OOCYTE DIFFERENTIATION IN THE SEA URCHIN, *ARBACIA PUNCTULATA*, WITH PARTICULAR REFERENCE TO THE ORIGIN OF CORTICAL GRANULES AND THEIR PARTICIPATION IN THE CORTICAL REACTION

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ABSTRACT

This paper presents morphological evidence on the origin of cortical granules in the oocytes of *Arbacia punctulata* and other echinoderms. During oocyte differentiation, those Golgi complexes associated with the production of cortical granules are composed of numerous saccules with companion vesicles. Each element of the Golgi complex contains a rather dense homogeneous substance. The vesicular component of the Golgi complex is thought to be derived from the saccular member by a pinching-off process. The pinched-off vesicles are viewed as containers of the precursor(s) of the cortical granules. In time, they coalesce and form a mature cortical granule whose content is bounded by a unit membrane. Thus, it is asserted that the Golgi complex is involved in both the synthesis and concentration of precursors utilized in the construction of the cortical granule. Immediately after the egg is activated by the sperm the primary envelope becomes detached from the oolemma, thereby forming what we have called the *activation calyx* (see Discussion). Subsequent to the elaboration of the activation calyx, the contents of cortical granules are released (cortical reaction) into the perivitelline space. The discharge of the constituents of a cortical granule is accomplished by the union of its encompassing unit membrane, in several places, with the oolemma.

INTRODUCTION

The eggs of echinoderms have claimed the attention of many cell biologists. Whereas investigators have recognized the unique value of eggs from organisms within the entire phylum, it seems that the eggs of the Echinoidea, the class to which sea urchins belong, possess characteristics that are particularly useful for obtaining answers to specific questions. Harvey (38) states: "the experimental work on sea urchin eggs has included every line of approach, cytology, embryology, physiology and biochemistry, and has been concerned with the solution of many fundamental problems." While a great deal has been learned about the eggs of organisms throughout the animal kingdom, much cytological information is still needed in an effort to understand further certain events during and immediately after oogenesis. For example, as oocytes of many organisms develop there appears, within the ooplasm, a population of bodies of varied sizes and internal configurations. Initially these structures are randomly distributed; however, as the oocyte approaches maturity they come

to lie in the peripheral ooplasm. Because of the position they occupy in the mature egg, these structures have been called cortical granules both in invertebrates and in vertebrates. In the piscine egg they have been referred to as cortical alveolae (see reference 87).

Harvey (39) was the first to call our attention to the cortical granules of the eggs of *Arbacia*. The granules were later described in cytological preparations by Hendee (40), and they have since been a topic of interest and controversy among cytologists, embryologists, and physiologists. The function of cortical granules in oocytes of some organisms remains unexplained (7, 36, 42). However, it is the consensus that the cortical granules of oocytes of a wide variety of organisms are involved in the initial phase of the multistep phenomenon of fertilization (see references 2, 4, 11, 32, 33, 76, 87).

Notwithstanding the numerous papers dealing with these structures, our knowledge concerning the origin of cortical granules is somewhat nebulous and fragmentary. In our efforts to understand those structural changes occurring during differentiation of oocytes and associated structures, we have found it desirable to inquire further into the genesis and fate of cortical granules in *Arbacia*. Therefore this paper explores the origin and ultrastructure of these ooplasmic structures and their participation in the cortical reaction.

MATERIALS AND METHODS

The principal organism used in this study was the sea urchin, *Arbacia punctulata*. For comparison, more limited observations were made on the origin of cortical granules in the following species of echinoderms: *Asterias forbesi* (starfish), *Ophioderma bievispinum* (brittle star), and the sea urchins *Echinarachnius parma* and *Strongylocentrotus purpuratus*. All of the forementioned organisms were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts during the months of June and July, with the exception of *Strongylocentrotus purpuratus*, which was procured from the Pacific Bio-Marine Company, Venice, California, during the months of March, April, and May.

For light microscopic analysis, ovarian tissue was fixed in the following fixatives: Ammerman's, Carnoy's, Champy's, and 10% buffered (pH 7.4) formalin. After dehydration, infiltration, and embedding in Paraplast, sections were made of the Ammerman's and Champy's fixed material and stained with Heidenhain's iron hematoxylin, or Mallory's triple stain. Paraplast sections made of tissue fixed in

Carnoy's were stained by the periodic acid–Schiff technique, toluidine blue, bromphenol blue, and Alcian blue (41). Tissue fixed in buffered formalin was stained with Perl's Prussian blue for the demonstration of iron (15).

For electron microscopy, tissue was fixed for 2 hr in a 2% solution of glutaraldehyde (pH 7.4) in seawater, or the paraformaldehyde-glutaraldehyde (pH 7.4) mixture recommended by Karnovsky (46, 74). After fixation the tissue was washed in seawater and postfixed in a 1% solution of osmium tetroxide dissolved in seawater. Rapid dehydration of the tissue through graded concentrations of ethanol was followed by infiltration and embedding in Epon (52). 1 μ sections of the Epon-embedded material were stained according to the recommendation of Ito and Winchester (43). Thin sections were stained with uranyl acetate, followed by the lead citrate stain of Venable and Coggeshall (83), and examined with a Philips 200 electron microscope.

For a study on the participation of the cortical granules in the cortical reaction, gametes from *Arbacia* were obtained either by the electrical stimulation technique of Harvey (38) or by injecting 0.5 cc of isosmotic 0.5 M KCl into the lantern coelomic cavity (80). Sperm and egg suspensions were fixed at intervals (3 sec–60 min) after insemination at 18°–20°C. Some of the fertilized eggs were permitted to develop to the two cell stage for study of the spatial relation and configuration of the so-called hyaline layer. The fertilized eggs and the two-cell stages of the embryo were fixed and processed for electron microscopy as outlined above.

OBSERVATIONS

Light Microscopy

The ovaries of sea urchins are aciniform structures. Histological preparations reveal that the outer limits of each ovary consist of a single layer of flat epithelial cells that rest on a basement lamina (*a*, Fig. 1). There are two layers of collagenous connective tissue (84, 85). One of these subtends the basement lamina of the epithelial cells; the other is adjacent to the developing oocytes (*OC*, Fig. 1). These layers of connective tissue are separated from each other by a stratum of smooth muscle fibers (*b*, Fig. 1).

The inner layer of the ovary has been called the germinal epithelium, a terminology about which there exists some controversy. The polemics center around the following questions: (*a*) do oogonia develop in the outer or inner epithelial layer, or (*b*) do they develop in some other organ (19, 20, 75, 79)? For lack of unequivocal information in the
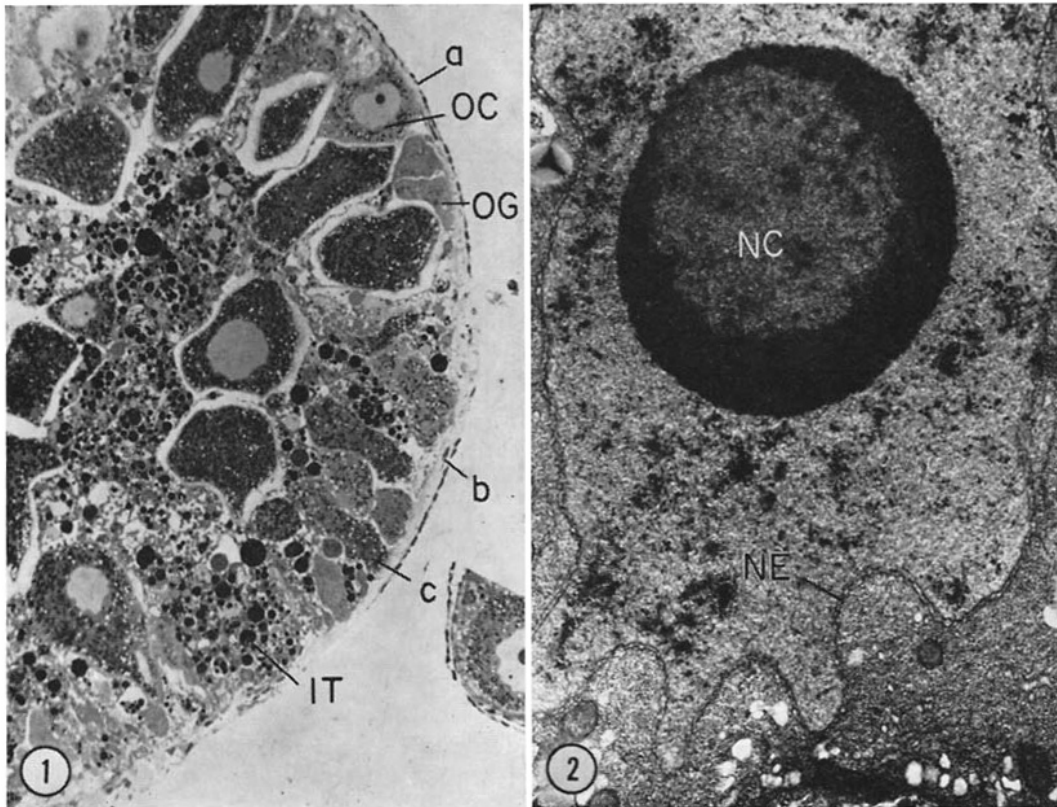
FIGURE 1 A photomicrograph of a portion of an acinus: a, outer epithelial layer; b, smooth muscle fibers and connective tissue; c, germinal layer comprised of oogonia (OG) and oocytes (OC). Note the interstitial tissue (IT). Epon-embedded, toluidine blue–stained. × 250.

FIGURE 2 An electron micrograph of a young oocyte showing its nucleus with a large nucleolus (NC) and nuclear envelope (NE). × 10,000.

forms investigated, the inner layer of the ovary will be referred to in this paper as the germinal epithelium.

Fig. 1 is a photomicrograph that depicts the germinal epithelium with oogonia (OG) and oocytes (OC) in different stages of development. At the time of spawning, the eggs are tightly packed together in the central part of the acinus. In this condition, they assume various shapes: elliptical, polygonal, or triangular. Admixed with the developing oocytes is some interstitial tissue (IT, Fig. 1) whose cellular processes are closely applied to the surfaces of oocytes (P, Fig. 4). Also found among the oocytes and interstitial tissue are various kinds of amebocytes (leukocytes). A large intranuclear crystalloid is frequently found in the amebocytes of Arbacia. The present study

confirms the observations by Karasaki (45) with respect to the structure and staining qualities of the intranuclear crystalloid.

