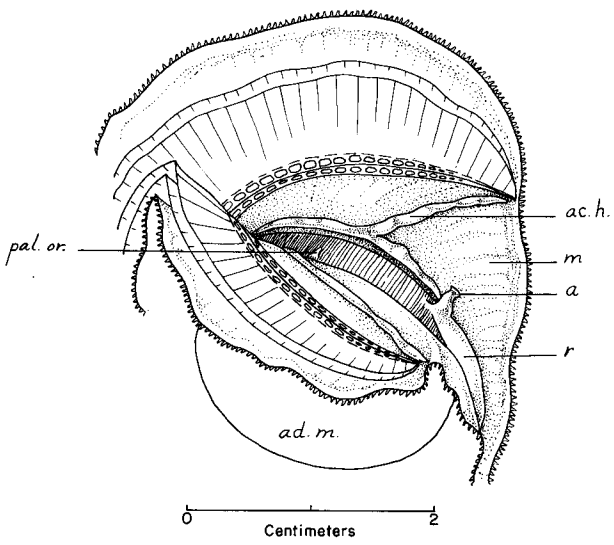

FIGURE 236.—The position of the accessory heart on the left of the cloacal wall of *C. virginica*. The epibranchial chamber was dissected, and the demibranchs of the right and left side pulled apart to expose the ventral side of the adductor muscle. The oyster was fully narcotized. The accessory hearts on both sides were fully expanded (only the part of the right accessory heart is shown). a.—anus; ac.h.—accessory heart on the left side; ad.m.—adductor muscle; m.—mantle; pal.or.—pallial organ; r.—rectum.

wall of the cloaca to the pallio-branchial junction (p.br.j.).

Under slight mechanical stimulation the delicate wall of the accessory heart collapses and the structure becomes invisible. This explains why the earlier investigators did not recognize it as an active organ and mistook it for ridges on the inner wall of the mantle (Rawitz, 1888; Kellogg, 1892).

The structure of the accessory heart of *C. virginica* (fig. 238) resembles that of the arteries of the mantle. The walls have a well-developed layer of longitudinal and circular muscles, but the endothelium lining is indistinct and is probably absent.

The pulsation of accessory hearts of *C. virginica* observed in winter at the Woods Hole laboratory was very irregular, not exceeding two to three times per minute at room temperature of 20° to 22° C., and was independent of the heart beat. During the summer the rate of contraction was six to seven times per minute. Hopkins (1934) states that in *C. gigas* the accessory heart beat at a slower rate than the average heart rythm of this species and the frequencies for right and left organs averaged 6.0 and 7.5 times per minute respectively.

The connection of the accessory heart to other vessels was studied by the following method of injection. Live oysters were kept for 24 to 48 hours in a refrigerator, then placed overnight in cold sea water with 5 percent magnesium sulphate. About 2 ml. of lithium carmine was injected, using the finest hypodermic needle. The preparation was rapidly rinsed in fresh water and immediately immersed in 95 percent ethanol, which precipitated the dye. The injected material remained inside the vessels and was not diffused or washed away by dehydration and clarifying agents (cedar oil or xylene). In this way several permanent preparations were obtained.

Dye injected into the ventral branch (fig. 237, v.br.) penetrated some distance into the circumpallial arteries of the right and left mantle lobes and into the small branches and capillaries of the efferent vein of the gills (ef.v.). The dorsal branch (d.br.) was found to extend along the wall of the cloaca: it does not "disappear into the excretory organs," as stated by Hopkins, but extends under the renal sinus to the dorsal part of the cloacal wall. The ramifications of the branch end in a number of capillaries which connect them with the dorsal portion of the efferent vein. The third or posterior branch (p.br.) follows the ventro-lateral border of the adductor muscle and gives many ramifications inside the cloacal wall.

Blood carried by the ventral branch of the accessory heart enters the pallial artery against the pressure produced by the principal heart. Under these conditions its penetration inside the artery must be limited, and at the end of the contraction wave some of the blood probably re-enters the branch. Movement of the blood inside the circumpallial artery can not be seen, but through the thin wall of the accessory heart one can observe the flushing of blood cells back and forth. Ramifications of the ventral and dorsal branches form capillaries which are in direct connection with the side vessels of the efferent vein. It can be assumed from the direction of the contraction waves that blood from the accessory heart moves toward the efferent vein of the gill and that part of the blood is flushed back as the impulse wave progresses along the wall of the branch.

