A CYTOLOGICAL STUDY OF THE CENTRIFUGED WHOLE, HALF, AND QUARTER EGGS OF THE SEA URCHIN *ARBACIA PUNCTULATA*

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ABSTRACT

While the ooplasmic components of centrifuged eggs of *Arbacia punctulata* do not stratify in homogeneous layers, we have obtained the following strata beginning with the centripetal end: lipid droplets, pronucleus, clear zone, mitochondria, yolk, and pigment. Whereas mitochondria may be found mingled with yolk bodies, we have never observed lipid droplets nor pigment bodies among any of the other inclusions. The so-called clear zone contains a heterogeneous population of inclusions: annulate lamellae, heavy bodies, Golgi complexes, and rod-containing vacuoles. The peripheral cortical granules of immature (germinal vesicle stage) and of mature eggs are not dislodged from the cortical ooplasm with the centrifugal force utilized. When the eggs are treated with urethane, prior to centrifugation, the cortical granules of mature eggs abandon their peripheral position.

Further centrifugation of the initially stratified eggs produces nucleated and nonnucleated halves and the centrifugation of the halves results in quarters. The cytology of the halves and quarters is discussed. The halves and quarters have been activated with either sperm or hypertonic sea water. With the exception of the nucleated halves, we were unable to obtain plutei larvae from the other fractions (red halves and quarters). We believe that the lack of development of the various fragments is a function of the balance of particular inclusions necessary for differentiation.

INTRODUCTION

Lyon (42, 43) was the first to make use of rather weak centrifugal forces to study the structure of the eggs of sea urchins. In addition to his observations on the stratification of the various ooplasmic components, he also attempted to analyze the function of different substances on development. Although studies involving the use of centrifugation of eggs had been done before (47) and after (8, 12, 13, 47, 48) those of Lyon, the construction of the microscope-centrifuge by E. N. Harvey and Loomis (27) effected a true renaissance in cytology and embryology (see also reference 23). In 1931, E. N. Harvey (26) made a study of living cells with the microscope-centrifuge and stated that it was an instrument "that will have many uses in the field of protoplasmic studies and experimental embryology." In connection with investigations dealing with embryology, the most outstanding experiments exploiting the microscope-centrifuge were those made by E. B. Harvey on the eggs of the sea urchin, *Arbacia punctulata* (21, 22, 24, 25). In a series of experiments, E. B. Harvey was able to demonstrate that the ooplasmic components may be stratified in order of their relative densities (25). The stratified egg may be pulled into nearly equal halves and the halves into quarters. She also re-

ported that each of these quarters, when fertilized, is capable of developing into a normal pluteus larva. The results obtained by E. B. Harvey with the microscope centrifuge inspired numerous investigators to use the centrifuge in their studies of eggs in search of answers to outstanding questions concerning: (*a*) organ-forming substances (11), (*b*) polarity (26, 38, 56), (*c*) surface active substance (26), (*d*) stored messenger ribonucleoprotein (5, 9, 15), (*e*) cleavage substances (54), and (*f*) distribution of enzymes (30). In view of the fact that investigators continue to search for morphogenetic agents (19, 32, 35, 36), and use nucleated and nonnucleated eggs of sea urchins to study protein synthesis (9, 15) and the like (see 20, 31), a detailed ultrastructural analysis of the centrifuged whole, half, and quarter eggs of *Arbacia* is warranted. The cytological data that follow should constitute the matrix upon which to construct other experiments aimed at understanding subcellular differentiation.

MATERIALS AND METHODS

Eggs of the sea urchin *Arbacia punctulata* were obtained from the Marine Biological Laboratory, Woods Hole, Mass. during the months of June, July, and August. The animals were induced to spawn by the electrical stimulating technique of Harvey (25). As recommended by Harvey (27), eggs were obtained from a single female for each experiment. The eggs were shed into sea water and the sperm were collected "dry" according to the methods recommended by Costello et al. (14).

The eggs were prepared by buoyant density centrifugation in sucrose-sea water gradients according to Harvey (25). The initial stratification was accomplished by layering the eggs over a 0.95 molal (*m*) isotonic sucrose solution in cellulose nitrate centrifuge tubes (5/8 inch diameter, 3 inches long), and centrifuging at about 9000 *g* for 10–15 min at 23°C. All centrifuging was carried out in the No. 40 angle head rotor of Beckman's ultracentrifuge, Model L-2 (Beckman Instruments, Inc., Fullerton, Calif.). After 20–30 min centrifugation, the whole stratified egg divides into a nucleated white half and a nonnucleated red half.

Separation of the white halves into a granular and a clear quarter, and the red halves into a yolk quarter and a pigment quarter, was accomplished by layering the white halves over a solution consisting of 3 parts of a 0.95 *m* isotonic sucrose solution, plus 1 part sea water, and red halves over 0.95 *m* isotonic sucrose solution, and centrifuging at 10,000 *g* for 30 min.

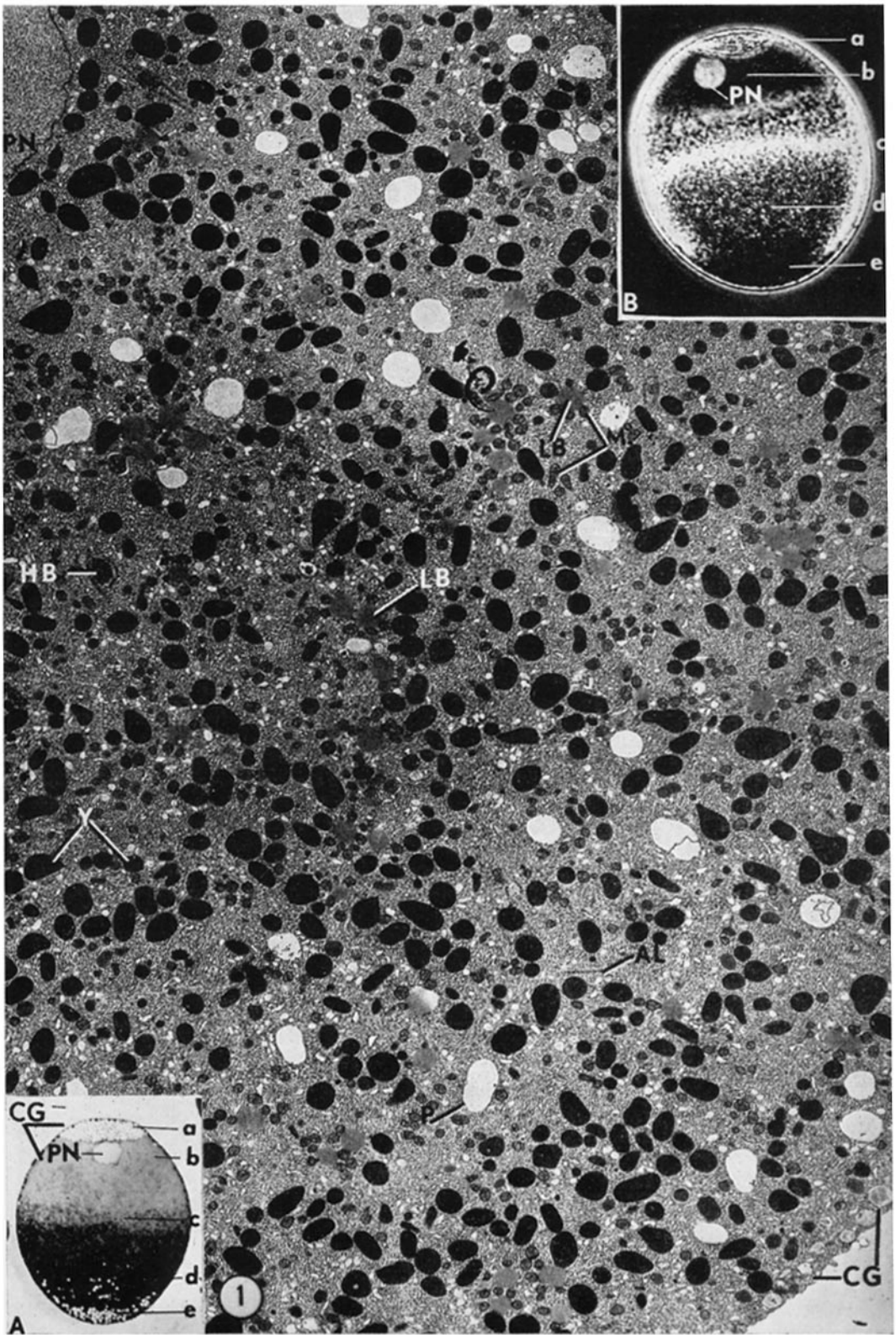
In an effort to study the effects of urethane on the centrifugal distribution of ooplasmic components, the mature eggs were treated for 5 min with 3% urethane in sea water at 20°–23°C. They were subsequently washed, layered over a 0.95 *m* sucrose solution, and centrifuged for 15 min at 23°C. The treatment with urethane does not kill the eggs, for they can be fertilized (many of them are polyspermic, 40) and can develop into what appear to be normal plutei.

Some of the halves and quarters were washed several times and fertilized with the collected "dry sperm" that had been previously diluted with sea water according to Costello et al. (14). The halves and quarters were also artificially activated with hypertonic sea water (3% sodium chloride/liter sea water) (25). Samples of the material inseminated by sperm or activated with hypertonic sea water were collected and fixed at 2 min intervals for a 30 min period, while some were permitted to develop overnight. Some of the activated nucleated quarters developed into multicellular ciliated structures in the time period.

In order to obtain a population of oocytes in the germinal vesicle stage, ripe female *Arbacia* were spawned by injecting 0.5 cc of isosmotic 0.5 M KCl into the lantern coelomic cavity (59). The spawned animals were then maintained in running sea water and fed the brown alga. *Laminaria* sp. 3 days after the initial KCl-spawing, the organisms were electrically stimulated (25) and the egg population (with many oocytes in the germinal vesicle stage) was layered over a 0.95 *m* isotonic sucrose solution and centrifuged for 30 min.