During the maturation of oogonia, an abundant supply of yolk and echinochrome pigment is formed within the ooplasm. A detailed description of how these deutoplasmic elements develop will not be presented here. Of special interest in the context of this paper, is the appearance of a population of certain spherical bodies within the ooplasm of young oocytes, which occurred before the advent of yolk or pigment bodies. These structures are initially randomly scattered within the ooplasm; however, in the mature egg they are peripherally situated. They are PAS-positive, are metachromatic after staining with toluidine blue, and give a positive reaction for Alcian blue. The
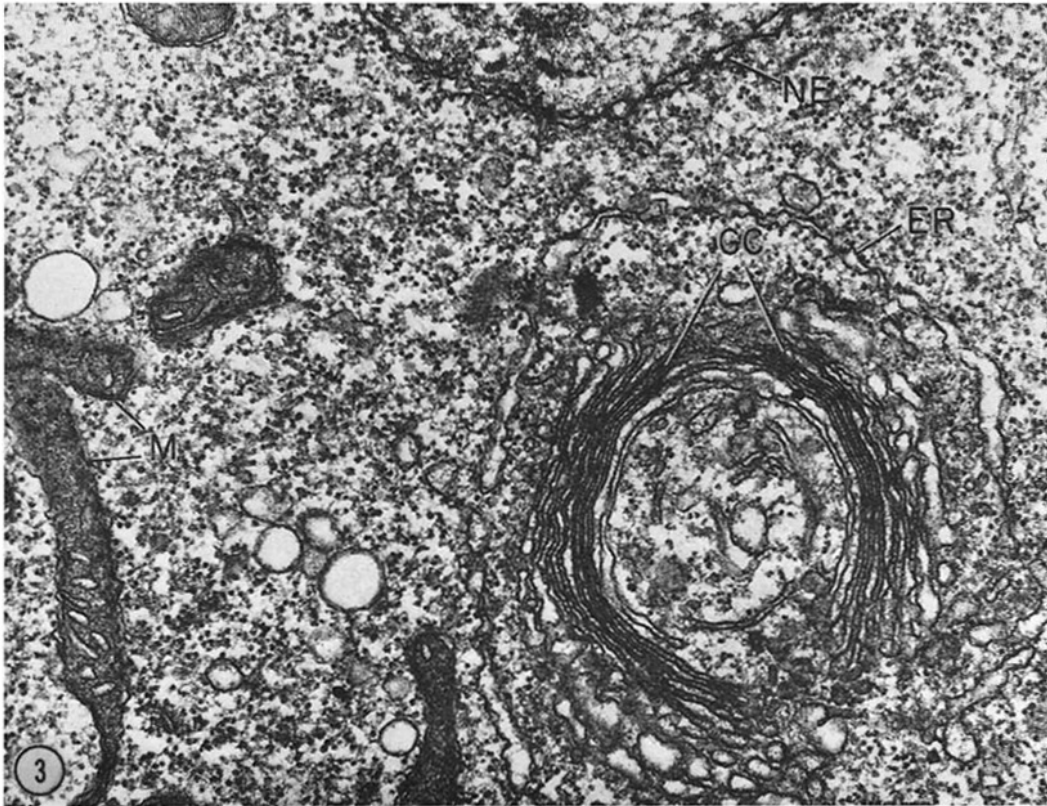
FIGURE 3 A portion of a young oocyte and illustrating nuclear envelope (*NE*), large Golgi complex (*GC*), mitochondria (*M*), and endoplasmic reticulum (*ER*). × 28,000.

aforementioned tinctorial properties indicate the presence of acid mucopolysaccharide. These bodies also give a positive reaction when stained with bromphenol blue, denoting the presence of basic protein, and they are interpreted as cortical granules. A similar reaction was obtained for the primary (vitelline) envelope. Superficial to the primary coat is a jelly-like substance which dissolves when it is in contact with seawater. This material was preserved in neither ovarian nor shed eggs prepared for electron microscopy.

Ultrastructure

OOGONIUM, OOCYTE, AND MATURE EGG: The nucleus of an oogonium is organized like that of early and late oocytes, i.e. it consists of a large bipartite nucleolus (*NC*, Fig. 2) surrounded by a granular nucleoplasm that is limited by a perforated nuclear envelope (*NE*, Fig. 2). In *Arbacia* and presumably in other sea urchins, meiosis of

the egg nucleus occurs within the ovary (38, 82). The haploid pronucleus of the mature egg is rather small (Fig. 9) when compared with the diploid nucleus of young and late oocytes.

Located in an adnuclear position within oogonia and very young oocytes is a large Golgi complex (*GC*, Fig. 3). The ooplasm of oogonia contains a host of ribosomal particles and few mitochondria. The rough endoplasmic reticulum makes its initial appearance in very young oocytes (*ER*, Figs. 3–5). As differentiation proceeds, there is an increase in quantity of the previously mentioned organelles, the appearance of the so-called heavy bodies (*HB*, Fig. 9) (see reference 3), and annulate lamellae (*AL*, Fig. 15). In addition to these organelles and inclusions, numerous vesicular bodies also appear within the ooplasm. The interiors of these bodies contain some rodlike structures (*VR*, Figs. 13, 14, 16–18).
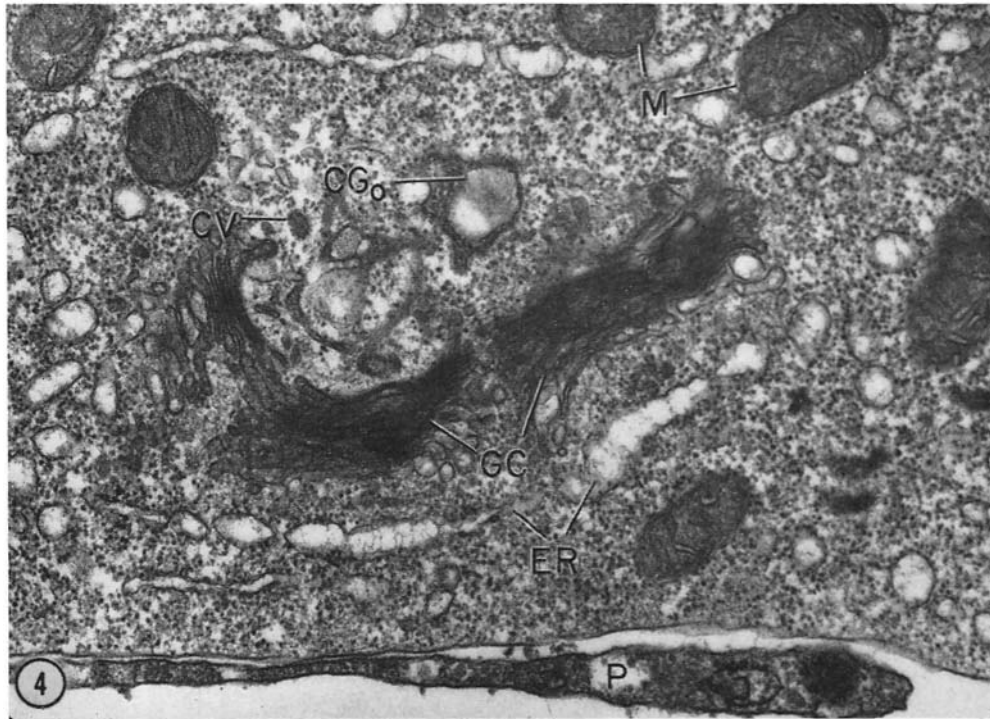
CORTICAL GRANULES: Each Golgi com-

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FIGURE 4 An electron micrograph of a small portion of a young oocyte. *GC*, Golgi complexes; *CV*, a coated vesicle; *CG₀*, a presumptive cortical granule; *ER*, endoplasmic reticulum; *M*, mitochondria. Note the cytoplasmic projection (*P*) of an interstitial cell closely applied to the nonmorphologically specialized oolemma of a young oocyte. × 28,000.

plex consists of a number of saccules arranged in parallel array. Many of the saccules contain a relatively dense substance (*GC*, Figs. 4, 5). The surfaces of the dilated-tip saccules possess a fuzzy coat. In the vicinity of the saccules are mitochondria, cisternae of the rough endoplasmic reticulum, and a host of vesicles of varied sizes and shapes. The vesicles contain a substance whose density is similar to that of the material within the saccules. Some of these vesicles also possess a fuzzy coat; they are thought to be produced by being pinched from the tips of the saccules of the complex. In Figs. 5 and 6 the structures labeled *CG₀*, and *CG1* are closely associated with the Golgi complex (*GC*). Structures designated as *CG₀* are here identified as presumptive cortical granules, while those marked *CG₁* are defined as miniature cortical granules.

The miniature cortical granule is encompassed by a unit membrane and contains two components of different density and consistency (*CG1*, Figs. 5, 6). The dense component, which is triangular

in shape, sometimes appears granular; however, it is often reticular. The less dense, usually ovoid portion commonly consists of a filamentous material. As the oocyte continues to differentiate, the cortical granules become randonly distributed within the ooplasm and increase in number and size (in *Arbacia* this size ranges from 0.5 to 1 μ in diameter). The dense component of the mature cortical granule is stellate and marginated by a variable number of less dense ovoid units. When the oocyte develops into a mature egg, the cortical granules are found primarily in the peripheral ooplasm (*CG*, Fig. 9). The tangential section through the periphery of a mature egg, illustrated as Fig. 7 (*CG*), reveals the spatial distribution of the cortical granules and other ooplasmic components. The unit membrane encompassing the contents of the mature cortical granule (*MC*, Fig. 10) is separated from the unit membrane (*OL*, Fig. 10) of the oolemma (see below) by a space of about 200 A.

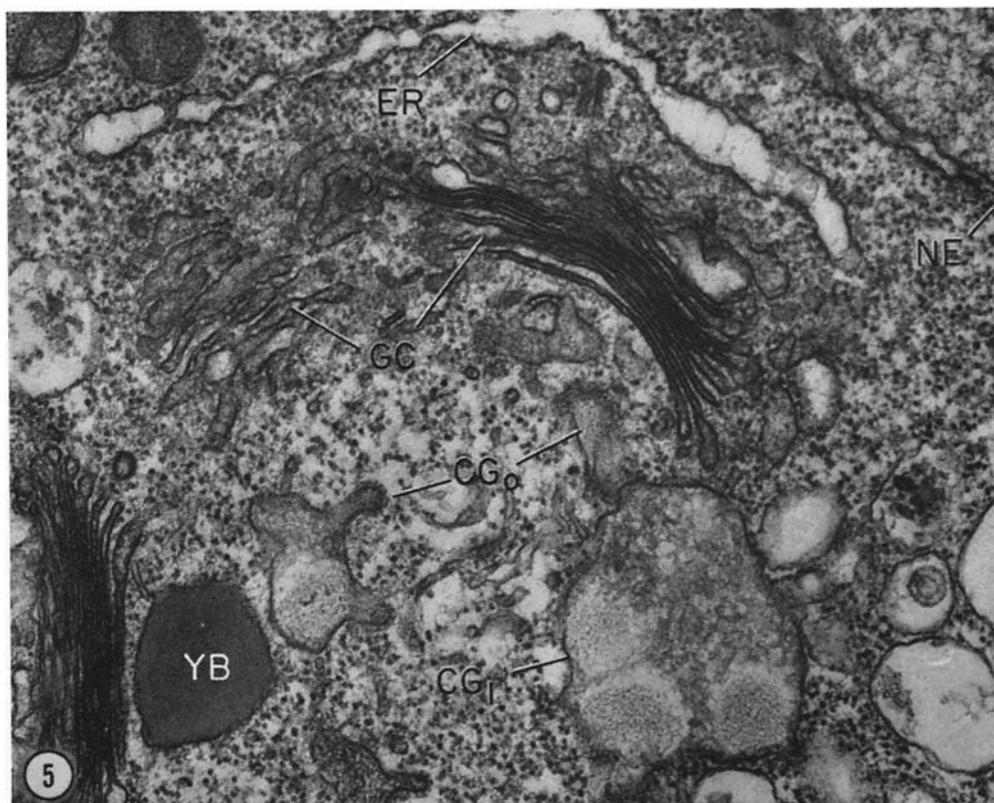The cortical granules of the species listed in the

FIGURE 5 Section through a young oocyte which shows nuclear envelope (NE), cisternae of endoplasmic reticulum (ER), Golgi complex (GC), presumptive cortical granules (CG$_o$), a miniature cortical granule (CG$_1$), and a yolk body (YB). × 40,000.