Oscillation of the blood in the mantle is the primary function of the accessory hearts. Their oscillatory movements facilitate the gaseous exchange and provide a means for efficient respiration. The location of the accessory hearts con-

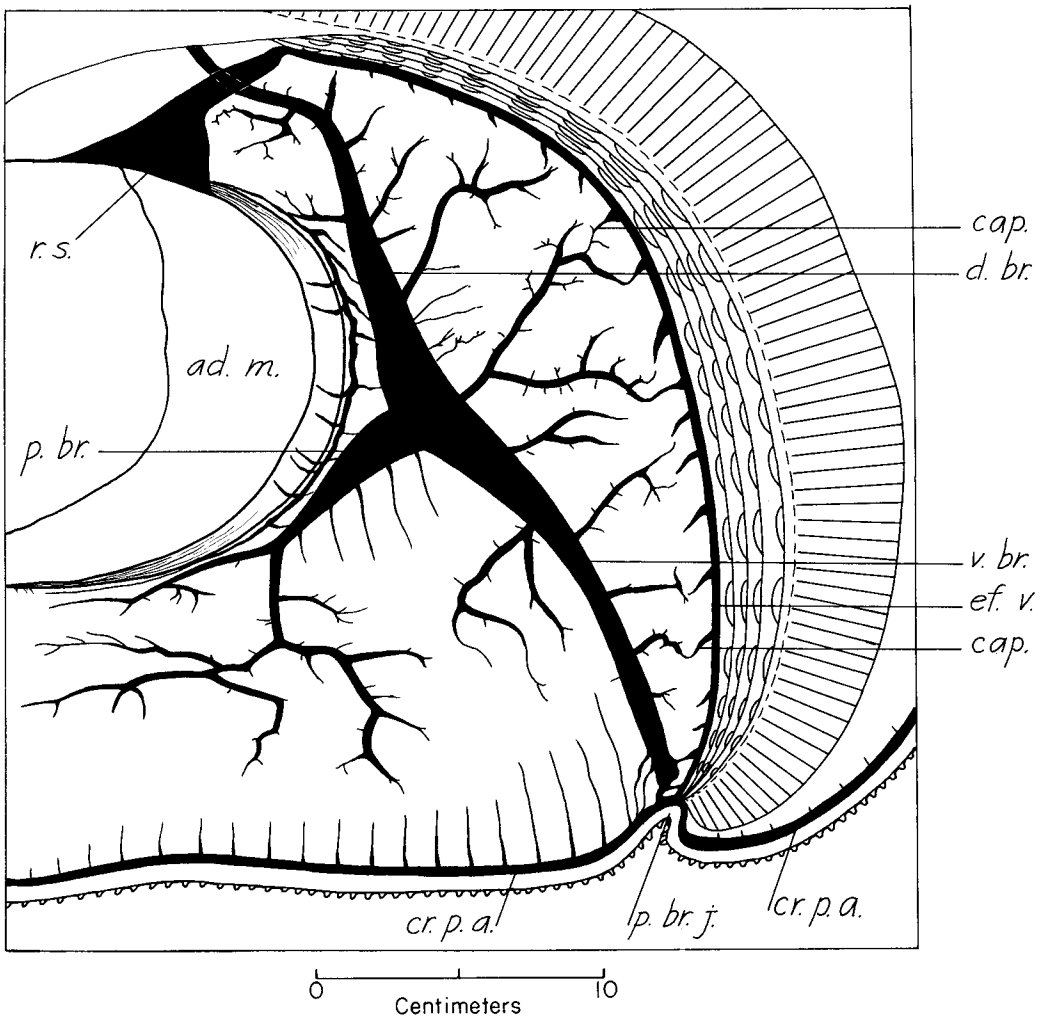

FIGURE 237.—Accessory heart of *C. virginica*. Drawing made from an injected preparation. ad.m.—adductor muscle; cap.—capillaries; cr.p.a.—circumpallial artery; d.br.—dorsal branch; ef.v.—efferent vein; p.br.—posterior branch; p.br.j.—pallio-branchial junction; r.s.—renal sinus; v.br.—ventral branch.

firms the opinion that the mantle and the wall of the cloaca play significant roles in the respiration of oysters.

The pulsation of the accessory hearts makes it possible for the blood of the pallial sinuses to enter the branchial efferent veins or to be forced into the gills through the lateral afferent veins. The pacemaker system and the nervous control of the accessory hearts have not yet been studied.

