organs are absent but the tentacles, especially the long ones, are well supplied with nerves branching out from the nerve which enters the base of the tentacle and is itself connected with the nervous system of the mantle (fig. 86).

The circumpallial nerve provides communication between the tentacles and the radial nerves. The structure of this nerve resembles that of a ganglion: numerous nerve cells of the types found in visceral and other ganglia (see p. 288) occupy the periphery of the nerve; its center consists of nerve bundles with occasional small ganglion cells.

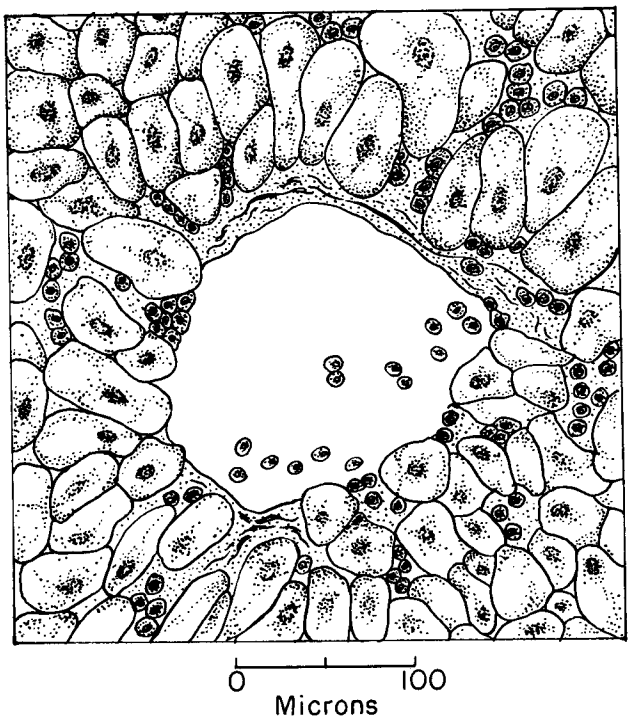

FIGURE 85.—Transverse section of a small vein of the mantle. Note the absence of endothelium and poorly developed elastic layer. Bouin, hematoxylin-eosin.

Close nerve contact between the muscles and other organs of the mantle is maintained through a fine nerve network which can be made visible by using the gold impregnation method (fig. 87). I have had no success in revealing it with vital stains.

PERIOSTRACAL GROOVE AND GLAND

The narrow space between the middle and the outer lobes of the mantle edge, called the periostracal groove (fig. 77, per.gr.), is lined with ciliated epithelium which is replaced at the bottom of the groove by glandular cells. The innermost part of the groove is called the periostracal gland (fig. 88), although it would have been more appropriate to refer to it not as a gland but as a secretory epithelial surface (Maximow and Bloom, 1930). This surface is covered with a single layer of glandular cells different in appearance and structure from the epithelial cells of the distal part of the groove. Unlike a true gland, it does not form a compact body extending under the surface of the groove and it has no duct. On transverse sections of the mantle edge the gland sometimes appears as a round structure surrounded by connective tissue. Examination of a

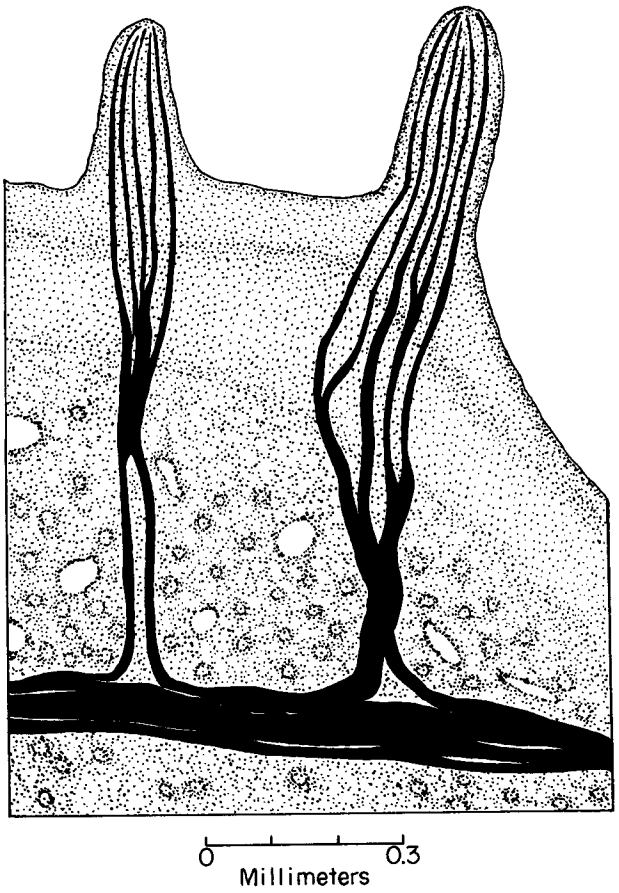

FIGURE 86.—Innervation of the tentacles of the middle lobe. Formalin 5 percent, gold impregnation. Whole mount.

series of sections shows, however, that this appearance is caused by the invaginations of the inner surface of the lobe. The periostracal gland is present in all lamellibranchs and was the object of many histological studies (Leenhardt, 1926; List, 1902; Moynier de Villepoix, 1895; Rassbach, 1912; Rawitz, 1888).

There is a conspicuous difference in the appearance of the cells along the two sides of the groove. Those lining the outer lobe (fig. 88, left side) are distended at the distal ends and taper toward the base into slender rootlike processes which, according to Rawitz (1888) who described them in the oyster, penetrate the underlying connective tissue. I was not able to reveal such rootlets in my material. None of these cells bear cilia, although the distal part of the groove, not shown in figure 88, is lined with ciliated epithelium. Typical goblet cells containing eosinophile granules, amoebocytes, and round mucus cells are present in the epithelial layer of both sides of the groove.

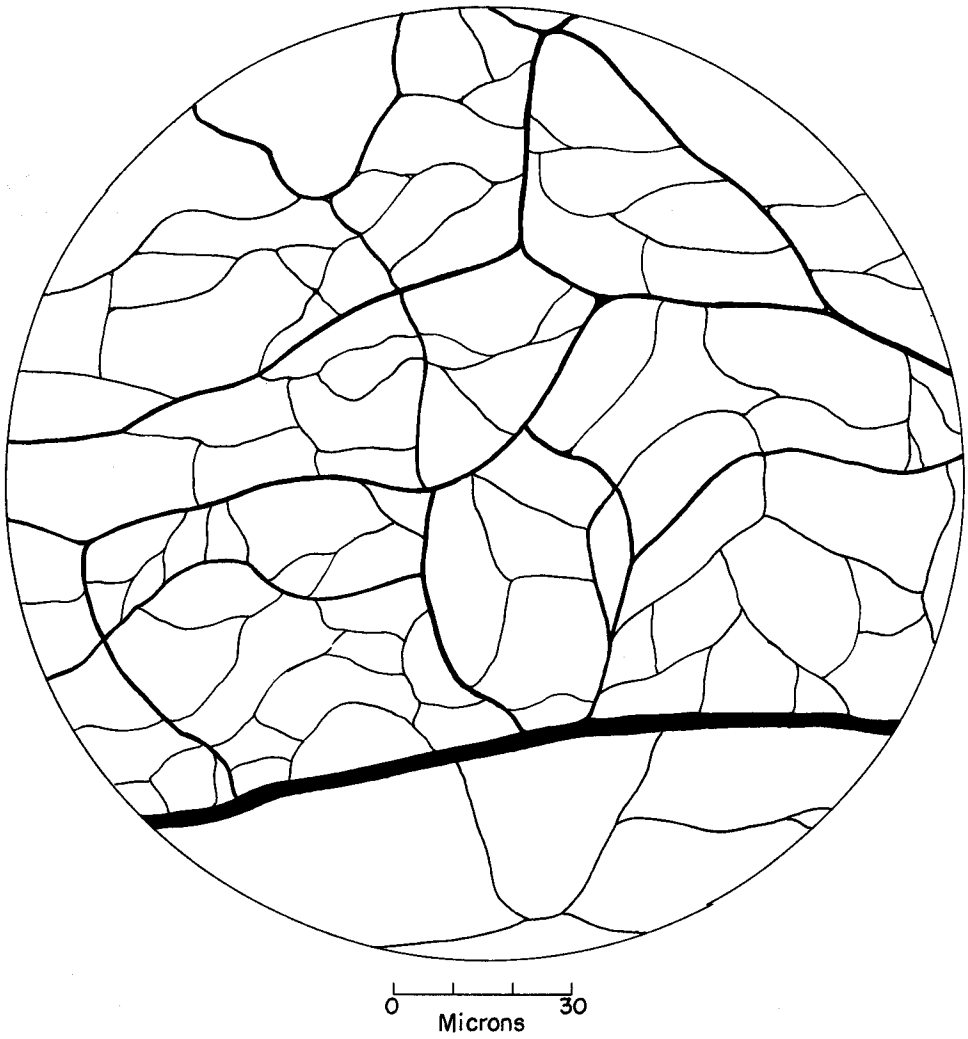

FIGURE 87.—Small area of mantle showing nerve net. Formalin 5 percent, gold impregnation. Whole mount. Camera lucida drawing.