All of the material from the above experiments was examined with phase-contrast optics, and also proc-

FIGURE 1 A section through a noncentrifuged *Arbacia* egg depicting the various ooplasmic components except the Golgi complex. *PN*, pronucleus; *HB*, heavy bodies; *Y*, yolk; *AL*, annulate lamellae; *P*, pigment; *LB*, lipid bodies; *M*, mitochondria, some of which are associated with lipid bodies; *CG*, cortical granules. Inset A is a photomicrograph of a centrifuged egg embedded in Epon, and inset B is a phase-contrast photomicrograph of a living centrifuged egg. *a*, lipid layer; *PN*, pronucleus; *b*, clear zone; *c*, mitochondria; *d*, yolk; *e*, pigment; *CG*, cortical granules. Fig. 1, × 3,000) after Anderson, see reference 2. Reprinted by permission from *The J. Cell Biol.*); inset A, × 400; inset B, × 600. Epon-embedded; toluidine blue-stained. × 250.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essed for electron microscopy. Eggs were fixed either according to Longo and Anderson (40) or in 3% glutaraldehyde dissolved in sea water to which was added 1% acrolein and 7% sucrose. The material was washed in sea water and postfixed for 1–1.5 hr in a 1% solution of osmium tetroxide solution in sea water. Following postfixation, the specimens were rapidly dehydrated in a graded series of ethanols, infiltrated, and embedded in Epon (41) (Shell Chemical Co., New York). Thick sections of Epon-embedded material were made and stained according to Ito and Winchester (34). Thin sections were cut with a Porter-Blum MT-1 or MT-2 microtome, stained with uranyl acetate followed by the lead citrate stain of Venable and Coggeshall (60), and examined with a Philips EMU 200 electron microscope.

OBSERVATIONS

The Mature Egg

The mature egg of *Arbacia* is about 73–75 μ in diameter and contains a pronucleus (Fig. 1, *PN*) with many peripherally located nucleolus-like structures. The ooplasm contains yolk bodies (Fig. 1, *Y*), lipid bodies (Fig. 1, *LB*), mitochondria (Fig. 1, *M*), some of which are clustered around lipid droplets, and dense particles, endoplasmic reticula, and annulate lamellae (Fig. 1, *AL*). Also observed are some spherical bodies with flocculent or filamentous interiors, rod-containing vacuoles, heavy bodies (Fig. 1, *HB*), Golgi complexes, pigment (echinochrome) bodies (Fig. 1, *P*), and peripherally located cortical granules (Fig. 1, *CG*) situated just beneath the oolemma (2).

Initially Stratified Egg

Light Microscopy

Fig. 1 (inset B) is a phase contrast photomicrograph of a living egg, and Fig. 1 (inset A) is a photomicrograph of a thick section of an Epon-embedded specimen. In each of these preparations the strata appear as follows: (*a*) lipid at the centripetal end, (*b*) a clear layer, (*c*) mitochondria, (*d*) yolk, and (*e*) pigment. The pronucleus (Fig. 1, *PN*, insets A and B) is always centrifugal to the lipid droplets comprising the oil cap; the so-called clear zone appears to have some unidentified dense inclusions. The cortical granules are immovable with the centrifugal force used (Fig. 1, *CG*; inset A).

Electron Microscopy

Each of the previously mentioned regions (*a–e*) of the centrifuged egg as shown by light microscopy was examined submicroscopically, beginning with the lipid droplets.

LIPID DROPLETS: The lipid layer consists of lipid droplets (Figs. 2, 3, *LB*). The droplets vary in diameter and each possesses a clear center and an electron-opaque cortical area. In addition to lipid droplets, the lipid layer also contains mitochondria (Fig. 2, *M*), numerous ribosomes, and a few cisternae of the rough endoplasmic reticulum (Fig. 2, *ER*). We have never found lipid droplets among the components of the other layers.

NUCLEUS AND CLEAR LAYER: The components of the nucleus show no stratification. The peripherally situated nucleolus-like bodies remain in intimate association with the nuclear envelope upon centrifugation. This is similar to the relationship between the cortical granules (Fig. 1, inset A, and Fig. 2, *CG*) and the oolemma upon centrifugation.

The so-called clear layer is rather complex in its composition. As shown in Fig. 3 its matrix contains numerous short and long cisternae of the rough endoplasmic reticulum (*ER*) and annulate lamellae (Fig. 6, *AL*). The cisternae of the endoplasmic reticulum often appear layered (44). Located randomly within the clear layer are many heavy bodies (Fig. 5, *HB*) and numerous Golgi complexes (Figs. 7–9, *GC*). Sometimes a cisterna of the endoplasmic reticulum is associated with certain Golgi complexes (Fig. 7). That portion of the cisterna of the endoplasmic reticulum facing the Golgi complex is occasionally evaginated (Fig. 7 [*]). This configuration of the cisterna of the endoplasmic reticulum and its association with the Golgi complex is a frequent observation during oogenesis at the time the cortical granules and protein-carbohydrate yolk bodies are being fabricated (2).

While the rod-containing vacuoles (Fig. 9, inset *RC*), like other organelles and inclusions, do not stratify in a definite layer, a large population of these structures may be found centripetal to the mitochondria as shown in Fig. 9 (*RC*).

The ground substance of the clear layer contains dense particles some of which may be glycogen, while others may be ribosomes.

MITOCHONDRIA: The mitochondria, the major portion of which is found centripetal to the rod-containing vacuoles, are similar in their ultrastructure to those found amongst, and clustered around, the lipid droplets, and those clustered around dense particles (Fig. 4, *M*). They have

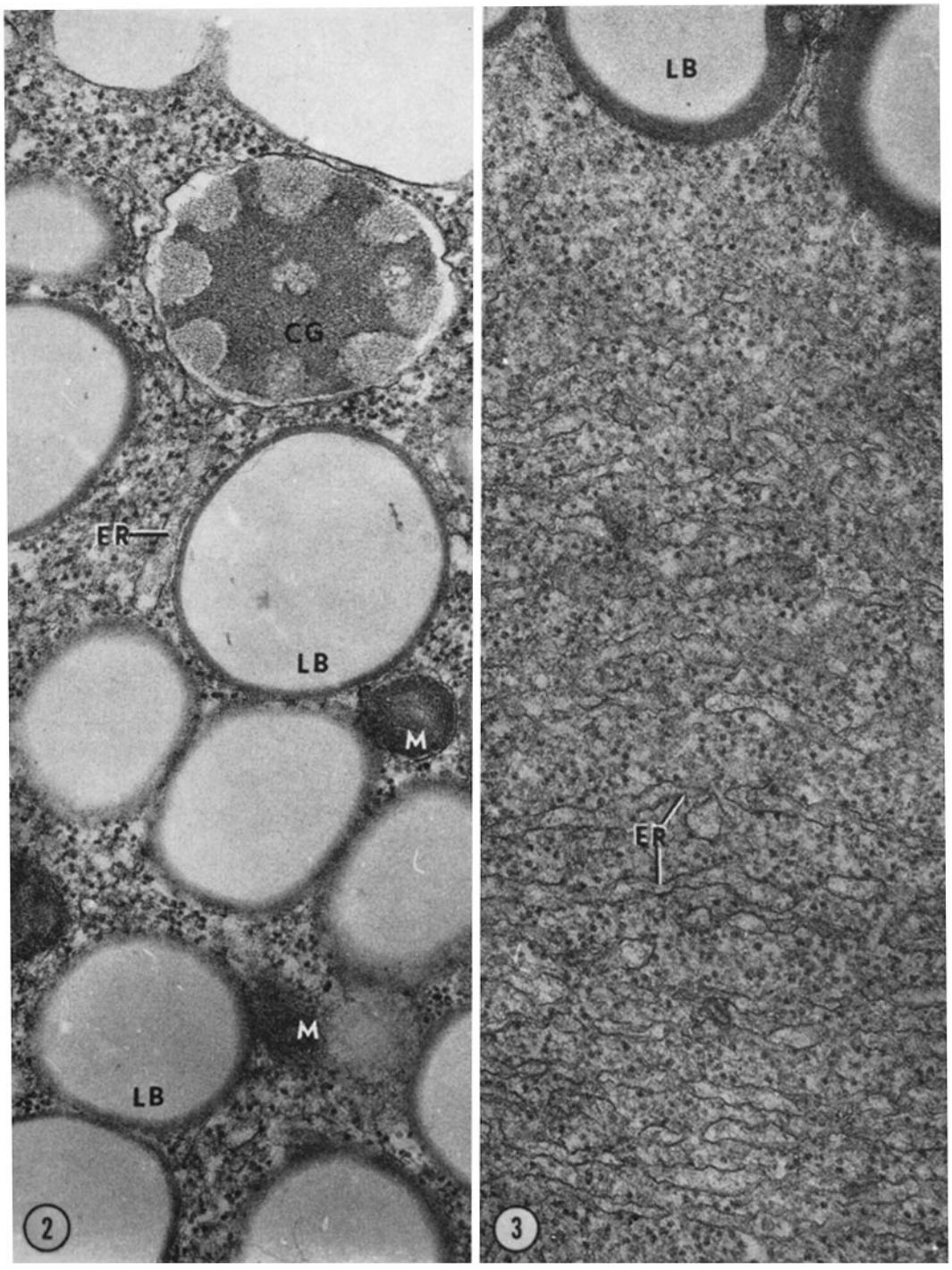


FIGURE 2 A section through the lipid layer. *CG*, cortical granule; *LB*, lipid bodies; *M*, mitochondria; *ER*, endoplasmic reticulum. × 50,000.

FIGURE 3 A section through the clear zone in the region of the nucleus showing a lipid body (*LB*), and endoplasmic reticulum (*ER*). × 50,000.

