CHAPTER XV

EGG, SPERM, FERTILIZATION, AND CLEAVAGE

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As early as 6 to 10 weeks after setting, young *C. virginica* of New England waters, then 6 to 8 mm. in height, develop primordial gonads of profusely branching tubules (Coe, 1932a). At this stage the germinal epithelium is a layer of morphologically undifferentiated cells; some of them will transform into larger cells to become ovocytes, i.e., the cells destined to develop into mature eggs. The smaller cells of the epithelium proliferate very rapidly and are recognizable as the male germ line, and eventually develop into spermatozoa. For several weeks the immature, or primary, gonad of an oyster remains nonfunctional and bisexual (ambisexual), for it contains both male and female germ cells which will transform into mature spermatozoa or ova during the following summer. In some individuals the primary bisexual gonad is retained until the second year, a delay which Coe (1932a, 1938) attributes to poor nutrition.

The more rapid multiplication of male germ cells suppresses the development of ovocytes and results in a predominance of males among the 1-year-old oysters and in the appearance of different degrees of intersexuality (predominance of the cells of one sex over the other). In the same brood which contains also distinctly ambisexual oysters there are, however, other young individuals in which the primary gonad develops directly into ovary or spermary. Local conditions on oyster beds apparently influence the tempo of changes. In the warmer waters at Beaufort, N.C., young oysters are more apt to develop directly into females than in the northern cold waters of New England. Coe (1938) found that the proportion of females to 100 males varied at the first breeding season between 37.1 and 48.8 at Beaufort; 5.6 and 24 at Milford, Conn.; and 3.3 and 12.5 at New Haven Harbor. The differences are not consistent with geographical latitude since the female to male ratio at West Sayville, Long Island, N.Y., was 31.2; at Delaware Bay 41.9; and at Apalachicola, Fla., 7.1. It is obvious that these variations cannot be attributed to temperature alone and are probably caused by a combined effect of environmental conditions.

Toward the end of the second breeding season the primary gonad is transformed into a definite ovary or spermary (fig. 291). The gametogenesis, i.e., complete transformation of the primordial germ cells into mature ova (ovogenesis) or spermatozoa (spermatogenesis), is a very complex process. The differentiation is accompanied by rapid multiplication of the new generations of cells which

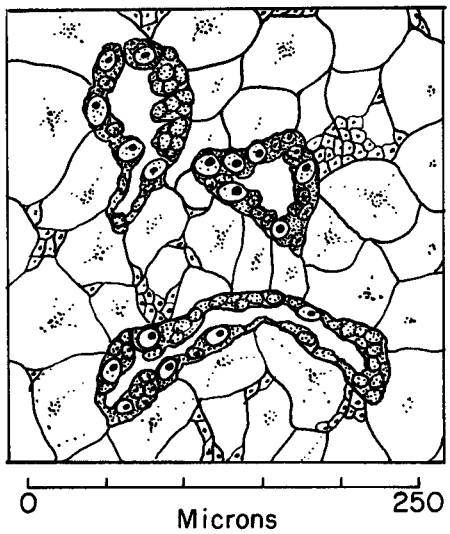

FIGURE 291.—Section of a gonad of *C. virginica* at an early stage of differentiation. End of March, Woods Hole, Mass. The larger, clear cells are ovogonia, the smaller ones are undifferentiated cells of germinal epithelium. Bouin, hematoxylin-eosin.

extend inward and fill up the lumen of the follicles. Early at this stage the sex cells become dense and opaque, a condition which interferes with cytological study.

Gametogenesis of *C. virginica* and *O. lurida* has been studied by Coe (1932a, 1932b, 1934, 1936, 1938) and that of the Australian rock oyster, *C. commercialis*, by Cleland (1947). Only the main points of this process were disclosed by these investigations.

OVOGENESIS

In all animals the primordial germ cells become distinguishable as primary ovogonia in the females or spermatogonia in the males after a certain number of divisions. After a period of quiescence they begin to divide again and give rise to secondary ovogonia or spermatogonia. After several generations the cells stop dividing and enter a growth period, which is more prolonged in the females than in the males. The growth period is characterized by a series of cytological changes, each differing from the preceding stage. The cells which will produce gametes are at this stage called auxocytes, from the Greek "auxesis" meaning growth, and are referred to as ovocytes in the female and spermatocytes in the male.

Ovogenesis in oysters begins with the appearance of enlarged cells in the germinal epithelium. These are the ovogonia, which in *C. virginica* and *O. lurida* are distinguished from other cells of the germinal epithelium by their relatively large nuclei with conspicuous nucleoli and loose chromatin network (fig. 292, og). The ovogonia usually lie next to the follicle wall, and their distal sides do not protrude into the lumen. Differences between the early ovogonia and indifferent residual cells (I) are not conspicuous. Examination of a series of sections and study of the sequence of changes in the appearance and structure of the cells are necessary to assure a positive identification.

After one or two divisions the ovogonia change in appearance as well as in size. This generation of female sex cells called ovocytes can be recognized by the presence of fibrillar mitochondrial bodies (sometimes called yolk nuclei), and by the spiremes of densely packed chromosomes (figs. 293 and 294). Their nucleoli become very conspicuous.

During the last stage of ovogenesis the ovocyte begins to grow rapidly, and the distal part, grossly enlarged and rounded, protrudes into the lumen of a follicle. At the same time the connection

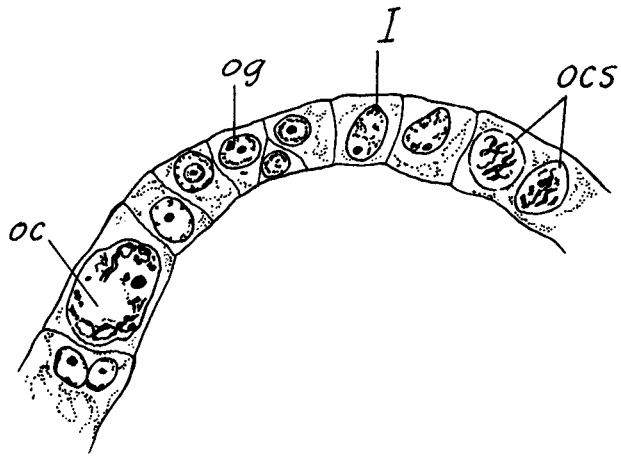

FIGURE 292.—Follicle wall of the ovary of *C. virginica*. I—indifferent residual cell; oc—residual ovocyte; ocs—two young ovocytes in synaptic phase; og—group of ovogonia. Redrawn from Coe, 1932a, fig. 9. Highly magnified.

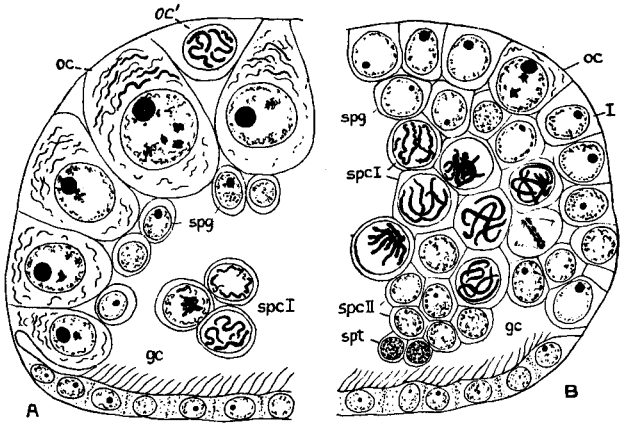

FIGURE 293.—Portions of two follicles of a bisexual gonad of 4-month-old *C. virginica*. A—predominantly female, and B—predominantly male follicle; gc—genital canal lined with ciliated cells; oc—large ovocyte; oc'—young ovocyte in spireme phase; spcI—primary spermatocytes in spireme phase; spcII—secondary spermatocytes; spt—spermatides. Photographically reproduced from Coe, 1932a, fig. 6. Highly magnified.

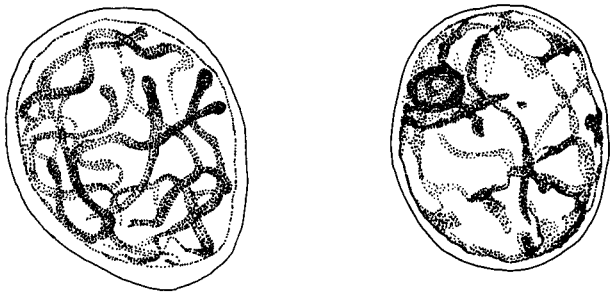

FIGURE 294.—Two young ovocytes at spireme stage in a mature ovary of *C. virginica*. Redrawn from Coe, 1932a, fig. 9. Highly magnified.

with the basal membrane of the follicle wall is narrowed to an elongated stem. The nucleus increases greatly in bulk, and the developing egg assumes a pear-shaped form. An accumulation of dark granules (mitochondria) at the proximal end of the cells may indicate that food for the growing ovocyte is obtained through the wall of the follicle. The granules are not pronounced in the ovocyte of *C. virginica* but are conspicuous in some other bivalves, particularly in *Sphaerium* (Woods, 1932). From the beginning of sexual differentiation to the final maturity of an ovum, the early ovocyte increases in volume more than 3,000 times.

Ovogenesis in the Sydney rock oyster *C. commercialis*, described by Cleland (1947), is somewhat different from the ovogenesis of the American species. At the earliest stage before the start of the growth phase an ovocyte of the rock oyster is a small cell, 4 μ to 5 μ in diameter. Two-thirds of the cell is occupied by a clump of chromosomes surrounded by a rim of clear cytoplasm. Cleland identifies this stage as a definite auxocyte, i.e., an ovocyte just before entry into the growth phase. At ths stage the cell has no nucleolus.

The definite auxocyte begins to grow and passes through three stages (called by Cleland Auxocyte I, II, and III) which differ in size and nuclear structure. Auxocyte I has a diameter of about 9 μ, with a relatively large germinal vesicle (6 μ) and a nucleolus of about 1.7 μ. The nucleus is centrally placed in the homogeneous cytoplasm with an excentric nucleolus which is not in contact with the nuclear membrane. Auxocyte II has diameter of 12.6 μ, with the germinal vesicle (nucleus) about 7 μ and nucleolus 2 μ to 3 μ. The cell is usually spherical with a centrally located germinal vesicle and chromosomes spaced more widely than in Auxocyte I. At this stage a group of granules appears at one pole of the nucleus. Auxocyte III is a spherical cell 20 μ in diameter, with the germinal vesicle measuring 11 μ and eccentrically located nucleolus of about 4.2 μ in diameter. The cell is separated from the wall and is free in the lumen of a follicle. Protein granules are abundant along the periphery of the cell where they are found in a mature egg. The mature ovocyte of *C. commercialis* has a diameter of about 38 μ. The nucleus is large, about 21 μ across; the nucleolus is 4.6 μ. The chromosomes are paired and are usually placed peripherally in the germinal vesicle but not in contact with the nuclear membrane. Crossing-over is frequently seen at this stage but the chromosomes are not coiled.

SPERMATOGENESIS

Spermatogenesis in the oyster is known primarily from the studies by Coe (1931) on the development of the gonad of young *O. lurida*. Comparison with the gonads of *C. virginica* shows that there is close agreement in the general features of the process in both species. Progressive stages of the formation of sperm begin with the undifferentiated gonia which line the inner wall of the gonad follicles. After a large number of descendants have been produced the spermatogonia can be distinguished from the ovogonia by their smaller size and position within the follicles. Ovogonia lie in a single row along the wall; the primary spermatogonia of *C. virginica* are found either singly or in groups between the ovogonia lining the wall and in the lumen (fig. 295).

In the hermaphroditic gonad of *O. lurida* a single primary spermatogonium divides several times to form a cluster of cells which become separated from the follicle wall and occupy a position toward the center of the lumen (fig. 296).

The number of divisions of spermatogonia presumably depends on the amount of nourishment available to the gonad. Coe estimates that in *O. lurida* each primary spermatogonium divides six to nine times to produce a cluster of 64 to 500 cells. In spite of the close contact the adjacent cells of the clusters are separate but are held together by a delicate noncellular secretion. Its

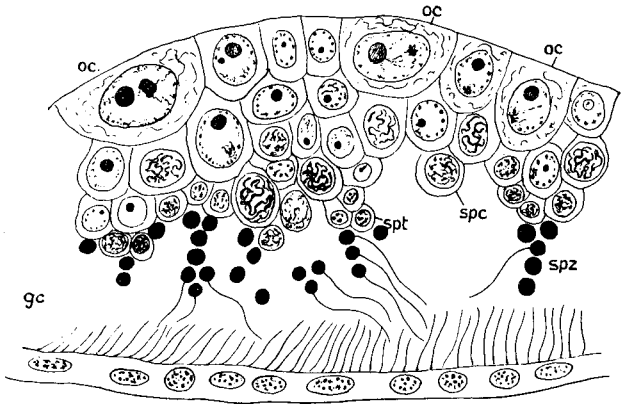

FIGURE 295.—Portion of bisexual gonad of young *C. virginica*. gc—genital canal; oc—ovocytes with spermatogonia filling the lumen; spc—spermatocytes; spt—spermatids; spz—spermatozoa. Photographically reproduced from Coe, 1934, fig. 5A. Highly magnified.

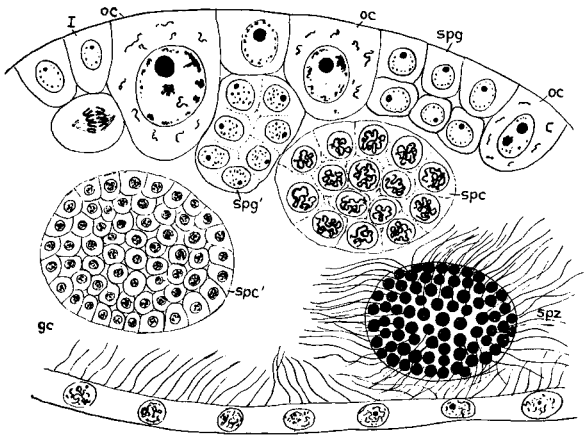

FIGURE 296.—Portion of an hermaphroditic gonad of *O. lurida*. gc—genital canal; I—indifferent cells; oc—ovocytes; spc—primary spermatocytes; spc¹—secondary spermatocytes; spz—spermatozoa united into a sperm ball. Photographically reproduced from Coe, 1934, fig. 5B. Highly magnified.

chemical nature has not yet been determined. In poorly preserved preparations the clusters sometimes have the appearance of syncytia with nuclei embedded in a common matrix. In both species all spermatogonia have a conspicuous nucleolus and loose chromatin reticulum. As the divisions proceed the diameter of the spermatogonia diminishes from about 6μ to 3μ or less at the last stage leading to the formation of primary spermatocytes. In *C. virginica* these cells are globular, each with a large nucleus resolved into slender threads (spiremes). This leptonema stage is frequently observed in the developing spermary but the conjugation of chromosomes (synapsis) has not been described with any detail. However, in reference to the spermatogenesis in *O. lurida*, Coe (1931) states that the leptotene stage "is followed by the usual process of synapsis."

The appearance of secondary spermatocytes is similar to that of the primary. In *C. virginica* they can be distinguished by their radial orientation in the lumen and small size. Different phases of spermatogenesis in *C. virginica* are shown in a semidiagrammatic drawing published by Coe (1932a) and reproduced in fig. 297. Meiotic divisions and the transformation of spermatids into mature spermatozoa have not been fully described for *C. virginica*. In a mature spermary the spermatozoa are always oriented with their tails toward the center of the lumen. The photomicrograph shown in fig. 298 shows the gradual increase in the number of male sex cells from the wall of the follicle toward the center. Successive stages of the spermatogenesis of *O. lurida* drawn by Coe are shown in fig. 299.