At the very bottom of the groove the tall epithelial cells are suddenly replaced by short cubical cells (fig. 88, right side) which extend a short distance along the inner side of the groove.

The material secreted by the periostracal gland accumulates at the bottom of the groove and in the majority of my preparations appears to adhere to the cells of the outer side (left side of figure 88). This, however, is the result of shrinkage caused by dehydration during the processing of slides. In preparations mounted in glycerin the conchiolin can be seen in close contact with the epithelium of both sides of the groove.

The function of the periostracal gland is to supply large quantities of the material required for new shell growth at the edge of the valves. The organic matrix (conchiolin) and foliated layers of calcite needed for increasing thickness of the valves, on the other hand, are secreted by the epithelium covering the entire outer surface of the mantle and in close contact with the inner surface of the valve. The epithelium consists of nonciliated cells which are cylindrical near the free margin of the mantle but become flattened and almost cubical in more proximal areas. Both conchiolin-secreting and calcium-secreting cells are present in this epithelium but their cytological differentiation by means of staining reactions or by precipitation of calcium oxalate is not reliable. Mucus cells and oval cells containing eosinophile granules also occur throughout the entire surface of the epithelial covering.

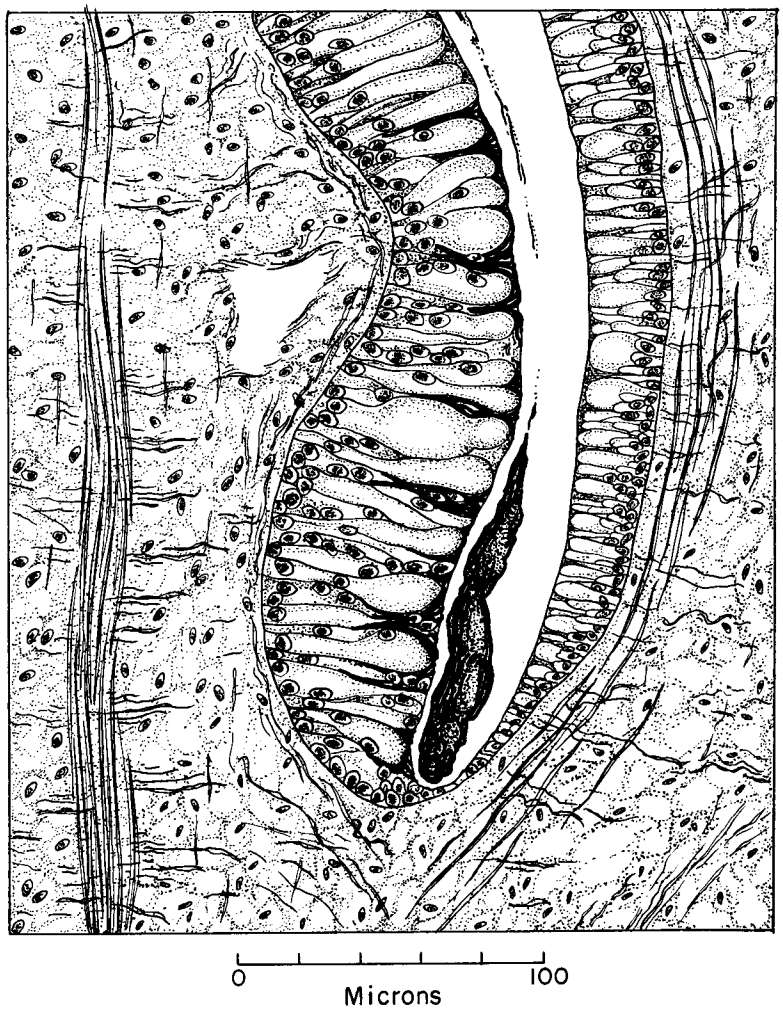

FIGURE 88.—Transverse section of the periostracal groove. The cells on the left side are distended with secretion; those at the very bottom are short, almost cuboid. The black mass at the bottom of the groove is conchiolin. Bouin, hematoxylin-eosin.

Owing to the presence of the conchiolin-secreting cells, the entire outer surface of the mantle is sticky and adheres closely to the inner surface of the shell. List (1902) advanced a theory, not well supported by observation, that in the Mytilidae the mantle adheres to the shell by means of fibrillae which originate in the myoepithelial cells and pass through the epithelium. Such an arrangement is not found in *C. virginica* or in *C. angulata*, and according to Leenhardt (1926) does not exist in *Mytilus*.

The epithelial layer along both surfaces of the mantle including its free edge contains alkaline phosphatase, an enzyme involved in the calcification of the shell. The presence of the enzyme can be demonstrated by the Gomori method (Gomori, 1939, 1943) based on the formation of insoluble calcium phosphate as a result of phosphatase action on sodium glycero-phosphate and calcium ions. Further treatment with 5 percent silver nitrate (or with cobalt nitrate) converts the calcium phosphate to silver (or cobalt) phosphate which turns black after exposure to light. Both reagents gave satisfactory results in demonstrating the localization of the enzyme in the epithelium of the mantle. The strongest reaction, judged by the opacity and width of the black layer, was found to occur along the edges of the mantle and in the area of the periostracal groove. Even the tips of the tentacles contained noticeable amounts of the enzyme (fig. 89). These obser-

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

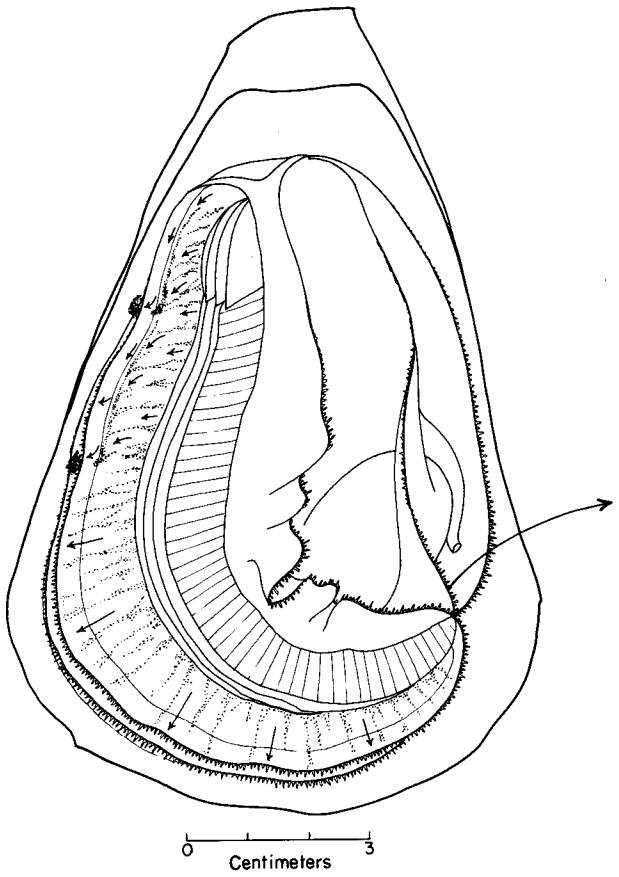

FIGURE 90.—Discharge areas of the mantle. Note the two large lumps of the discarded material at the edge of the mantle which mark the boundaries of the principal discharge area. Small arrows indicate the direction of ciliary currents. The clear path along the periphery of the mantle in the upper left 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