Materials and Methods section of this paper are formed in a manner similar to that indicated above for *Arbacia*. Although the cortical granules of each investigated species are bounded by unit membranes and contain two structural components, the organization of the internal components varies according to species (see reference 78). For example, Fig. 8 depicts the cortical granule of *Strongylocentrotus purpuratus* which contains a compact filamentous unit that is often eccentrically placed. Emanating from the compact unit (CS) are a variable number of lamellar structures (LS), each of which is composed of a granular material. Each lamella is associated with its neighbor by fine filaments.

Oolemma

During oocyte differentiation, the oolemma becomes specialized by the formation of micro-

villi (MV, Figs. 9, 12). In *Arbacia*, the microvilli are short and few in number when compared with the large number of rather slender ones produced by the oolemma of the oocytes of the brittle star, *Ophioderma*. It should be pointed out that microvilli are rarely found on those areas of the oolemma overlying a cortical granule in a mature *Arbacia* egg.

Associated with the morphologically specialized surface of the oolemma is the primary (vitelline) envelope composed of a substance which has a matted appearance. In *Arbacia* and *Strongylocentrotus* the primary envelope is produced in scant amounts (PC, Fig. 12 inset c), whereas in *Asterias* it is produced in rather large quantities.

FERTILIZATION: Inset a Fig. 12 is a phase-contrast photomicrograph of a mature living egg. Fig. 9 (inset) shows the initial contact of the sperm with the egg immediately after insemination. After
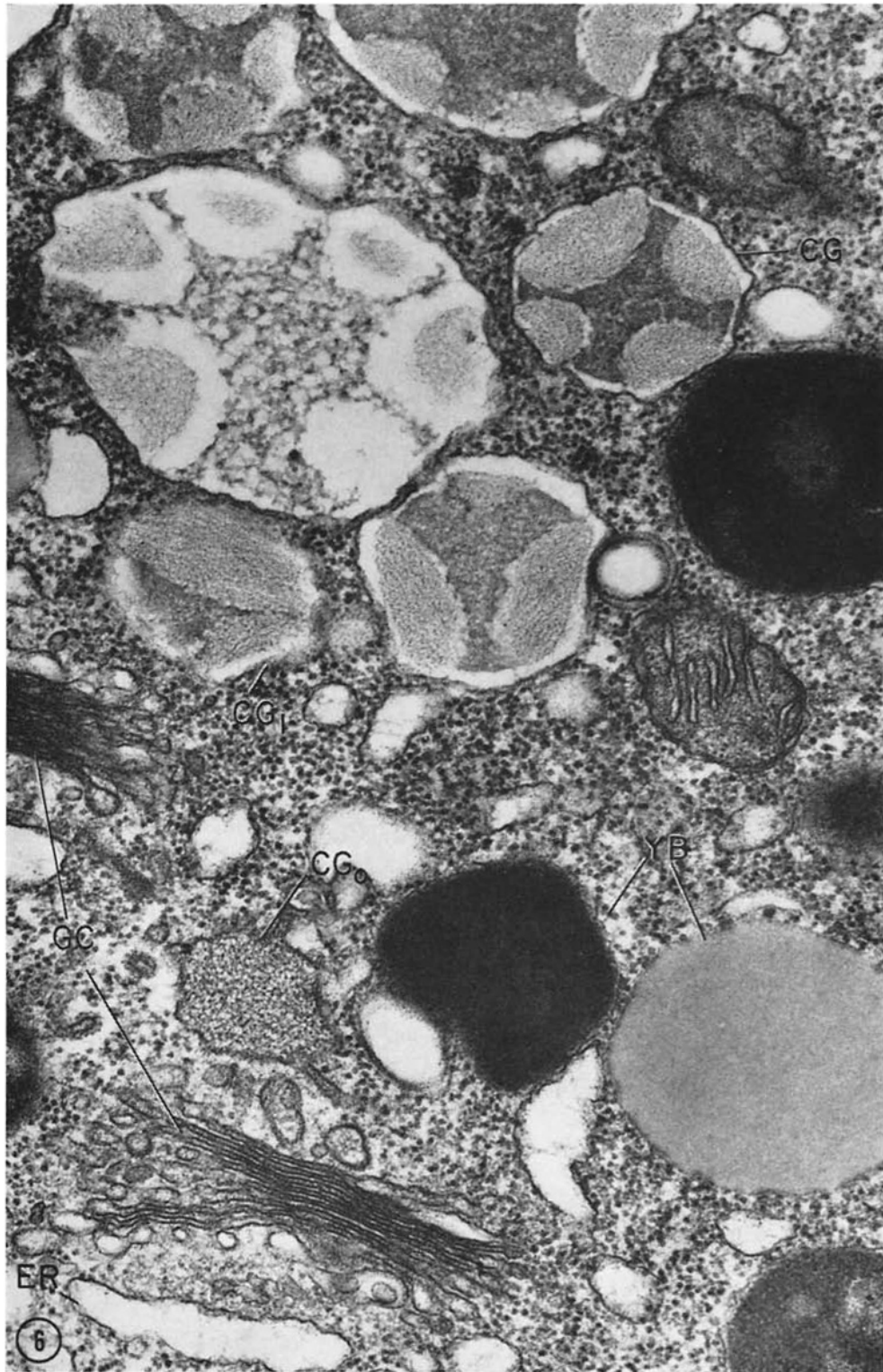
FIGURE 6 A section through the ooplasm of a young oocyte; *GC*, Golgi complex; *CG_o*, presumptive cortical granules; *CG₁*, miniature cortical granule, *CG*, cortical granule; *ER*, cisternae of the endoplasmic reticulum; *YB*, yolk bodies. $\times$ 42,000.
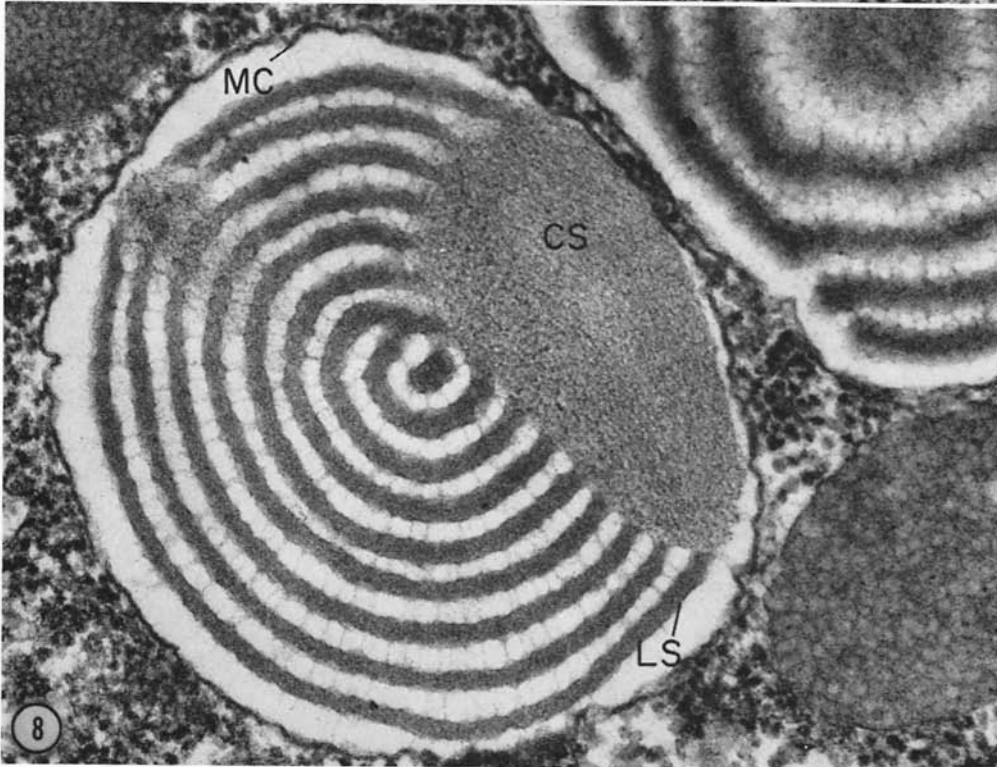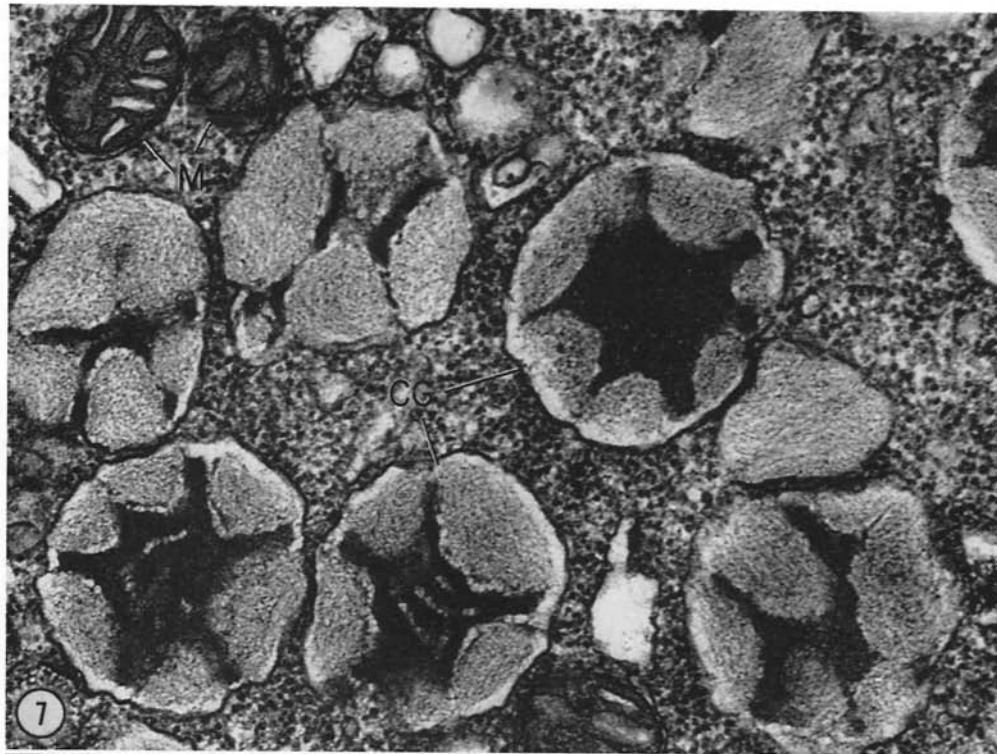
FIGURE 7 A tangential section through the peripheral ooplasm of a mature egg illustrates cortical granules (*CG*) and mitochondria (*M*). × 42,000.