Secondary spermatocytes of *O. lurida* are held together in spherical masses. Close contact by the spermatids continues during their transformation into spermatozoa; in the sperm ball of a mature oyster the tails radiate from the center. Each sperm ball is composed of from 200 to 2,000 spermatozoa originating from a single spermatogonium (Coe, 1932b). During mitotic divisions the "prophase, metaphase, and telophase are all of typical appearance, with a delicate spindle of the usual form" (Coe, 1931). Because of the crowded condition of the metaphase and anaphase plates, Coe was unable to determine the chromosome number which he states "is not very large." In two diagrammatic drawings of spermatocyte division Coe (1931, fig. 3, E and F) figures 10 chromosome pairs. This is the most common number of chromosomes found by Cleland at the two- and four-cell stage of cleavage in the fertilized egg of *C. commercialis* (Cleland, 1947). The number of chromosomes seen during the cleavage of *C. virginica* eggs is discussed later (p. 345).

STRUCTURE OF THE MATURE EGG

Eggs in the mature ovary of *C. virginica* are pear-shaped and compressed. Many of them are attached to the follicle wall by long, slender peduncles; others are free in the lumen ready to be moved to the genital canals and discharged (fig. 300). The long axis of the eggs varies from 55μ to 75μ depending on their shape; the width at the broadest part measures from 35μ to 55μ, and the diameter of the nucleus is from 25μ to 40μ. The oblong shape is retained for some time after the discharge of eggs into water but gradually the egg becomes globular and denser. Under the transmitted light of a microscope the nucleus appears as a large, transparent area surrounded by densely packed granules (fig. 301). In a globular egg the nucleus cannot be seen unless it is cleared in glycerol or other clarifying reagents (fig. 302).

Eggs of oysters living under marginal conditions in water of salinity less than 10 °/₀₀ frequently become cytolyzed upon their removal from the ovary; the nuclei appear larger than those of normal eggs. Only 1 or 2 percent of these eggs is fertilizable. The delicate primary or "vitelline" membrane surrounding the unfertilized egg is

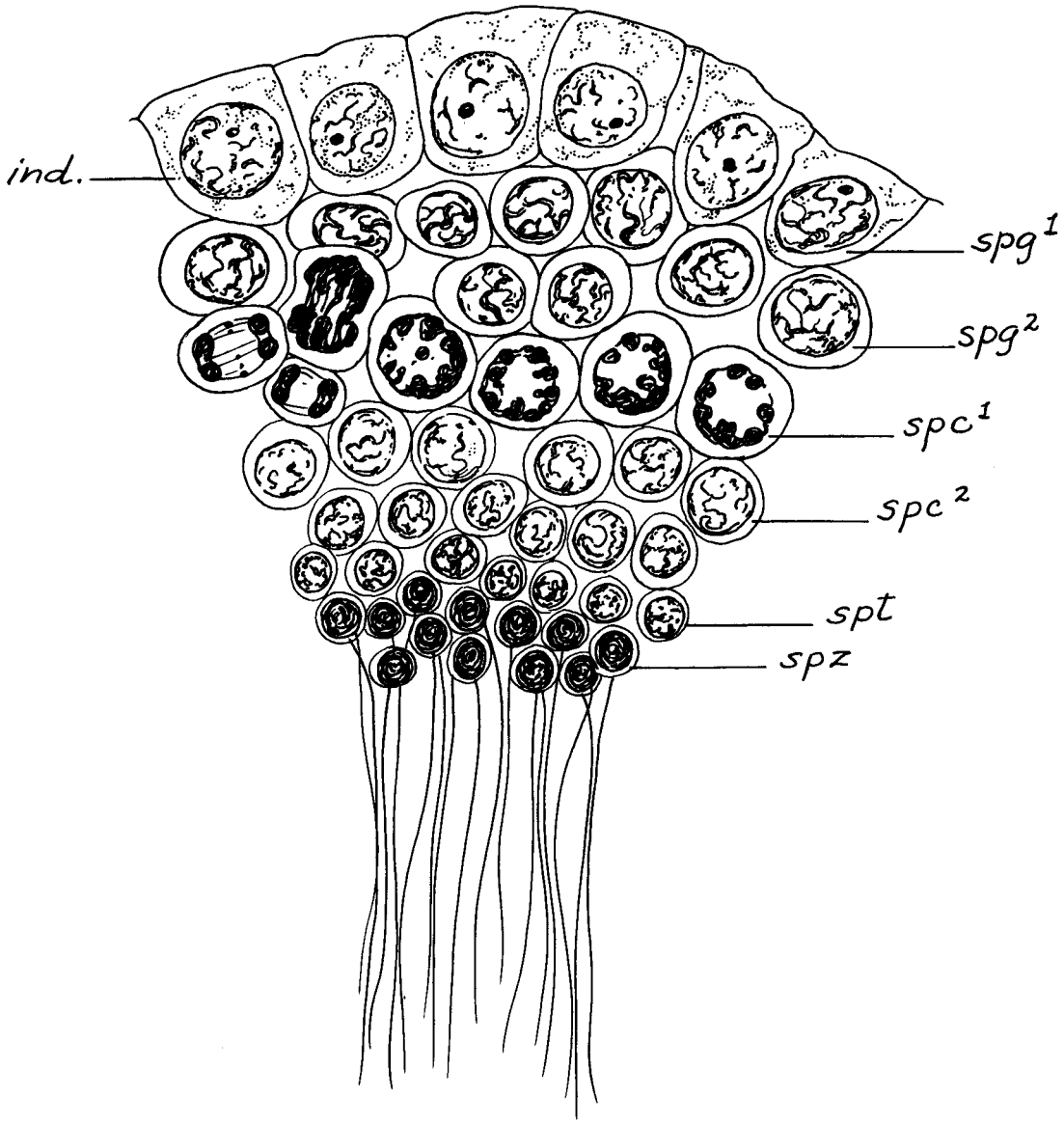

FIGURE 297.—Mature spermary of *C. virginica*. ind—indifferent cells; spg¹ and spg²—primary and secondary spermatocytes; spt—spermatids; spz—mature spermatozoa. Redrawn from Coe, 1932a, fig. 8.

secreted by the egg itself while it is still in the ovary. Raven (1958) states that in some cases the vitelline membranes of *Ostrea*, *Mytilus*, *Dreissensia*, and *Dentalium* are thrown off soon after shedding. I have not seen this happen in the eggs of *C. virginica*.

CYTOPLASMIC INCLUSIONS

Cytoplasmic components of an oyster egg are not well known primarily because the ultrastructure has not been studied by electron microscopy. Certain types of minute granules can be seen, however, in examination of live eggs under high magnification of phase contrast lenses; by applying vital and metachromatic stains; by centrifuging whole eggs or their homogenates in order to separate various components and study their staining reactions. For descriptions of the techniques used in modern cytology the reader is referred to the textbooks on cytology and microscopic histochemistry (Gomori, 1952; DeRobertis, Nowinski, and Saez, 1960; and others). The yolk constitutes the major part of the eggs of marine bivalves. Quantitative data on the amount of yolk in oyster eggs are lacking, but for *Cumingia tellinoides* and *Mytilus californianus* Costello (1939) found that

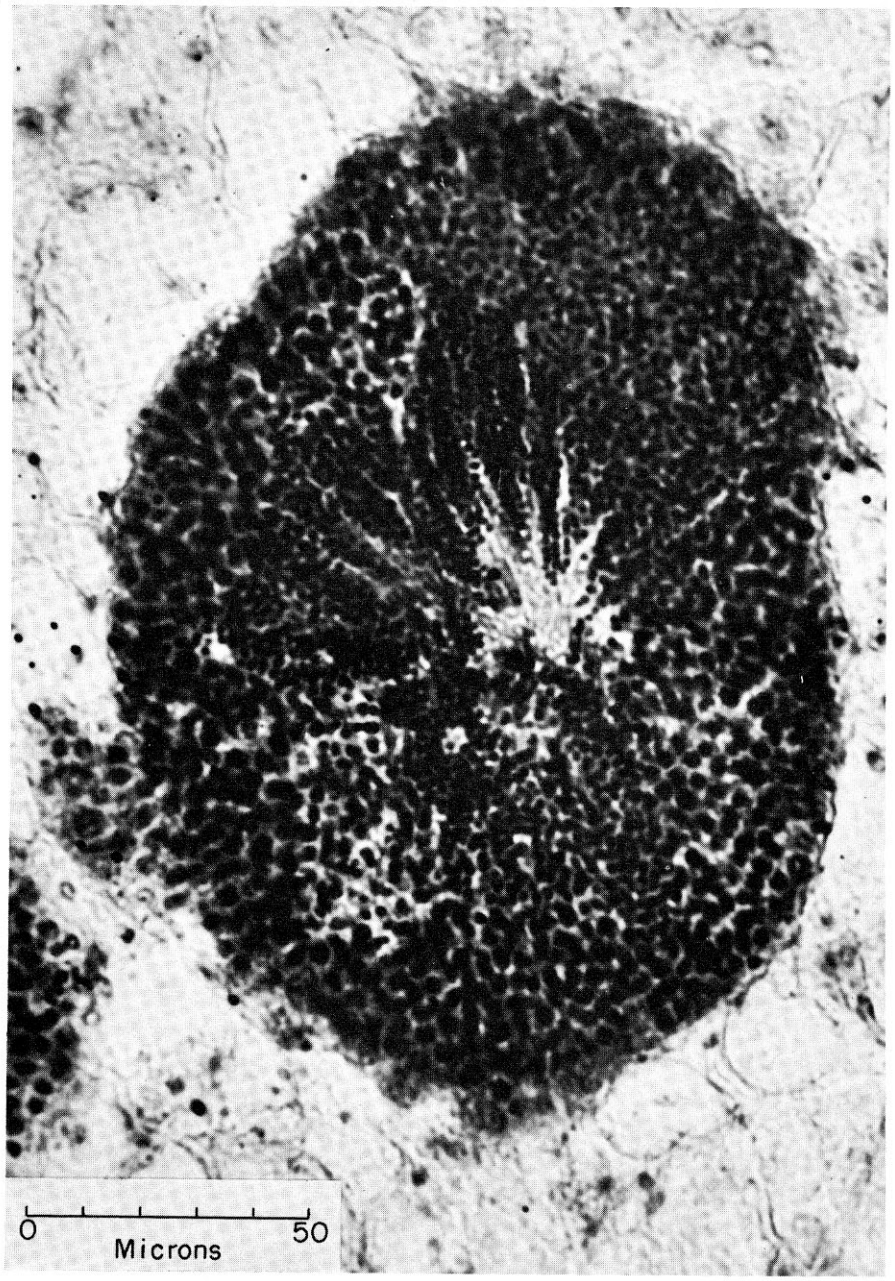

FIGURE 298.—Photomicrograph of a cross section of one follicle of a fully mature spermary of *C. virginica*. Hematoxylin-eosin.

yolk forms 35 and 31 percent respectively of the total egg volume. The estimates were obtained after a centrifugal force of 20,000 (*Cumingia*) and 4,800 (*Mytilus*) times gravity had been applied to the unfertilized eggs. In the cytoplasm of the eggs of the two species the relative volumes of hyaline zone were 42 and 55 percent and of the oil 10 and 14 percent.

The yolk of molluscan eggs is made of two types of granules, one of proteid and the other of fatty materials. In cytological literature the distinction between the proteid yolk and fatty yolk is not always made clear. In descriptions of the cytoplasmic inclusions of an egg based on light microscopy some authors apply the term exclusively to protein granules, while others, including Gatenby (1919), Gatenby and Woodger (1920), and Brambell (1924) in their studies of the

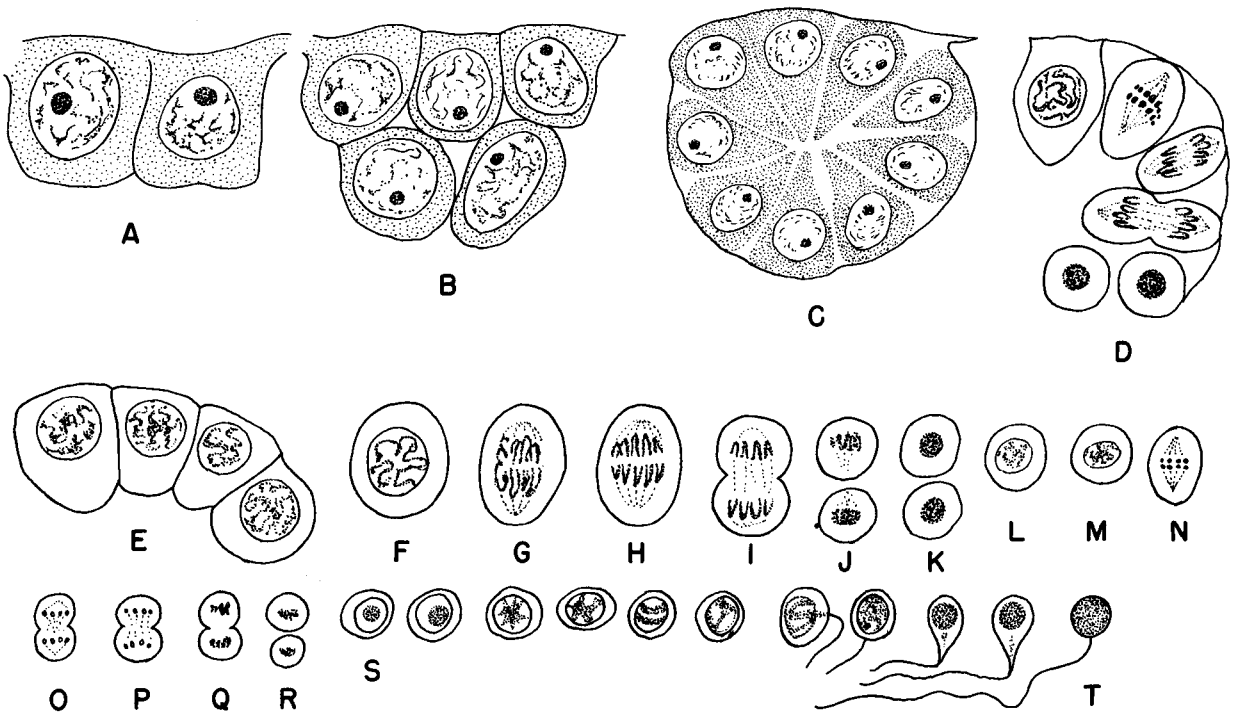

FIGURE 299.—Diagram of successive stages of spermatogenesis in *O. lurida*. A—two indifferent germ cells on the wall of the gonad; B—small group of spermatogonia, with reticular chromatin and conspicuous nucleoli; C—small group of secondary spermatogonia; D—division of secondary spermatogonia to form spermatocytes; E—primary spermatocytes with slender chromosomes; F to K—division of primary spermatocytes; L to R—division of secondary spermatocyte; S to T—transformation of spermatid into the mature spermatozoon. Redrawn from Coe, 1931, fig. 2.

gametogenesis in the gastropods *Helix*, *Limnaea*, and *Patella*, identify the protein granules as "mitochondria" and restrict the term yolk to fatty inclusions.