THE BLOOD

There are two distinct groups of blood corpuscles in bivalve mollusks, the hyaline cells and the granular amoeboid cells. The latter are frequently called granulocytes because of the large number of granules in their cytoplasms, or amoebocytes and phagocytes because of their ability for amoeboid movements and phagocytosis. The hyaline cells are not entirely devoid of granules but they are very sparse. These cells also display amoeboid movement but are much less active than the granular cells. Both types of cells are present in the oyster.

Samples of blood for examination may be obtained by puncturing the pericardial wall with a fine glass pipette and drawing the desired volume of blood. In the same manner blood may be obtained directly from the ventricle or auricles. Some blood cells are always present in the shell liquor and on the surfaces of the gills and mantle. A fair sample of cells can be obtained by scraping these tissues with cover slips or by drawing the pipette along them. For examination of live

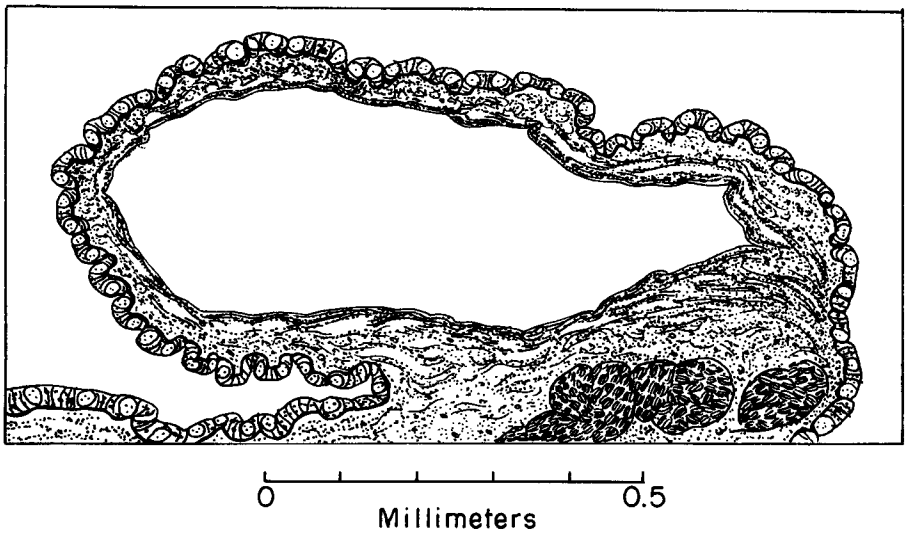

FIGURE 238.—Transverse section of the accessory heart of *C. virginica* preserved in widely expanded state. Kahle, hematoxylin-eosin.

cells the sample may be placed in a moist chamber or a small quantity of blood may be dropped in a glass dish with sea water of the same salinity from which the oysters were taken. Under these conditions the cells of *C. virginica* may remain alive for about 6 days and can be used for studies or classroom demonstration (Breder and Nigrelli, 1933).

For smear preparations drops of blood should be left on slides until the cells begin to expand. When a desired state of expansion has been attained, the preparation is fixed in Bouin III for a few minutes or in chromic or osmic acid (liquid or fumes). Satisfactory preparations may be obtained by using Romanowsky's, Leishmann's, Giemsa's, and McNeal's tetrachrome stains made in a solution of absolute methyl alcohol. These reagents fix and stain the cells in one operation.

COLOR OF BLOOD

The blood of the oyster is colorless and contains no respiratory pigments such as the hemoglobin in vertebrates or hemocyanin found in snails and cephalopods. In semipopular books on oysters a statement is sometimes found about the presence of hemocyanin in oyster blood. To clarify this question, a composite sample of blood and pericardial fluid was collected from six adult *C. virginica* and submitted for spectrophotometrical analysis, which was kindly performed in George Wald's laboratory at Woods Hole. The following is the report received from Wald:

"The pH (of the sample) was 7.33. The absorption spectrum showed specific absorption in the visible region corresponding to the hemocyanin band at about 570 mμ. Hemocyanin possesses also a very high, sharp absorption peak at about 340 mμ., some 20 to 30 times as intense as the absorption in the visible spectrum. This therefore constitutes a very delicate test for the molecule. This also did not appear in the spectrum though a small band was found at lower wavelengths, at about 327 mμ.

"The 570 and 340 mμ. absorptions are found in oxyhemocyanin; both are abolished in the reduced condition. As an added test therefore this sample of oyster blood was reduced with sodium hydrosulfite. The ultraviolet absorption at about 327 mμ. instead of being depressed, rose greatly. I do not know what this substance is, but quite certainly it is not hemocyanin."