FIGURE 8 A section of a late oocyte of *Strongylocentrotus purpuratus* illustrates portions of two cortical granules, one of which shows a compact structure (*CS*) associated with lamellar units (*LS*). The lamellar units are associated with each other by fine filaments. Note the unit membrane (*MC*) of the cortical granule. × 90,000.
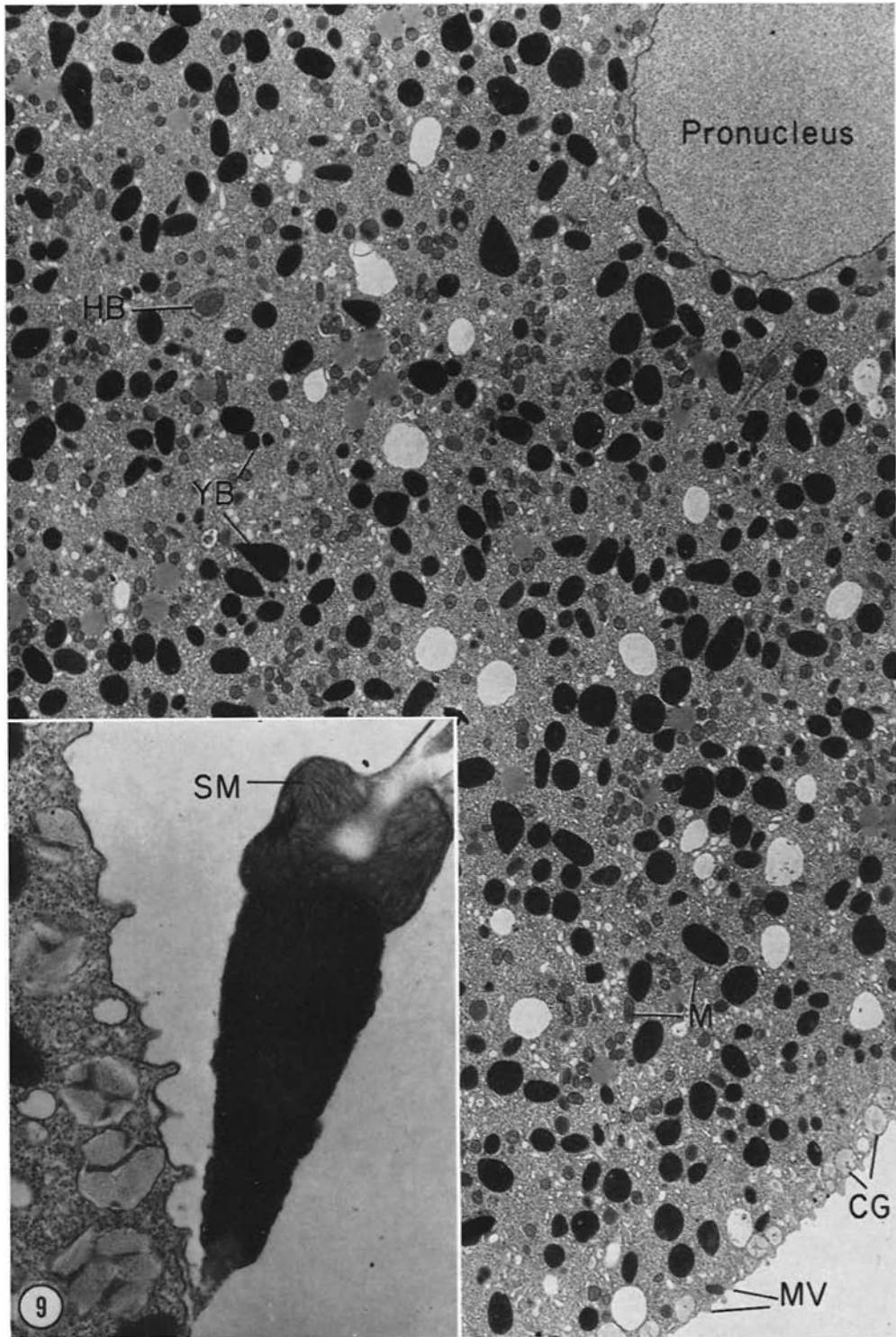
FIGURE 9   A section of a mature egg. Pronucleus; *HB*, so-called heavy bodies; *YB*, yolk bodies; *M*, mitochondria; *CG*, cortical granules; *MV*, microvilli. Inset shows the initial contact of a sperm with the egg's surface. *SM*, sperm mitochondrion. Fig. 9, × 5,000; inset, × 16,000.
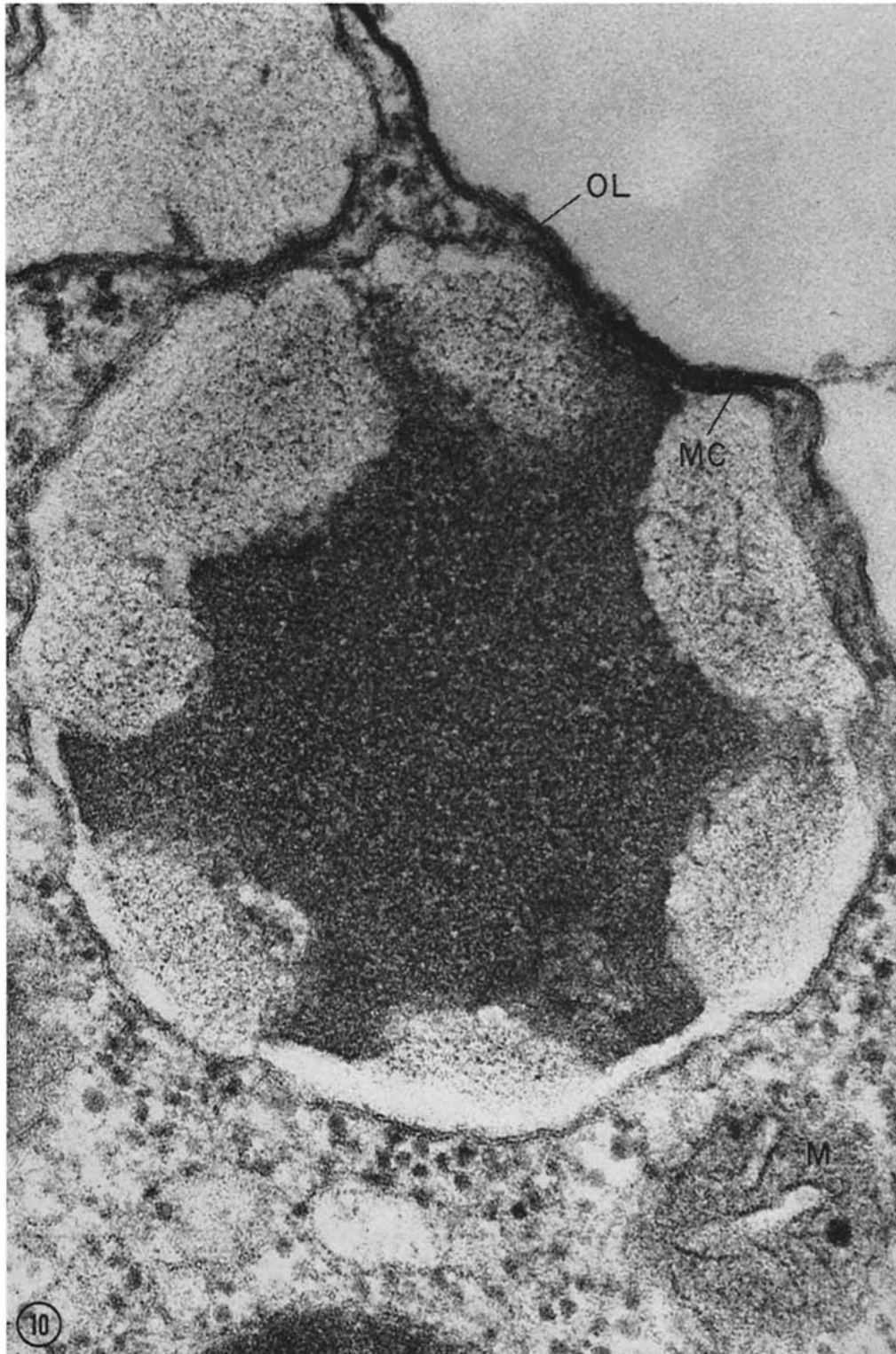
FIGURE 10   A section through a mature cortical granule. *MC*, membrane of cortical granule; *OL*, oolemma; *M*, mitochondrion. × 140,000.

FIGURE 11   A section illustrating the release of acrosomal material (*AM*) by the sperm (*S*) and portions of discharged cortical granules (*CG**). *PC*, primary coat; *CG*, intact cortical granule; *YB*, yolk body. × 42,000.
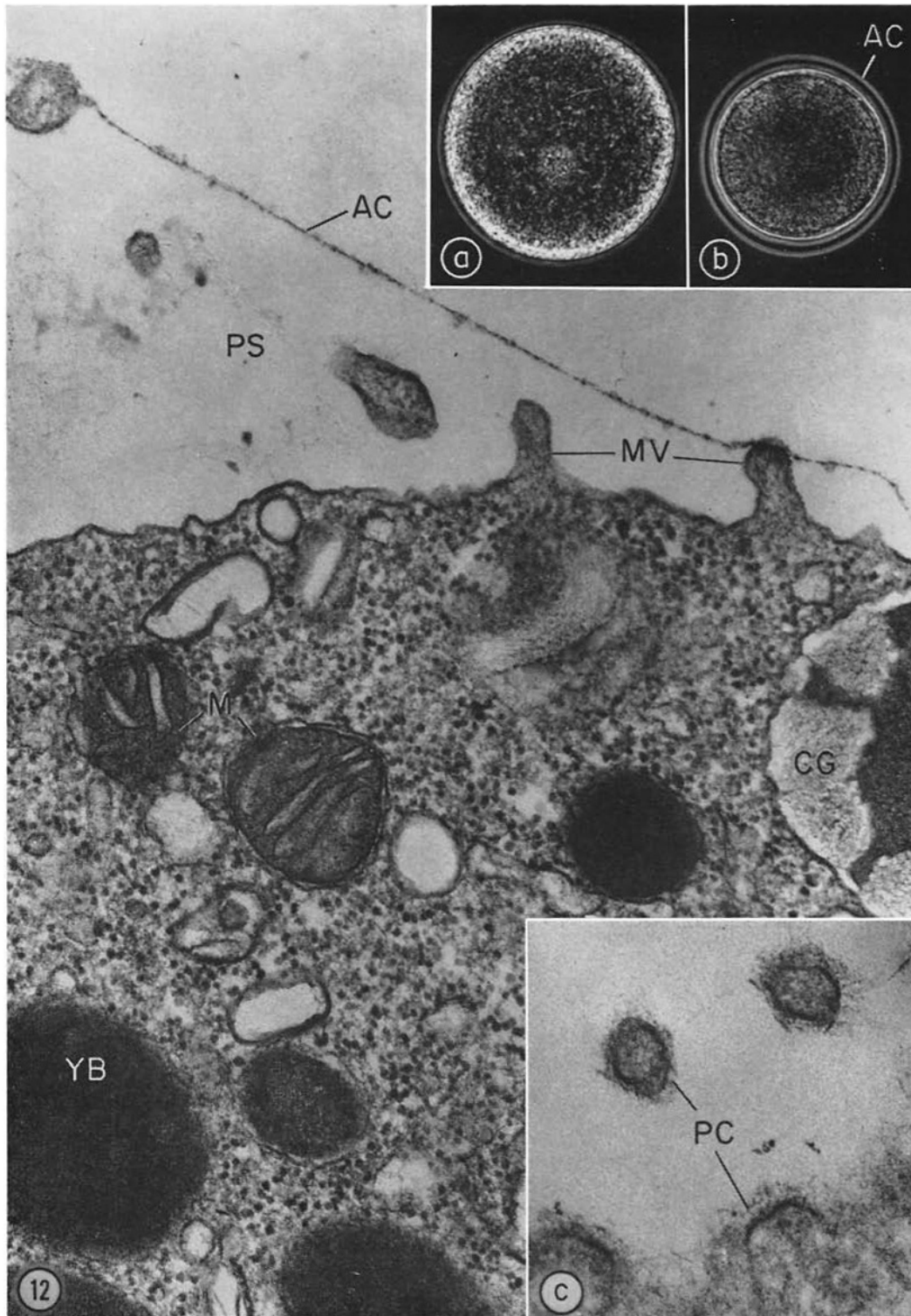
FIGURE 12   A section of an activated egg showing activation calyx (*AC*), perivitelline space (*PS*), microvilli (*MV*), yolk body (*YB*), and a portion of a nonactivated cortical granule (*CG*). Inset *a* is a phase-contrast photomicrograph of a living mature egg. Inset *b* is a phase-contrast photomicrograph of a living fertilized egg showing the complete activation calyx (*AC*). Inset *c* is an electron micrograph of a tangential section of a mature egg illustrating the primary coat (*PC*). *M*, mitochondrion. Fig. 15, × 70,000; inset *a*, × 800; inset *b*, × 700; inset *c*, × 80,000.
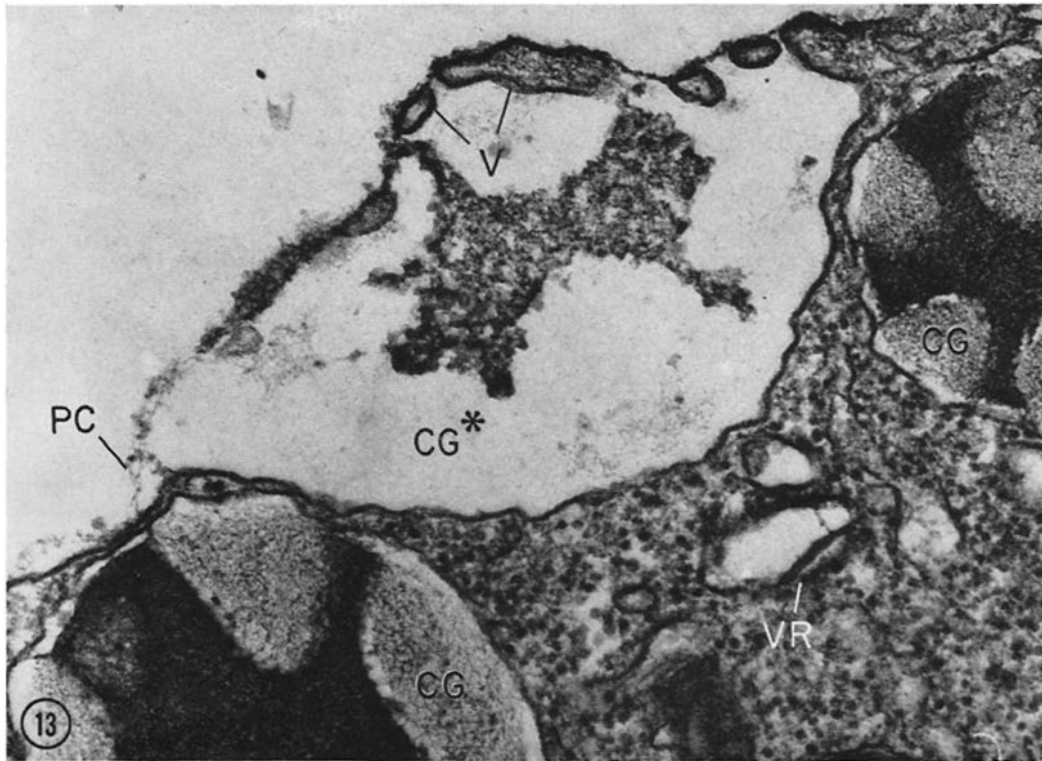
FIGURE 13 A section through a portion of the surface of an activated egg showing the primary coat (PC), two intact cortical granules (CG) and a partially released one (CG*). Note the membrane-bounded vesicles (V) at the top of the partially released cortical granules and vesicles with rodlike structures (VR). × 61,000.