The role of the mitochondria in the formation of yolk has not been fully resolved. According to Rai (1930), the fatty yolk in the eggs of *C. cucullata* is formed directly from the Golgi vesicles as it is in ascidians, *Helix*, and other invertebrates; mitochondria do not participate in the vitellogenesis, and albuminous yolk is absent in the egg of the Indian oyster (*C. cucullata*). This view is in agreement with the conclusion of Worley (1944), who found no protein yolk in the eggs of *Mytilus* and *Ostrea*. The question is not settled because apparently the cytologists have no clear agreement on the difference between the protein yolk and mitochondria.

A study of cytoplasmic inclusions was made by Cleland (1947, 1951), who separated the granules found in egg cytoplasm of the oyster by differential centrifugation following homogenization. Mature eggs were suspended in a solution of 0.2 M potassium chloride and 0.02 N sodium citrate buffered to pH 7.5. Homogenates were obtained by blending the suspension in an electric blender surrounded by an ice jacket. By centrifuging the samples of homogenates at different speeds the following types of granules were obtained: P granules or protein yolk; L granules or lipid yolk; M granules or mitochondria; and S or submicroscopic granules. The P granules obtained by Cleland's technique are spherical and can be stained by Janus green B in the test tube. In the living egg these granules are located along the periphery and absorb Nile blue stain. After being centrifuged at 5,000 times gravity for 5 minutes they form a thick centrifugal layer with a sharp upper boundary. Alpha or lipid yolk granules are also spherical. They occupy the central part of the living egg. In the centrifuged egg they form a centripetal layer with a sharp lower boundary. Alpha granules of phospholipid and neutral fat can be recovered from the supernatants of homogenate suspensions. M granules or mitochondria in the live centrifuged egg form a thin, rather loose layer above the P granules and stain both with Janus green B and Nile blue.

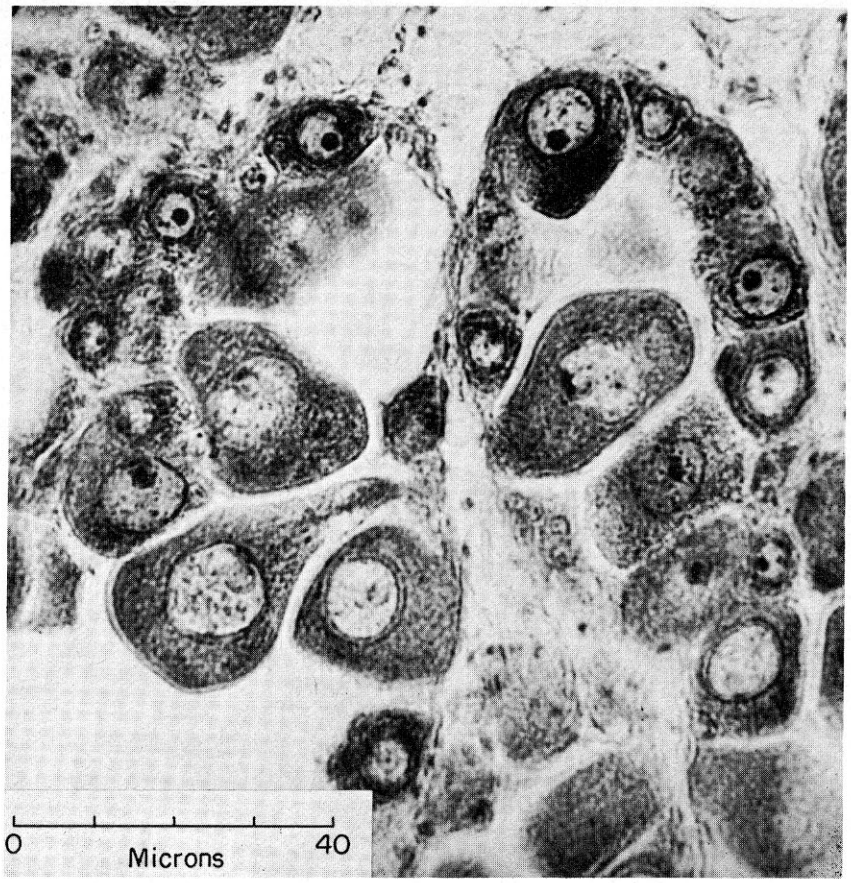

FIGURE 300.—Photomicrograph of eggs in the follicles of the ovary of *C. virginica* at the beginning of the spawning season. Ovocytes and small indifferent cells line the wall; mature eggs are either free or connected to the wall with long peduncles. Kahle, hematoxylin-eosin.

Cleland states that in the uncentrifuged mature egg they are unrecognizable. From homogenates the M granules can be separated by centrifuging for 10 minutes at 10,000 times gravity. Cytoplasm also contains submicroscopic or S granules (according to Cleland's terminology), which are separable by applying a centrifugal force of 20,000 times gravity for 30 minutes. These S granules are probably homologous to mammalian microsomes, i.e., the submicroscopic ribonucleoprotein particles which are considered to be the major sites of protein synthesis (a discussion of this problem is found in Shaver, 1957, and Novikoff, 1961b).

With the exception of pure lipid granules, the cytoplasmic components of the egg of *C. commercialis* show an increasing content of nucleic acid with decreasing size of granules, the ground cytoplasm containing the highest concentration of nucleic acid. Cleland's observations need to be corroborated, using the eggs of different species of oysters.

The formation and composition of yolk in the eggs of animals other than bivalves have been studied by many investigators, frequently with different and sometimes contradictory results. As Brachet (1944) stated nearly 20 years ago, the problem cannot be resolved at present. This uncertainty about yolk and other granules still persists and probably will continue until the ultrastructure of the marine egg is thoroughly explored by electron microscopy.

Examination with the light microscope of ripe, unfertilized, and unstained eggs of *C. virginica* discloses a multitude of tightly packed minute granules in the cytoplasm which obscure the inner portion of the egg. The granules appear to be uniformly distributed around the nucleus (fig.

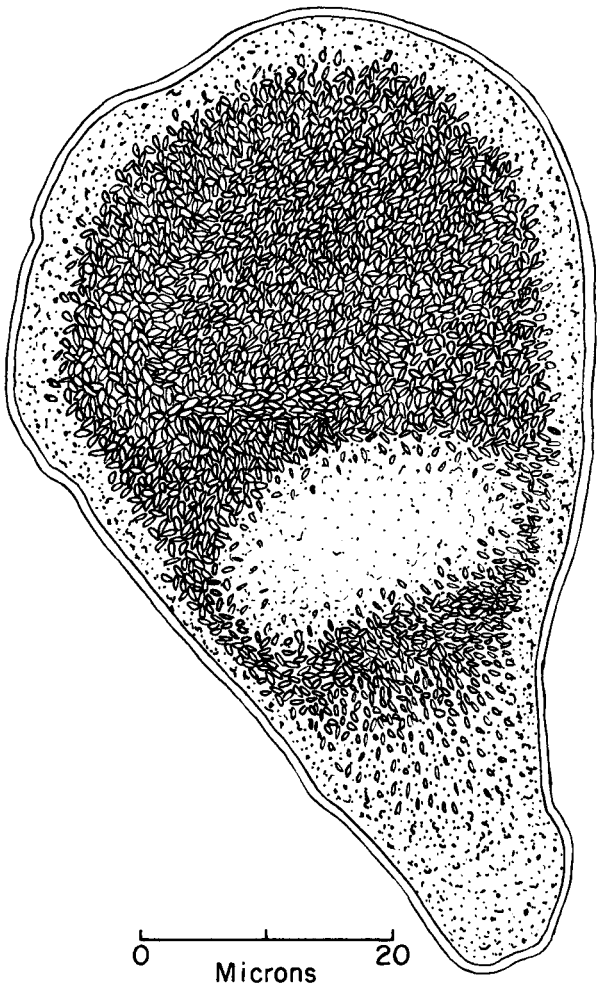

FIGURE 301.—Camera lucida drawing of live unfertilized egg of *C. virginica* in sea water. Germinal vesicle not visible under yolk granules.

301). The eggs are devoid of pigment. Oil globules of different sizes can be made visible under high magnification by gently pressing the egg under a coverslip; by using fat-staining dyes (Sudan II, III, or Black Sudan B) they become conspicuous (fig. 303). Under the effect of dye (dissolved in weak alcohol) the small granules of lipid yolk, stained dark red or black, gradually fuse into large globules and penetrate the vitelline membrane and a slight pressure will force them through it (fig. 304). The size of the globules increases during the time that the preparation remains under the microscope. These artifacts are due to the fusion of globules under the effect of dye.

The mitochondria of *C. virginica* can be stained by a 0.5 percent solution of Janus green in sea water. They appear as small rodlike structures uniformly distributed in the subcortical layer of the egg (fig. 305). The origin of fatty or lipid yolk in *C. virginica* has not been studied. In *Mytilus* eggs the lipid of the yolk apparently arises in an intimate association with the Golgi apparatus (Worley, 1944). In *Lymnaea* (Bretschneider and Raven, 1954) they are formed in certain parts of the protoplasm independently of cell structures visible under the light microscope.

In the eggs of the Bombay oyster, *C. cucullata*, which are similar to those of *C. virginica*, the fatty yolk, according to Rai (1930), is formed directly from the Golgi vesicles. Mitochondria exist in the eggs of this species in the form of very minute granules forming a circumnuclear ring. Later they grow in size and are more or less uniformly distributed. This conclusion is in agreement with the observations of Gatenby and Woodger (1920), who found that in *Helix* and *Limnaea* the Golgi elements gradually spread throughout the ovocyte and probably take part in the formation of yolk bodies. They found no evidence that part of the mitochondrial constituents of cytoplasm metamorphose into yolk.

Oyster eggs placed for 5 minutes in a dilute solution (1 to 25,000 or 1 to 30,000) of toluidin blue 0 and washed in sea water are colored metachromatically. Pasteels and Mulnard (1957) found

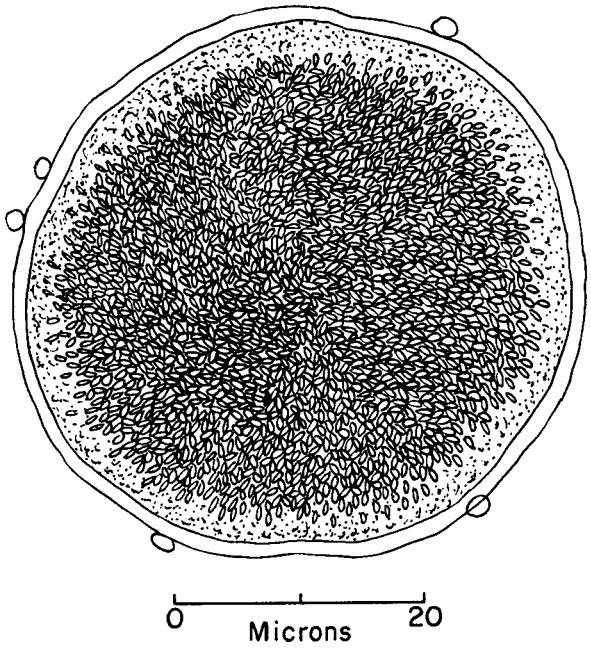

FIGURE 302.—Camera lucida drawing of live egg of *C. virginica* a few minutes after fertilization.

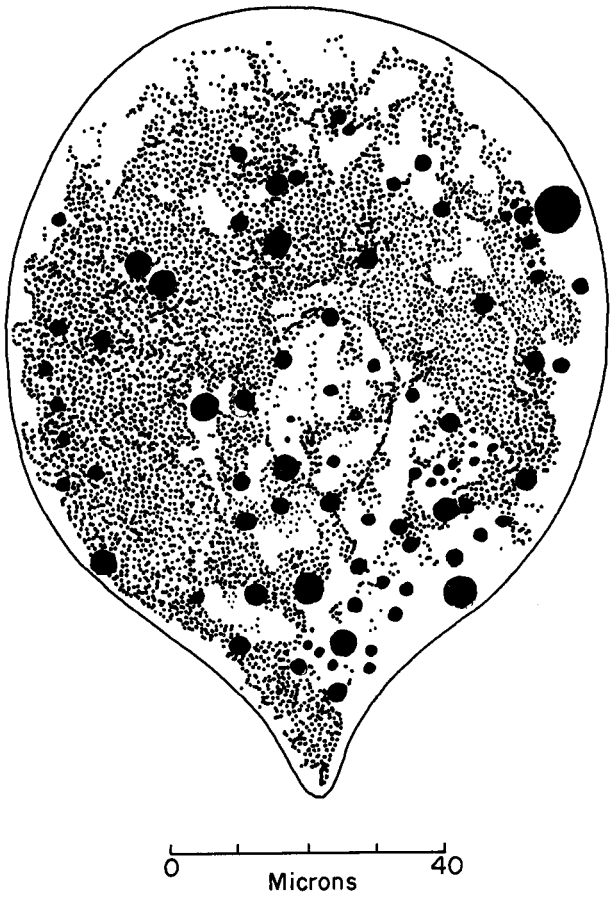

FIGURE 303.—Oil globules in the unfertilized egg of *C. virginica* after staining in Sudan III. Whole mount. Drawing made from a photomicrograph.

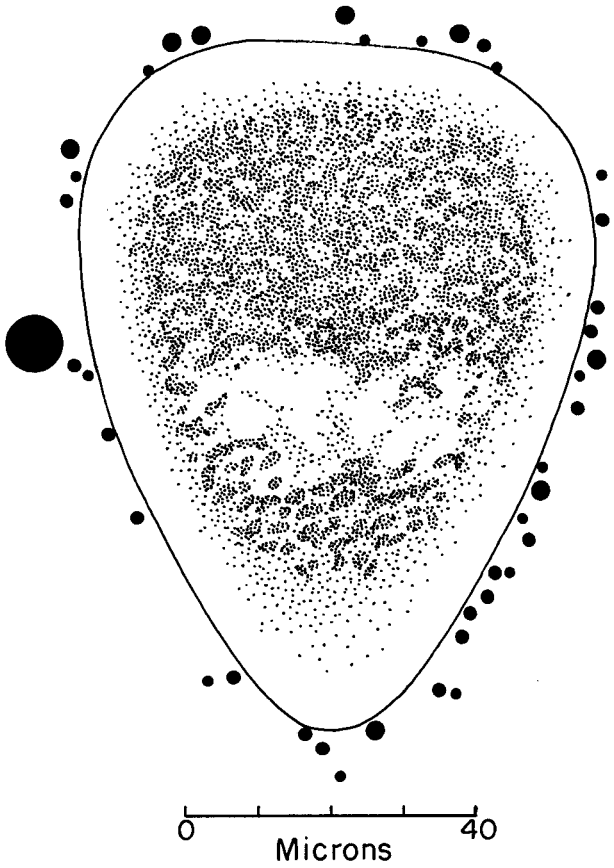

FIGURE 304.—Unfertilized egg of *C. virginica* stained with Black Sudan B; slight pressure on a coverslip forces the oil globules through the vitalline membrane. Drawn from life.

that the development of eggs of the Portuguese oyster, *C. angulata*, is not affected by toluidin blue used in such dilute solution for only a short time. The dye is fixed at the level of the small granules, which the cytologists designate as alpha granules, uniformly distributed in the cytoplasm between the yolk vesicles. Later in the development of a fertilized egg, new and larger granules, called beta granules, appear at the time of prophase. Their higher metachromasy is acquired at the expense of the alpha granules. Subsequent studies (Mulnard, Auclair, and Marsland, 1959) have suggested that the beta granules are related to the Golgi complexes of the eggs.