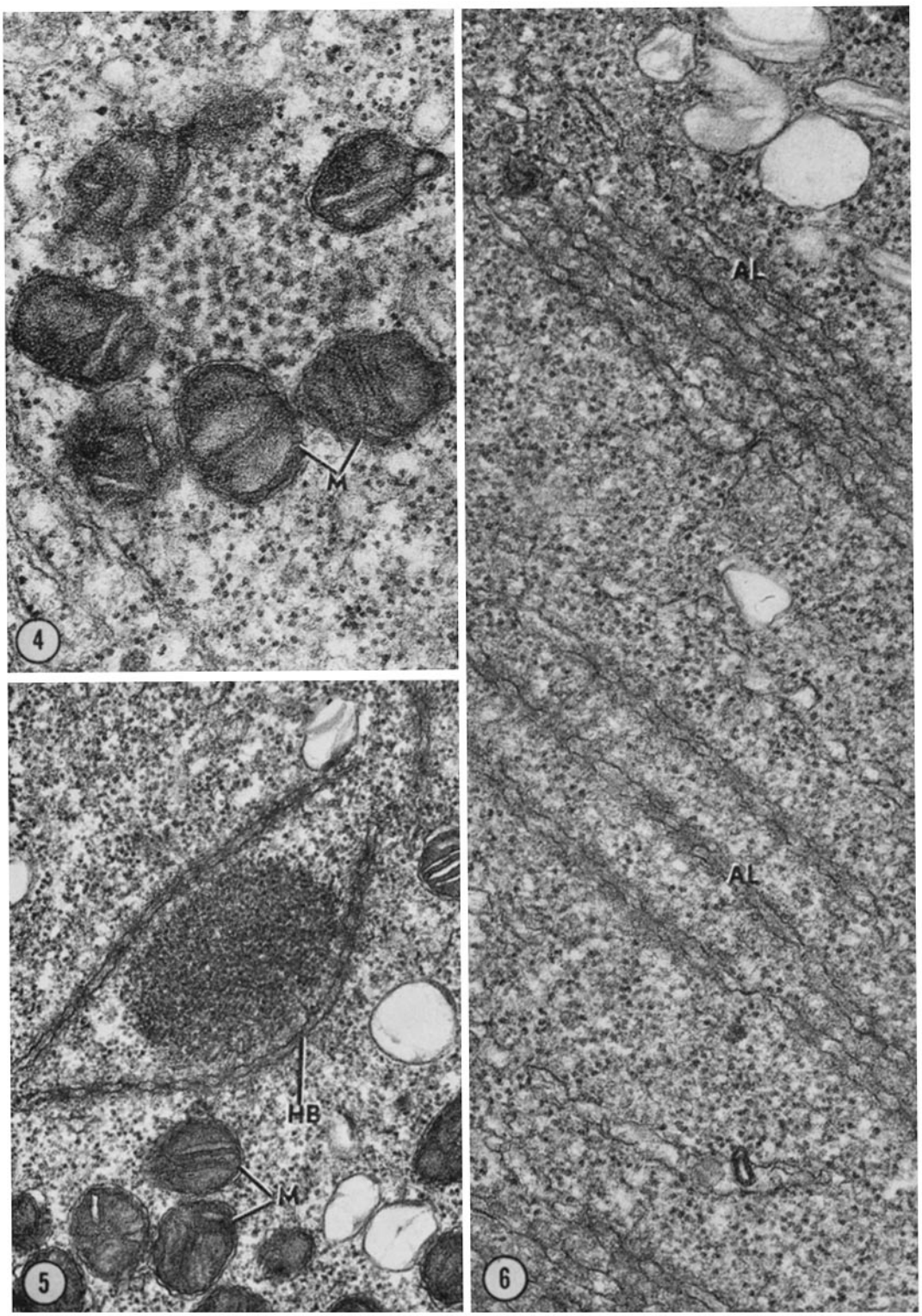
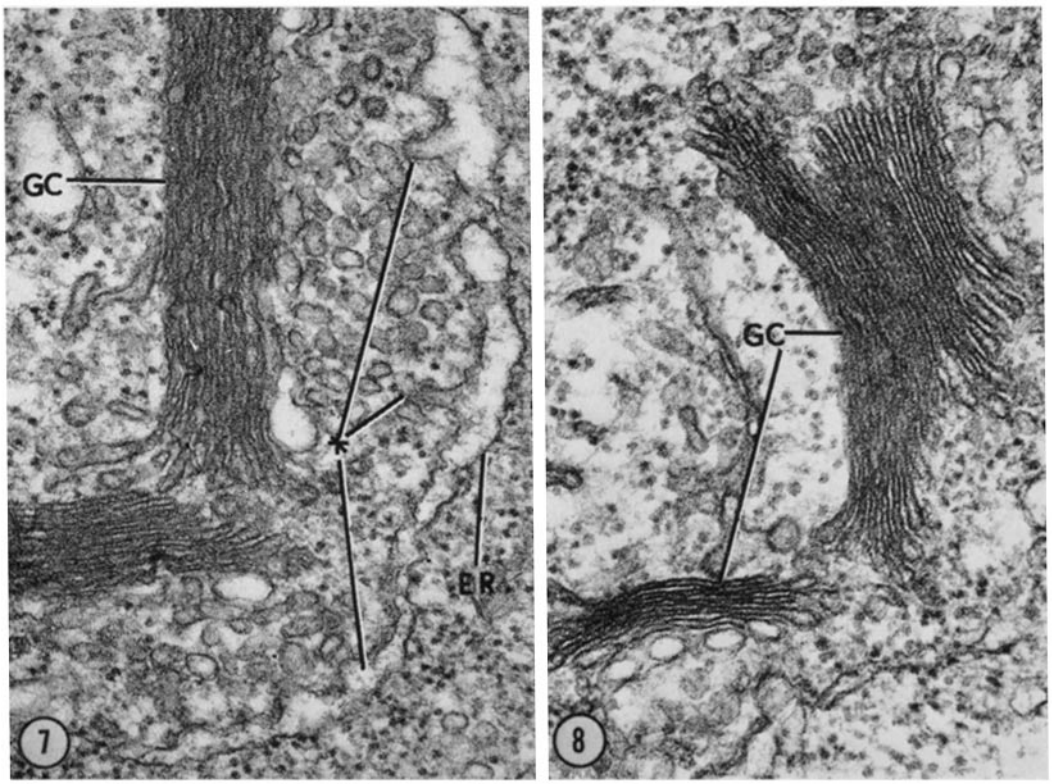


FIGURE 4 A section showing mitochondria (*M*) associatd with a cluster of dense granules. × 50,000.

FIGURE 5 A section through the clear zone centripetal to mitochondria (*M*), showing a heavy body (*HB*). × 50,000.

FIGURE 6 A section through the clear zone showing annulate lamellae (*AL*). × 50,000.



FIGURES 7 and 8 Sections through the clear zone illustrating Golgi complexes (*GC*). Fig. 7 shows a Golgi complex associated with a cisterna of the endoplasmic reticulum (*ER*). Note that the portion of the endoplasmic reticulum facing the Golgi complex is evaginated (*). Fig. 7, × 60,000; Fig. 8, × 50,000.

well-developed, transversely oriented cristae. No clue has been found for the existence of two morphological types of mitochondria in the mature egg, nor have we observed the mitochondria to form two layers. In immature eggs (germinal vesicle stage), however, a large number of the mitochondria are dumbbell shaped (Fig. 13, *MC*), and others contain regions composed of a filamentous material (Fig. 12, *MF*). These filaments may be mitochondrial deoxyribonucleoprotein.

YOLK BODIES: Admixed with the compact yolk bodies (Fig. 10, *Y*) are Golgi complexes (Fig. 11, *GC*), mitochondria, rod-containing vacuoles, and endoplasmic reticulum (Fig. 11, *ER*).

PIGMENT BODIES: The pigment bodies, whose contents are not retained when prepared by techniques of electron microscopy, are limited by a membrane (Figs. 14 and 22, *P*) and are not associated with filaments, as reported by Parpart (50). In association with the pigment bodies may be found most of the regularly occurring organelles and inclusions such as mitochondria (Fig. 14, *M*) and yolk (Fig. 14, *Y*).

Halves

WHITE NUCLEATED AND RED NONNUCLEATED HALVES: When the initially stratified egg is centrifuged for about 30 min it becomes dumbbell shaped (Fig. 15) and subsequently breaks in the region of the mitochondria, producing a nucleated (*PN*) white half (Fig. 16, ~60–63 μ in diameter) and a nonnucleated red half (Fig. 17, ~53–56 μ in diameter). While certain organelles and inclusions are more numerous in one half than in the other, one can find, for example, yolk in the white half and Golgi complexes in the red half.