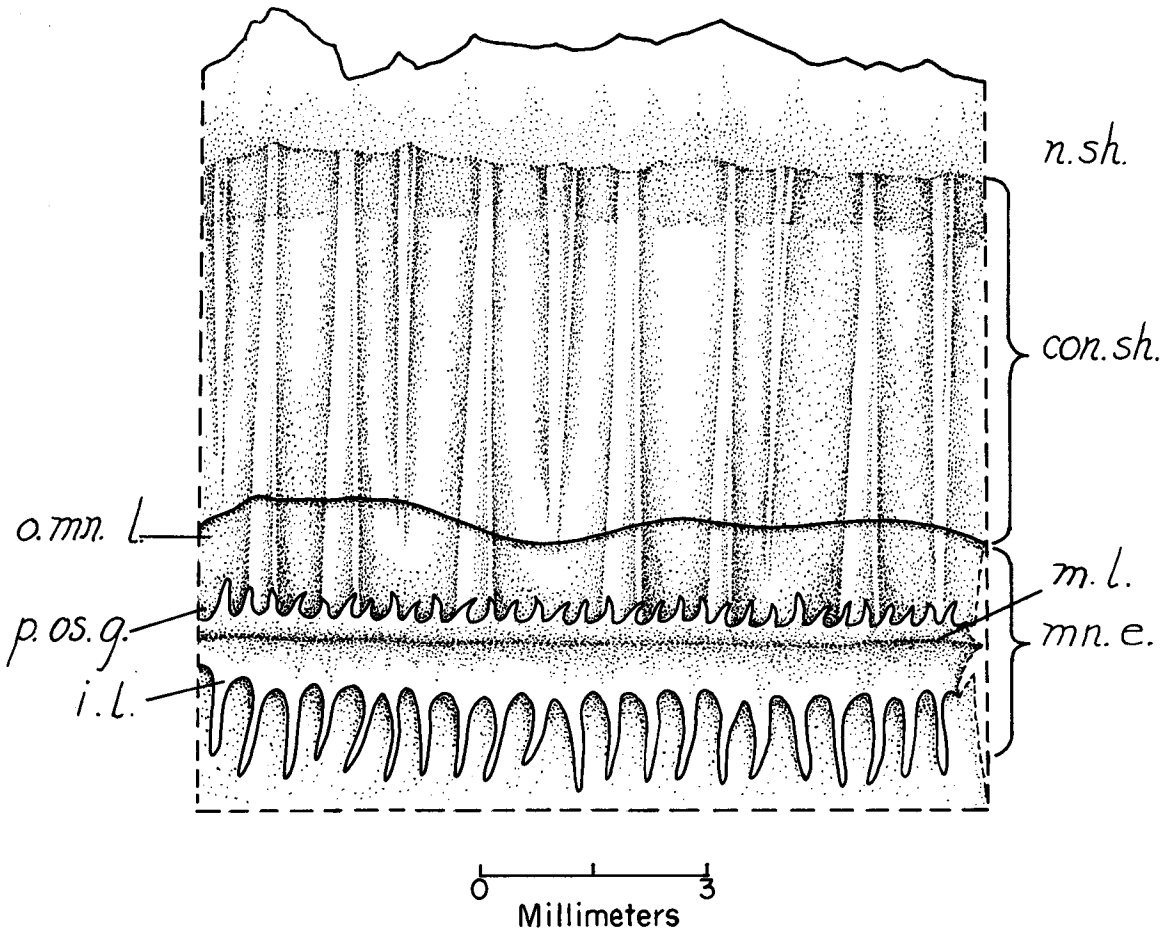

FIGURE 91.—Small area of the mantle edge with the adjacent part of the newly secreted shell viewed from above. The mantle was exposed by cutting off a piece of the opposite valve, and the oyster was placed in sea water under a binocular microscope. con.sh.—conchiolin sheet; mn.e.—edge of the mantle; 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

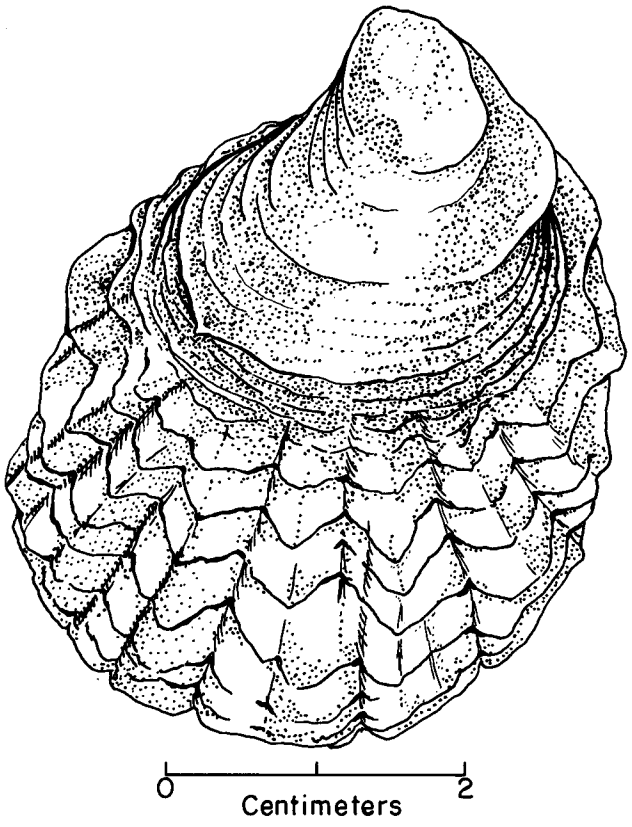

FIGURE 92.—New shell growth formed during 1 year along the periphery of the valve of an adult oyster from Long Island Sound planted in the Oyster River, Chatham, Mass. The newly formed shell is recognizable by zigzag lines of the material; its width is greatest along the ventral edge.

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

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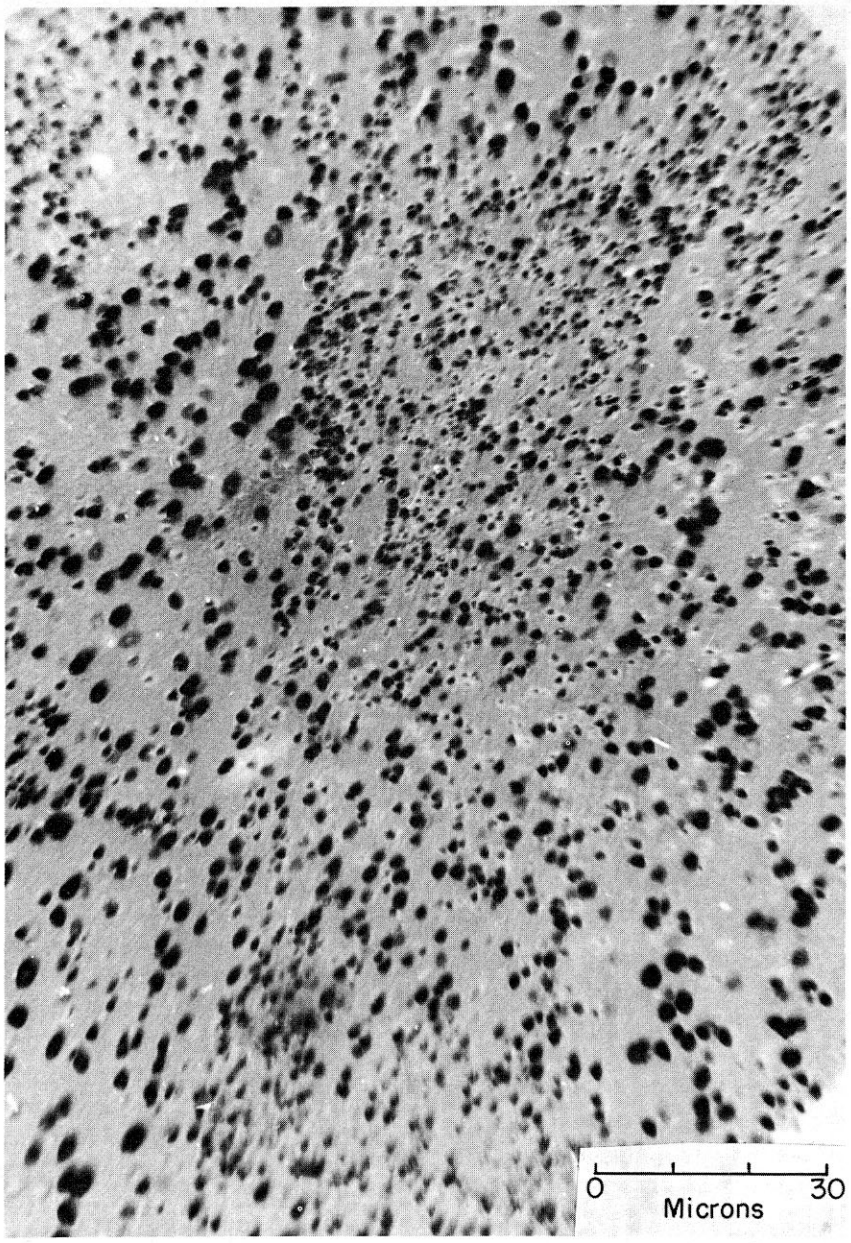

FIGURE 93.—Small granules (calcospherites) in conchiolin shortly after its secretion by the mantle. Black and white enlargement of a Kodachrome photograph taken with polarized light.