The alpha granules of the unfertilized eggs of *C. angulata* can be separated from the yolk vesicles by centrifuging; they are displaced in the direction of the centrifugal force (Pasteels and Mulnard, 1957), while the beta granules at the pronucleus stage of the fertilized egg are moved in the centripetal direction. The alpha and beta particles of Pasteels and Mulnard probably correspond to the P and L granules of Cleland. Personal observations show that in ripe but unfertilized eggs of New England *C. virginica* stained with toluidin blue, elements corresponding to the alpha particles of Pasteels assume a lavender color while mitochondria and other smaller granules are bluish. The nucleolus is also of bluish color. After 10 minutes of centrifuging at 4,000 times gravity the yolk granules of the stained eggs concentrate at the lower (centrifugal) pole, while the alpha particles of lavender hue and slightly bluish mitrochondria are at the opposite pole (fig. 306).

Metachromatic granules have been described in the eggs of various bivalves. They were found in *Barnea candida* (Pasteels and Mulnard, 1957); *Mactra* (Kostanecki, 1904, 1908; *Mercenaria* (*Venus*) *mercenaria*, *Mytilus edulis*, and *Spisula solidissima* (Worley, 1944; Kelly, 1954, 1956;

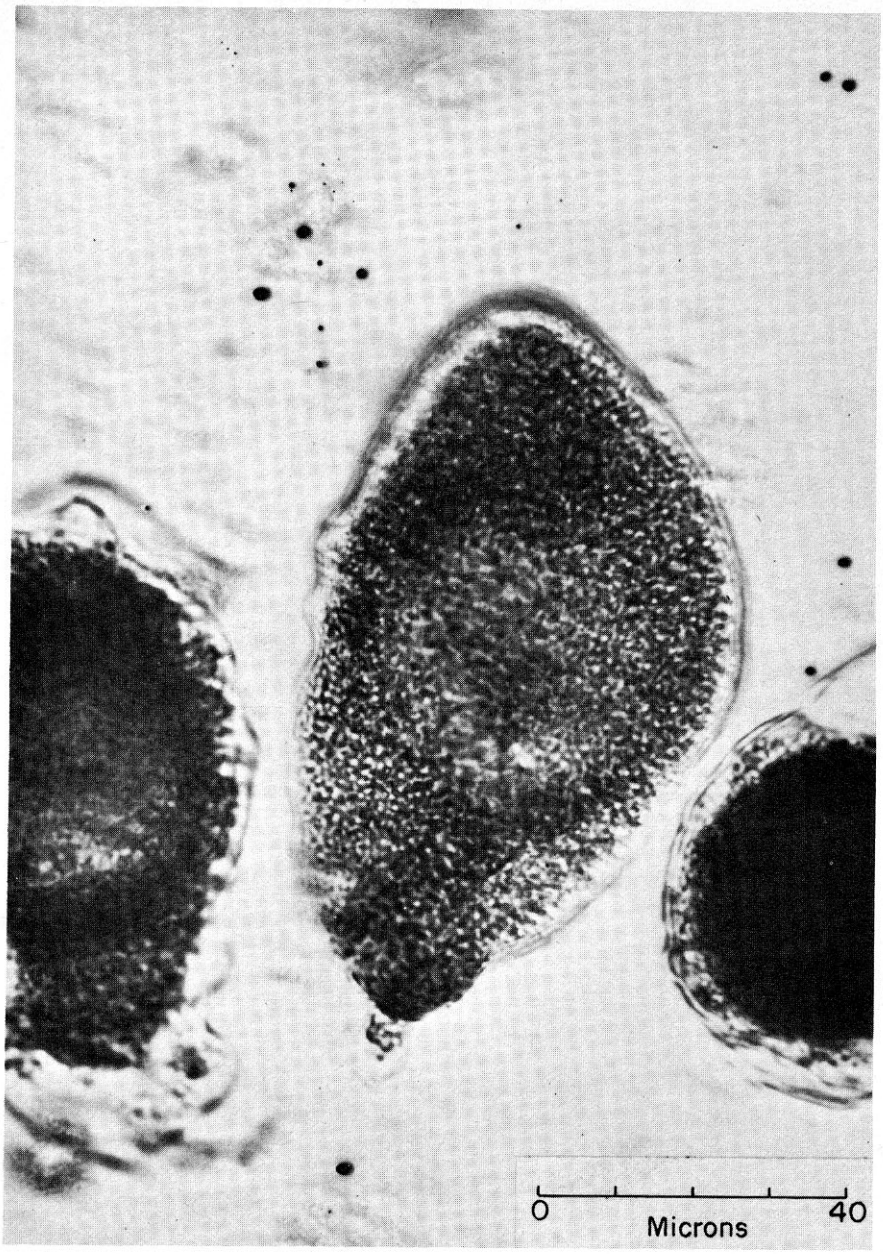

FIGURE 305.—Photomicrograph of an unfertilized egg of *C. virginica*. Janus green. Whole mount. Small rodlike inclusions seen along the periphery are mitochondria stained green; larger yolk granules are dark.

Allen, 1953; and Rebhun, 1960). The role of these almost submicroscopical bodies is not clear, but there is no doubt of their importance in the physiology and development of eggs. Recent publications of Dalcq (1960), Brachet (1960), and Rebhun (1960), and the reviews given by Novikoff (1961a, 1961b) should be consulted for ideas concerning the possible role of these elements in the morphogenesis of mosaic eggs in which they are concentrated in the posterior blastomeres.

Eggs of the surf clam *S. solidissima* contain a heparinlike blood anticoagulant which was also extracted from the tissues of this clam (Thomas, 1954). Whether substances with similar activity are present in oyster eggs is not known.

The nucleus of a mature egg is surrounded by a nuclear membrane which can be clearly seen on sectioned and stained preparations of the ovary (fig. 300). A spherical, dense nucleolus is excentrically located; its diameter varies from 4 μ to 6 μ.

FIGURE 306.—Unfertilized egg of *C. virginica* centrifuged for 10 minutes at 4,000 times gravity after staining with toluidin blue. Dark yolk granules are at the lower (centrifugal) pole while the mass of small inclusions consisting of lavender particles and bluish mitochondria are at the opposite end of the egg.

The chromosomes appear as fine threadlike structures near the nuclear membrane. Using methyl green and pyronin B stains, Kobayashi (1959) found that the nucleolus in the eggs of *C. gigas* and *O. laperousi* consists of two parts, the karyosome,

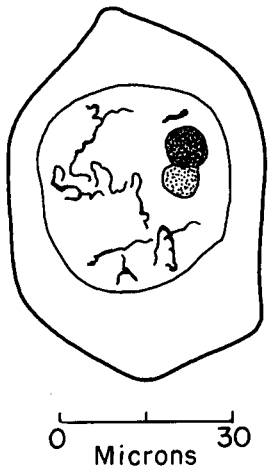

FIGURE 307.—Ovocyte of *O. laperousi* stained with methyl green and pyronin B after Navashin fixation. Dark globule is the karyosome, light shaded area is the plasmosome. From Kobayashi, 1959.

shown in solid black in fig. 307, and the plasmosome, lightly shaded, in close contact with each other. The definite KP axis (Karyosome-Plasmosome) of the unfertilized egg changes after fertilization by the turning of the karyosome around the plasmosome. The existence of this axis in the eggs of other species of oysters has not been described.

STRUCTURE OF THE MATURE SPERMATOZOON

The spermatozoon of bivalve mullusks appears under the microscope (Franzén, 1956; Lenhossék, 1898; Retzius, 1904) to consist of an oval or round head with a pointed front, a middle piece at the lower end of the head, and a long tail (flagellum) with a narrow "end piece" which is longer than the width of the head. The middle piece consists of four, sometimes five, oval-shaped bodies clustered around the tail; and a minute "central granule" or centriole located in the center at the point of attachment of the tail. The sharply outlined oval bodies are mitochondria; they are strongly osmiophilic and can be deeply stained with rosanilin. The head of the spermatozoon is formed by a compact nucleus capped with the apical body or acrosome with a pointed tip (perforatorium), which apparently assists the spermatozoa in penetrating the egg membrane at fertilization (Wilson, 1928). The features listed above may be seen in properly fixed and stained preparations of the sperm of *C. virginica* and in live spermatozoa examined with phase contrast oil immersion lenses. In live preparations the nucleus appears to be dark while the acrosome and middle piece are light (fig. 308). The center of the spermatozoon head is occupied by an axial body, a relatively large, light-refracting structure which is separated from the acrosome.

The dimensions of normal, uncytolyzed spermatozoa have been measured by means of an eyepiece micrometer of a light microscope. The head varies from 1.9 μ to 3.6 μ in length (median value 2.7 μ) and between 1.0 μ and 2 μ in width. The tail is from 27 μ to 39 μ long (median value 36 μ). The tails are usually slightly curved; specimens with straight tails are rarely found.

Electron microscopy reveals much greater complexity in the structure of the spermatozoon (Galtsoff and Philpott, 1960). Study was made of small sections of ripe spermary preserved in cold 1 percent osmium tetroxide buffered to pH

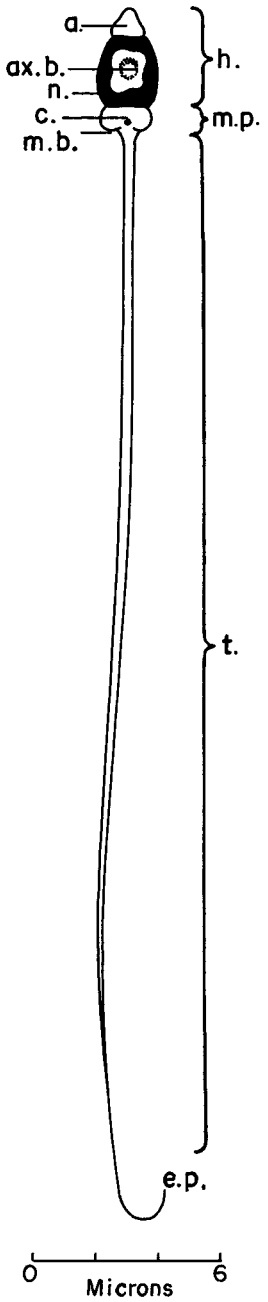

FIGURE 308.—Live spermatozoon of *C. Virginica* examined under light microscope with phase contrast oil immersion lens. A—acrosome; ax. b.—axial body; C—centriole; e.p.—end piece of tail; h.—head; m.p.—middle piece; mt.—mitochondrial bodies; N—nucleus; t.—tail.

7.2–7.4 and embedded in a mixture of three parts butyl and one part methyl methacrylate. To increase the contrast, some of the material was placed in 1 percent alcoholic phosphotungstic acid for 5 to 6 hours before embedding. The embedded material was sectioned on Philpott's microtome (Philpott, 1955), using a diamond knife. Since the preserved pieces consisted of a multitude of spermatozoa arranged in a central mass with their tails pointing outward, the individual spermatozoa were always cut at random in different planes, regardless of how the embedded tissue may have been oriented on a microtome block. The resulting electron micrographs showed a number of sperm heads cut at different planes and many transverse sections of tails (fig. 309). The entire structure of the head was diagrammatically reconstructed by bringing various elements together and placing them in their relative positions (fig. 310). The oval-shaped head consists of slightly granular, homogeneous material covered with an osmiophilic membrane made of two layers. The apical portion of the nucleus is occupied by a caplike acrosome of highly osmiophilic substance. The acrosome is clearly separated from the nucleus by a sharply defined membrane. An egg-shaped body in the central part of the nucleus extends from the apex of the acrosome almost to the base of the nucleus. This structure, named axial body (Galtsoff and Philpott, 1960), has a central stem of fibrous material which emerges from the flattened bottom and extends about two-thirds of the total length of the axial body. The indented base of the nucleus is near the base of the axial body. The caved-in space formed by this indentation consists of material of lesser electron density and extends under the nucleus to the upper surface of the centriole, which is surrounded by four mitochondrial bodies. Only two of them are shown in fig. 310.

The centriole of the sperm of *C. virginica* is a hollow, cylindrical structure with walls made of nine bands; these can be seen in cross section (fig. 311). The side view (fig. 310) shows that the centriole is formed in several alternating and slightly constricted layers which connect with the four mitochondrial bodies. These bodies have the typical appearance of twisted lamallae enclosed in a membrane which encompasses the centriole and continues over the tail.

The tail consists of a pair of axial filaments surrounded by a ring of nine double filaments spaced at equal intervals along the periphery (fig. 312). The filaments are interconnected by delicate strands. The axial filaments begin near the basal plate (fig. 310) where the tail emerges. Radial trabeculae connect the ring filaments to the outside wall of the tail and form nine separate

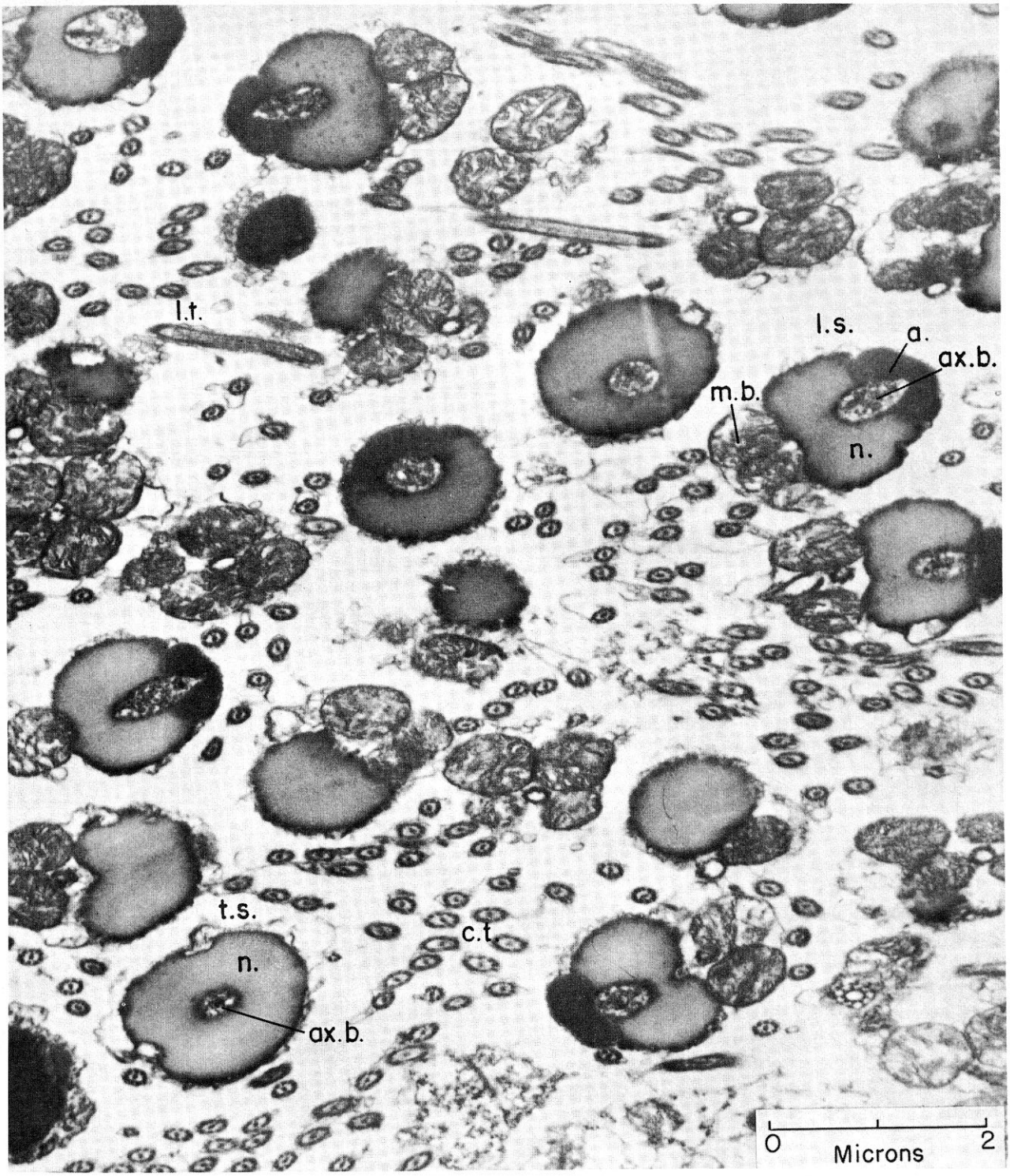

FIGURE 309.—Electron micrograph of a section of ripe spermary of *C. virginica*. Longitudinal and transverse sections of sperm heads and tails can be seen in various parts of the micrograph. A—acrosome; ax. b.—axial body; c.t.—cross section of tails; l.s.—longitudinal section of sperm head; l.t.—longitudinal section of part of a tail; m.b.—mitochondrial bodies; N—nucleus; t.s.—transverse section of head.