When the white and red halves separate, the

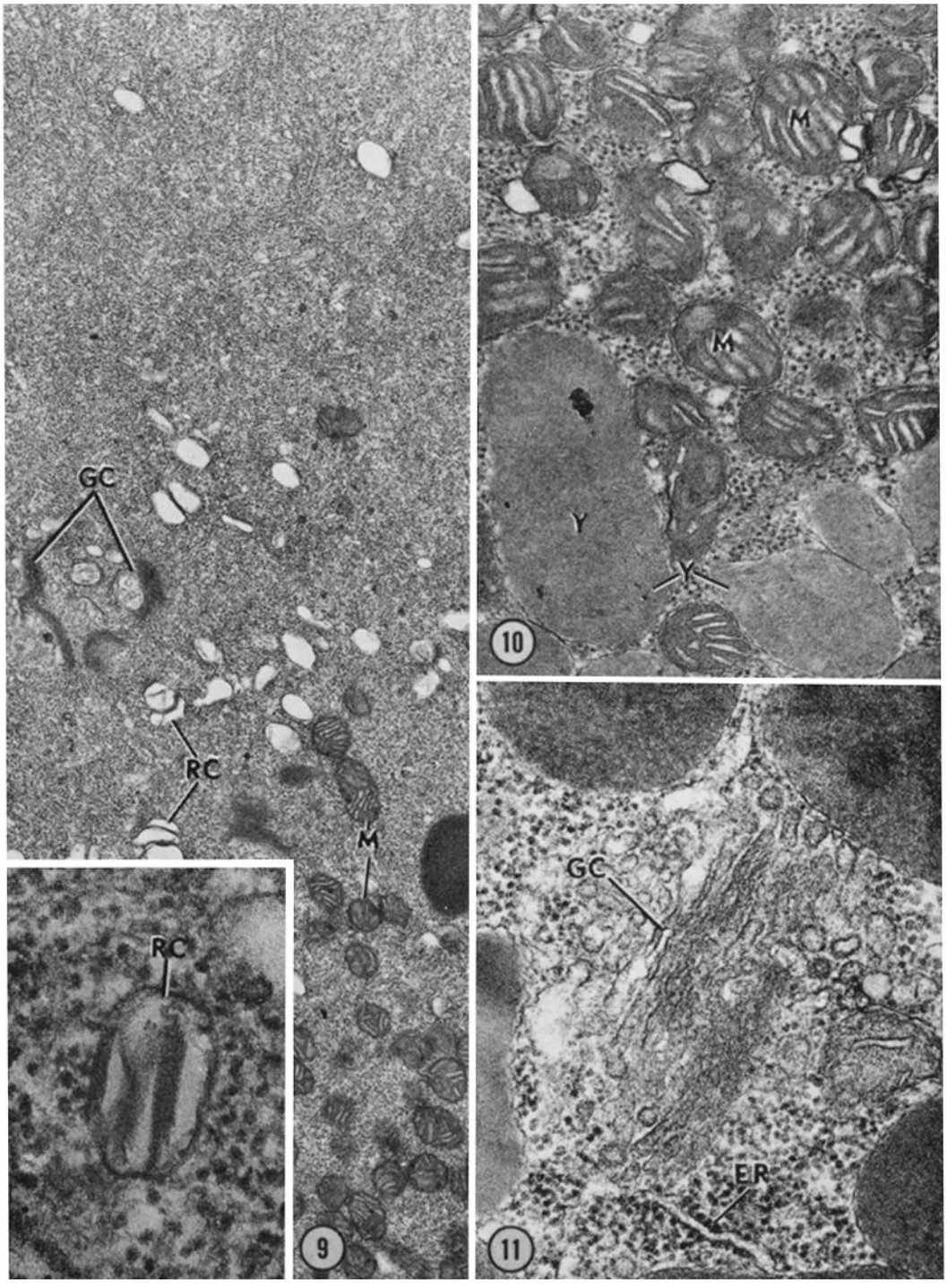
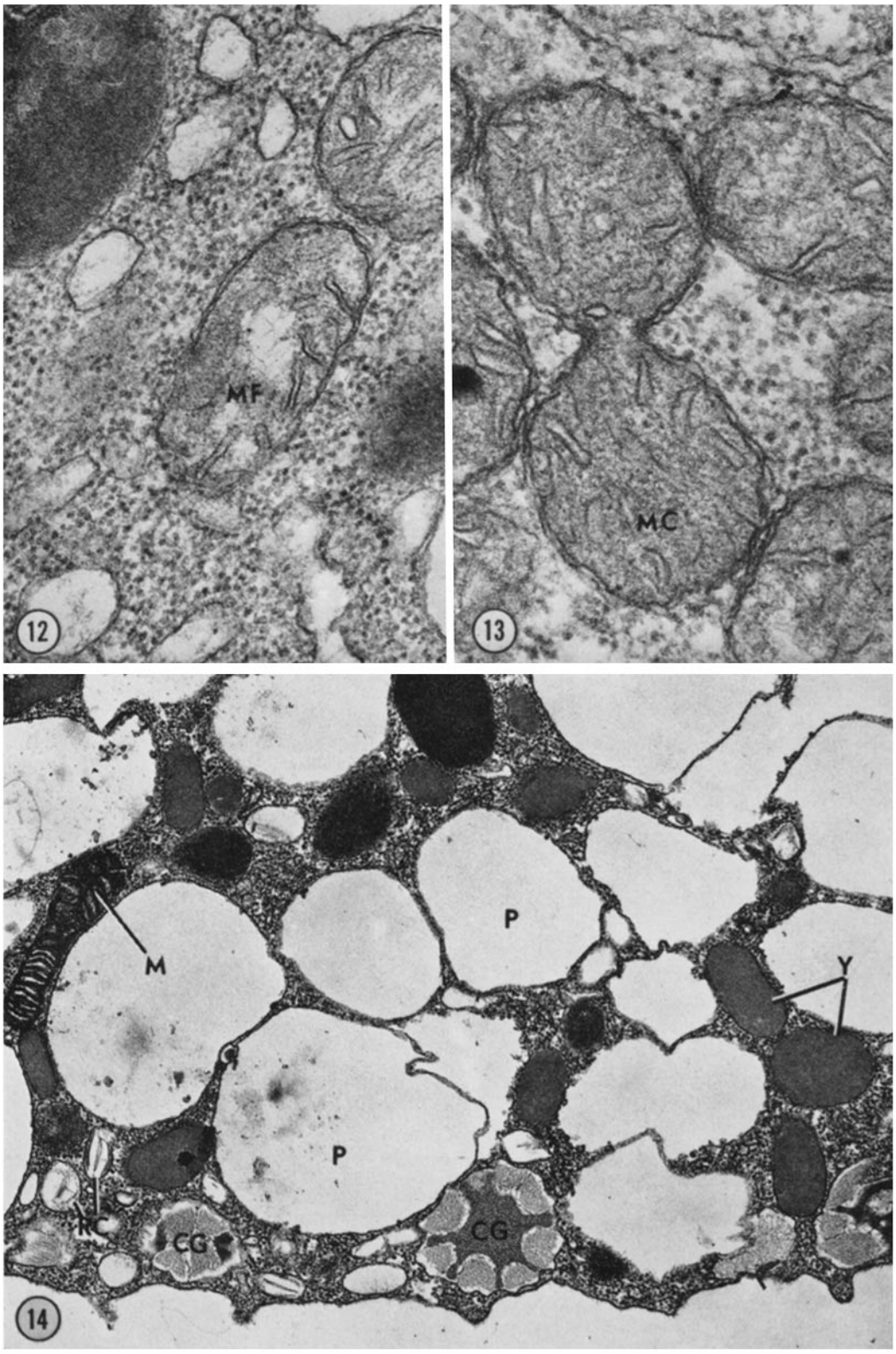


FIGURE 9 A section through the clear layer showing the rod-containing vacuoles (*RC*, and inset). Note the Golgi complexes (*GC*) and the centrifugally located mitochondria (*M*). Fig. 9, × 50,000; inset, × 90,000.

FIGURES 10 and 11 A section through the mitochondria (*M*) and the yolk (*Y*) layers. A Golgi complex (*GC*, Fig. 11) is seen among the yolk bodies. Cisternae of the endoplasmic reticulum (*ER*) may be seen among the yolk bodies and mitochondria. Fig. 10, × 30,000; Fig. 11, × 50,000.



FIGURES 12 and 13 Sections through ovarian oocytes illustrating a mitochondrion containing filaments (*MF*, Fig. 12) and one showing a constriction (*MC*). Fig. 12, × 50,000; Fig. 13, × 90,000.

FIGURE 14 A section through the pigment layer (*P*). *M*, a mitochondrion; *Y*, yolk bodies; *RC*, rod-containing vacuoles; *CG*, cortical granules. × 20,000.

centrifugal ooplasmic matrix of the white half contains mostly mitochondria, while the ooplasmic matrix of the centripetal end of the red half contains endoplasmic reticulum (Fig. 18, *ER*), mitochondria (Fig. 18, *M*), and Golgi complexes (Fig. 18, *GC*).

It is interesting to note that during and prior to stretching into a dumbbell shaped egg, no unusual features of the oolemma were observed. Occasionally one sees a few subsurface cisternae in the region of the break. Regardless of the fragment, the oolemma, at the point of breakage, always re-forms a few of the short microvilli characteristic of the oolemma of the mature egg (Fig. 18, *MV*).

Quarters

CLEAR NUCLEATED AND GRANULAR NONNUCLEATED QUARTERS: When white halves are centrifuged for 30 min at about 10,000 *g*, one obtains a clear quarter (~54–56 μ in diameter) and a granular quarter (~40–42 μ in diameter).

The clear quarter contains numerous lipid droplets (Fig. 19, inset *LB*) and a centrally located pronucleus (Fig. 19, inset *PN*). The clear quarter also contains clusters of Golgi complexes (Fig. 19, *GC*), mitochondria (Fig. 19, *M*), numerous rod-containing vacuoles (Fig. 19, *RC*), annulate lamellae (Fig. 19, *AL*), and peripherally located cortical granules (Fig. 19, *CG*).

While the granular quarter contains a variety of organelles and yolk (Fig. 20, *Y*), it is distinguished by an abundance of mitochondria (Fig. 20, *M*). The few cortical granules (*CG*) are found in the peripheral ooplasm.

YOLK QUARTER: The yolk quarter (~50–52 μ in diameter) is dominated by yolk bodies (Fig. 21, *Y*); however, other organelles such as mitochondria (Fig. 21, *M*) and endoplasmic reticulum (Fig. 21, *ER*) are also present.

PIGMENT QUARTER: The pigment quarter (~30–32 μ in diameter) contains some yolk bodies (Fig. 22, *Y*), mitochondria, and rod-containing vacuoles (Fig. 22, *RC*). Like the yolk quarter, the pigment quarter contains cortical granules (Figs. 21 and 22, *CG*). It is interesting to note that the quarters appear to have many fewer cortical granules than the halves. As shown in this study, the cortical granules are not dislodged from the cortical ooplasm by the centrifugal force utilized. They were, however, dislodged after treatment with urethane and moved centripetally (see below). Possibly, during centrifugation the cortical granules move centripetally in the cortical ooplasm, and therefore the quarters have few granules.

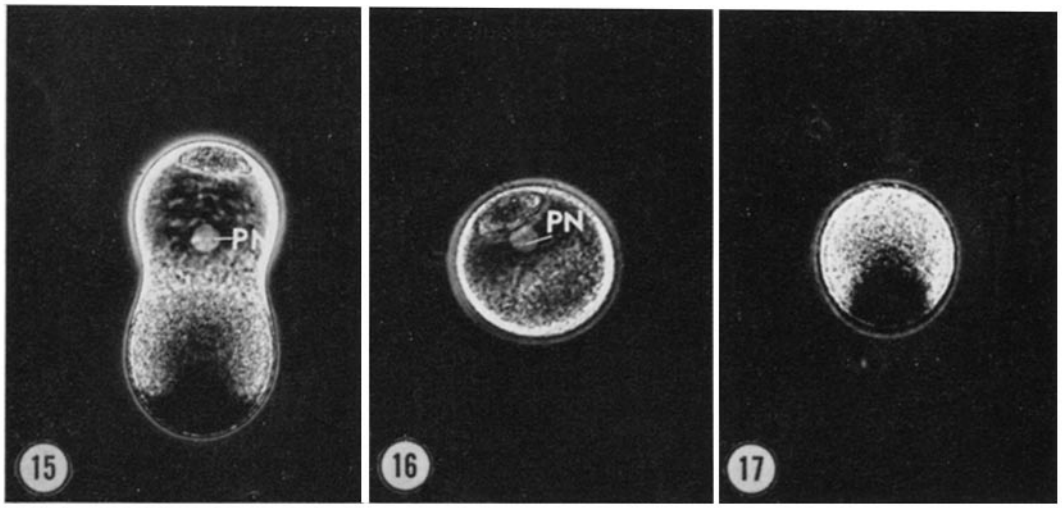
CORTICAL GRANULES OF GERMINAL VESICLE STAGE: It has already been stated that the cortical granules are not dislodged by the centrifugal force utilized. This appears to be the case not only for the mature eggs but also for oocytes in the germinal vesicle stage. Fig. 23 insets A and B are photomicrographs of centrifuged oocytes in the germinal vesicle (*GV*) stage. Those cortical granules already in the peripheral ooplasm appear to remain in the cortex. The cortical granules scattered within the ooplasmic matrix appear to stratify near the lipid droplets (Figs. 23 [inset B] and 24, *CG*).

Unlike the nucleolus-like structures in the mature egg, the nucleolus of oocytes of the germinal vesicle stage is found within the centrifugal end of the nucleoplasm (Fig. 23 insets A and B, *NC*). Within the ooplasmic matrix of the centrifuged oocytes are lipid bodies (Figs. 23 and 24, *LB*) and mitochondria (Figs. 23 and 24, *M*).

EFFECTS OF URETHANE ON THE DISTRIBUTION OF CORTICAL GRANULES: While many effects are produced by treatment of the mature egg with urethane, only the one specifically related to the cortical ooplasm will be reported here (40). When eggs are treated for 5 min with 3% urethane in sea water at 20°–23°C and centrifuged for 15 min, the cortical granules

FIGURES 15–17 Phase-contrast photomicrographs of living material. Fig. 15 shows an egg centrifuged for 30 min, illustrating its dumbbell appearance. *PN*, pronucleus. Figs. 16 and 17 represent a nucleated (*PN*) white half and a nonnucleated red half, respectively. Figs. 15–17, × 600.