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-

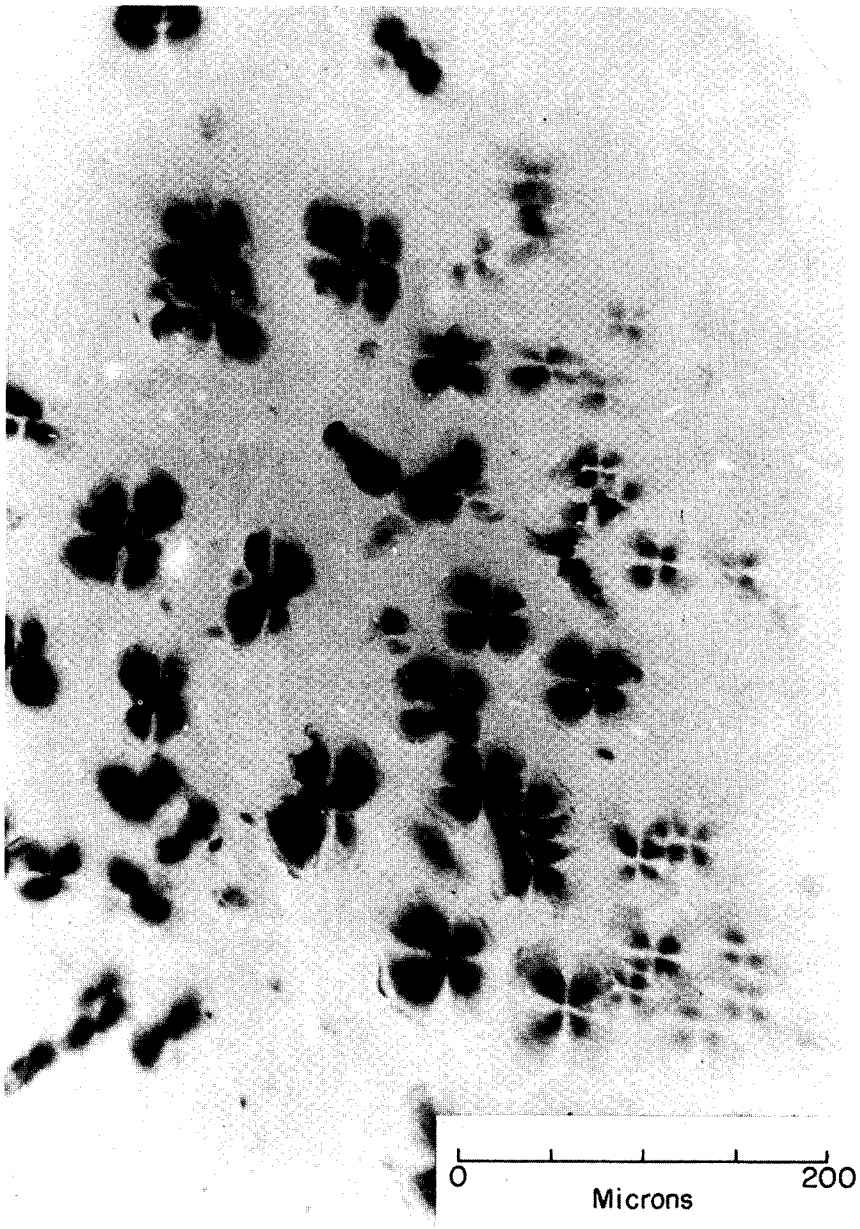

FIGURE 94.—Calcite crystals of new oyster shell about 24 to 36 hours after its formation. Black and white enlargement of a Kodachrome photograph taken with polarized light.

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

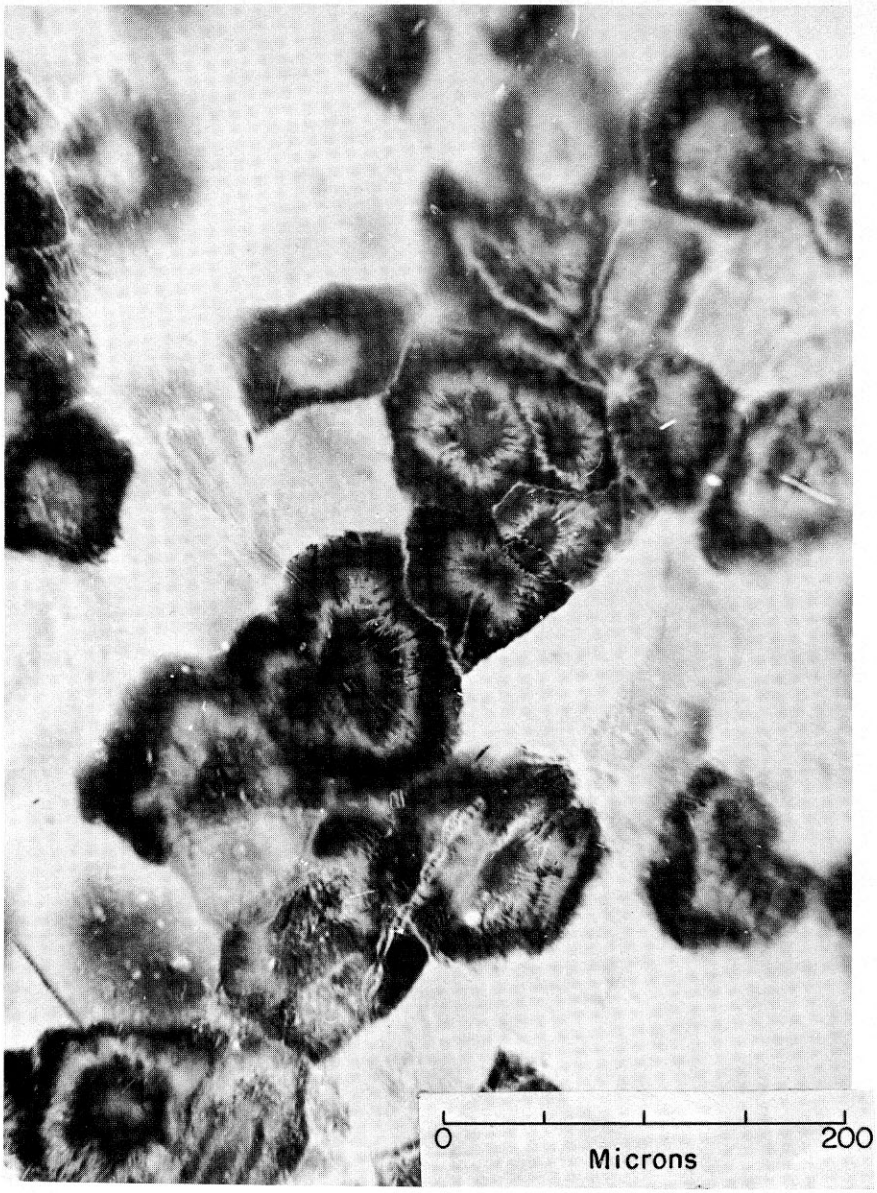

FIGURE 95.—Early stage of the formation of prismatic layer. Photographed with polarized light.

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

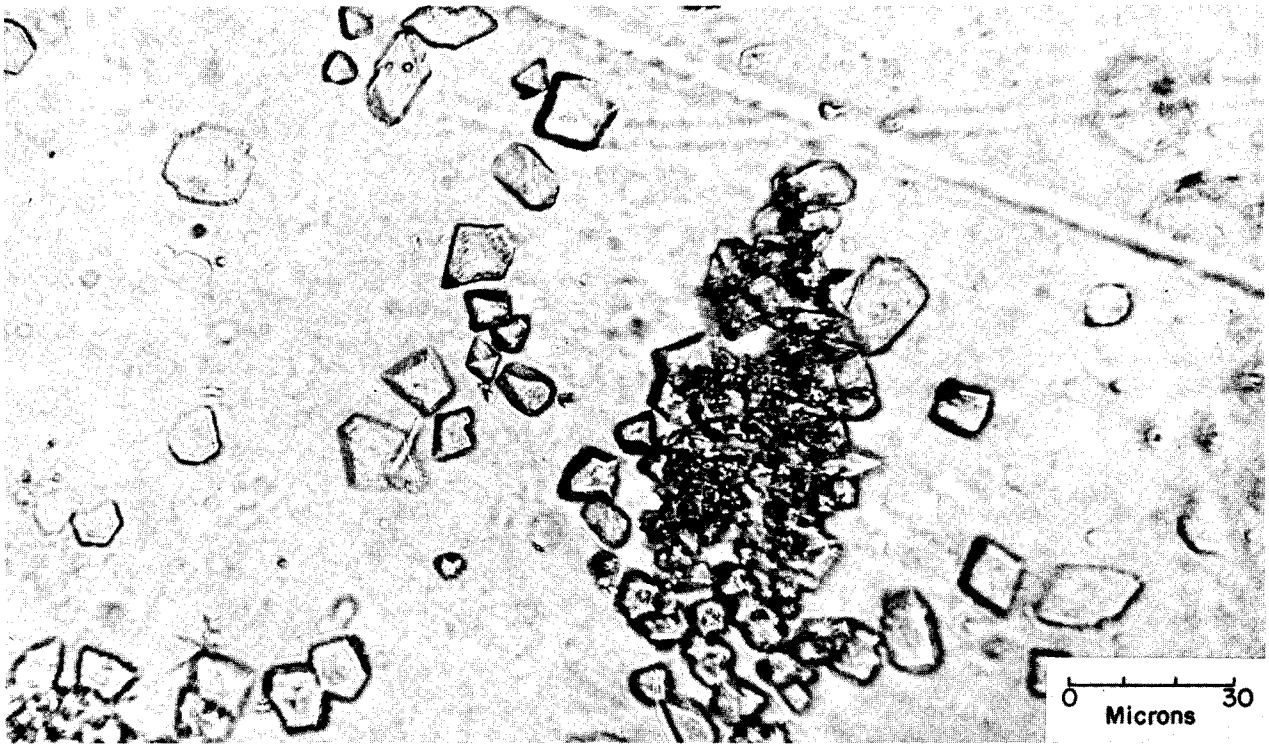

FIGURE 96.—Photomicrograph of a piece of new shell of *Crassostrea virginica* taken 28 hours after the beginning of calcification. Small calcite crystals are randomly distributed, and calcospherites scattered over the entire field of view are in places densely packed between the larger crystals.

