A CYTOLOGICAL STUDY OF THE RELATION OF THE CORTICAL REACTION TO SUBSEQUENT EVENTS OF FERTILIZATION IN URETHANE-TREATED EGGS OF THE SEA URCHIN, *ARBACIA PUNCTULATA*

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ABSTRACT

Eggs of the sea urchin, *Arbacia punctulata*, treated with 3% urethane for 30 sec followed by 0.3% urethane and inseminated are polyspermic and fail to undergo a typical cortical reaction. Upon insemination the vitelline layer of urethane-treated eggs either does not separate or is raised only a short distance from the oolemma. 1–6 min after insemination, almost all of the cortical granules remain intact and are dislodged from the plasmalemma. Later (6 min to the two-cell stage) some cortical granules are released randomly along the surface of the zygote. Not all zygotes show the same degree of cortical granule dehiscence; most of them experience little if any granule release whereas others demonstrate considerably more. The thickness of the hyaline layer appears to be directly related to the number of cortical granules released. Subsequent to pronuclear migration, several male pronuclei become associated with the female pronucleus. Later the male and female pronuclear envelopes contact and the outer and the inner laminae fuse, thereby forming the zygote nucleus. The male pronuclei remaining in the cytoplasm increase in size and progressively migrate to, and fuse with, the zygote nucleus. By 60 min some zygotes appear to contain only one large zygote nucleus which subsequently enters mitosis. Other zygotes possess a number of male pronuclei which remain unfused, and later these pronuclei along with the zygote nucleus undergo mitosis. There does not appear to be a direct relation between the number of cortical granules a zygote possesses and the above mentioned dichotomy.

The importance of the cortical reaction during fertilization has been recognized for approximately 60 yr and has been of considerable interest to numerous biologists (see Wilson, 1925; Monroy, 1965). Recent fine structural investigations (Endo, 1961 *a* and *b*; Anderson, 1968; Millonig, 1969) of the cortical reaction in echinoids have demonstrated that this initial aspect of fertilization essentially consists of the following events: the separation of the vitelline layer from the surface of the egg to form the activation calyx (fertilization membrane; see Anderson [1968] for a discussion), the wave-like release of the cortical granules starting from the point of gamete fusion, the development of the hyaline layer, and the release of rot-like structures into the perivitelline space.

Sugiyama (1956) claims that eggs "narcotized" with agents such as urethane fail to exhibit a

cortical reaction during insemination or artificial activation. A similar loss of the cortical response has also been reported in eggs treated with uncouplers of oxidative phosphorylation or with hydrostatic pressure (Okazaki, 1956; Whiteley and Chambers, 1960). The possibility that sea urchin eggs may be activated without a cortical reaction is of great importance since various investigators claim that an inhibitor of the initiation of development (e.g., cleavage), which is present in the egg cytoplasm, is eliminated during the release of the cortical granules (Runnström and Immers, 1955; Runnström, 1966). On the other hand, studies by Motomura (1952, 1954) and Kojima (1969) indicate that sea urchin eggs may cleave without breakdown of the cortical granules.

Allen (1954 *a*, 1958) and Allen and Hagström (1955 *a* and *b*) contend that eggs partially fertilized in glass capillaries, i.e. treated so that only a portion of their surface undergoes a cortical response, possess an "unfertilized" and a "fertilized" cytoplasm corresponding to the areas along the egg's surface which have or do not have intact cortical granules. In partially fertilized eggs, development of the sperm aster, migration of pigment granules and the pronuclei, and pronuclear fusion are restricted to fertilized cytoplasm. Allen (1958) suggests that many of these results are due to influences emanating from the cortex of the activated egg.

In an effort to more fully understand the various structural changes occurring during fertilization, we have found it desirable to undertake a study of urethane-treated eggs. The present study explores the relation of the cortical response to later events of fertilization and attempts to put to rest the notion that the release of the cortical granules of the sea urchin plays a significant role in events of fertilization other than during the cortical reaction.