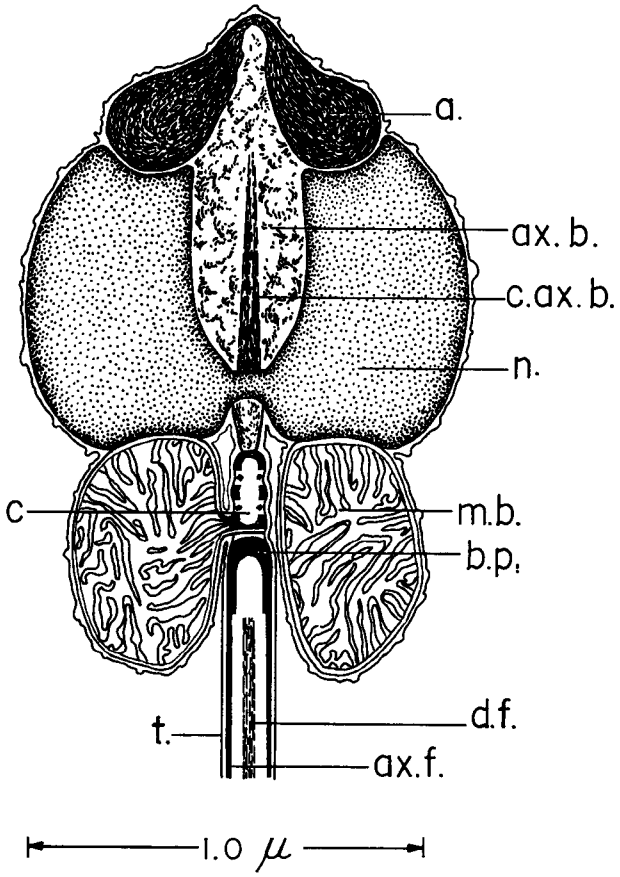

FIGURE 310.—Reconstruction of the sperm head of *C. virginica* made from a large number of longitudinal sections. a.—acrosome; ax. b.—axial body; ax. f.—axial filament of the tail; b.p.—basal plate of the tail; c.—centriole; c. ax. b.—core of the axial body; d.f.—double filament on the periphery of the tail; m.b.—mitochondrial body; n.—nucleus; t.—proximal part of the tail.

compartments filled with material of lesser electron density. The central radial strands are similar to the "spokes" described by Afzelius (1955) for the sperm of the sea urchin *Psammechinus miliaris*. They are not present in the proximal portion of the tail where there are no axial filaments, but otherwise the ultrastructure of the sperm tail is similar to that of cilia and flagella of various animals and plants.

FERTILIZATION

The spawned eggs of *C. virginica* and *C. gigas* are heavier than water and quickly sink to the bottom. The time they remain in suspension may be prolonged by horizontal currents and upward movements of the water, and consequently the chances of fertilization are increased. Because spawning is usually initiated by the males, the water into which the eggs are discharged already contains active spermatozoa and fertilization takes place within a few minutes following ovulation. It is obvious that the success of reproduction of an oyster population in which spawning is mutually stimulated by the discharge of sex cells is dependent on close proximity of the sexes and their simultaneous response to spawning stimuli.

Eggs and sperm secrete substances called gamones which play an important role in fertilization. Secretion from an unfertilized egg has a significant effect on spermatozoa. This effect can be observed if a suspension of eggs is permitted to stand for 15 to 20 minutes and the supernatant fluid is decanted or filtered and added to the suspension of sperm. The resulting so-called "egg water" (Lillie, 1919) causes the agglutination of sperm. To observe the agglutination reaction with the naked eye, a drop of egg water must be added to a sperm suspension, which shortly forms irregular lumps (fig. 313). Under a high-power light microscope one sees that the heads of agglutinated spermatozoa stick together to form large aggregates (fig. 314).

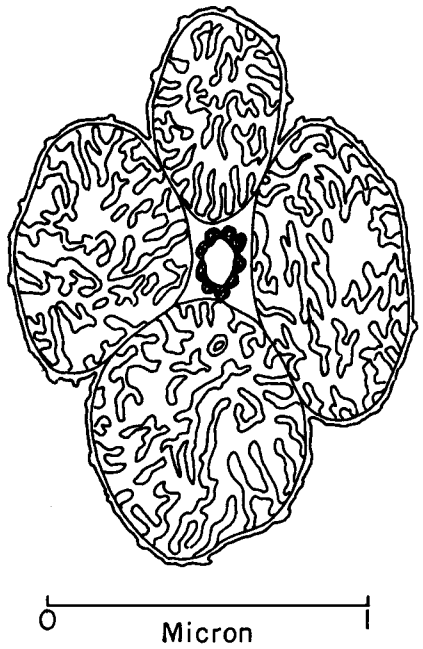

FIGURE 311.—Drawing based on electron micrographs of the cross section of the lower part of the middle piece of the spermatozoon of *C. virginica*. Centriole, at the center, is surrounded by four mitochondrial bodies.

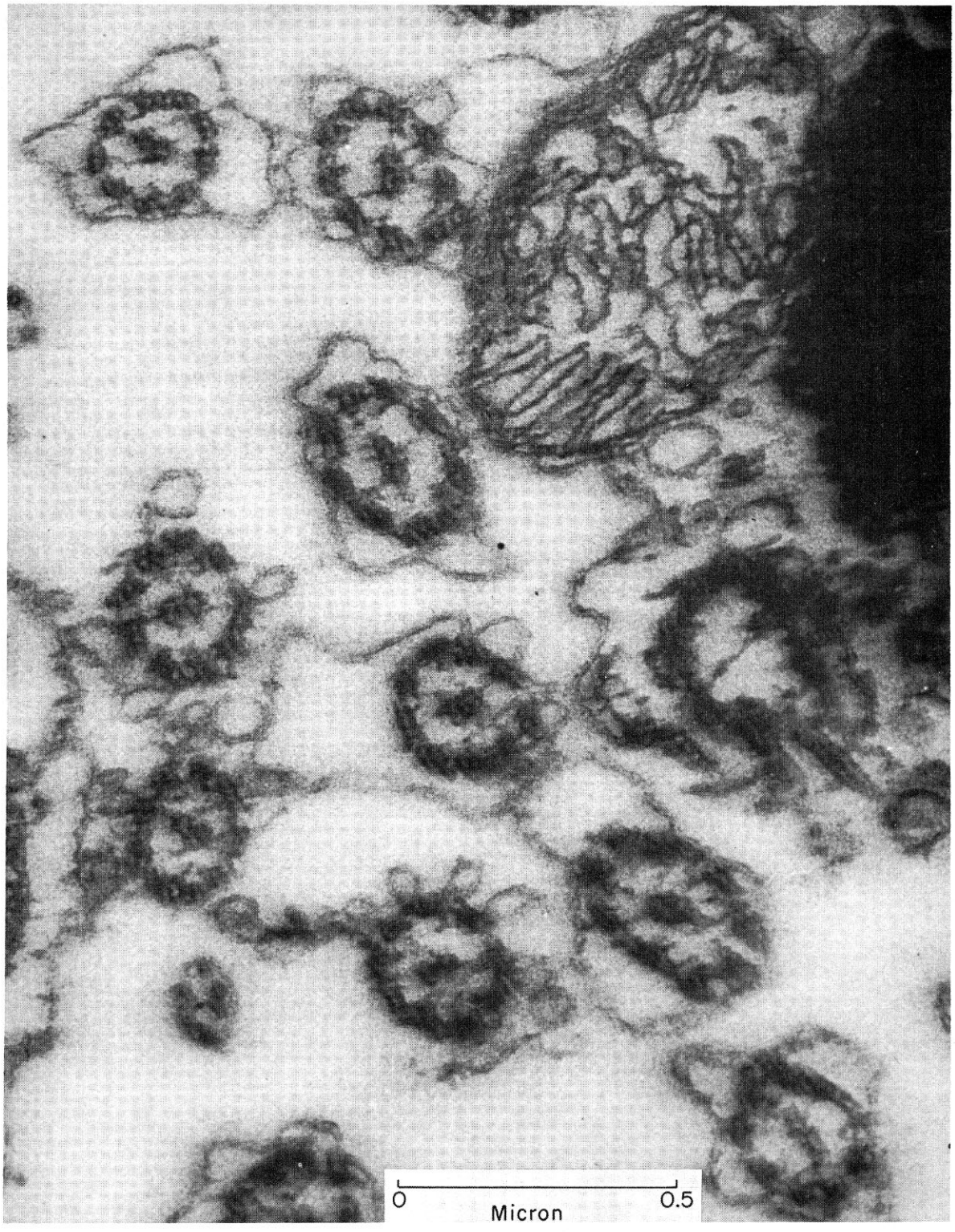

FIGURE 312.—Transverse section of the tails of oyster sperm of *C. virginica* slightly below the level of the middle piece. Electron micrograph.

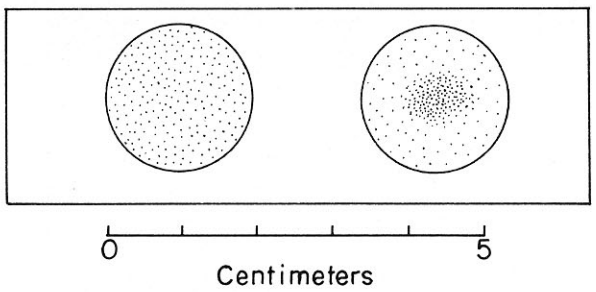

FIGURE 313.—Sperm suspension of *C. virginica* on a slide in sea water (left) and in sea water containing a small quantity of egg water (right). Drawn from life. Natural size.

Agglutination also occurs in the sperm of *C. gigas* and in *O. circumpicta* Pilsbry. Terao (1927) experimented with the latter species using egg water made by mixing 0.55 ml. of ripe eggs in 9 ml. of sea water and removing the eggs by centrifuging and filtering after they had stood for 20 minutes. The filtrate caused the isoagglutination of sperm even in a dilution of 1 to 10 millions. Heteroagglutination by the egg water of *O. circumpicta* has been observed in the suspensions of sperm of the bivalve *Arca*, sea urchin *Toxocidaris tuberculatus*, and starfish *Luidia quinaria*.

Lillie (1919) regarded the sperm agglutinating factor he discovered in *Arbacia* eggs as an essential to fertilization, and to the active substance of egg water he gave the name fertilizin. Tyler (1948) identified fertilizin with the jelly substance of the egg and on the basis of experimental data concluded that the presence of the jelly coat has a favorable effect on fertilization. By biochemical analysis of sea urchin eggs, Vasseur (1948a, 1948b) determined the composition of the jelly coat and found that 80 percent of it consists of polysaccharide and 20 percent of amino acids. The substance was found to exert a heparinlike action in a blood-clotting system (Immers and Vasseur, 1949). After removing the jelly coat with acidified water, Hagström (1956a, 1956b, 1956c, 1956d) found that the rate of fertilization was higher than in the presence of the coat. It is, therefore, apparent that the jelly coat is not essential for fertilization.

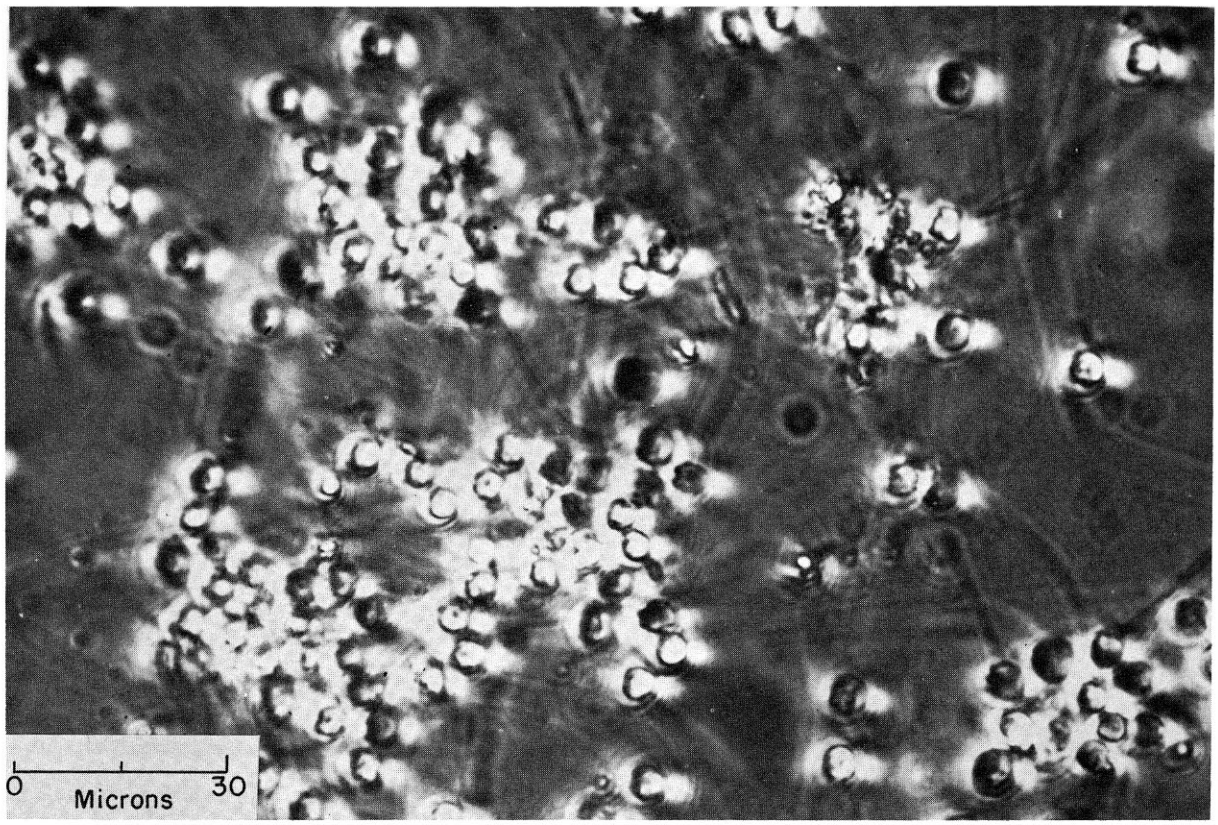

FIGURE 314.—Photomicrograph of sperm of *C. virginica* agglutinated by egg water of the same species. Phase contrast oil immersion.