$(C_6H_5O_7)_2Ca_3+4H_2O$. 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

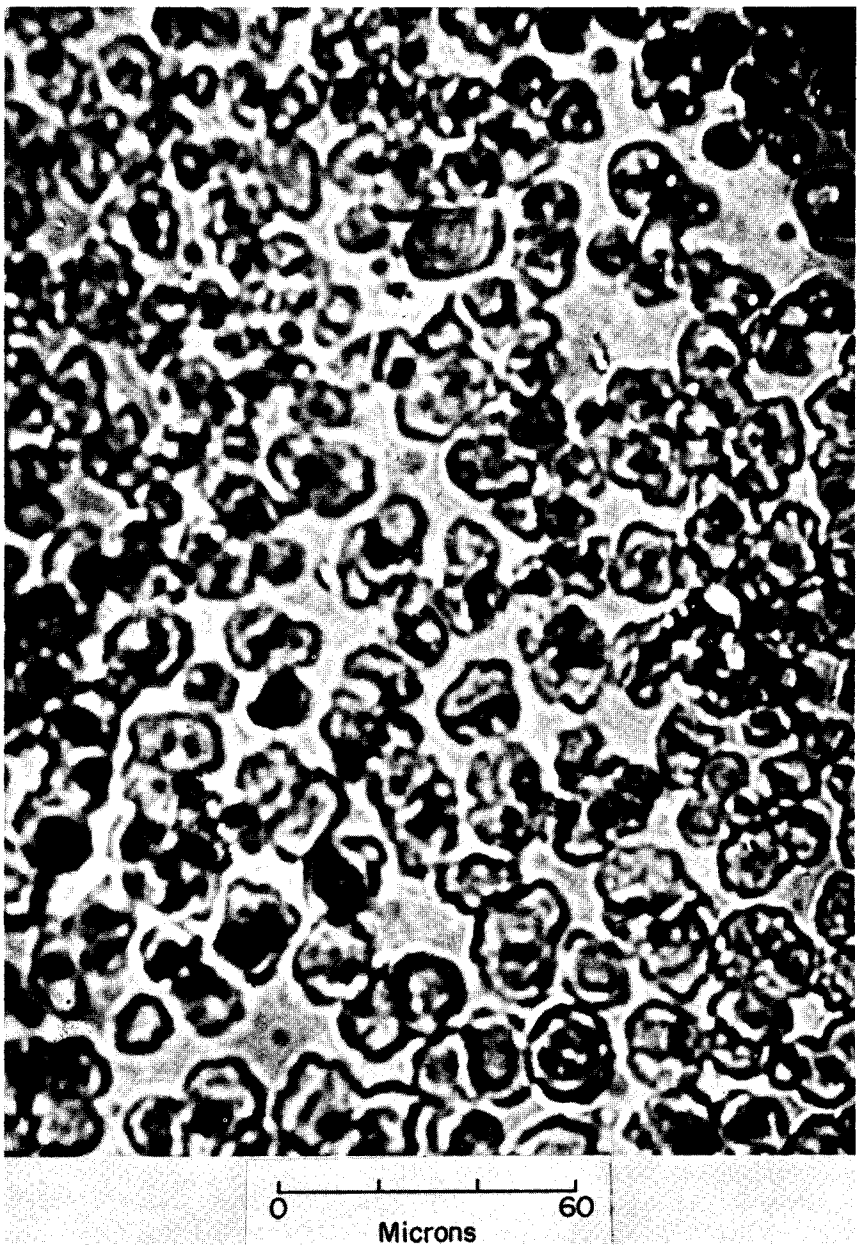

FIGURE 97.—Calcite crystals deposited on a piece of conchiolin. Photomicrograph taken 38 hours after the secretion of conchiolin has started.

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

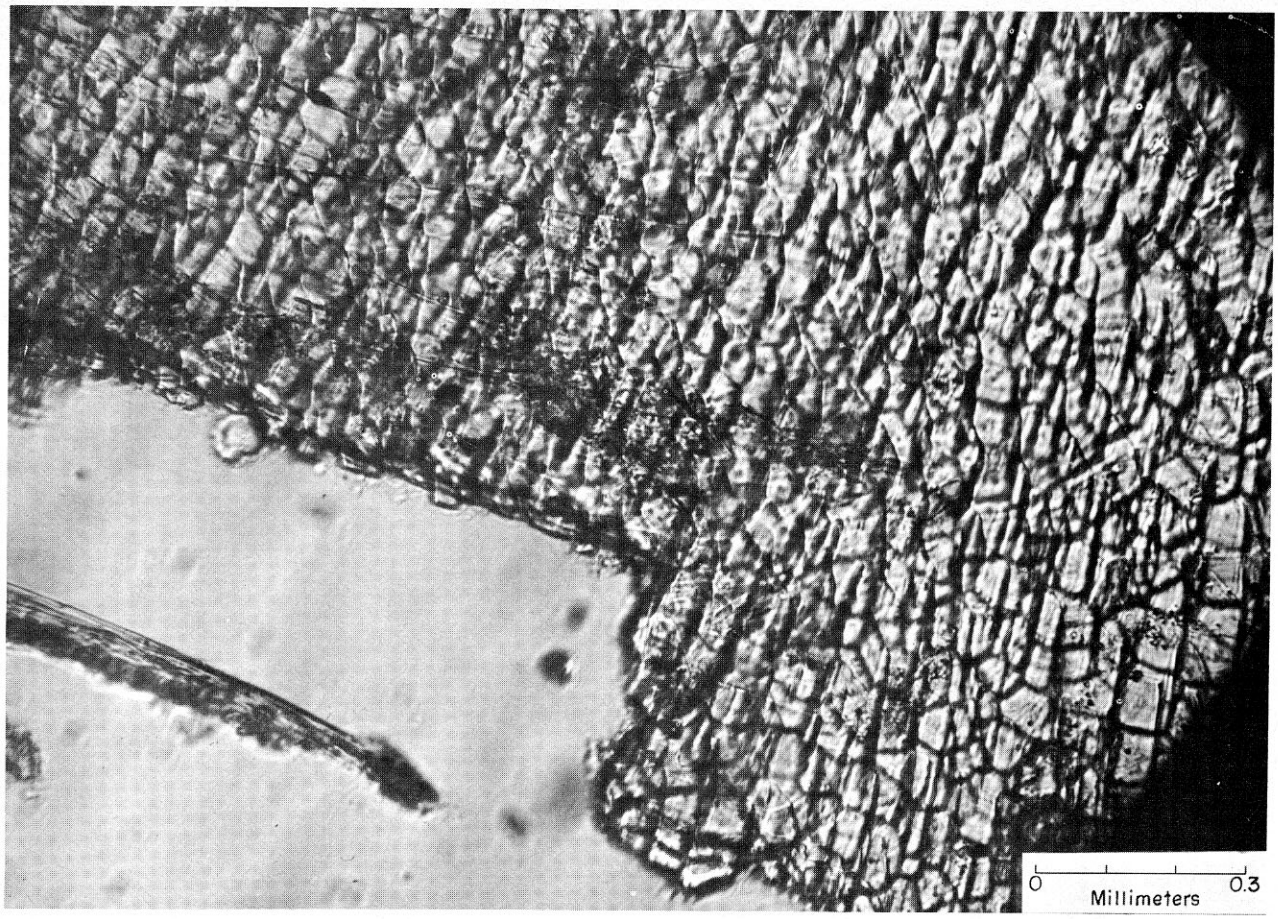

FIGURE 98.—Prismatic layer of the new growth of shell at the edge of the mantle. Four to five days old. Photomicrograph of an unstained whole mount.