the initial contact of the sperm with the egg, the acrosome undergoes what has been described as the acrosomal reaction (see references 28, 31, 35). Such a reaction was observed in material fixed 3–4 sec after insemination (AM, Fig. 11). Shortly after, or at the time of the liberation of the acrosomal substance, there occur (a) the formation of the so-called fertilization membrane (see Discussion), (b) the release of the contents of cortical granules, and (c) the fusion of the sperm with the egg.

FERTILIZATION MEMBRANE: Fig. 12 is an electron micrograph of an egg fixed about 30 sec after insemination. Here the primary envelope becomes disjoined from the surface of the oolemma, thereby forming the fertilization membrane (AC). When the membrane is elevated, an area, known as the perivitelline space, is produced between it and the egg (PS, Figs. 12, 15–18). The fertilization membrane is not formed over the entire sur-

face of the egg simultaneously. Often, however, it is initiated at the point of sperm-egg fusion and, with time, progresses around the circumference of the egg (see reference 54). About 3 min after union of the gametes the fertilization membrane (AC) appears over the entire egg as shown in the phase-contrast photomicrograph featured as inset b in Fig. 12. Subsequent to the entrance of the sperm contents into the ooplasm the fertilization membrane becomes thicker. Figs. 17 and 18 (AC) illustrate this membrane at 6 min after insemination. The membrane retains this appearance up to about 12 min following insemination (see below).

RELEASE OF THE CONTENTS OF CORTICAL GRANULES: Figs. 13 and 14 show cortical granules in various stages of their dehiscence. The outer portion of the cortical granules show membrane-bounded vesicles (V) of varied sizes. Some of these vesicles become closely associated
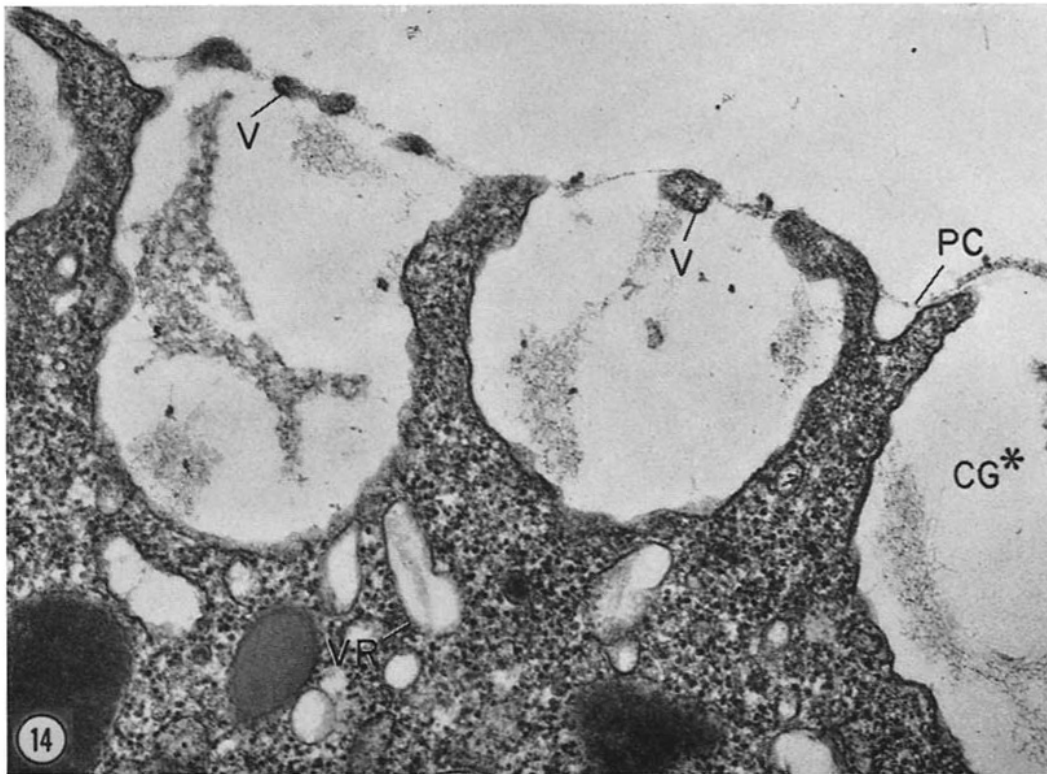
FIGURE 14   A section through an activated egg showing three partially released cortical granules (*CG**).
PC, primary coat; *V*, membrane-bounded vesicle; *VR*, vesicle containing rodlike structures. × 40,000.

with the inner aspects of the fertilization membrane (*V*, Fig. 16). The primary envelope labeled *PC* in Figs. 13 and 14 was presumably fixed while it was in the process of being detached from the oolemma. Eventually, the contents of the cortical granules (*CG**, Figs. 15, 16) come to lie within the perivitelline space. When the contents of a cortical granule appear within the perivitelline space the major portion of the membrane limiting the cortical granule is now confluent with a portion of the original oolemma. This "new" membrane (see Discussion) possesses some invaginations whose cytoplasmic side is coated (*PV*, Fig. 15 inset). These invaginations are interpreted as initial stages of micropinocytosis. Just prior to the completion of the cortical reaction, i.e. release of the components of the cortical granules into the perivitelline space, the sperm fuses with the egg (*PMS* ↔ *PME*, Fig. 16) (see references 24–26). The egg responds to this fusion by the production of the fertilization cone (*FC*, Fig. 16).

About 7 or 8 min after the initial release of the

contents of the cortical granules into the perivitelline space, a stratum is formed known as the hyaline layer (37). Initially, it is composed of what appears to be fine filaments organized in a reticular pattern (*HL*, Figs. 19, 21). During later stages of the maturation of the fertilized egg, the hyaline layer becomes thicker; however, it displays the same structural organization as previously described. Prior to cleavage, the hyaline layer is within the perivitelline space around the fertilized egg. At the two cell stage the hyaline layer follows the contour of the blastomeres; it is not found between blastomeres.