THE HYALINE CELLS

These cells with clear cy ɜm containing but few granules are of uniform shape, varying only in size from 5 to 15 μ. When examined alive they are usually spherical (fig. 239), have a distinct cell membrane, and are of pale yellow-green color. Because of their high refractive quality they stand out sharply in the field of view of the microscope. The slow movement of the cells can be noticed if the preparation is watched intently for 30 minutes or longer. One of these cells, under continuous observation in the Woods Hole laboratory for 45

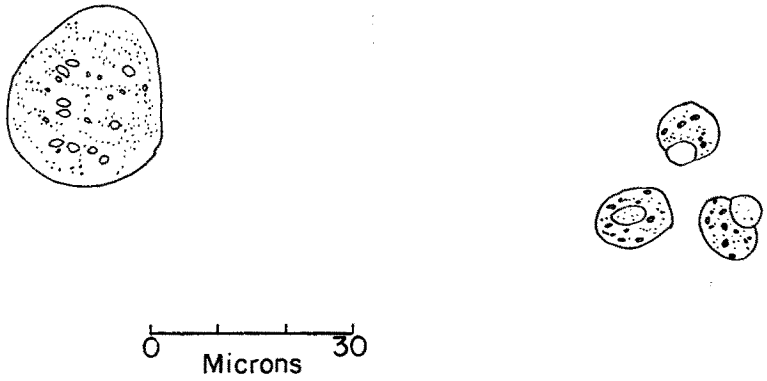

FIGURE 239.—The hyaline blood cells of *C. virginica*. Very small cells on the left; normal cells on the right. Camera lucida drawing of live cells on glass.

minutes changed its shape four times from round to oval and back again. The movement is extremely gradual and consists mainly in bulging of one side of the body. The nucleus is not visible in the live cells and rarely can be seen in stained preparations. The cells are basophilic, staining reddish-purple with Romanowsky's stain. The nucleus stains the same color as the cell.

The hyaline cells comprise about 40 percent of the total number of blood cells in a sample. This is an average of a number of samples taken from the oysters of Long Island Sound and of Chesapeake Bay in which blood was drawn from the pericardium, heart, and shell liquor. In the oysters in good, healthy condition, the proportion of hyaline cells varied from 25 to 64 percent, but the differences were not consistent and did not seem to be affected by the origin of the oysters or by the part of the body from which the sample was taken.

THE GRANULAR CELLS

The granular cells or the amoebocytes vary greatly in shape, size, and behavior. This undoubtedly is due to their pronounced ability for amoeboid movement. In live contracted state they measure about 6 μ in diameter, but they expand and spread to a much larger size. When fresh blood drawn from the oyster by a pipette is spread on a glass slide, many blood cells form aggregates or clumps. This aggregation or agglutination results from the adhesiveness of the cell membranes, which stick on contact with one another (Drew, 1910). In a quiescent stage the cells are usually round and motionless. In about half an hour they begin to expand and separate from the clump. By the end of the first hour the amoeboid movement becomes active and the cells disperse themselves and form concentric rings around the clump.

The cytoplasm and the granules of a moving amoebocyte (fig. 240) flow slowly from the center of the cell out to the edge and push the cell membrane out, forming a pseudopodium. During the formation of very narrow pseudopodia the cytoplasm appears to flow out with the granules arranged in single file. Contraction seems to be affected all at once over an entire cell area, and the action can be quite sudden. In withdrawing, the cytoplasm sometimes leaves behind it a colorless and empty membrane. Fine hyaline projections called "bristle pseudopodia" (fig. 240, right) may remain extended from the membrane and some can be traced back to it. This seems to confirm the argument of Goodrich (1920) that the bristle type pseudopodium is a fold or thickening in the membrane and not a physiologically active part of the cell body.

Clots of blood cells are often observed in injured blood vessels and the connective tissue surrounding small arteries of the mantle, and can be produced by intercardiac injection of tissue extracts. Infiltration of connective tissue by amoebocytes and intravascular blood clots is usually found in watery green oysters from polluted water (fig. 241).

There is no true coagulation of the oyster blood. The coalescence and clot formation of blood cells outside of the body is the result of the entanglement of amoebocytes by the bristlelike pseudopodia or by the strands of hyaline ectoplasm (fig. 242).