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

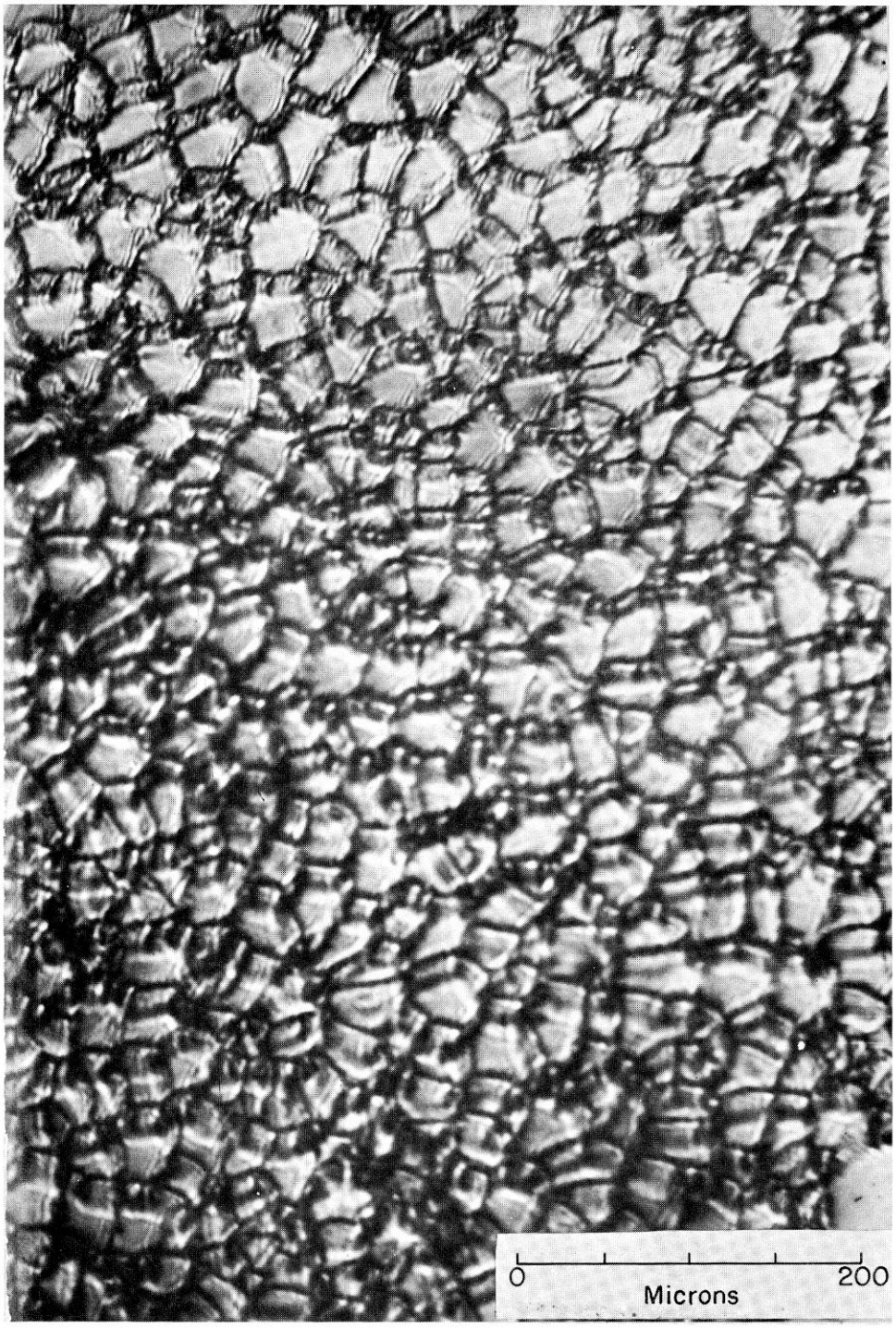

FIGURE 99.—Photomicrograph of organic meshwork of prismatic layer of shell after decalcification in weak hydrochloric acid. Note that the outlines of the capsules retain the shape of the mineral prisms.

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-

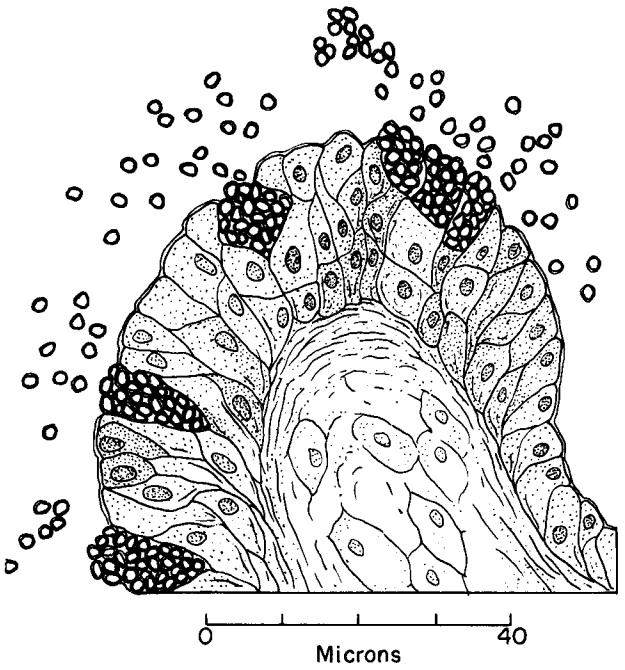

FIGURE 100.—Calcium containing granules discharged by the epithelial cells at the edge of the mantle of *C. virginica*. Neutral formalin 3 percent, alizarin.

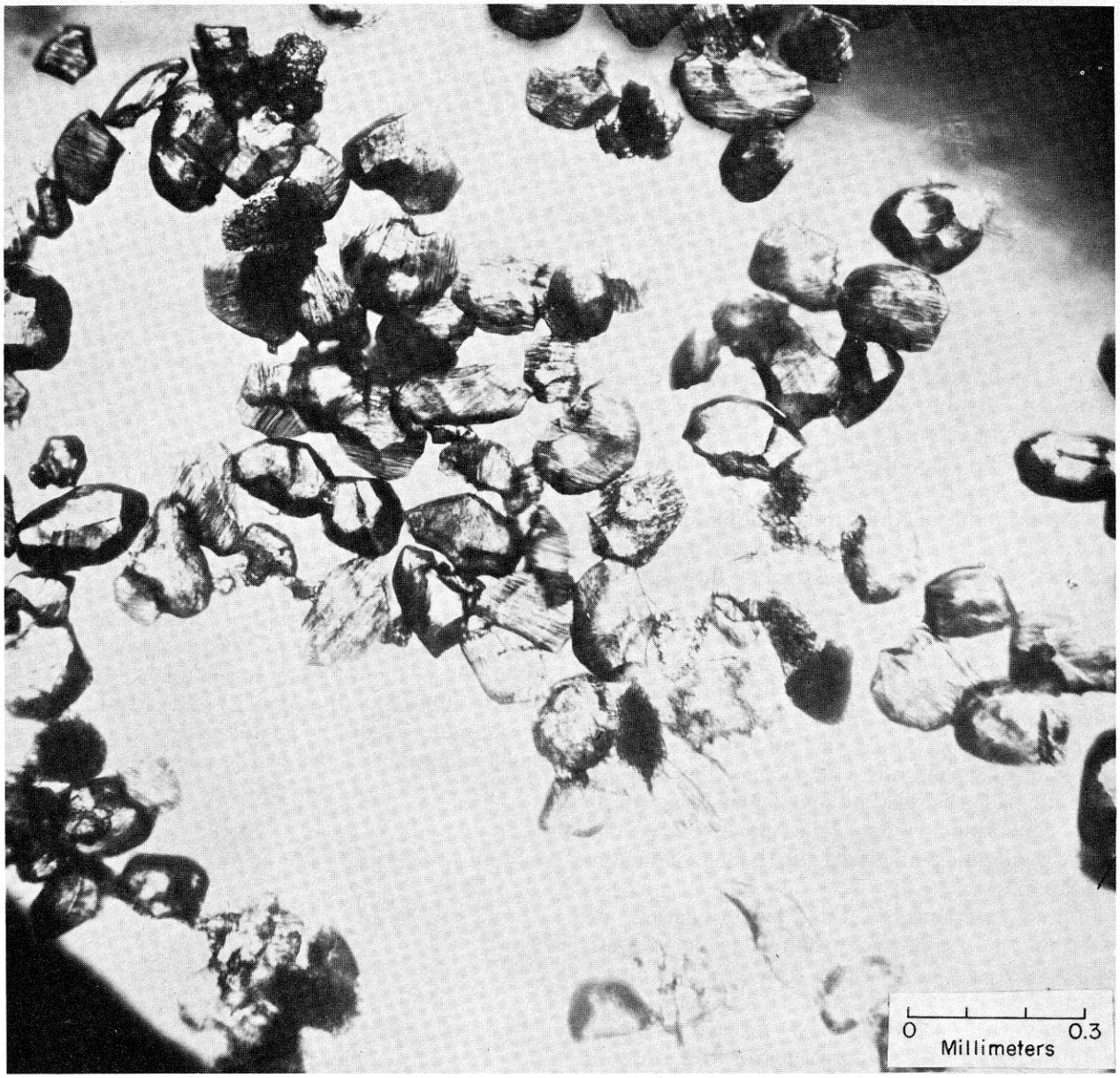

FIGURE 101.—Photograph of crystals of a mixture of calcite and gypsum formed in the mantle cavity of *C. virginica*.

chemical reactions show that the secretion of calcium is not confined to special sites but takes place over the entire edge and outer surface of the mantle. The intensive coloration of the granules by alizarin suggests that they contain a considerable amount of calcium, probably bound in organic compounds of the globules. Amoebocytes present in the material secreted by the mantle also may be involved in the mobilization of calcium during the formation or repair of shells.

Sometimes the mineral crystals formed by the mantle are not incorporated in the conchiolin but accumulate in the pallial cavity and are eventually ejected. On several occasions fairly large quantities of a white powdered material were found in front of the discharge areas of oysters which were kept in glass trays in running sea water in the laboratory. The material consisted of crystals (fig. 101) which, according to the X-ray analysis kindly performed by Marie Lindberg of the Geochemistry and Petrology Branch of the Geological Survey of the U.S. Department of the Interior, were found to consist of a mixture of

calcite and gypsum (hydrous calcium sulfate), with the latter present only as a minor constituent. The oysters appeared to be normal in every respect and showed good growth of shells. The presence of gypsum is of interest since it is not a normal constituent of oyster shell. What particular disturbance in the calcium metabolism produced its formation is unknown.