RELEASE OF THE RODLIKE STRUCTURES FROM VESICULAR UNITS: Attention has already been called to the vesicular bodies that contain rodlike structures. During differentiation of the oocyte these structures do not acquire a specific position within the ooplasm; they are randomly distributed. *After* the completion of the cortical reaction many of the vesicular bodies are found closely associated with the new plasma
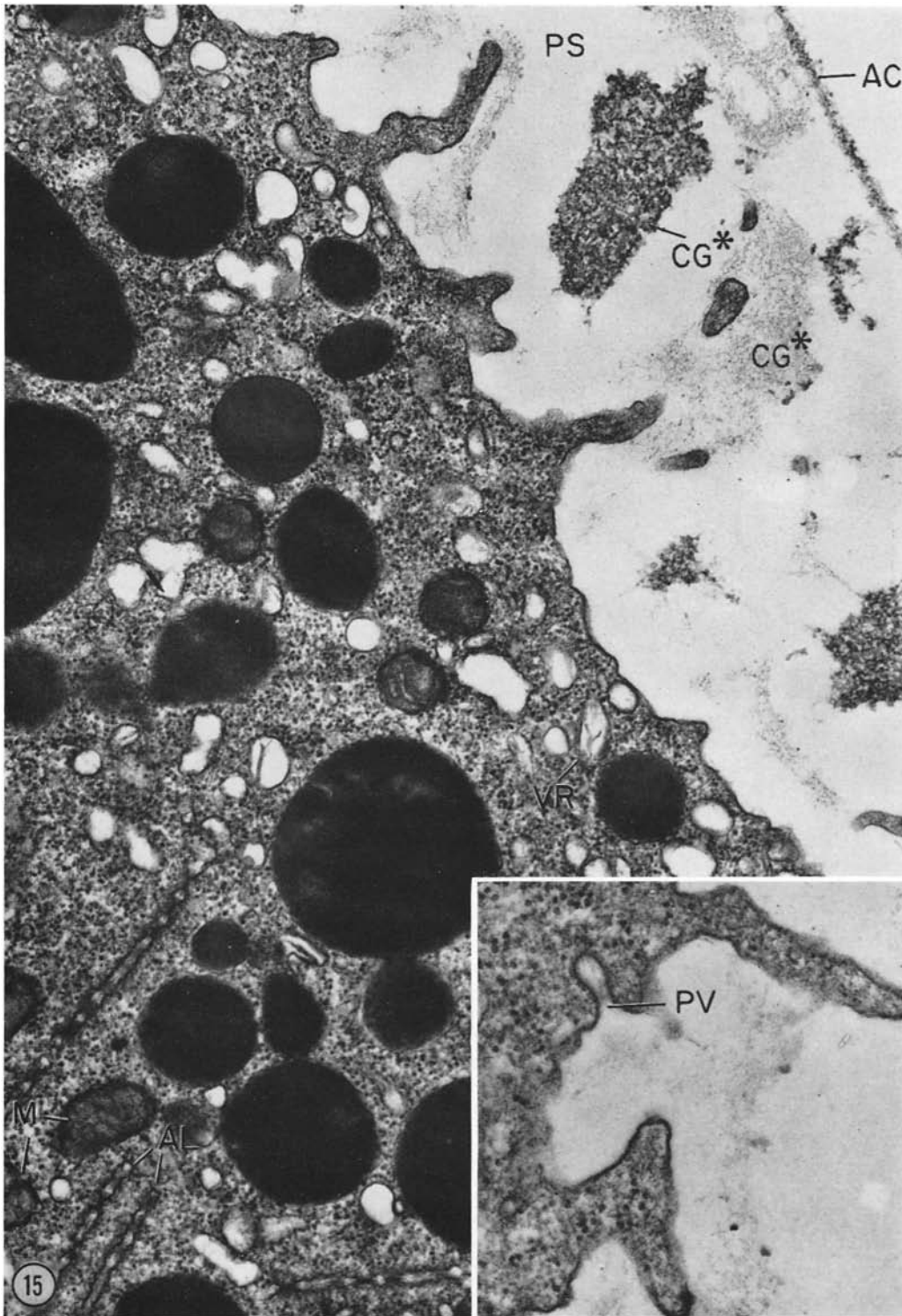
FIGURE 15 A section through the surface of a fertilized egg. *AC*, activation calyx; *PS*, perivitelline space; *CG**, dense and less dense portions of discharged cortical granules; *VR*, vesicles with rodlike structures; *PV*, pinocytotic invagination (see inset); *M*, mitochondria; *AL*, annulate lamellae. Fig. 15, × 27,000; inset, × 42,000.

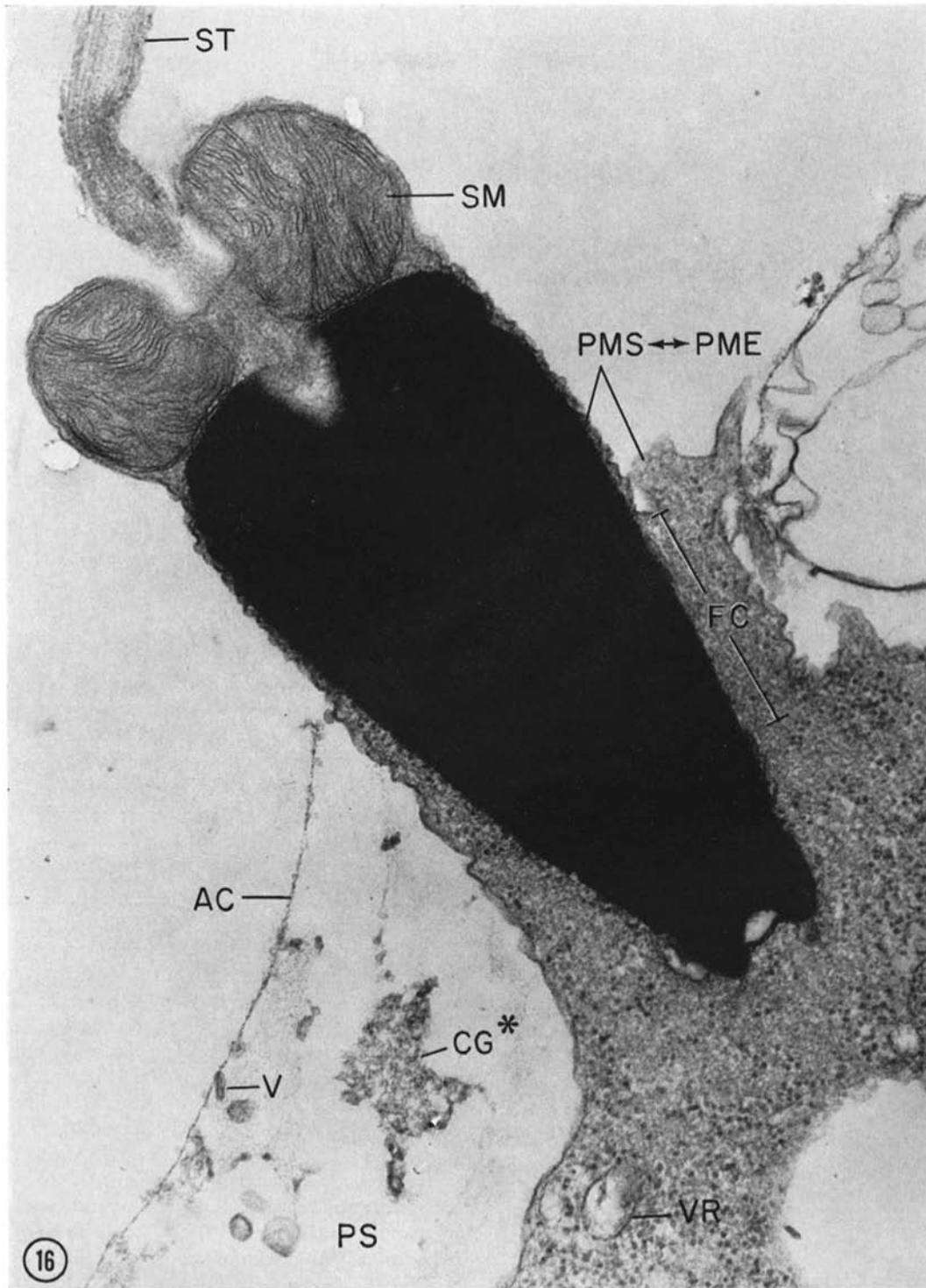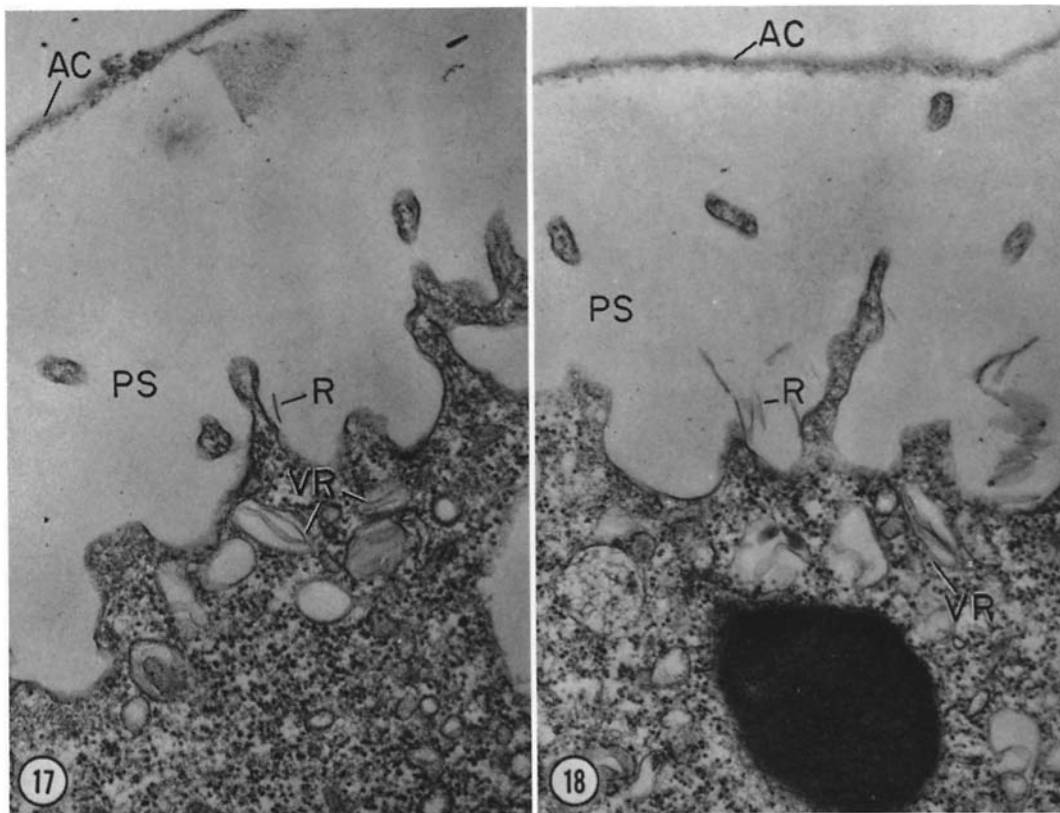FIGURE 16   A section through the fertilization cone (*FC*) where the plasma membrane of the activated egg is continuous with that of the sperm (PMS ↔ PME). *ST*, sperm tail; *SM*, sperm mitochondrion; *AC*, activation calyx; *CG**, dense portion of a discharged cortical granule; *V*, membrane-bounded vesicle; *VR*, a vesicle containing rodlike structures; *PS*, perivitelline space. × 42,000.

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FIGURES 17 and 18 Sections through the periphery of two activated eggs 6 min after fertilization. *AC*, activation calyx; *PS*, perivitelline space; *R*, rods within the perivitelline space; *VR*, vesicles containing rodlike structures. × 42,000.

membrane (*VR*, Figs. 15, 17, 18) of the fertilized egg. In *Arbacia* the rodlike structures display an axial periodicity (*R*, Fig. 20) and were first noted within the perivitelline space 6 min after insemination (*R*, Figs. 17, 18; also see Fig. 19). The rods of *Arbacia* may be equivalent to those of Japanese sea urchins described by Endo (33) and Runnström (72). Presumably the membrane of the vesicles fuses with the membrane of the fertilized egg, and contents of the vesicles are released into the perivitelline space. Extrusion of the rods of these vesicles appears to be akin to the secretory process like that observed in merocrine glands (65). Some of these rods become enmeshed within the constituent (s) of the hyaline layer. The vesicles containing the rods are found not only in oocytes and mature eggs but also within the cytoplasm of blastomeres (two cell stage), and they

are apparently released from these cells during this early stage of embryonic differentiation.

CHORION: Fig. 21 is an electron micrograph of the fertilization membrane (*AC*) 12 min (at about 22°C) after insemination. This report is not concerned with the fusion of male and female pronuclei; however, it is important to point out that the illustration presented as Fig. 21 was made from a fertilized egg whose pronuclei were just beginning the process of fusion (Longo, F., and E. Anderson. Unpublished observations). After 14 min the pronuclei have fused and the fertilization membrane appears as two dense lines separated by a homogeneous area (*CH*, Fig. 22). This layer may now be referred to as the chorion (see Discussion). As the zygote (single cell, 2N nucleus) continues to differentiate there is an increase in the thickness of this stratum (*CH*, Fig. 19). This increase is not a simultaneous one, for the stratum

appears to thicken in several places. 25–30 min after insemination and during the two cell stage, the chorion (*CH*) appears to be like that shown in Fig. 23. The chorion is a tough layer and is closely associated with the blastomeres (*CH*, Fig. 25); however, it may be easily cut away from the embryo (*CH*, Fig. 24). It is composed of two tri-laminar structures (outer *ol*, Fig. 23, and inner *il*) each of which is composed of two 25–30 A dense lines separated by a gap of approximately 50 A. The inner and outer trilaminar structures are separated from each other by a space of about 500–600 A.