This view agrees with the conditions found in oyster eggs which have no jelly coat.

According to the modern view discussed in the review of the problem by Runnström, Hagström, and Perlmann (1959), the jelly coat not only fails to improve fertilization but impedes it by acting as a sieve. Its action may be considered as an elimination process by which the number of spermatozoa capable of attaching to the cytoplasmic surface is substantially reduced.

Fertilizin of sea urchin eggs has two distinct properties: it agglutinates sperm suspension and activates the motility of free, single spermatozoa. Both of these properties are present in the fertilizin of an oyster egg.

ACROSOMAL REACTION

Spermatozoa of various invertebrates have been found to carry a substance of protein character, probably a lysine, capable of dissolving the vitelline membrane of the egg. Such lysine is present in the sperm of the giant keyhole limpet, *Megathura crenulata* (Tyler, 1939), in the sperm of *Mytilus*, where it is probably located in the acrosome (Berg, 1950; Wada, Collier, and Dan, 1956), and in other marine animals (Tyler, 1948, 1949).

Upon contact with the surface of an egg, the spermatozoon undergoes a so-called acrosomal reaction, which is described as the deterioration of the surface of the acrosomal region of the head followed by a projection of a stalklike filament. The acrosomal reaction and the discharge of the filament have been observed in starfish, holothurians, mollusks, and annelids. The reaction was studied by Colwin, A. L., and L. H. Colwin (1955) and Colwin, L. H., and A. L. Colwin (1956) in the annelid *Hydroides hexagonus* and enteropneust *Saccoglossus kowalewskii*. Using electron microscopy, the Colwins revealed many interesting details of the penetration of the spermatozoon into egg cytoplasm. In pelecypod mollusks the discharge of the acrosomal filament was observed in *Mytilus* and in the three species of oysters, *C. echinata*, *C. nippona*, and *C. gigas* (Wada, Collier, and Dan, 1956; Dan and Wada, 1955). The reaction can be induced by egg water as well as by the contact of a spermatozoon with the egg surface. The first sign of acrosomal reaction in oyster sperm is the flattening of the anterior surface of the spermatozoon. At the same time the head becomes extremely adhesive, the acrosome membrane bursts, and the filament is discharged. The reaction can be observed when a small drop of live sperm suspension is placed on a cover slip, a minute quantity of egg water is added, and the cover slip then inverted on a slide. The preparation is examined with phase contrast oil immersion lens using anisol (Crown oil) of refractive index 1.515 instead of cedar oil.

The acrosome reaction of *C. virginica* is similar to that observed by Dan and Wada in three other species of oysters. Under the effect of egg water the head becomes swollen and rounded and the filament is ejected from the acrosome. The discharged filament is wider than the tail and is about three to four times longer than the length of the head (fig. 315). In my observations only a small number of oyster spermatozoa suspended in egg water discharged acrosomal filaments.

The exact role of the filament in the fertilization of oyster eggs is still unknown. Investigations

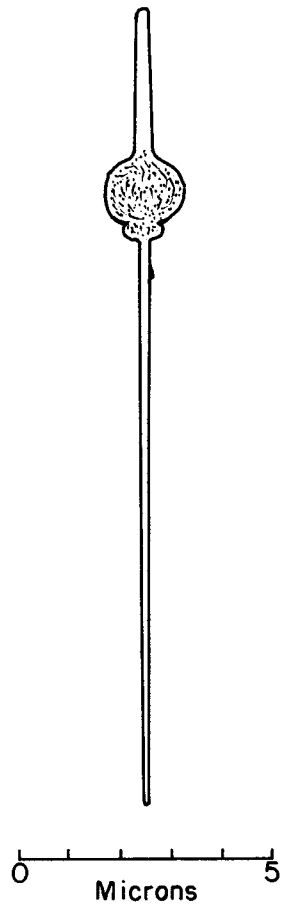

FIGURE 315.—Diagrammatic drawing of acrosomal reaction in the spermatozoon of *C. virginica* produced by egg water. Only a part of the sperm tail is shown in the drawing. Drawn from live preparation.

with eggs of other invertebrates suggest that the acrosome region of a spermatozoon is active during the first stages of fertilization and that it carries a lysine which facilitates the attachment of the spermatozoon to the egg membrane and its penetration into the cytoplasm.

The old view that spermatozoa penetrate the egg by a mechanical action of screw-borer movements of the pointed end (the perforatorium) has been abandoned. It is now generally accepted that the action of the sperm head is primarily chemical and that probably several enzymes are carried by the acrosome. Readers interested in the problem of fertilization are referred to comprehensive reviews of this subject by Runnström, Hagström, and Perlmann (1959), Colwin, A. L., and L. H. Colwin (1961a, 1961b), and Colwin, L. H., and A. L. Colwin (1961).

FERTILIZATION OF EGG

Eggs for fertilization experiments may be obtained in the laboratory by stimulating a single female spawn as described in chapter XIV. A suspension of eggs pipetted off the bottom of a laboratory tank is free of blood and other body fluids. Eggs may also be taken directly from the ovary by cutting off small slices from the surface of the gonad and mincing or shaking them in sea water. Cutting into the underlying layer of digestive diverticula should be avoided to prevent contamination with body fluids. The eggs must be washed several times in filtered seawater by decanting or by filtration through a fine sieve until the suspension is free of tissue cells and debris. After being in sea water for a short time, the eggs change their shape and become globular but their large germinal vesicles remain clearly visible (fig. 316).

A sperm suspension may be obtained by any one of three methods. Male spawning can be induced by raising the water temperature or by adding a small amount of thyroid suspension, and live spermatozoa collected as they are discharged through the cloaca; small pieces of ripe spermary can be excised and the spermatozoa liberated in sea water by shaking; or a very ripe spermary can be pressed gently with the fingertip and the spermatic fluid pipetted as it comes from the gonoduct. Concentrated sperm suspension must be diluted for fertilization. I found it convenient to make a standard suspension using 0.2 g. of gonad material in 50 ml. of sea water and then

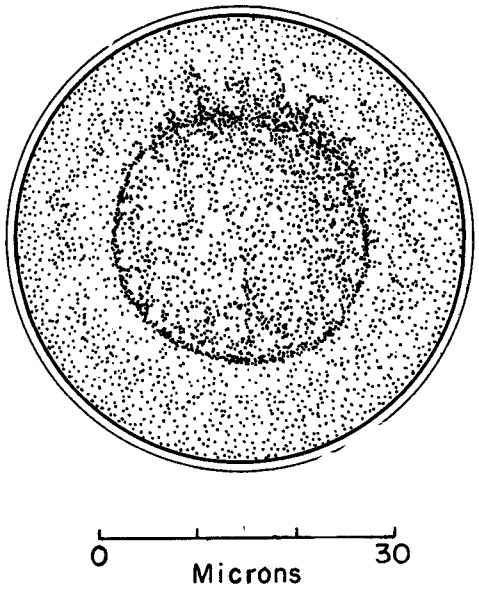

FIGURE 316.—Camera lucida drawing of naturally spawned but unfertilized egg of *C. virginica*.

diluting it, using 0.5 ml. for 100 or 150 ml. of water containing eggs.

Although several spermatozoa may attach themselves to an egg, (fig. 317), only one penetrates the cytoplasm. The others, called supernumeraries, eventually are cast off when cleavage

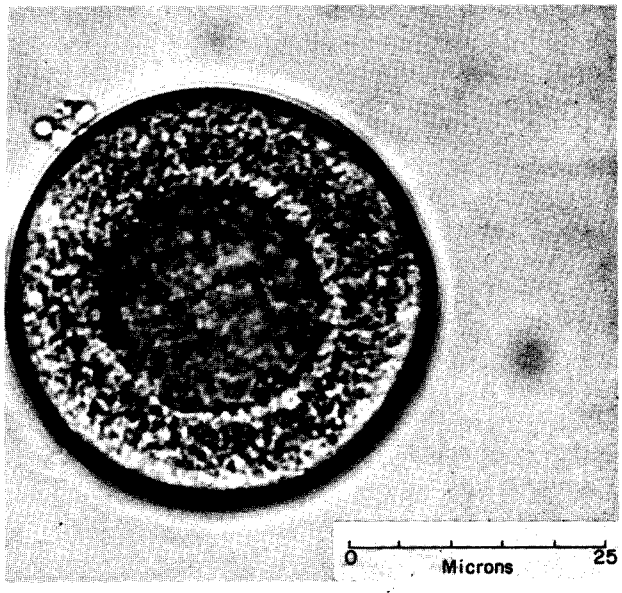

FIGURE 317.—Photomicrograph of a fertilized egg of *C. virginica* a few minutes after the formation of the fertilization membrane. Several spermatozoa are attached to the egg membrane but only one will penetrate it. The germinal vesicle is intact. Contrast phase oil immersion lens.

begins. If the sperm suspension is too concentrated, many spermatozoa enter one egg and cause polyspermy, a condition which may interfere with normal development of the egg.

The spermatozoon which succeeds in penetrating the egg's surface undergoes great changes. Its acrosome region becomes swollen and disrupted and the tail loses its motility; the head gradually penetrates the egg membrane as the sperm moves deeper into the cytoplasm. At the same time the fertilized egg contracts and assumes a globular shape if it was not round before; the cytoplasm becomes so dense that the germinal vesicle is no longer visible under the layer of yolk granules. A few seconds after the sperm head touches the egg's surface a thin, transparent fertilization membrane is elevated and under the light microscope appears to be homogeneous. This membrane apparently is formed from the pre-existing vitelline membrane and is underlined by a layer of subcortical particles (fig. 318). The two layers are optically separated. It is generally accepted (Runnström, 1952) that in *Arbacia* and many other species the fertilization membrane originates from the vitelline membrane because it fails to form after the vitelline membrane has been removed with potassium chloride, trypsin, or urea. No experimental work of this type has been done on oyster eggs.

AGING OF EGGS AND SPERM

The longevity of eggs of marine invertebrates, i.e., their ability to form fertilization membrane and undergo cleavage, was observed in the sea urchin (*Arbacia*) and in other common species (Harvey, 1956). Oyster eggs also undergo aging changes and lose their ability to be fertilized. This has been demonstrated in a number of tests made in the Bureau's shellfish laboratory at Woods Hole. Because of wide individual variability in fertilization capacities only one female and one male were used in each series of tests. The following technique was used: Suspension of eggs was made by shaking 0.5 g. of ripe ovary tissue in about 200 ml. of sea water: eggs released by this action were permitted to settle on the bottom and the supernatant water was decanted; the remaining eggs were rinsed twice in sea water and transferred to a beaker filled with 500 ml. of filtered sea water. The beaker was kept half submerged in running sea water to prevent heating to room temperature. Samples of eggs were taken for fertilization every hour during the first 4 hours, then at 2-hour intervals for the next 6 hours, and finally one sample was taken each time after 12 and 24 hours. Eggs were collected at random from the bottom of the beaker and placed in a finger bowl in 100 ml. of filtered sea water. To fertilize them 0.5 ml. of dilute stock suspension of sperm was used; the water was gently stirred

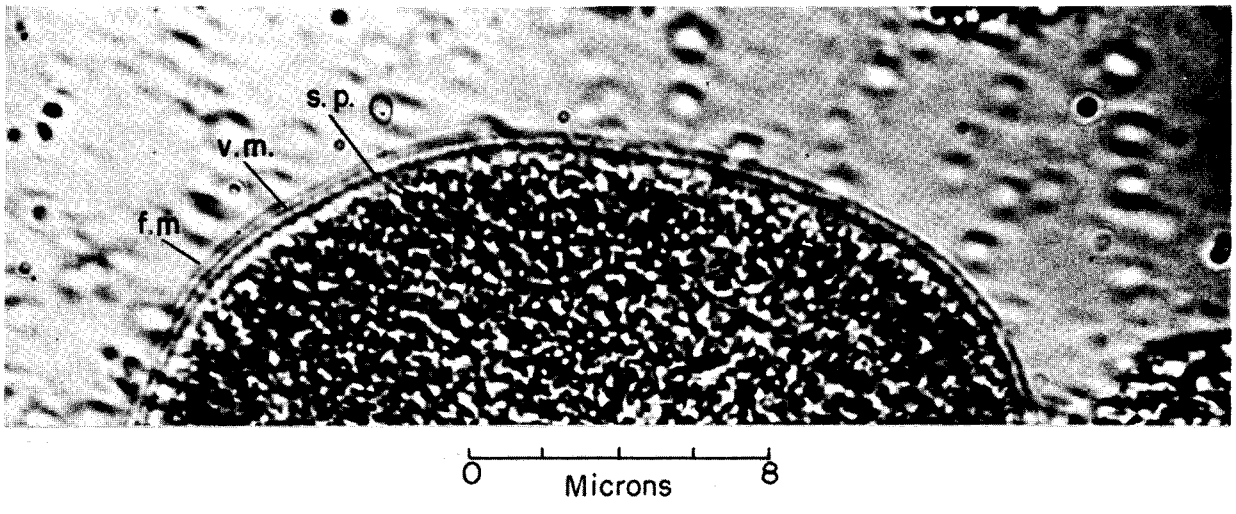

FIGURE 318.—Photomicrograph of a portion of fertilized egg of *C. virginica* shorly after the attachment of sperm. Fertilization membrane (f.m.) (outside layer) is underlined by the vitelline membrane (v.m.) with a dense row of subcortical particles (s.p.). Live preparation. Oil immersion phase contrast lens.

to obtain uniform distribution of sperm, and the finger bowl set aside for 5 hours, half submerged in running sea water. At the expiration of this period a sample was taken for examination and cleaved and uncleaved eggs were counted. In each case 300 eggs of the sample were examined. All tests were made in water of 31 to 32‰ salinity and 20.8° to 21.4° C. During the first 4 hours of aging the percentages of cleaved eggs declined from 90 to 70. After 5 or 6 hours the percentages dropped to 60. Then the fertilizability decreased to about 20 percent in 10 hours, and only a few eggs cleaved normally after 12 and 24 hours of aging.

It is common knowledge among embryologists that the fertilizing power of spermatozoa is not decreased if sperm is kept at 10° to 12° C. in a concentrated suspension in a tightly closed container. This is also true for oyster sperm. Its fertilizing power is affected by dilution and increased temperature. At room temperature in a dilute suspension, the spermatozoa lose their fertilizing ability within 4 to 5 hours. However, in a concentrated suspension, protected from evaporation, and stored in a refrigerator at about 10° C. the sperm remains active and retains its full fertilizing power for 24 hours and possibly longer.

The effect of cold storage on the fertilizability of eggs is not known. On several occasions ripe females with intact shells were kept for 3 to 4 days in a refrigerator (about 10° C.) and after that were successfully used in spawning experiments. The effect of cold storage on eggs of the excised ovary has not been studied.

POLARITY OF EGG

The polarity of all molluscan eggs apparently is determined while they are still attached to the wall of the ovarian follicle. Presumably the side on which the food reaches the growing ovocyte becomes the vegetative pole of the mature egg (Raven, 1958).