SOURCES OF CALCIUM

It has been suggested (Pelseneer, 1920; Galtsoff, 1938) that lamellibranchs may remove calcium directly from sea water. Pelseneer (1920) cites an example of a young *Anodonta cygnea* which in 2 months removed all the calcium from 5 l. of water in which it was kept. Definite proof of the direct absorption of calcium by the oyster mantle is given by the experiments with *C. virginica* (Jodrey, 1953) in which radioactive Ca^{45} was used. Calcium turnover was also studied by Hirata (1953) in mantle-shell preparations made by cutting off the adductor muscle and the visceral organs, and leaving the intact mantles spread over their respective valves. The mantle remained alive for several days and deposited the shell material, although at a lower rate than does the intact oyster. Jodrey placed a mantle preparation in 500 ml. of aerated sea water with a Ca^{45} activity of 5.8 microcuries. At least part of the calcium of the newly formed shell substance came directly from the sea water, and the deposition of calcite took place in tissue isolated from the circulatory and digestive systems. The experiments also demonstrated that the greater portion of calcium in the mantle appears to be inert. Only 2.5 percent of the total calcium content was renewed every 24 minutes, the turnover being 0.6 mg. of calcium per minute per gram of mantle. In addition to entering the mantle directly calcium can be taken up by other organs of the oyster and transported to the mantle (Wilbur, 1960).

MINERALOGY OF CALCIUM CARBONATE IN MOLLUSCAN SHELLS

Calcium carbonate is known to occur in 12 mineral forms (Prenant, 1924), but only three of these have been found in animals. In the shells of mollusks, calcium carbonate usually occurs as calcite and aragonite. There are many species in which both minerals occur together although in different parts of the shell. Prenant (1928), who contributed much to the study of calcification, found that besides calcite and aragonite the animal tissue may contain small spheres (sphaerolithes) or tiny needles of the mineral called "vaterite", after the mineralogist Vater who discovered it. Vaterite was reported to be present in the connective tissue of certain gastropod mollusks, cestodes, and trematodes, and in the fat tissue of insects (Diptera). Its presence in the tissues of the oyster has not been reported.

The various forms of calcium carbonate secreted by animal tissue can be identified by their crystallographic properties, birefringence, density, and chemical reaction. Some of these distinctive characteristics are summarized in table 12, taken from Prenant (1924).

Impurities always present in material secreted by living forms can sometimes make the mineralogical identification of calcium carbonate doubtful. Calcite and aragonite can be distinguished by means of the polarizing microscope. Calcite crystals examined under crossed nicols give a brilliant picture of various colors, and a distinct black cross appears when the optical axis is aligned parallel to the axis of the microscope (fig. 94.) In the case of aragonite, hyperbolic arched lines appear instead of the black crosses. Exact identification of minerals can of course be made by X-rays, but this method is rarely available to the biologist.

Among various chemical identification methods the Meigen color reaction can be most easily employed (Bøggild, 1930, p. 238). In a weak solution of cobalt nitrate aragonite becomes violet, the intensity of coloration increasing as the solution is warmed. Calcite, however, remains pale blue even in a heated solution.

The conditions under which a mollusk secretes calcium carbonate in a specific mineralogical form are not at present understood. It is reasonable to presume that the organic matrix of the shell is somehow involved in this process. Roche, Ranson, and Eysseric-Lafon (1951) found that in the shells of mollusks consisting both of calcite and aragonite the conchiolin associated with the calcite of the prismatic layer had higher concentrations of glycine and tyrosine than were present in the nacre of the same shell consisting of aragonite (see ch. II, p. 41). The causal relationship between the mineralogical forms of carbonate and amino acids of its conchiolin has not been demonstrated.

A hypothesis that carbonic anhydrase, an enzyme present in the tissues of the mantle, plays

TABLE 12.—*Distinctive properties of principal mineral forms of calcium carbonate found in invertebrates* [1]

Name	Chemical composition	Optical System	Birefringence	Index of refraction	Density	Meigen reaction
Calcite	$CaCO_3$	Rhomboedric, uniaxial	Strong (0.172)	1.658–1.486	2.714	Negative.
Aragonite	$CaCO_3$	Monoclinic, biaxial	Slightly weaker, (0.156)	1.686–1.530	2.95	Positive.
Vaterite	$CaCO_3$	Sphaerolithes, optically negative	Weak	About 1.55	2.5–2.65	Do.
Amorphous	$CaCO_3$	Isotropic		Near 1.5	2.25–2.45	
Hydrated carbonate	$CaCO_3\ 6H_2O$	Prisms or Monoclinic tablets	Near 0.085	About 1.5	1.777	

[1] From the data published by Prenant, 1927.

an important role in the formation of calcium deposits in molluscan shells has been advanced by Stolkowski (1951). According to this theory the enzyme exerts its effect by orienting the calcium carbonate molecules in the aragonite crystal lattice. The action of carbonic anhydrase in this admittedly very complex process is not, however, satisfactorily explained and should be more thoroughly investigated before its role in the formation of aragonite or calcite in mollusk shells is definitely established. In its present state the hypothesis fails to explain the existence of shells in which both aragonite and calcite are present. Recently Stenzel (1963) reported that in the shells of *C. virginica* aragonite covers the areas of attachment of the adductor muscle, the imprint of Quenstedt's muscle, and is found in the ligament.

Another explanation of the formation of the less stable aragonite instead of calcite suggests that strontium and magnesium carbonates influence the formation of aragonite in shell. Some support to this idea is found in the fact that in vitro the crystallization of aragonite is facilitated by strontium and lead salts. This observation made by Prenant (1924) apparently influenced Trueman's (1942) hypothesis that strontium, magnesium, and probably other salts found in living mollusks influence the crystallization of aragonite.

That there may be some correlation between the predominance of the particular mineralogical form of calcium carbonate and the temperature of the surrounding water has recently been suggested by some geologists. Through quantitative X-ray analysis of shells they have demonstrated that in certain polyclad worms (Serpulidae) and in some gastropods and pelecypods (*Mytilus, Volsella, Pinctada, Anomia,* and others) the concentration of aragonite in shells increases with increasing temperatures (Epstein and Lowenstam, 1953; Lowenstam, 1954). In *Mytilus*, for instance, only the shells of warm water species are composed entirely of aragonite, whereas those from colder waters contain varying amounts of both calcite and aragonite. This interesting ecological observation does not, however, provide a clue to the nature of the biochemical processes which control the predominance of one or another crystallization system.

RATE OF CALCIFICATION

The calcification rate of the left valve of *C. virginica* is significantly higher than that of the right one, as can be readily seen by examining newly formed shells. The calcareous material deposited by the left mantle is thicker and heavier than that deposited during the same time by the right mantle (Galtsoff, 1955). I made the following observations on shell growth rate of adult *C. virginica*. After the new growth of shell along the valve edge was carefully removed the oysters were placed in tanks abundantly supplied with running sea water. About 2 months later the areas of newly deposited shells on each valve were measured with a planimeter, carefully removed from the shell, rinsed in distilled water, dried in air, and weighed. The results are summarized in table 13. In every case the amount of calcified material deposited over a unit of area was considerably greater on the left

TABLE 13.—*Areas of new growth and rate of deposition of shell material by* C. virginica *in mg. per day per cm.² during April to June 1954, Woods Hole, Mass.*

Oysters	Area of new shell	Weight per cm.²	Deposition per cm.² pay day	Days under observation	Ratio weight of left to weight of right valve
	Cm.²	*Mg.*	*Mg.*	*No.*	
Five-year-old, Narragansett Bay					
Left valve	5.80	156.0	2.8	55	2.6
Right valve	5.16	59.3	1.1		
Adult, Narragansett Bay					
Left valve	7.1	123.0	1.8	68	6.2
Right valve	7.7	19.9	0.3		
Adult, Narragansett Bay					
Left valve	6.1	74.2	1.09	68	2.9
Right valve	8.8	25.5	0.37		
Two-year-old, New Hampshire					
Left valve	3.68	163.8	2.98	55	3.2
Right valve	4.20	52.0	0.95		
Very old, New Hampshire					
Left valve	6.83	71.2	1.3	55	2.2
Right valve	7.35	33.0	0.6		

valve (lower) than on the right one (upper), the difference varying from 2.2 to 6.2 times.

The rate of deposition of calcified material by the surface of the mantle may also be studied by inserting between the mantle and shell small pieces of plastic or other nontoxic material of known area and weight. Results obtained with this method vary greatly. Observations made on 16 adult oysters at Woods Hole during the period of August 9 to 20, 1953, show that in 15 oysters the daily rate of shell deposition per square centimeter varied from 0.4 to 2.1 mg. One oyster deposited 14.2 mg. in 2 days or 7.1 mg. per day. The amounts of shell material deposited by 20 Narragansett Bay oysters kept in laboratory tanks for 68 days during the period of April to June varied from 0.1 to 0.79 mg. of shell substance per day cm.^{2}. In some of these oysters the presence of the plastic material induced pathological conditions which resulted in the formation of leathery capsules similar to the blisters frequently found on the inside of shells near the adductor muscle. The formation of such blisters was accompanied by deposition of calcite greatly in excess of the rate of calcification under normal conditions.