The granules of live amoebocytes are usually yellowish-green, with the color much more pronounced in green oysters. The staining affinities of blood cells have been studied by several in-

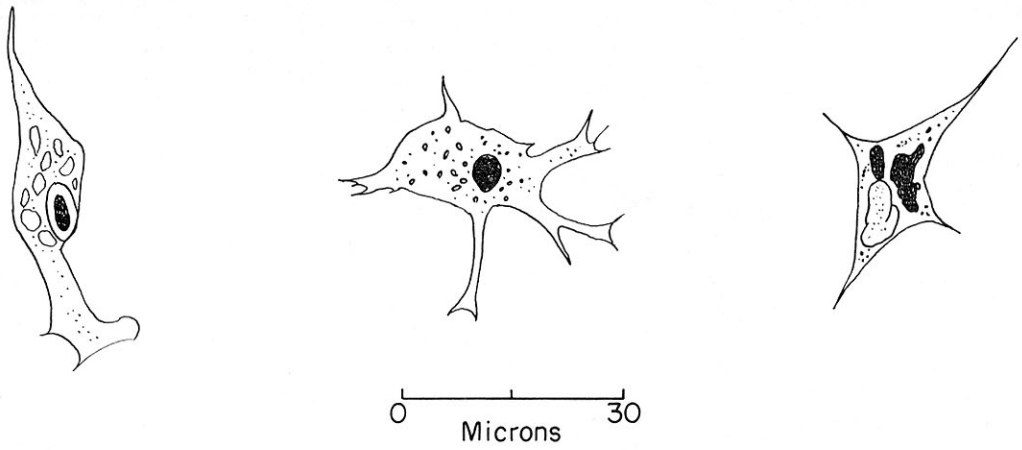

FIGURE 240.—Amoebocytes (granular cells) of *C. virginica* observed in vivo. Camera lucida drawings of live cells on glass.

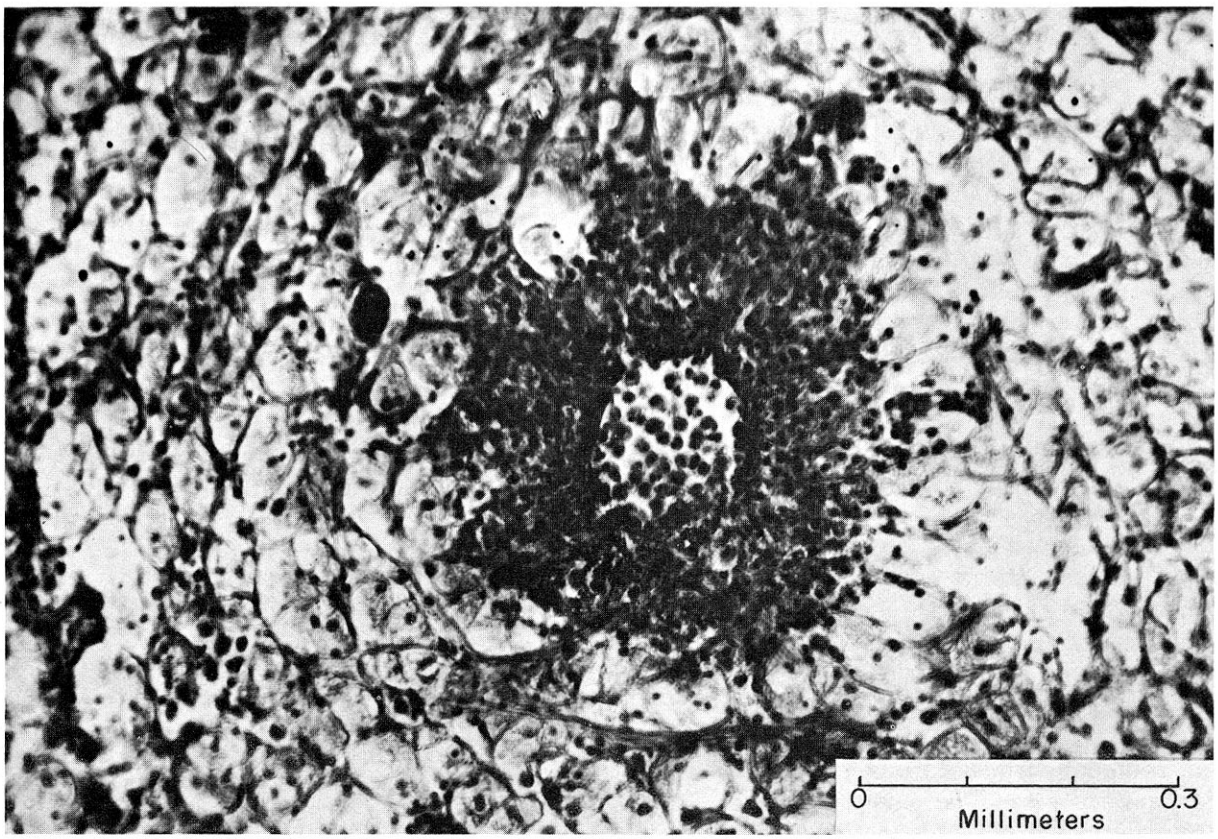

FIGURE 241.—Intervascular blood clot and infiltration of amoebocytes in the mantle of green *C. virginica*. Bouin, hematoxylin-eosin.