FIGURE 18 A section through the centripetal portion of the nonnucleated red half. *MV*, a microvillus; *GC*, Golgi complexes; *RC*, rod-containing vacuoles; *M*, mitochondria; *ER*, endoplasmic reticulum. × 20,000.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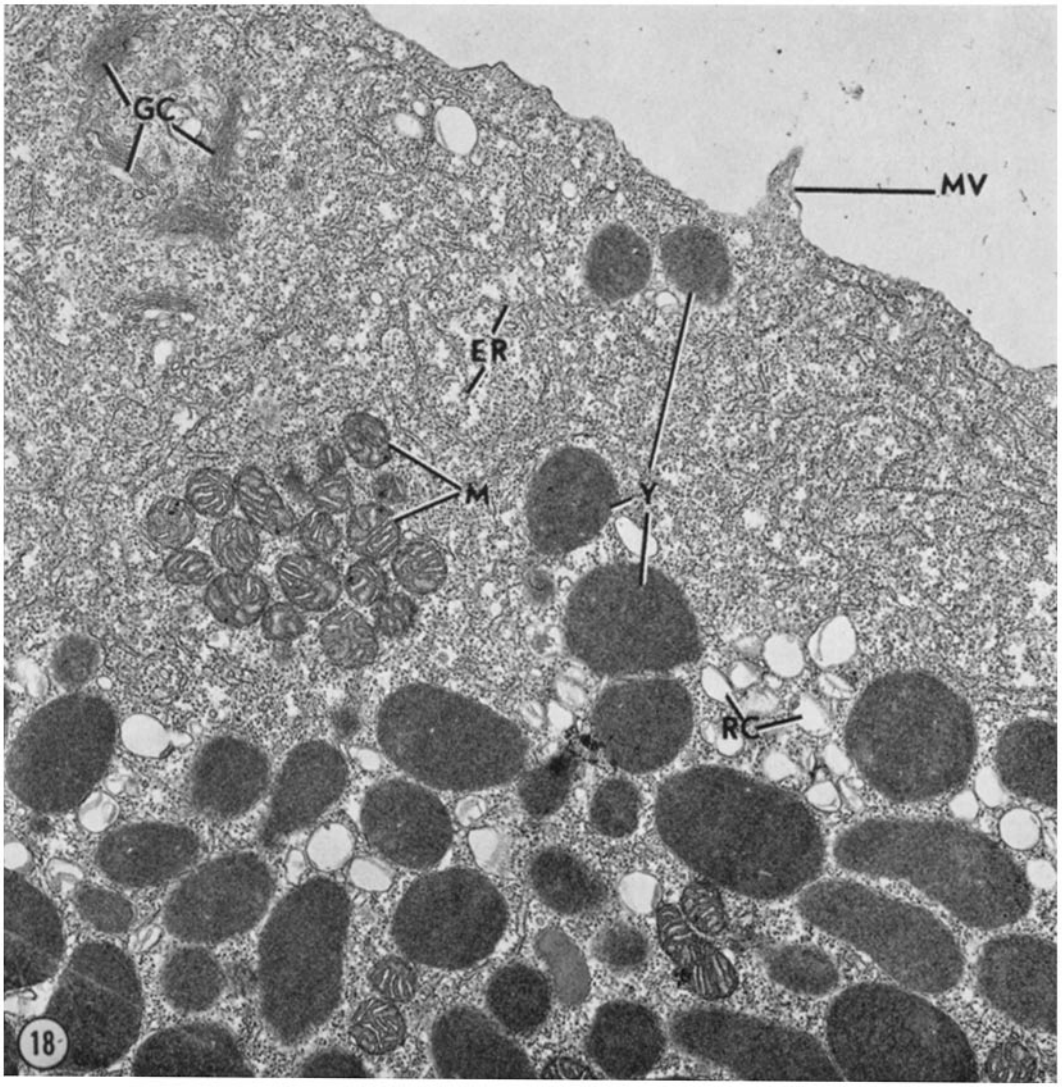
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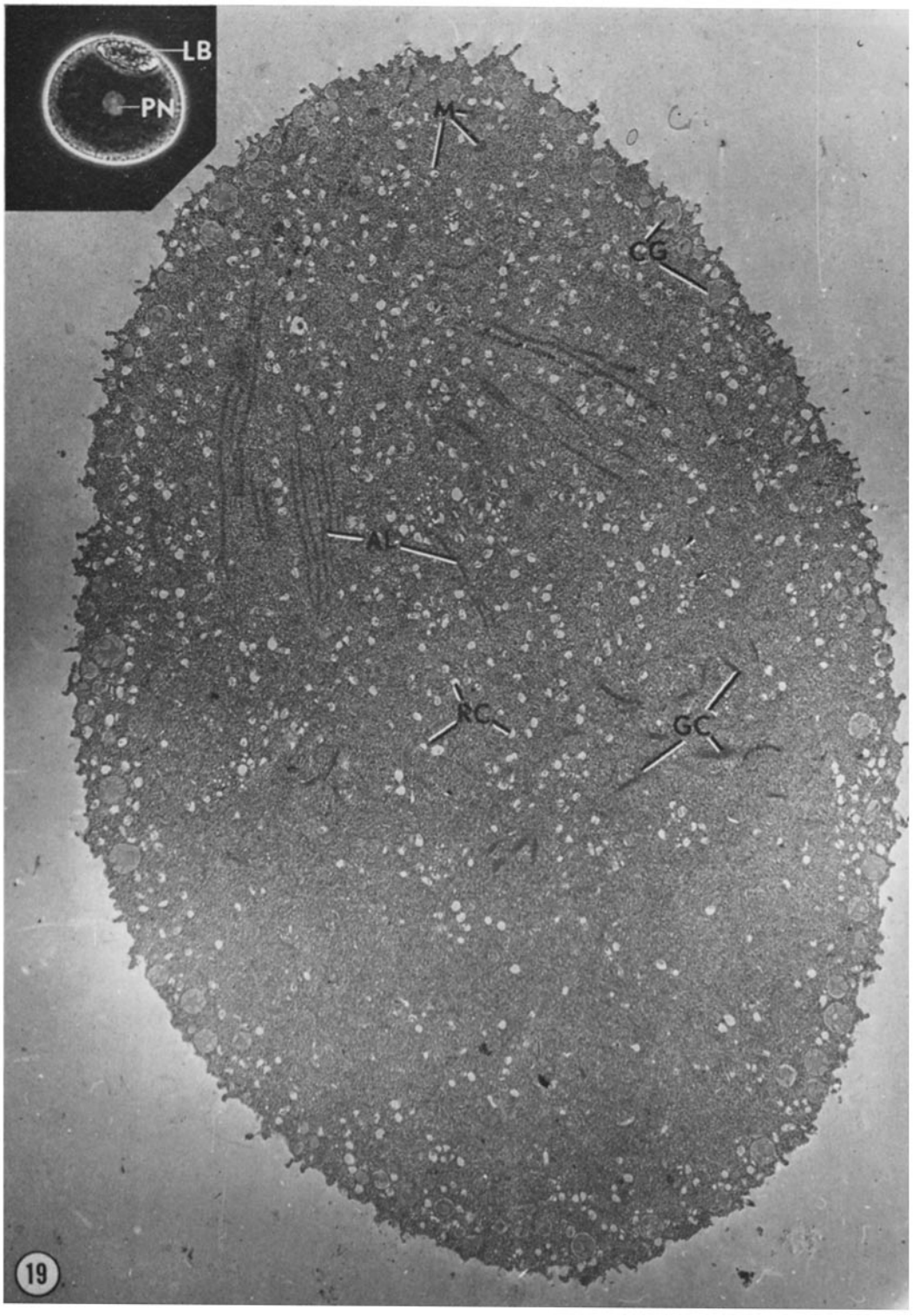


FIGURE 19 Inset in Fig. 19 is a phase-contrast photomicrograph of a living nucleated (*PN*) clear quarter. Fig. 19 is an electron micrograph of a section through a nucleated clear quarter. *LB*, lipid droplets (inset); *CG*, cortical granules; *AL*, annulate lamellae; *RC*, numerous rod-containing vacuoles; *GC*, Golgi complexes; *M*, mitochondria. × 3,000; inset, × 600.