Seasonal variation in rate of shell deposition over the inner surface of the valves was also studied, using 20 adult oysters for each set of determinations. Observations were continuous from June 1954 until the end of February 1956. To avoid possible injury to the mantle while introducing pieces of plastic, the oysters were fully narcotized in magnesium sulfate solution and insertions made when the mantle was completely relaxed and did not respond to touch. Thin sheets of plastic were cut into rectangular pieces 0.5 cm.^{2} in area and weighed before inserting them under the mantle, their weight varying from 5.5 to 6.0 mg. Some of the pieces introduced were ejected by the oysters, but losses were minimized when the insertion was made under full narcosis. The treated oyster was then marked and placed on its left valve in a large tray supplied with running sea water. The temperature of the water was recorded twice a day. Each set of 20 oysters was kept in water as long as the seasonal rise or fall of water temperature did not exceed 2.5° C.

To obtain measurable quantities of shell deposits the pieces of plastic were left inside the oysters for a longer time in winter and in August, after the completion of spawning, than during the rest of the year. The number of days the oysters with inserted pieces were left undisturbed varied as follows: from 10 to 16 days in April to July; from 25 to 30 days in August; from 13 to 18 days in September to November; for 30 days in December; and 70 days in January to March. Observations were continued for 14 months. No shell was formed in January to March except in a few oysters in which the mantle was injured during insertion. These samples were not included in the data plotted in fig. 102. Laboratory observations showed that shell opening and feeding of the oysters at Woods Hole are as a rule temporarily reduced after the discharge of sex products which takes place late in July and early in August. Unequal time intervals in observing shell deposition do not affect the validity of the results since the rates of shell formation shown in figure 102 are expressed as weights of shell deposited per cm.^{2} in 1 day.

At the end of each period the oysters were removed, the pieces of plastic recovered, rinsed in distilled water, dried at 55° C., and weighed. The results summarized in figure 102 are shown as medians (Md.) of the rate of shell deposition per cm.^{2} per day, and as lower (Q_1) and upper (Q_3) quartiles.

The curves show two periods of accelerated shell growth in Woods Hole water, one in May to June and another in October, and no shell growth during winter from December to the end of April when the temperature of the water varied between 1° and 2° C. These observations are in agreement with many field data and with the experiences of practical oyster growers of the North Atlantic states, who found that oysters grow more rapidly in the spring and in the autumn and cease to grow when the water temperature drops to about 5° C. The relatively low rate of shell deposition during the summer is attributable to the inhibitory effect of fully developed gonads. Observations frequently made in the Woods Hole laboratory show that shell growth in the winter will begin within 24 hours after the transfer of oysters from the harbor to much warmer sea water in the laboratory.

Under normal conditions no shell is deposited in winter. In several instances, however, large amounts of shell material were secreted over an area of the mantle which was apparently injured by the insertion of plastic. One of these cases is shown in figure 103. In this oyster a heavy pocket of shell material was deposited on the valve over

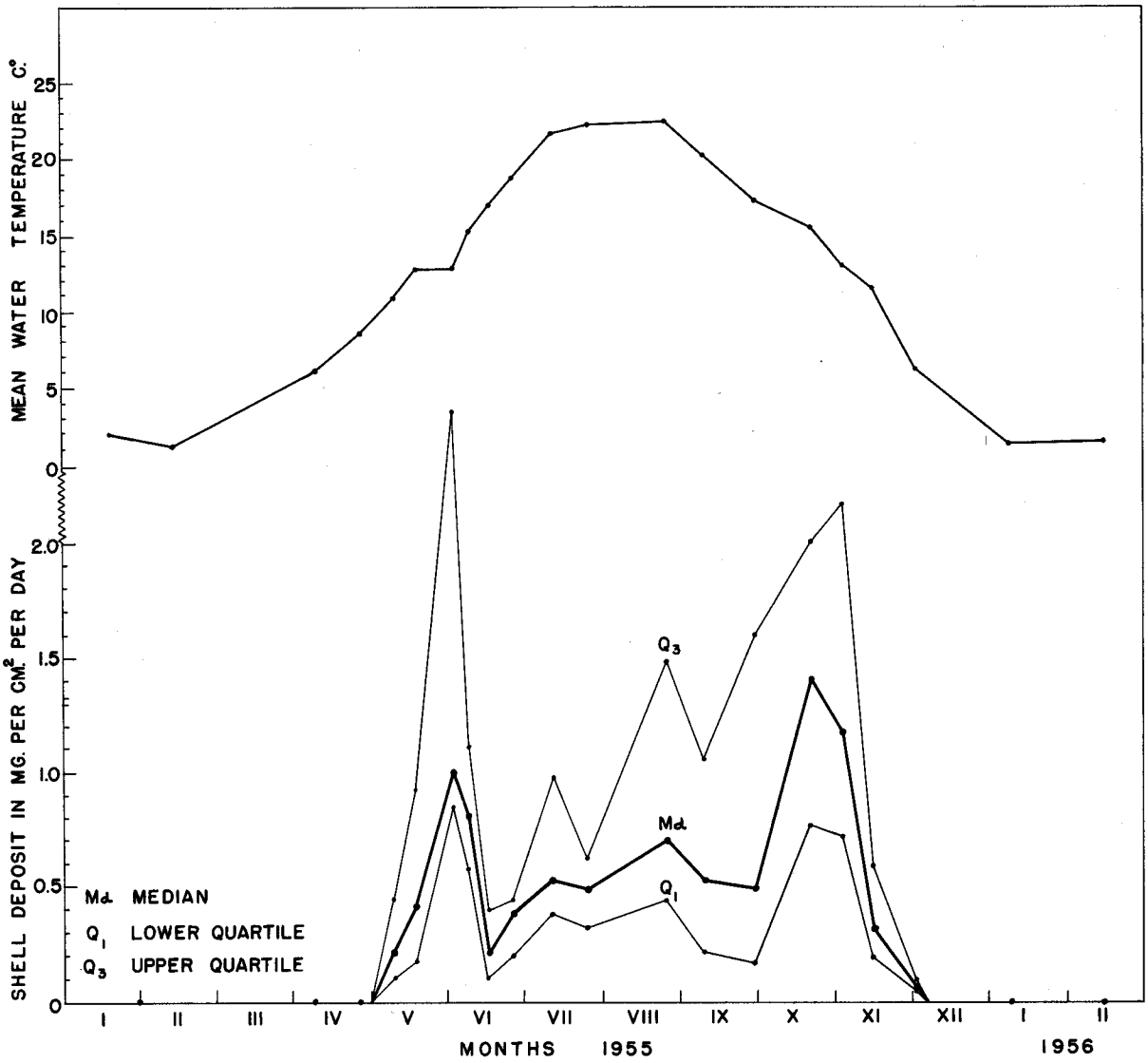

FIGURE 102.—Seasonal changes in the rate of deposition of shell material over the inner surfaces of left (lower) valves.

the area occupied by a piece of plastic, and a shell ridge was formed along the edge of the mantle, which was withdrawn a considerable distance back from its normal position. It can be deduced from these observations that injury to the mantle stimulates the shell secretion and that deposition may take place even at low temperatures when normal shell growth is inhibited. This would indicate that the enzymatic system involved in shell deposition is always present and may become active in response to pathological conditions in spite of the inhibitory effect of winter temperatures.

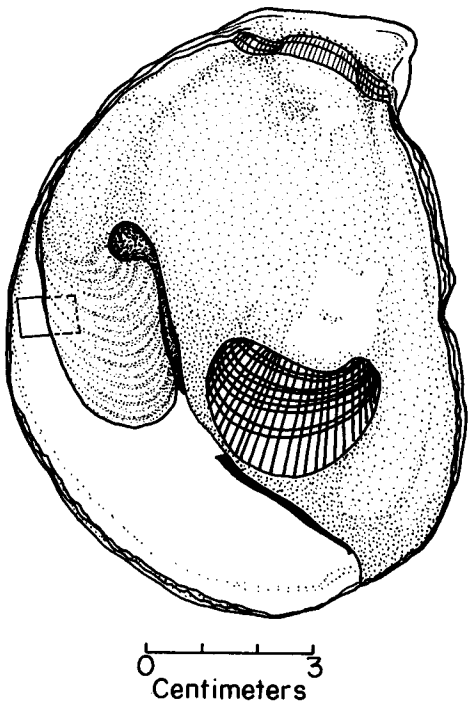

FIGURE 103.—Abnormal deposition of shell material along the edge of the mantle (black line) and over the piece of plastic quadrangle, which was completely encapsulated in a pocket of newly secreted shell. Mantle is shown by stippled area. Winter observation at Woods Hole.

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