vestigators with somewhat different results. Kollmann (1908) found that marine lamellibranchs have acidophilic granules, while those of fresh water mollusks are amphophilic. The granules of *O. edulis* (Takatsuki, 1934a) are neutrophilic with a tendency to become stained vitally by basic dyes. The amoebocytes of *C. circumpicta* (Ohuye, 1938) have eosinophilic or amphophilic cytoplasm and basophilic granules. In a blood smear preparation of *C. virginica* examined in the Bureau's shellfish laboratory the granules stained reddish-purple to dark blue with polychrome

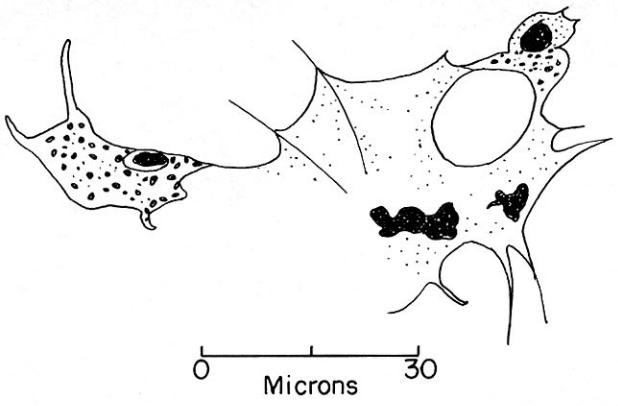

FIGURE 242.—Beginning of coalescence of blood cells of *C. virginica*. Camera lucida drawings of live preparations.

methylene blue mixture (Ramanovsky stain). Methylene blue alone stained the granules very poorly. In Ehrlich triacid stain a few granules were blue, indicating a neutrophilic reaction. In my preparations the blood cell granules never took up eosin, which is very acid stain.

The oval-shaped nuclei of the amoebocytes can be seen easily in a stained preparation. The nucleus is usually located slightly off the center of the cells in a pocket devoid of granules.

Some of the amoebocytes accumulate iron, copper, zinc, and manganese. The presence of heavy metals can be detected by treating the sectioned tissues with ammonium sulfide, which blackens the metals inside the cells (see: chapter XVII).

The following enzymes have been found in extracts of amoebocytes: amylase, glycogenase, lipase, protease, and a complete oxidase system (Yonge, 1926; Takatsuki, 1934a).

Phagocytic activity of amoebocytes is very pronounced. It can be demonstrated by injecting into the mantle or gill cavity various suspensions such as olive oil (stained with Sudan), carborundum, colloidal carbon, carmine, saccharated iron oxide, and cultures of diatoms or *Chlorella*. Some of the suspended particles may be picked up by the amoebocytes which are always present on the surface of the gills and the mantle. Ingestion of iron particles was observed in the Woods Hole laboratory by adding a suspension of iron saccharate to the shell liquor and treating the samples of tissues or smears with ferricyanide solution to produce Prussian blue reaction. Phagocytosis can also be observed in live amoebocytes placed in sea water on glass slides. Frequently under this condition the amoebocyte approaching a bacterium reverses its movement and turns aside. The cause of this failure of phagocytosis has not been determined. According to Bang (1961), who described the phenomenon in *C. virginica*, it was impossible to assign the failure to a particular combination of bacteria and amoebocytes because repeated observations gave inconsistent results. He concluded that there was probably an undiscovered factor in phagocytosis in oyster blood which was responsible for this variation in behavior.

Tripp (1960) found that various species of living bacteria and yeast cell injected in the tissues of *C. virginica* were rapidly destroyed extracellularly and by phagocytes. Bacterial spores were removed from tissues at a much slower rate.

At the beginning of phagocytosis of an uniflagellate bacterium, observed by Bang with the electron microscope (fig. 243), many filamentous pseudopods extend from the cell's surface and entangle the flagellum which is coiled around them while the bacterium remains outside the amoebocyte's body.