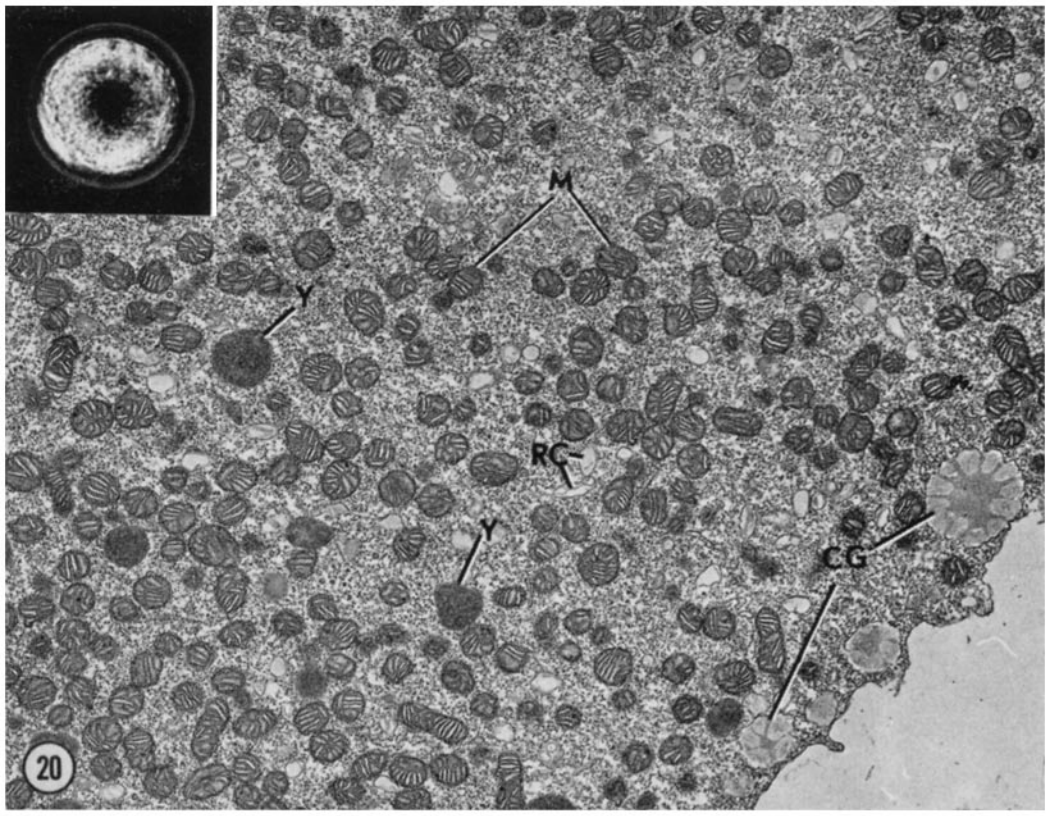


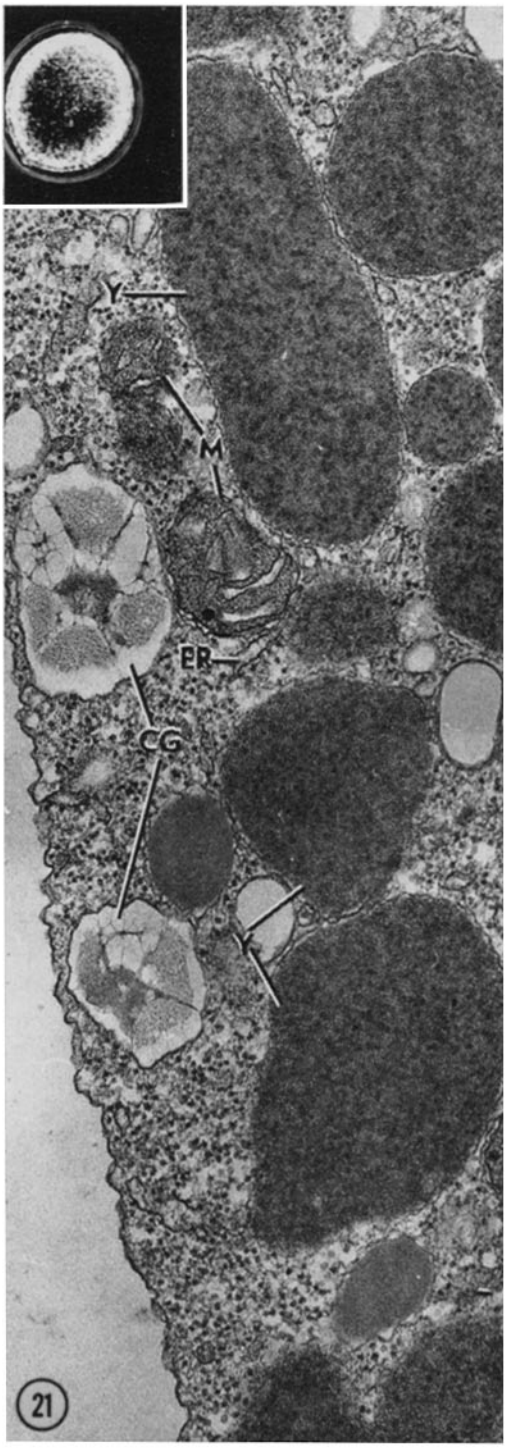
FIGURE 20 A section through a granular quarter (inset is a phase-contrast photomicrograph of a living granular quarter). *M*, mitochondria; *RC*, rod-containing vacuoles; *Y*, yolk bodies; *CG*, cortical granules. × 15,000; inset, × 600.

abandon their position and appear as a stratum (Fig. 25, inset *CG*). The cortical granules are found associated with other organelles such as mitochondria (Fig. 25, *M*), ribosomes, rod-containing vacuoles (Fig. 25, *RC*), and elements of the endoplasmic reticulum.

High magnification images of the peripheral ooplasm without cortical granules reveal no unique substructure. The cortical ooplasm devoid of cortical granules contains many ribosomes but appears to be lacking in mitochondria and other organelles.

Activated Halves and Quarters

It is well known that fertilization is a multistep phenomenon, and in the sea urchin eggs the cortical reaction is one of the initial steps of activation. As indicated above, we activated the halves and the quarter eggs with both sperm and hypertonic sea water. Fig. 27 shows the centrifugal end of a nucleated clear quarter with lipid droplets (*LB*), and Fig. 26 shows the centripetal end of the same quarter activated with hypertonic sea water. Here there is an activation calyx (2) and the contents of the few cortical granules have been released into the perivitelline space, thereby forming a relatively thin hyaline layer (Fig. 26, *HY*). The perivitelline space also shows rod-like structures (*R*) released from the rod-containing vacuoles (Fig. 26, *RC*). The microvillous surface (*MV*) of activated nucleated clear quarters appears much like that of a sperm-activated mature egg (2). In all of our observations of fragments activated with sperm or hypertonic sea water, the cortical response was evident, i.e., the cortical granules were dehisced. We have not been able to demonstrate the presence of a spermatozoan within the ooplasm of any of the quarters examined. It



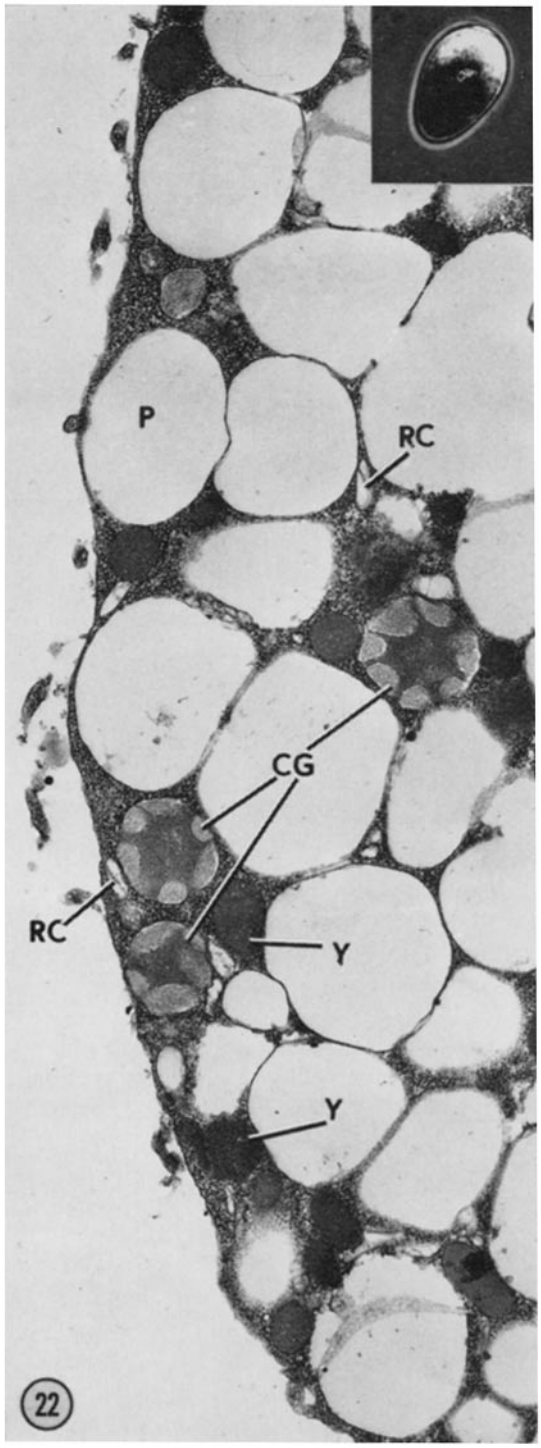


FIGURE 21 Section through a yolk quarter (inset is a phase-contrast photomicrograph of a living yolk quarter). *Y*, yolk bodies; *M*, mitochondria; *CG*, cortical granules; *ER*, endoplasmic reticulum. Fig. 21, × 30,000; inset, × 600.

FIGURE 22 Section through a pigment quarter (inset is a phase-contrast photomicrograph of a living pigment quarter). *P*, pigment bodies; *Y*, yolk bodies; *RC*, rod-containing vacuoles; *CG*, cortical granules. Fig. 22, × 18,000; inset, × 600.

is possible that the cortical reaction of the quarters studied was initiated by the sucrose, for some of the eggs initially centrifuged displayed the cortical reaction.

From the numerous observations made during this study, it is evident that *only* the activated *nucleated white halves* produce what appear as normal plutei larvae. As indicated before, all quarters may be activated by hypertonic sea water, i.e., each demonstrates a cortical reaction. When some nucleated quarters (activated with hypertonic sea water) were isolated, they were observed to undergo cleavage (Fig. 28, inset A), and subsequently to develop into multicellular ciliated (*C*) structures (Fig. 28, inset B) that remind one of miniature blastulae. In fact, the cellular architecture of the multicellular ciliated structure is reminiscent of that which has already been described for the blastula developed from sperm-inseminated eggs (E. Anderson, unpublished observations). Suffice it to say that the cells comprising the multicellular ciliated structure contain the regularly occurring organelles, for example mitochondria (Fig. 28, *M'*), and endoplasmic reticulum (Fig. 28, *ER'*), and the abundant lipid droplets (Fig. 28, *LB'*), so characteristic of the nucleated quarters. The cells are held together apically by septate desmosomes (Fig. 28, inset C, *SD*). Numerous gap junctions are encountered between the main body of the cells and their rather long pseudopodial extensions.

DISCUSSION

Harvey (25) reported that the ooplasmic components of the egg of *Arbacia* stratify into *uniform layers* in order of their relative densities. These layers, beginning with the centripetal end, were reported as follows: lipid, nucleus, clear zone, mitochondria, yolk, and pigment. The present report has shown that, while it is true that the previously mentioned regions contain the particular inclusion in question, it is also true that each region may contain elements of the other. One exception to this observation is the fact that lipid droplets which occupy the extreme centripetal end and pigment bodies which occupy the extreme centrifugal end were never observed in other regions. As indicated by those few accounts dealing with the centrifuged whole eggs and the present one, the clear zone is the most heterogeneous one of all (3, 18, 44, 51, 56, 58). While other studies have revealed the presence of annulate lamellae, Golgi bodies, and heavy bodies within the matrix of the clear zone, no one has shown the distribution of the population of rod-containing vacuoles (2) subsequent to centrifugation. The centrifuge technique has also substantiated Harvey's claim that the stratified *Arbacia* eggs may be pulled into nearly equal halves, and the halves into quarters.

Lansing et al. (37) stated that centrifuged eggs of *Arbacia* "contain two layers of what appear to be mitochondria, one in the centripetal lipid and the other just above the yolk granule layer" (17, 49). This statement by Lansing has been repeated and discussed in the literature many times. The discussion has centered around the question concerning the validity of different kinds of mitochondria. It has been shown by this study that the mitochondrial population of the *mature* egg is a rather homogeneous (i.e., morphologically) population, and there is no structural evidence for the existence of two (structural) types of mitochondria (46). We were able, however, to demonstrate that mitochondria obtained from eggs in the *germinal vesicle* stage may be dumbbell shaped and possess a region composed of fine filaments. It is thought that the mitochondria found amongst the lipid droplets of mature eggs are the same structurally, and presumably functionally, as are those found in the other parts of the egg. As we have indicated previously, in the mature uncentrifuged egg, mitochondria are frequently found clustered around lipid droplets and dense granules (33, 39, 55). We believe that this association is retained during centrifugation, much like the association of certain Golgi complexes and cisternae and endoplasmic reticula, and may account for the presence of mitochondria among the lipid droplets.