DISCUSSION

Origin of Cortical Granules

This study has shown that as oogonia differentiate into oocytes, the first recognizable constituent of the ooplasm is the cortical granule. Staining procedures reveal that these cortical structures contain acid mucopolysaccharide and protein; this finding confirms previous histochemical studies (4, 55, 56). When very young oocytes were examined with the electron microscope the organelle conspicuously associated with cortical granules is the Golgi complex. Each component comprising the Golgi complex, i.e. saccules and companion vesicles, is filled with a rather dense homogeneous substance. A constant feature of the aforementioned organelle is the presence of miniature forms of "mature" cortical granules. Mitochondria are found in the vicinity of the Golgi complex, and a noticeable characteristic is the presence of cisternae of rough endoplasmic reticulum. These morphological observations encourage the suggestion that the Golgi complex plays a major role in the production of cortical granules (8). Here one might think of the genesis of cortical granules as commencing within the vesicular component of the Golgi complex that is presumably derived from the saccules by being pinched from their tips. These vesicles, which are thought to contain the precursor(s), may be viewed as presumptive cortical granules. The presumptive cortical granules subsequently increase in diameter by coalescing with others and thereby produce larger ones. During further differentiation they enlarge and assume a position in the peripheral ooplasm immediately beneath the oolemma.

If the hypothesis set forth is true, namely that the vesicular component of the Golgi complex

contains the precursor(s) of the cortical granule, one must assume that either all or a portion of the chemical components are fabricated within the saccules of the Golgi complex. In recent years investigators have concerned themselves with the function of the Golgi complex. In connection with this, Caro (21) and Caro and Palade (22), for example, coupled the techniques of radioautography and electron microscopy to elicit information concerning the function of this organelle in the acinar cells of the pancreas. Their studies revealed that the Golgi complex is a site for protein concentration (also see reference 44). Caro and Palade found that the protein concentrated by the Golgi complex was transferred to this organelle after it had been fabricated within the cisternae of rough endoplasmic reticulum. Utilizing the protocol outlined by Caro and Palade, other investigators have presented evidence that the Golgi complex is not only capable of sequestering protein but is involved in the production of polysaccharides (68, 69). While similar experiments have not been carried out in this study, it does not seem unreasonable to suggest that the protein component of the cortical granule is synthesized by the rough endoplasmic reticulum and transferred to the Golgi complex where it becomes complexed with the polysaccharide fabricated by the Golgi complex. The concept presented above is similar to that reported for the origin of cortical granules in other organisms (1, 12, 15, 47, 77). For different views concerning the origin of cortical granules the reader is referred to references 53 and 73.

Electron micrographs accompanying this paper disclose that vesicles near the saccules of the Golgi complex possess a surface coat. In this connection, it has been demonstrated many times and in a wide variety of cell types that, during the process of micropinocytosis, the pits and invaginations of the plasmalemma which are destined to form vesicles also possess a coat on their cytoplasmic side. The suggestion has been made that the coated pits, routed to become cytoplasmic vesicles, are regions on the plasmalemma that are specialized for the uptake of certain substances, for example protein (5, 71). Moreover, if one examines electron micrographs published by investigators long before attention was called to coated vesicles, one finds coated vesicles commonly associated with the saccules of the Golgi complex. There is relatively little micropinocytotic activity on the oolemma of the
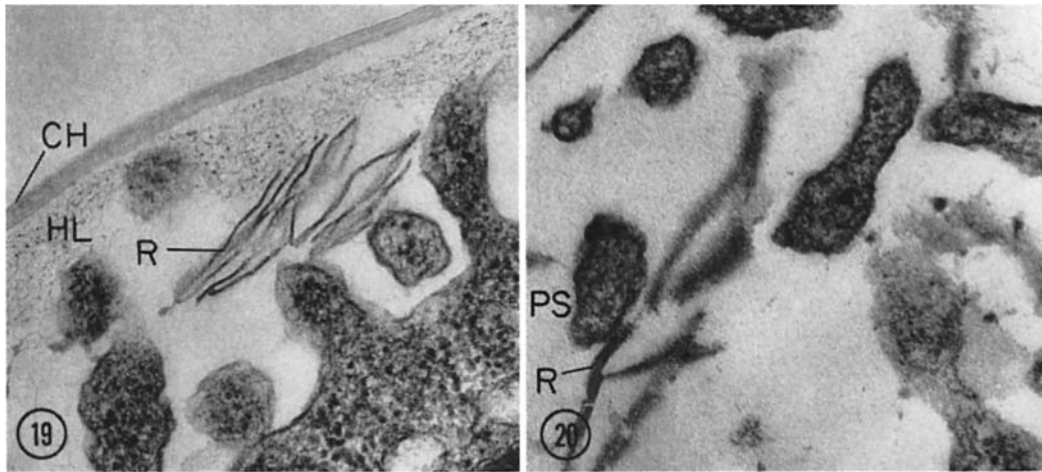
FIGURES 19–20 Small portions of the surface of an egg 30 min after insemination. *CH*, chorion; *HL*, hyaline layer; *R*, rods within the perivitelline space (*PS*). Fig. 19, × 43,000; Fig. 20, × 80,000.

oocytes investigated in this study. Therefore, it is thought that the coated vesicles in the vicinity of the saccules of the Golgi complex do not originate from the oolemma but rather from the Golgi complex. In reference to the latter, Anderson (6) has stated that "it does not seen unreasonable to assume that the flattened sacs of the Golgi complex, after sequestering certain classes of substances, may be induced to undergo evaginations with the production of aveolate and /or nonaveolate vesicles. In other words, *it would appear that the Golgi membranes are selective* and the stimulus(i) and mechanism(s) for the formation of Golgi vesicles may be equivalent to that which operates in the plasma membrane."

Fertilization Membrane

During the differentiation of oocytes of *Arbacia* and other organisms the oolemma acquires a homogeneous, sometimes filamentous coat. This coat in *Arbacia* is composed of acid mucopolysaccharide and is the primary envelope (10). Shortly after the sperm comes into contact with the egg the so-called fertilization membrane is produced (see reference 27). Initially this membrane is none other than the primary envelope. Whether the envelope lifts up from the oolemma or the egg shrinks away from it is obscure. Both interpretations have been offered (57). Of interest is the structure of this layer to which the term membrane has been applied. In reference to the nomenclature "mem-

brane", Bennett (18) has pointed out that the term membrane has been used to indicate many things; for example (a) tissue membranes, (b) cellular membranes, (c) basement membranes (basement laminae), and (d) cytoplasmic membranes. Morphologically, the structure that is disassociated from the surface of the egg following sperm activation would be similar to what Bennett has categorized as a basement membrane (basement lamina). Moreover, as a result of a comparative cytological study of material on the surfaces of plasmalemmas, Bennett (17) found that these surface coats are all rich in polysaccharides. He assigned the inclusive term "glycocalyx" to these surface coats, a term that indeed applies to the primary envelope (vitelline envelope) of sea urchin oocytes. It is obvious that the structure of the layer that initially defines the outer limits of the perivitelline space does not conform to the configuration of a unit membrane (70). While the terminology "fertilization membrane" is venerable, the appellation "membrane" does not, morphologically, identify it properly. Since activation is one of the initial phases in the multistep phenomenon of fertilization and since the resulting disjoined structure was the original glycocalyx, it is therefore proposed that the descriptive name "activation calyx" be given to what was formerly called the fertilization membrane.

It is well known that the activation calyx may appear not only by sperm activation but also by
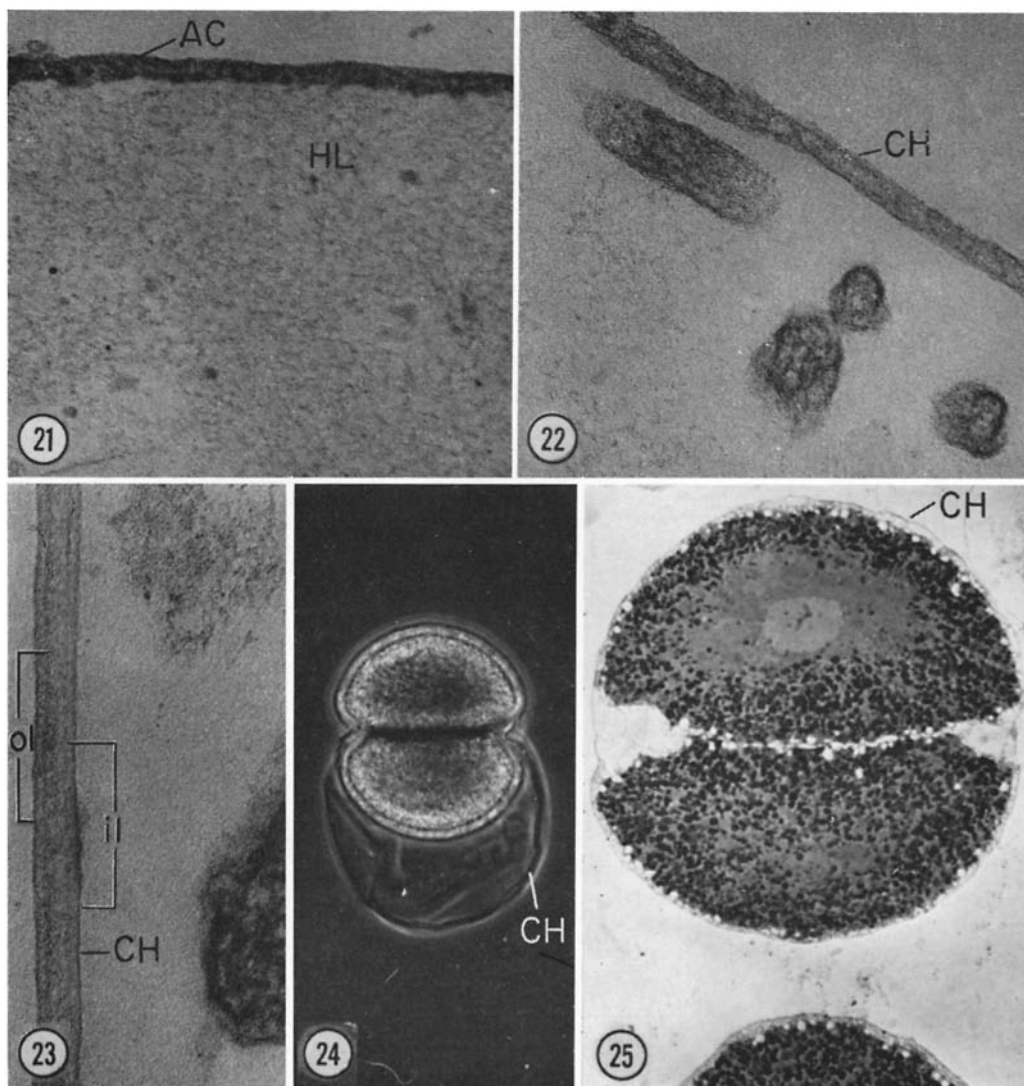
FIGURES 21–23   Fig. 21 shows the activation calyx *(AC)* 10–12 min after insemination and the hyaline layer *(HL)*. Fig. 22 illustrates the chorion *(CH)* 14 min after insemination. 30 min after insemination up to the two-cell stage of the embryo the chorion *(CH)* appears to be like that indicated in Fig. 23. It consists of an outer *(ol)* and an inner *(il)* trilaminar structure separated by a relatively wide space. Figs. 21 and 22, × 105,000; Fig. 23, × 135,000.