The metabolic gradient along the egg axis is indicated by a concentration of cytochrome oxidase which Kobayashi (1959) detected with M–Nadi reaction; the activity of the enzyme was observed using Gräff's modification of this method. (The reader not familiar with the reaction and its significance in cytochemical research is referred to the publications of Danielli (1958), Deane, Barrnett, and Seligman (1960), and to a review by Novikoff (1961a, p. 308).) In brief, the localization of oxidative enzymes and their presence in mitochondria can be determined by the staining reactions. The rate of respiration of eggs of *C. virginica* increases with fertilization by a factor of 1.4 (Ballentine, 1940) but in the eggs of the Sydney rock oyster, *C. commercialis*, the rate of respiration increases only at the onset of the first cleavage (Cleland, 1950).

CLEAVAGE

The spermatozoon may enter the oyster egg anywhere. Its path inside the egg cytoplasm toward the nucleus has not been described, and cytological details of the process leading to the fusion of the female and male pronuclei have not been studied. It is probable that the major features of these events are not different from those found in other mollusks. Maturation divisions occur in the oyster egg after the elevation of the fertilization membrane. The germinal vesicle (ovocyte nucleus) breaks down and moves toward the egg's periphery. At temperatures of 22° to 24° C. the first polar body is formed within 25 to 50 minutes after the addition of sperm. The reduction of the number of chromosomes probably takes place during the first meiotic division. This is to a certain extent corroborated by an examination of fertilized eggs of *C. virginica* stained in toto with Feulgen reagent or with acetic orcein. Unfortunately the results are not consistent enough to draw a final conclusion and the question remains unanswered, awaiting a complete cytological study.

The second polar body is formed shortly after the first, within 45 to 70 minutes after fertilization (at 22.5° to 24° C.). The two polar bodies remain attached to the surface of the egg (fig. 319) until the completion of cleavage and emergence of the trochophore.

The first cleavage following the formation of the second polar body divides the egg meridionally into two unequal cells designated as AB and CD (fig. 320). The inequality of the blastomeres is due to the occurrence of a polar lobe. Because the egg appears to consist of three cells this stage received the name trefoil.

The plane of the second division, also meridional, is at a right angle to the first. Both blastomeres divide synchronously and separate into the four quadrants. In fig. 321, drawn from a photograph of a cleaving egg taken from the animal pole, the position of the spindles indicates the plane of new

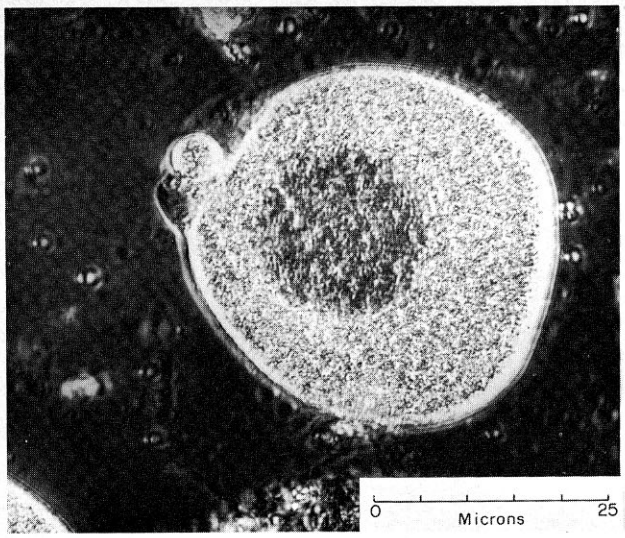

FIGURE 319.—Photomicrograph of a live fertilized egg of *C. virginica* after the formation of two polar bodies (top of egg). High-phase oil immersion lens.

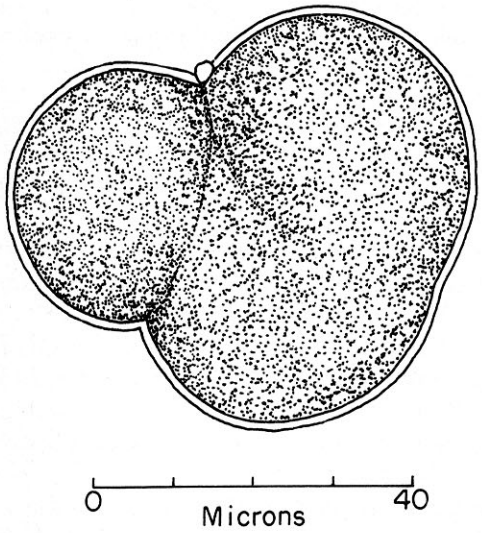

FIGURE 320.—First cleavage division of the egg of *C. virginica* 70 minutes after fertilization. Blastomere AB (left) and CD (right). Polar body on top.

furrows which will intersect the egg into four cells, A, B, C, and D. At this stage the mitotic figures are fairly large and the chromosomes are in a favorable position for examination. In the best sectioned preparations of the cleaving egg, eight daughter chromosomes were counted at the beginning of anaphase (fig. 322). It would be, however, premature to state that the diploid number of chromosomes in *C. virginica* is 8 because on other preparations 7, 9, and 10 were counted.

The third division of each quadrant cuts the cells in the equatorial plane and separates the

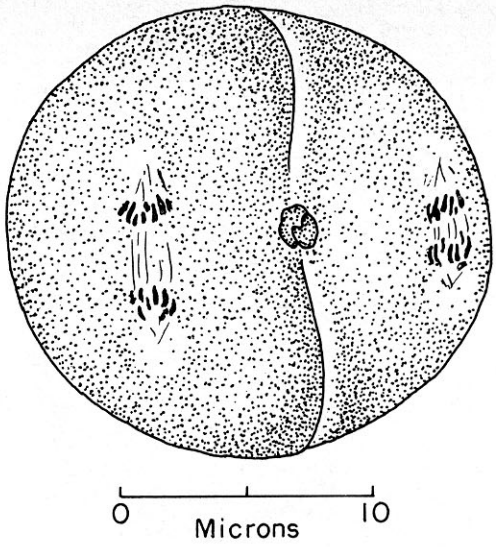

FIGURE 321.—Beginning of second cleavage of the egg. Viewed from the animal pole. Whole mount, Kahle, Feulgen stain.

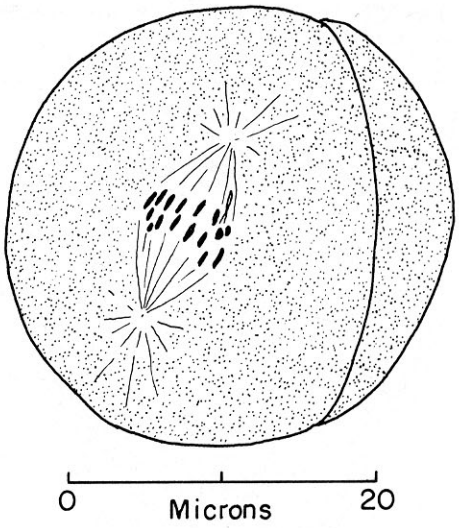

FIGURE 322.—Section of an egg of *C. virginica* at the second cleavage. Beginning of anaphase. Kahle, Heidenhain iron-hematoxylin.

first quartet of micromeres, small cells at the animal pole, from the macromeres, or larger cells at the vegetal pole (fig. 323).

At the fourth and fifth cleavages, resulting in 16- and 32-cell stages, the micromeres overgrow the macromeres. Only one of the macromeres is visible in the figure 324 and two in 325, which show a side view of an oyster egg at these two stages of development.

Cell lineage, or tracing the developmental history of the cleavage blastomeres through to

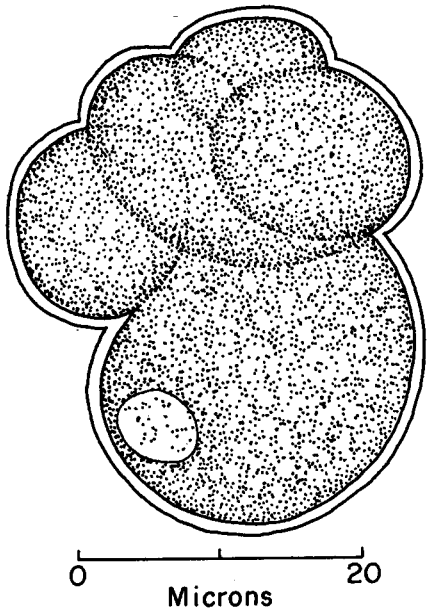

FIGURE 323.—Third division of fertilized egg of *C. virginica;* side view. Separation of micromeres (on top) from macromeres (bottom). Only one macromere is seen in the plane of view. Live egg.

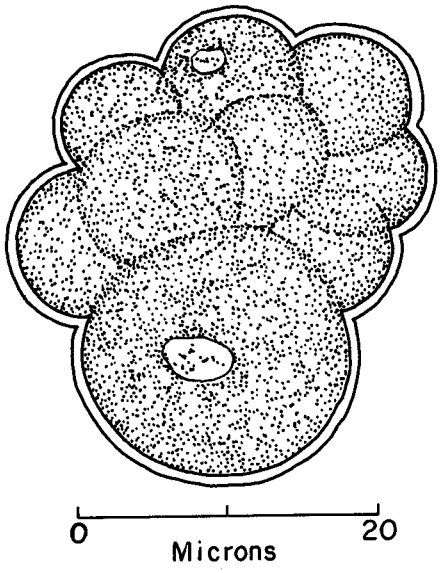

FIGURE 324.—Fourth cleavage of egg of *C. virginica* and the formation of 8 micromeres (2d quartet). Side view. Drawn from photomicrograph of live cell.

their ultimate fates as parts of the larva or adult, was first described by Whitman (1878) for the egg of *Clepsine*, and followed by Wilson (1892) for the egg of *Nereis*. The works of Lillie (1895) on the development of Unionidae, Conklin (1897, 1908) on *Crepidula* and *Fulgur*, Meisenheimer

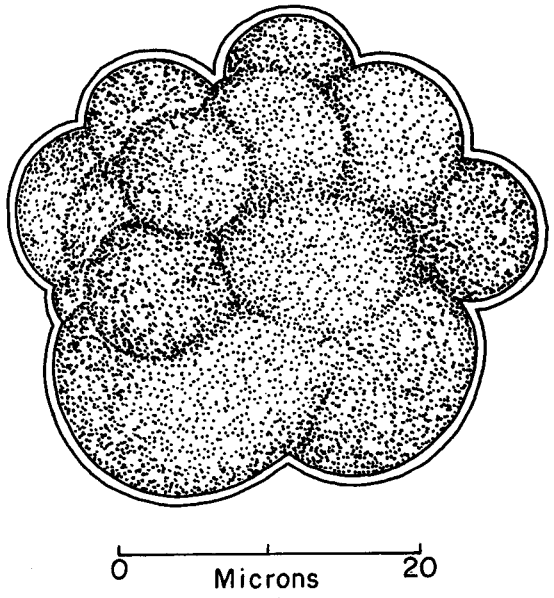

FIGURE 325.—Fifth cleavage of egg of *C. virginica* and the formation of the third quartet of micromeres (32-cell stage). Side view. Drawn from photomicrograph of live cell.

(1901a, 1901b) on *Dreissensia* and *Cyclas*, and Wilson (1904a, 1904b) on *Dentalium* and *Patella* constitute major contributions to the embryology of mollusks.

The nomenclature of the cleavage blastomeres, as developed by Wilson (1892), was progressively modified by Conklin (1897), Mead (1897), and Child (1900); the present system is based largely on the work of Robert (1902) on the development of the *Trochus* egg. The system is a combination of letters and numbers by which the blastomeres are identified.

The first four cells or macromeres are designated as A, B, C, and D; in the majority of cases studied D is the largest of the four and is situated at the side which will develop into the posterior portion of the embryo. When the first four blastomeres divide, their daughter cells are denominated 1a and 1A, 1b and 1B, 1c and 1C, and 1d and 1D, the small letters in each case referring to the micromere and the capital letter to the macromere.

In successive divisions 1A divides into 2a and 2A, 1B into 2b and 2B, and so on. When the micromeres divide, 1a is divided into $1a^1$ and $1a^2$, the superscript 1 denoting the daughter cell which is nearest to the animal pole and superscript 2 the one nearer to the vegetal pole. The nomenclature is capable of indefinite expansion, but certain confusion arises when the two daughter cells resulting

from the division of one cell lie at an equal distance from the pole. In this case the letters r for right and l for left are used. The practice is, however, not generally followed.

Descriptions of various types of cleavage can be found in volume 1 of MacBride (1914). The equal and unequal cleavages in the spirally cleaving eggs of annelids and molluscs are discussed by Costello (1955) in chapter 2 of Willier, Weiss, and Hamburger (1955).

During division the micromeres of a quartet, viewed from the animal pole, become slightly displaced because the spindles of the dividing cells (not shown in fig. 324 or 325) occupy an oblique position with respect to the egg's axis. At the following divisions the plane of separation of daughter cells is oriented approximately at a right angle to the preceding divisions. The pattern of such cleavage is called spiral. It gives rise to an irregular morula (sterroblastula according to Korschelt and Heider, 1895, from the Greek "sterros" meaning firm) found in annelids (*Nereis*), in some bivalves (Ostreidae, *Teredo*), and in gastropods (*Crepidula, Fulgur, Nassa*), and others. In all cases the sterroblastula arises from an unequal cleavage during which the micromeres overlie the macromeres, and at each division are slightly displaced to the right (dexiotropic cleavage) or to the left (laeotropic cleavage). Sometimes, as in the case of *Dreissensia*, the second dexiotropic cleavage is followed by a third dexiotropic cleavage after which the normal alternating course is established (Meisenheimer, 1901a). In the case of oyster eggs, as shown by Fujita (1929) for *C. gigas*, the cleavage is laeotropic.

The multicellular stages of a *C. virginica* egg are reached in the course of the sixth and ensuing cleavages (figs. 326 and 327) during which the micromeres divide much more rapidly than the macromeres, become progressively smaller and overgrow the vegetal pole. Approximately at this stage the sterroblastula of an oyster is formed.

Gastrulation begins with epibolic extension of the micromeres. At 22° to 24° C. the stage shown in fig. 328 is reached within 4 to 6 hours.

The cell lineage of *C. virginica* has not been studied; the stages of development of eggs of the species shown in figures 320 to 328 are similar to those previously described by Brooks (1898); Horst (1882) for *O. edulis;* Seno (1929) for *O. denselamellosa;* Hori (1933) for *O. lurida;* Yasugi (1938) for *O. spinosa,* and *C. gigas.* Yasugi

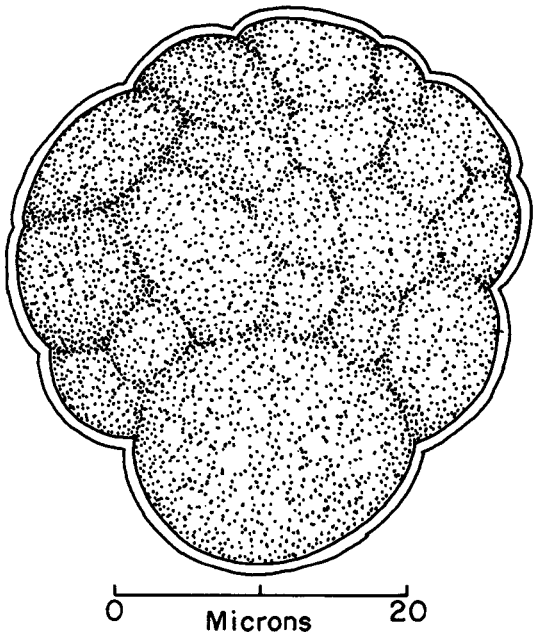

FIGURE 326.—Formation of sterroblastula in *C. virginica* egg; of the four macromeres only two are visible from the side. Micromeres begin to overgrow the vegetal pole. Drawn from photomicrograph of live egg.