As we have already pointed out, the centrifuge has been a much utilized embryological and cytological tool. Some cytologists have suggested that the centrifugation method may be applied to the *Arbacia* egg to study organellogenesis, particularly the origin and development of the Golgi complex (7). To utilize the centrifuge technique on the eggs of *Arbacia* to answer questions concerning the origin of organelles would necessitate devising techniques that take into account the lack of homogeneity of the fragments.

Egg Cortex

With the centrifugal forces used during this study it was shown that the cortical granules of the stratified, whole, half, and quarter eggs are not dislodged from their peripheral position. When the whole egg is treated for 5 min with 3% urethane prior to centrifugation it was found that most of the cortical granules abandon their peripheral position and form a stratum. An examination of the cortical ooplasm prior to and after centrifugation revealed no structure to which one could assign the function of maintaining the cortical granules within the peripheral ooplasm. Moreover, no unique structure(s) was found in the cortex after the eggs had been centrifuged after urethane treatment. What then could be responsible for maintaining the cortical granules within the peripheral ooplasm? In connection with this, it is well known from the findings of Chambers (10) and others that the cortical ooplasm of eggs and many other cell types has a much higher viscosity than the remainder of the cell (6). This is particularly evident in the case of *Arbacia* when other inclusions, except the cortical granules, appear to stratify readily. Presumably, when eggs are treated with urethane the viscosity of the cortical cytoplasm decreases (28).

The literature is replete with experiments designed by investigators to test the morphogenetic importance of the cortex and we will not attempt to review the voluminous literature concerning the cortex (1, 4, 36). Curtis (16), for example, has found that the cortex of amphibian eggs is concerned with gastrulation, and Runnström (54) suggests that in the sea urchin the cortex is involved in fertilization and cleavage (54, 61). A definition of the cortex of the egg of *Arbacia* is difficult to construct, for it is not clear whether or not the cortex consists of only cortical granules or cortical granules plus some other structures (29). Mercer and Wolpert (45), utilizing the electron microscope, found no differentiated cortical region in the eggs of sea urchin *Psammechinus*. Those authors suggested that the immobility of cortical granules upon centrifugation "depends on continuity of the membrane of the granule with the surface membrane of the egg." A previous study (2) and the present one do not substantiate the findings of Mercer and Wolpert. As indicated above, the treatment of the egg with urethane does not inhibit its development. We have found that the cortical granules of the eggs of *Arbacia* are not closely associated with the inner aspect of the oolemma after urethane treatment (40). Moreover, when the egg is inseminated, only a few granules participate in the cortical reaction. Nonetheless, development continues until a pluteus is formed (E. Anderson, unpublished observations). What, then, are we to consider the cortex and its significance in morphogenetic events during embryogenesis in *Arbacia punctulata*? It is obvious that a meaningful definition (structural and functional) of the cortex of eggs in general is still in the resolving phase (4, 52).

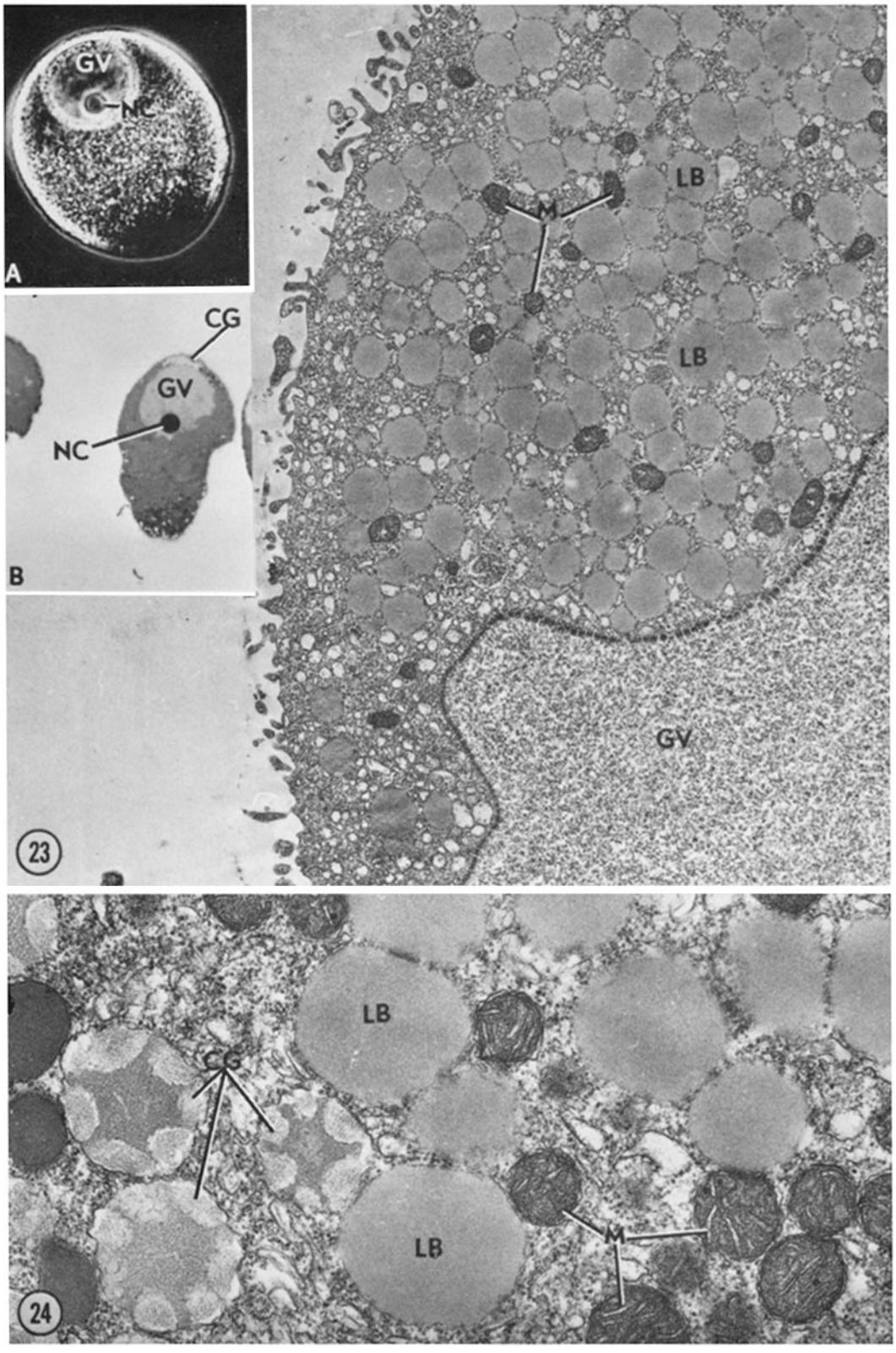
Development of Halves and Quarter Eggs

Much has been written concerning the expendability of certain organelles during the development of halves and quarter eggs of *Arbacia*. In connection with this, E. N. Harvey (26) wrote the following: "E. B. Harvey (1932–1938) in an exhaustive study of sea urchin eggs has shown that all the half and quarter fragments can be fertilized and will develop, even those with male nucleus alone, some to free swimming plutei. *None of the formed elements are essential to early development.*" (See reference 49 for further discussion.)

In our studies we were not able to get the high percentage of development for the various fragments as presumably was the case for the work reported by Harvey and associate. It should be remembered that in all cases our fragments were isolated so that we could be sure that we were dealing with a particular fragment, and that the possibility of being contaminated was decreased.

FIGURE 23 Centrifuged oocytes (also insets A and B) in the germinal vesicle stage. *GV*, germinal vesicle; *NC*, nucleolus; *CG*, cortical granules; *LB*, lipid bodies; *M*, mitochondria. Fig. 23, × 40,000; insets A and B, × 600.