FIGURES 24–25   Fig. 24 is a phase-contrast photomicrograph of a two cell stage embryo showing the chorion *(CH)* partially torn away from the blastomeres. Fig. 25 is a section of an Epon-embedded, toluidine blue–stained embryo which illustrates the intact chorion *(CH)*. Fig. 24, × 500; Fig. 25, × 640.

the action of strychnine (59), butyric acid, KCl, NaCl, and distilled water (51, 58, 61). In regard to the formation of the activation calyx by different chemical agents, a significant similarity exists between this phenomenon and the induced lifting, by certain chemical substances, of the pellicle (also an extraneous coat or glycocalyx) of the pigmented protistan ciliate, *Blepharisma undulans.* Nadler (62) noted that when *Blepharisma* is exposed to strychnine, morphine sulphate, and a

variety of other chemicals the pellicle is detached from the surface of the organism and discarded. The shedding of the pellicle is accomplished without interfering with the kinetosomes of cilia that are located within the cortical cytoplasm. Shortly after the lifting of the pellicle there is a release of the pigment granules (48). The release of these granules is reminiscent of the release of cortical granules during the cortical reaction by eggs investigated in this study (see below). Thus one sees that a significant parallelism exists between the induced lifting of the primary envelope by chemical agents and the induced lifting of the pellicle of *Blepharisma*. If one removes the activation calyx of the egg of *Arbacia* and subsequently refertilizes it, a second glycocalyx, which is necessary to form the activation calyx, is not produced; however, sperm do enter the egg (81; see also reference 63). On the other hand, once *Blepharisma* sheds its glycocalyx (pellicle) another one may be synthesized. It is understandable why a second glycocalyx is not synthesized by the fertilized egg, since this ability "is lost as an immediate consequence of the fertilization reaction" (49). When one thinks of fertilization, one is reminded of the statement by Moore (58) that "fertilization is a complex series of reactions which, if once completed, exclude all possibilities of repetition." In regard to the induced lifting of the glycocalyx in both the egg and *Blepharisma* one might ask the following question. Is there a physiological parallelism underlying the detachment of the glycocalyx in these two different cell types?

Chorion

Subsequent to its elevation, the activation calyx becomes augmented, i.e., it thickens and hardens. Some investigators have referred to the amplified activation calyx as the fertilization membrane. For example, in their book, Costello et al. (29) state that "this membrane (activation calyx) hardens and thickens during five minutes (after egg insemination) and, after alteration, is called the fertilization membrane." (Words in parentheses are added by the author.) In the electron micrographs presented in this paper, the activation calyx, after the fusion of male and female pronuclei and prior to and during the two cell stage, is composed of an inner and an outer trilaminar structure separated by a 600 A wide interspace. We have found that at about 10–12 min following insemination the male and female pronuclei begin to fuse and that by 14 min a 2N nucleus has been formed. This is

the earliest stage of a new generation: hence a zygote. We have already stated elsewhere that if the term chorion is to be retained it should only be applied to the "protective covering surrounding an embryo" (10). Therefore, the augmented activation calyx following the formation of the zygote nucleus should be referred to as the chorion. Whether the thickening and hardening of the activation calyx are due to materials released from the cortical granules, from those vesicles containing the rodlike structures, or from both is unknown. Experiments designed to ascertain the facts are now in progress.

Cortical Reaction

It has already been shown that the cortical granules of the mature egg are found in the peripheral ooplasm. When the sperm activates the mature egg the cortical reaction ensues and progresses in successive stages around the egg (54). The long bibliographic lists appended to the works of Allen (4), Runnström (73), and Tyler and Tyler (82) indicate the interest that biologists have taken in the cortical granules and the cortical reaction. This literature will not be reviewed here. Suffice it to say that during the cortical reaction the contents of the cortical granules are expelled into the perivitelline space (27, 30, 32–34, 67). How might this be accomplished? The data gathered in the present study support the following interpretation. As indicated earlier, the portion of the oolemma associated with the cortical granule is usually devoid of microvilli and thereby leaves a nonmorphologically specialized area of the oolemma associated with the cortical granule. It was also noted that the unit membrane encompassing the cortical granule is separated from the oolemma by a space of about 200 A. Shortly after insemination, the apposing membranes of the cortical granule and oolemma fuse and undergo vesiculation much like that described by Barros et al. (16) between membranes of two different cell types, i.e., the mammalian acrosome reaction. According to Barros et al., "the term membrane vesiculation is used . . . to denote the occurrence of multiple unions between two cellular membranes lying in close apposition, with the formation first of a double-walled fenestrated layer and ultimately of an array of separate membrane-bounded vesicles." As a result of the release of the contents of the cortical granules, a portion of the membrane limiting the cortical granule becomes a part of the

plasmalemma of the fertilized egg. Moreover, the over-all surface area of the fertilized egg is increased since it is obvious that more membrane is added than is lost as a result of the union of membranes. There is apparently no increase in cell volume (82). The conclusion, that a portion of the encompassing membrane of the cortical granule fuses with portions of the oolemma in several places, is drawn in spite of the fact that sections depicting the initial fusion have not been obtained. Such an achievement would be extremely difficult since the process is obviously a rather rapid one. The diagrammatic representation illustrated as Fig. 26 recapitulates the events associated with the *cortical reaction*. Other investi-

gators, contributing to the analysis of how the cortical granules release their contents, have presented varied interpretations (12, 13, 33, 47, 60, 86).

All of the cortical granules that are produced by the oocytes are not released during the cortical reaction (see reference 4); some are retained by blastomeres of the gastrula (2). The significance of this is unknown. One might speculate that when the oocyte obtains a full complement of these granules the over-all quantity may exclude the possibility of all of them becoming located adjacent to nonmicrovillous studded areas of the oolemma. It is possible that the nonmicrovillous portion of the oolemma is physiologically different
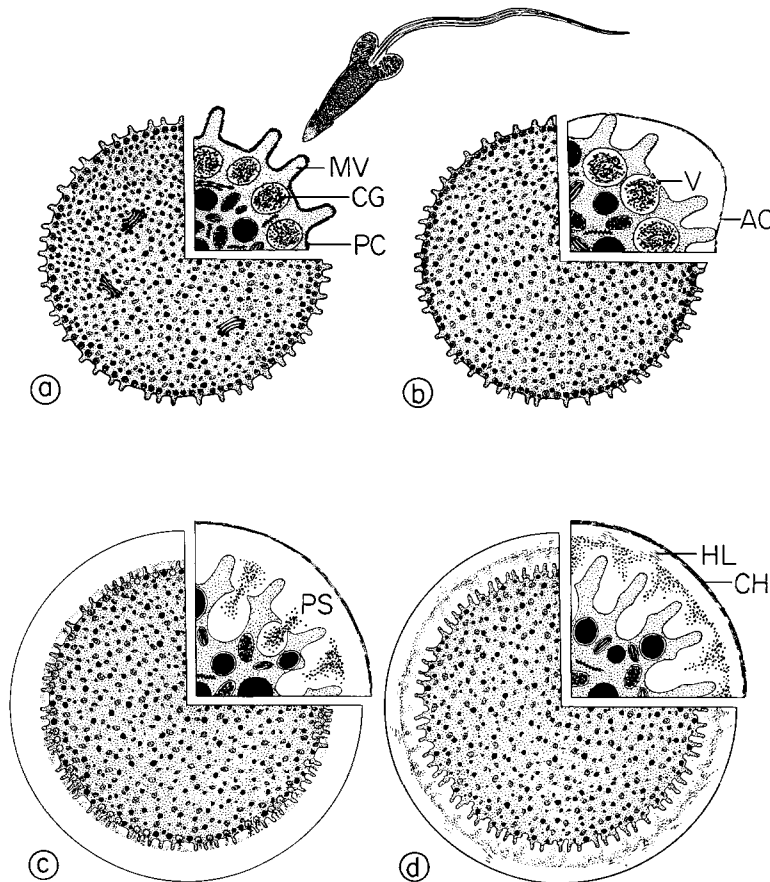

FIGURE 26 A schematic representation of the events associated with the cortical reaction. *a*, Activation of the egg by the sperm: *MV*, a microvillus; *CG*, cortical granule; *PC*, primary coat. *b*, Lifting of primary (vitelline) coat to form the activation calyx (*AC*) and the union of the membrane of the cortical granules with that of the oolemma thereby forming vesicular structures (*V*). *c*, Release of the contents of the cortical granules within the perivitelline space (*PS*). *d*, The thick chorion (*CH*) and hyaline layer (*HL*).

from microvillous areas. If this be the case, one can further conceive that such a difference would permit fusion between that portion of the oolemma and the membrane encompassing the cortical granule. In any event, once the initial steps of fertilization commence one sees that the original oolemma becomes a mosaic (see references 28, 57). In the fertilized egg one can envision that its mosaic plasmalemma might be composed of the following: (a) portions of the original oolemma; (b) portions of the membrane that encompassed the cortical granules; originally derived from the Golgi complex; (c) portions of the plasmalemma of the sperm; and (d) portions of the membrane limiting the vesicles that contain the rodlike structures. It is not known how long the mosaic nature of the membrane is retained, nor what significance the mosaic membrane may have on the future of the embryo. It is conceivable that such a mosaic plasmalemma may be important in the initial cleavage of the zygote (9).

Cortical Granules of Other Organisms

It has been well established that the cortical granules in the eggs of a number of different organisms do indeed participate in the cortical reaction (4, 11, 57, 77, 82, 87). In the polychaetous annelid, Sabellaria, the contents of a majority of the cortical granules are released when the egg comes into contact with seawater (64, 66). Pasteels (66) suggests that the release of cortical granules is independent of the fertilization phenomenon. In some forms, for example the amphineuran mollusc Mopalia muscosa (Anderson, E. Unpublished data.), the pelecypod Mytilus edulis (42), and the brachiopod Terebratalia transversa,[1] specific bodies come to lie in the peripheral ooplasm when oocytes become mature eggs. In these and other forms there is no visible cortical reaction when the egg is inseminated. Humphreys (42) found that a

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[1] Long, J. Personal communication.

major portion of the population of cortical granules in Mytilus is retained at least to gastrulation. The cortical granules in Terebratalia persist, near the surface of the ectodermal cells, until late larval life.[1] In many eggs, the cortical granules are composed of an acid mucopolysaccharide and protein. Long[1] has found that the cortical granules in Terebratalia possess some interesting cytochemical properties. They display a positive reaction with bromphenol blue, tyrosine (see reference 50), and alkaline fast green. The tyrosine reaction is blocked by prolonged iodination at high pH, and the coloration with alkaline fast green is blocked by prior diamination with nitrous acid. The granules do not show reaction with the Feulgen reagent, pyronine, periodic acid–Schiff, Alcian blue, oil red 0, methanol fast blue 2S for phospholipids, and Sakaguchi's reaction for tryptophane. On the basis of these results, Long tentatively suggests that the granules of Terebratalia contain a histone-like protein.

It is apparent, from what has been presented here, that a number of functions presumably will eventually be found for those structures that come to lie in the peripheral ooplasm of a mature egg and are referred to as cortical granules. In some eggs these structures clearly participate in the cortical reaction and could be classified as the cortical granules of fertilization. When the function of those cortical granules that do not participate in the cortical reaction during fertilization becomes known, perhaps the nomenclature selected could reflect their function.

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