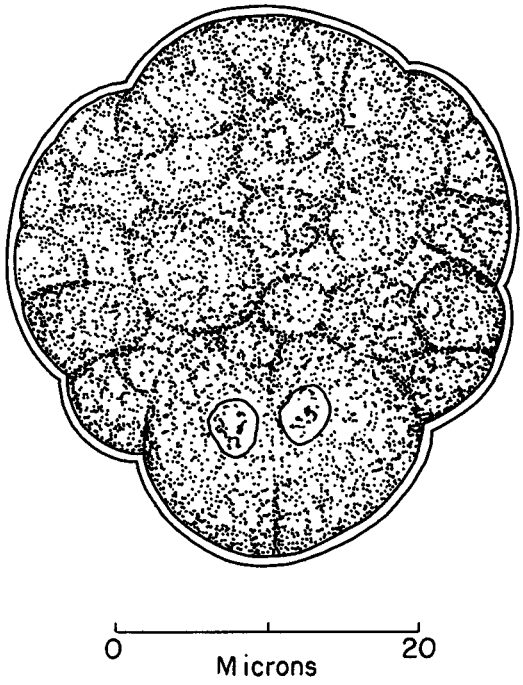

FIGURE 327.—Advanced sterroblastula stage of *C. virginica*. One of the macromeres has divided into two daughter cells of equal size. Drawn from photomicrograph of live egg.

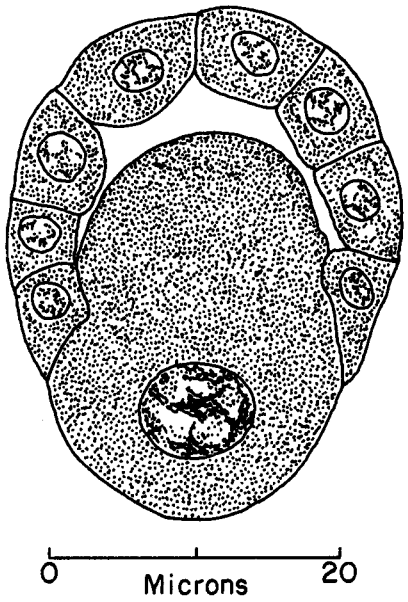

FIGURE 328.—Early stage of gastrulation in the egg of *C. virginica*. Seen at a section of an egg cut along its axis. Heidenhain, iron-hematoxylin.

found that equal cleavage can be induced artificially in eggs of the Japanese oyster by centrifuging for 2 minutes at 1,500 r.p.m. and at the centrifuge radius of 14 cm.

Fujita (1929) gives a brief account of the cell lineage of the eggs of *C. gigas* and states that the mode of cleavage of this species is identical to that of *C. virginica*. The main features described by him are as follows. The first polar body in the fertilized egg of *C. gigas* appears 15 minutes after insemination. At the two-cell stage (fig. 329) the two blastomeres of unequal size, AB and CD, are separated along the meridional plane. Their position corresponds to the anterior (Ant.) and posterior (Pst.) ends of the embryo. The second division, also meridional, separates the four blastomeres A, B, C, and D (fig. 329b).

A and B represent the anterior, and C and D the posterior halves of the embryo, while B and C form its left and A and D its right halves (fig. 329b). The ensuing cleavage starts with the blastomere B and proceeds in laeotropic order to C, D, and A; the resulting daughter cells, the micromeres a_1, b_1, c_1, and d_1, retain the shape of the mother cells but are smaller. The macromere D and micromere d_1 are respectively the largest. The four daughter cells a_1 through d_1 form the first quartet of micromeres located between the macromeres on the dorsal side of the embryo.

The 12-cell stage is initiated by the division of the macromere D; the ensuing larger cell d_2 (fig. 329c) is generally known as the first somatoblast X. (In the system of nomenclature used by American and European embryologists (see p. 346) the 1D cell gives rise to 2d and 2D and the 2d is the X cell.).

The cleavage is continued laeotropically, and the second generations of micromeres a_2, b_2, and c_2 are smaller than the first macromeres. They lie on the outside of the macromeres. The third cleavage of macromeres A, B, and C continues in laeotropic order and results in the micromeres a_3, b_3, and c_3; they are larger than other micromeres. After the third cleavage the macromeres make no further contribution to the formation of micromeres and in the course of development become the entoderm. The first somatoblast (X cell) gives rise to many organs of ectodermal origin. At the 18-cell stage of the embryo the position of cell X and its first divisions mark the beginning of the transition from spiral to bilateral symmetry (fig. 329d).

The mesoderm begins to form at about the 32-cell stage with the appearance of cell 4d, the second somatoblast, also designated as cell M. In bivalves the cell M remains at the surface for a long time, then divides into the two mesodermal teloblasts which sink into blastocoel (Raven, 1958, p. 117). The formation of mesoderm in *C. gigas* has not been followed in detail, but as a rule the mesoderm bands in bivalves remain rather rudimentary (Raven, 1958). Fujita states that the establishment of the three germinal layers in *C. gigas* is completed at the 30-cell stage (fig. 329 e and f).

The gastrula stage is reached in 4 to 6 hours. The cell lineage of *C. gigas* is generally comparable to that described for other bivalves (see: Raven, 1958, p. 70), but for details the reader should consult Fujita's (1929) original text and his drawings.

In about 4 to 6 hours after fertilization, an egg of *C. virginica* reaches the stage (fig. 330) when a few large cilia become visible at the vegetal pole, the oval-shaped body is covered with very

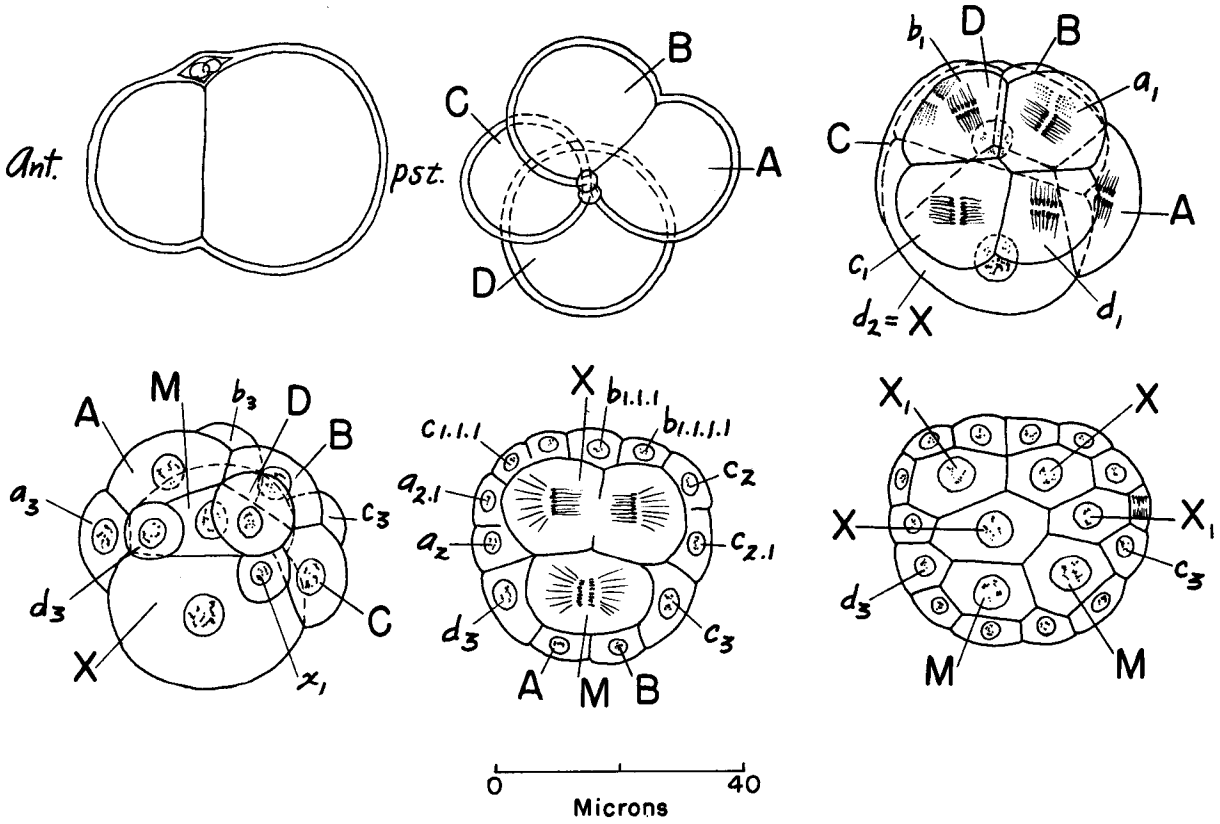

FIGURE 329.—Several stages of development of the egg of *C. gigas*. Redrawn from Fujita, 1929. a—Two-cell stage, Ant.—anterior, Pst.—posterior ends; b—Four-cell stage, formation of blastomeres A, B, C, and D; c—12-cell stage and the formation of the first somatoblast (cell X), viewed from the animal pole; d—embryo viewed from vegetative pole after the formation of the mesomere M; e—cleavage of mesomere M and the first somatoblast X, posterior view optical section; f—advanced stage of development showing the arrangement of the mesomeres M, M, and the somatoblasts, X, X, X_i, X_i, posterior view optical section. Cleavage nomenclature as given by Fujita.

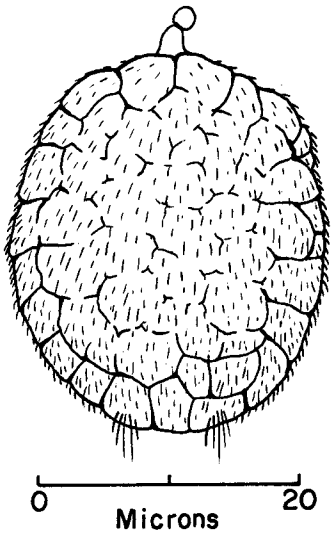

FIGURE 330.—Larva of *C virginica* ready to hatch. Drawn from photomicrograph of live larva.

fine ciliation, and two polar bodies still remain attached to the animal pole. The beating of the cilia is not coordinated at this stage, and the movements of the larva are irregular and spasmodic. A few minutes later a girdle of powerful cilia is formed, the polar bodies are lost, and the larva begins to swim upward (fig. 331). In a finger bowl containing cleaving eggs, the newly hatched larvae appear as white columns rising from the layer of fertilized eggs on the bottom of the container (fig. 332). The larvae can be pipetted off easily, transferred into larger containers and provided with suitable food.

The time required to complete the development of an oyster egg varies, depending on condition of eggs, temperature, salinity, oxygenation of water, and other environmental factors. Records of three sets of observations made in the Woods

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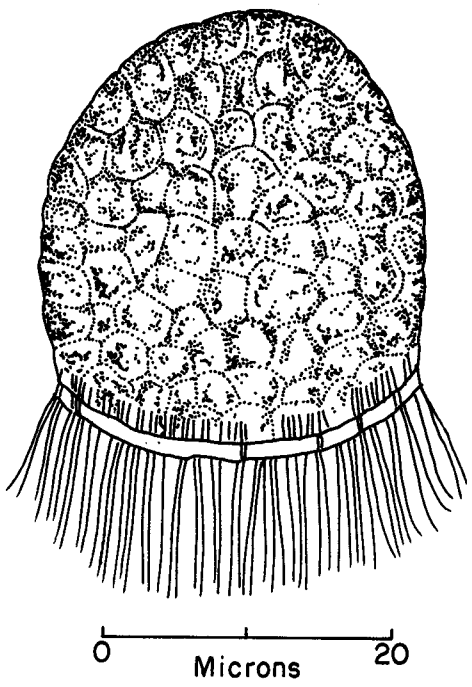

FIGURE 331.—Larva of *C virginica* at the time of its emergence 6 to 6½ hours after fertilization. Drawn from a photomicrograph of a live larva.

Hole laboratory at room temperatures varying from 22.5° to 24.5° C. and salinity of water of 32.2 °/₀₀ are given in table 37. To obtain records of rates of development at different temperatures, several hundred artificially fertilized eggs were placed in each Syracuse dish filled with fresh sea water and covered to prevent evaporation. The debris was removed, and the water contained no unfertilized or cytolyzed eggs.

TABLE 37.—*Observations on the time required for artificially fertilized eggs of* C. virginica *to reach trochophore stage*

All observations were made at Woods Hole in July at room temperatures varying from 23° to 25° C. The time required to reach different stages varied in different groups of eggs. The observations are arranged in two groups: A and B, which differ primarily in the duration of time required to reach rotating blastula and trochophore stages.

Stage of development	STAGES	
	A	B
Fertilization membrane	5 min	10 to 25 min.
First polar body	40 min	25 to 52 min.
Second polar body	1 hr. 10 min	40 to 65 min.
First cleavage	1 hr. 12 min	45 min.
Second cleavage		52 to 120 min.
Third cleavage	2 hr. 10 min	55 to 195 min.
Morula stage		135 min.
Rotating blastula	4 hr	6 hr. 30 min.
Trochophore	5 hr	8 to 9 hr.

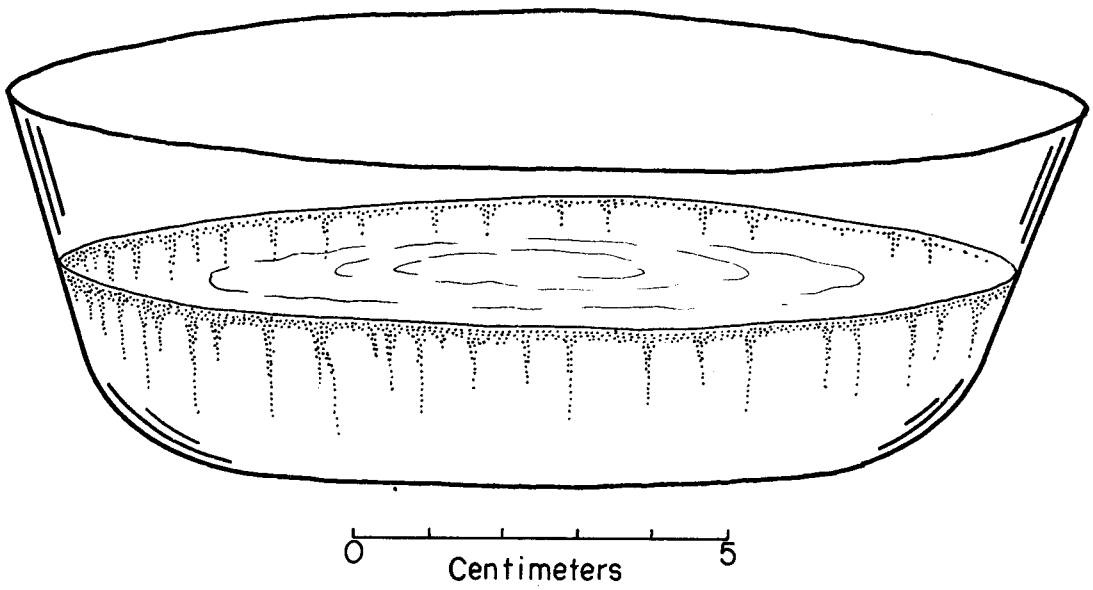

FIGURE 332.—The emergence of larvae of *C. virginica* from fertilized eggs kept in a finger bowl. The free-swimming larvae form columns, which tend to disperse at the surface. Drawn from life.

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