FIGURE 24 A section through the cortical granule region shown at *CG* in the inset (B) in Fig. 23. *CG*, cortical granules; *M*, mitochondria; *LB*, lipid bodies. × 40,000.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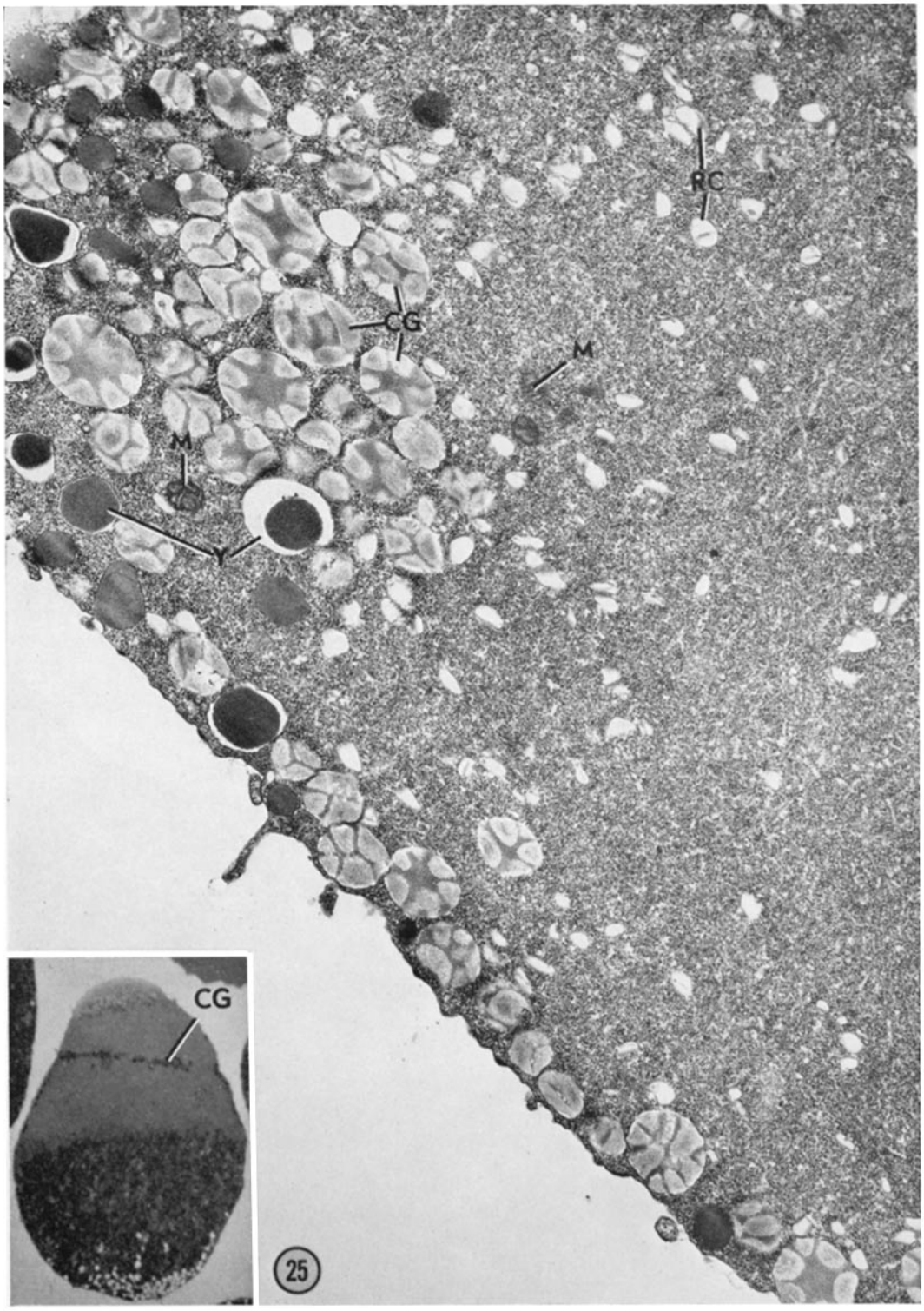
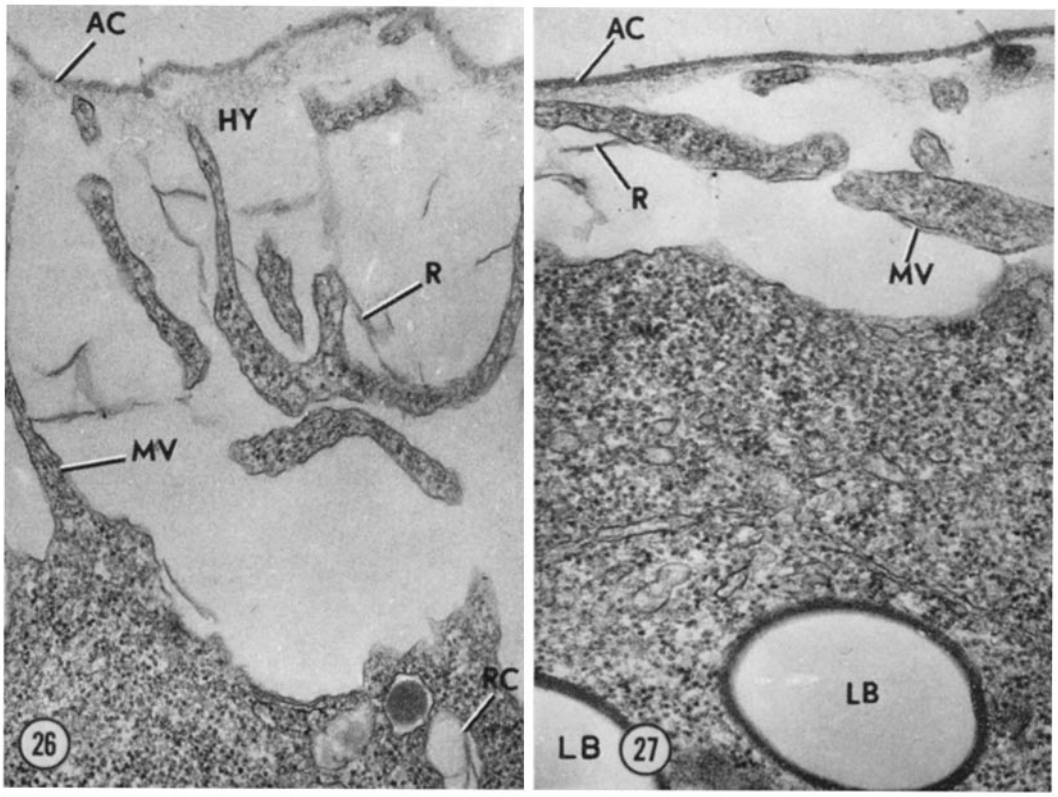


FIGURE 25 The inset of Fig. 25 shows an Epon-embedded, toluidine blue-stained egg that had been treated with 3% urethane for 5 min prior to being centrifuged at 9000 *g* for 15 min. Note the stratum of cortical granules (*CG*). The electron micrograph is a section through the region of the cortical granules. *CG*, cortical granules; *Y*, yolk bodies; *RC*, rod-containing vacuoles; *M*, mitochondria. Fig. 25, × 40,000; inset, × 600.



FIGURES 26 and 27 Sections through activated nucleated clear quarters. *AC*, activation calyx; *MV*, microvilli; *HY*, hyaline layer; *RC*, vacuole containing rod-like structures; *LB*, lipid bodies. *R*, rod-like structures. Figs. 26 and 27, × 30,000.

Although we obtained the various strata and fragments of eggs in the test tube as reported by Harvey, examination of the contents of the centrifuge tubes of each experiment indicated that other fragments also occur often. In developmental studies it is important to examine carefully the respective fragments used in each experiment. White halves are identified in development by the lack of pigment in the larva (pluteus); however, contamination of the pigmented halves with whole eggs cannot be ruled out by simple observations of the embryo, since both would be pigmented.

In presenting a rationale for using the centrifuge method for the study of eggs, Harvey (25) suggested that it obviates other methods such as cutting individual eggs with a glass needle (20, 57). Harvey further indicated that the centrifuged method insures a much more accurate division of the egg into parts of known structure than the "old haphazard and harmful method" of shaking the egg into various parts. In our discussion on the mature egg, we called attention to the random distribution of the various ooplasmic components. Tennent et al. (57) studied the development of egg fragments obtained by *cutting the egg* of the sea urchin *Lytechinus* with a glass needle, and found that the smallest fragment that formed a larva (pluteus) was slightly less than ¼ the original volume of the egg. In connection with this, it is obvious that, in our studies and those of others, the random distribution of inclusions is lost following centrifugation. Moreover, we have shown that each fragment investigated did not contain a homogeneous population of any one of the ooplasmic components; however, the fragments did contain differential amounts of each component. It is difficult at the moment to offer an explanation for the low percentage of development of the fragments other than the

nucleated half. It is possible, as indicated by Raven (53), that "A normal proportion of various cytoplasmic substances is required for normal development. If this 'histochemical equilibrium' is broken, the harmonic development of the larvae is disturbed."

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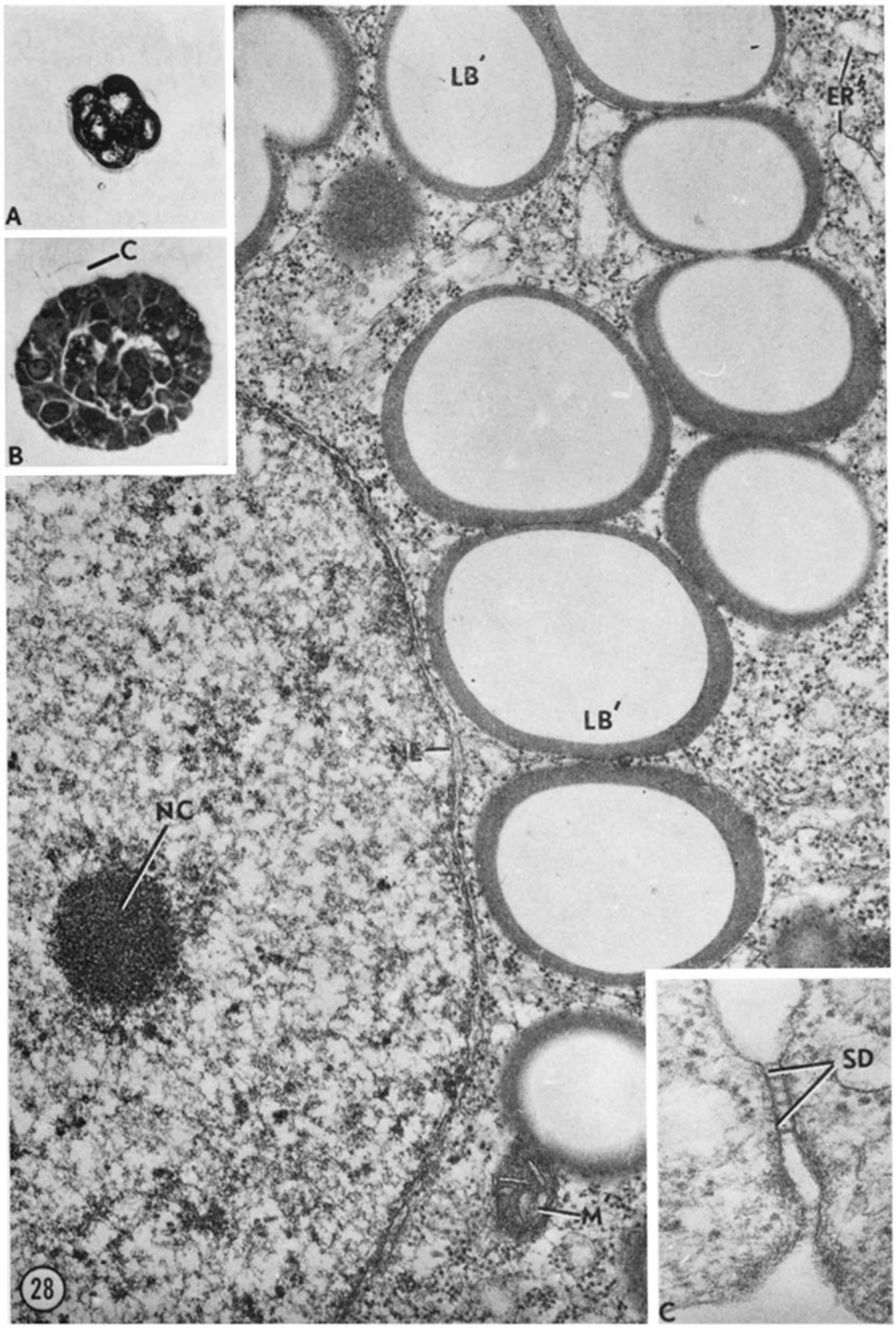
FIGURE 28 Inset A is a phase-contrast photomicrograph of a living, cleaving nucleated quarter. Inset B is an Epon-embedded, toluidine blue-stained multicellular ciliated (*C*; cilium) structure that resulted from a nucleated quarter. The cells of the multicellular ciliated structure are held together by septate desmosomes (*SD*, inset C). Fig. 28 shows some of the cellular components of the multicellular structure illustrated in inset B. *NC*, nucleolus; *NE*, nuclear envelope; *M'*, mitochondrion; *ER'*, endoplasmic reticulum; *LB'*, lipid bodies. Fig. 28, × 60,000; insets A and B, × 600; inset C, × 50,000.


A
C
B
LB'
ER
LB'
NE
NC
M
28
SD
C

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