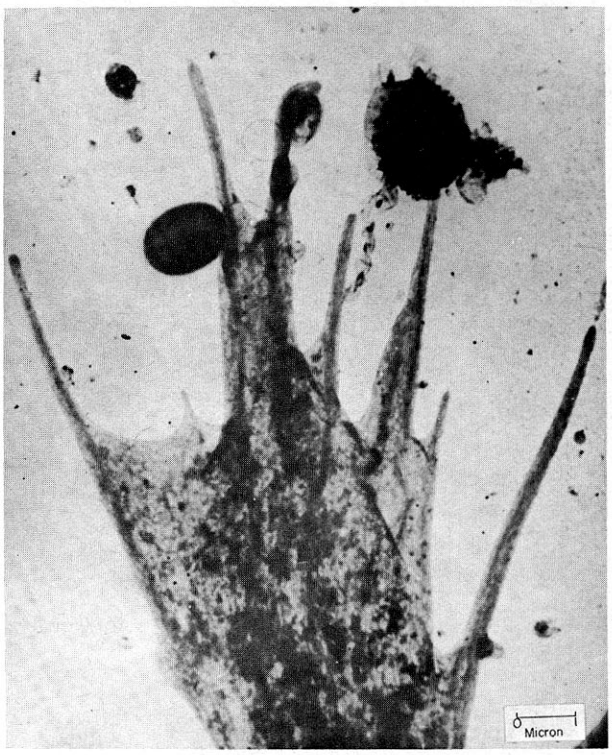

FIGURE 243.—Electron micrograph of a periphery of one amoebocyte which spread out on a collodion film and was fixed with osmium vapor. Courtesy of F. B. Bang.

SPECIFIC GRAVITY OF BLOOD

The osmotic pressure of body fluids of bivalves is about equal to that of the surrounding water so it may be expected that the specific gravity of blood approximates that of the water. For determining the specific gravity of blood or of pericardial fluid, the falling drop method of Barbour and Hamilton (1926) has been used. The procedure consists of timing a drop of fluid of uniform size as it falls a distance of 30 cm. through a mixture of xylene and bromobenzene in a vertical glass tube of exactly 7.5 mm. in diameter. The time is recorded with a stopwatch accurate to one-tenth of a second. The speed of falling of a drop of the sample is compared with that of a drop of the same size of standard potassium sulfate (K_2SO_4) solution of known density. By using an alignment chart (supplied with the instrument), correction is made for room temperature; the specific gravity of the sample can be calculated with an accuracy of 1 times 10^{-4}. The source of error caused by variations in the size of drops is minimized by using an automatic Guthrie pipette controller. The method is simple, rapid, and gives consistent results. In this way the specific gravity of blood was determined for oysters taken from various environments.

A series of tests was also made to record changes that occurred in oysters placed in diluted sea water and in those exposed to air. The blood collected from the ventricle with a glass pipette was centrifuged for 20 minutes at 1,200 r.p.m. to separate blood cells from plasma. For brief storage the sample of plasma was kept in a paraffin coated container from which portions were taken for determination. Observations were made at the time of full sexual maturity of the oysters in the middle of July and were repeated 2 weeks later at the completion of spawning. All tests were made at 22° C. and salinity 31.0–31.5 °/₀₀. The oysters were collected from Wellfleet Harbor, Mass., but remained in the laboratory tanks for about 3 weeks before the tests. The specific gravity of blood during the July 15 to 18 period varied from 1.0252 to 1.0262; in the tests made after spawning between July 28 and 31 the specific gravity of blood varied from 1.0258 to 1.0259. The results are close to those reported by Yazaki (1929) for *O. circumpicta* in which the specific gravity of blood in the summer specimens varied between 1.025 and 1.029.

No significant changes were found in the blood of oysters kept for 72 hours in the refrigerator at temperatures varying from 4.5° to 7.5° C. At the end of the test the specific gravity of the blood of the refrigerated mollusks was 1.0258; and in the controls which were kept in running sea water at 21° to 22° C. the blood was 1.0259.

A gradual decrease in specific gravity occurred in the oysters kept in running sea water of diminishing salinity. The results of this experiment are shown in table 31.

In highly diluted water shell movements of some of the oysters were abnormal and most of the time they remained closed. In these oysters the specific gravity of the blood after 72 hours of exposure to salinity of 9 to 12 °/₀₀ was relatively high (1.0138 and 1.0178) compared to the specific gravity of 1.0092 in the oysters which stayed open for more than 50 percent of the total time. It may be deduced from these experiments that the oysters kept in water in which the salinity was reduced from 31–32 °/₀₀ to 16.7–17.7 °/₀₀ attained the osmotic equilibrium of blood in about 120 hours.

TABLE 31.—*Decrease in the specific gravity of cell-free blood of the oyster,* C. virginica, *in water of lowered salinity*

Salinity (°/₀₀)	Time			
	24 hours	48 hours	72 hours	120 hours
31–32, control	1.0259	1.0259	1.0259	1.0259
16.7–17.7	1.0145	1.0143	1.0143	1.0127
9–12	1.0199	1.0103	1.0092	(*)

*Observations discontinued after 72 hours.

SEROLOGY

Serological reactions between several mollusks were studied by Makino (1934), who experimented with the following species: bivalves—*Meretrix meretrix, Paphia philipinarum, Ostrea (Crassostrea) gigas, Arca inflata;* gastropods—*Turbo cernutum, Haliotis gigantea, Rapana thomasiana;* cephalopods— *Sepiella japonica* and *Polypus variabilis.* In these tests the extracts of tissues in physiological saline solution were injected intraperitoneally or subcutaneously into rabbits to obtain the antisera. Injections were repeated for 7 days using doses which increased from 0.5 to 5 grams. One ml. of extract and 0.1 ml. of antiserum were used in performing precipitation tests, and the tube was set aside for 1 hour at 37° C. Positive reaction was obtained with all the species. *Ostrea* antiserum reacted very strongly with *Meretrix* and *Paphia* and less strongly with

Turbo, Haliotis, and *Rapana*. It is interesting to note that *Arca*, which belongs to the phylogenetically low order of Protobranchia, reacted very strongly not only with *Meretrix* and *Ostrea*, but also with the gastropods *Turbo, Haliotis*, and *Rapana*.

Wilhelmi (1944) applied the precipitation reaction to the problem of determining the relationship of the mollusca to other invertebrates. Using a technique similar to that employed by Makino, he made tests between two species of *Busycon, Pecten irradians, Nereis, Limulus*, and *Asterias forbesi* and concluded that, serologically, mollusca are more closely related to annelids than to any other group. At present this work has historical interest only, since it is obvious that no broad speculations about the relationship of various phyla should be made on the basis of a few tests made with only six species belonging to four different phyla.

The existence of serological differences in five bivalves (*Anadara inflata, A. laretu, Pecten yessoensis, Ostrea* (*Crassostrea*) *yessoensis*, and *O. circumpicta*) was demonstrated by Tomita and Koizumi (1951). In this work the serum was obtained by centrifuging the blood withdrawn from the auricles of the mollusk. Antisera were obtained by injecting rabbits with increased doses, starting with 1 ml. and adding 1 ml. each time until 5 ml. were given on the 5th day. Blood was taken on the 9th day after the last injection. In homologous precipitation tests with *C. gigas*, i.e. using the antiserum against the antigen of the same species, positive reaction occurred in 1:16 dilution of antiserum with 1:1280 dilution of antigen.

Finer differences between closely related species were detected by absorption tests. When a cross reaction is obtained in a test of an antiserum of one species against the serum of a related organism, it is assumed that the second organism possesses a chemical substance common with the homologous substances of the first one. If after the absorption the serum still reacts with homologous substance, it is considered that the antiserum contained antibodies to two or more chemical components including the one which is common to both. Using this method Tomita and Koizumi found that absorption with *C. gigas* antigen removed from *C. circumpicta* serum all antibodies for *gigas* but not for *circumpicta*. In another test *circumpicta* removed from *gigas* antiserum all antibodies for *circumpicta* but not for *gigas*. The authors' interpretation is that there are some common antigens between *C. gigas* and *C. circumpicta* but that each also has its own specific antigen. The investigators also found that *Anadara* (*Arca*) has all the antigens possessed by *C. gigas* plus its own specific antigen. This is in accord with the generally accepted view that *Anadara* (*Arca*) is a phylogenetically primitive form. Fresh-water *Anodonta* showed no affinities with any other species tested in this work.

The application of absorption technique enabled Numachi (1962) to show that the four local races of *C. gigas*—Hokkaido, Miyagi, Hiroshima, and Kumamoto—have some antigenic differences that are in accord with their geographic isolation.

Application of serological tests is a very promising method for studies of racial differences among oysters. At present it is not known whether the observed antigenic differences are hereditary characteristics or are caused by differences in local environment and particularly in the diet of oysters from different localities.

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