MATERIALS AND METHODS

Eggs and sperm of *Arbacia punctulata*, acquired from the Marine Biological Laboratory, Woods Hole, Mass., during the months of June and July, were obtained according to procedures previously described (Longo and Anderson, 1968). In an effort to obtain a high percentage of synchronously developing embryos after urethane treatment, we found it necessary to employ eggs from a single female for each experiment. A similar situation has also been reported by Harvey (1936) and Longo and Anderson (1970 *a*). After they had been twice washed in sea water, the eggs were treated with 3% urethane in sea water for 30–40 sec and subsequently inseminated according to the method prescribed by Just (1939). At 2 min postinsemination, fresh sea water was added to the fertilized eggs so that the final concentration of urethane was 0.3%. Control preparations consisting of eggs treated in the same manner except for the incubation in urethane were inseminated with the same sperm dilution.

To obtain a sequence of development from fertilization to cleavage, eggs were collected and fixed at 2-min intervals for a 20 min period, then at 5-min intervals for 60 min after insemination. In addition, unfertilized eggs incubated in 3% and 0.3% urethane were fixed at periodic intervals up to 10 min. We have referred to insemination as the introduction of spermatozoa into the egg suspension, and this marks the zero point from which all timing measurements are made.

Eggs and zygotes were prefixed for light- and electron-microscopy for 1.5 hr in a solution of 3% glutaraldehyde, 1% acrolein, 2% sodium citrate, and 7% sucrose in sea water which is adjusted to pH 7.6 with sodium hydroxide, washed in sea water with 2% sodium citrate for 2 hr, and postfixed in a 1% solution of osmium tetroxide in sea water for 1.5 hr. After fixation, the eggs and zygotes were rapidly dehydrated in a graded series of ethanol, infiltrated, and embedded in Epon (Luft, 1961). Thin sections were cut with a Porter-Blum MT-2 ultramicrotome, stained with uranyl acetate followed by the lead citrate stain of Venable and Coggeshall (1965), and examined with the RCA EMU-3H electron microscope. Thick sections (1 μ) made for general light microscopy were stained according to the method of Ito and Winchester (1963).

RESULTS

Eggs from various females react differently to the 3%–0.3% urethane treatment, i.e., some appear to undergo a cortical response after incubation while others do not. This difference, however, could be eliminated by altering the urethane concentration and/or the time of incubation for each group of eggs. In most instances, more than 98% of the eggs incubated in 3% followed by 0.3% urethane failed to show a typical cortical response. In addition, eggs treated in such a manner also become polyspermic. The controls underwent the same structural events previously described for normal development (Longo and Anderson, 1968; Anderson, 1968).

The Unfertilized Urethane–Treated Egg

Eggs exposed to 3% urethane in sea water for 30–40 sec and fixed demonstrate an increase in diameter and measure approximately 80 μ. Eggs

not treated in urethane and fixed measure about 74 μ in diameter. The cortical granules of urethane-treated eggs appear to be dislodged from their intimate association with the oolemma (Fig. 1, *CG*) and also exhibit an increase in size, measuring approximately 1.2 μ in diameter. In addition, the normally less dense, ovoid portions of the cortical granules which are peripherally located appear to condense and become separated by a 55–75 mμ space from the stellate central portion and the surrounding membrane of the cortical granule (Fig. 1). The cortical granules of untreated eggs measure about 0.8 μ in diameter.

The female pronucleus is spheroidal and appears morphologically equivalent to that of the untreated egg. Other than the cortical granules, the various organelles and inclusions found within the ooplasm do not appear to be affected after this 30–40 sec incubation.

After a 2 min incubation in urethane (3% followed by 0.3%), the female pronucleus and various organelles of the cytoplasm undergo structural changes which may eventually lead to the degeneration of the unfertilized egg. Since similar changes of the female pronucleus and cytoplasm also occur in the fertilized, urethane-treated egg prior to pronuclear fusion, the following description will pertain to both: With continued exposure to the 0.3% urethane, there appears to be a reduction in the amount of annulate lamellae. The annulae lamellae that constitute a part of a heavy body disappear, leaving only the granular matrix (Fig. 4, *Inset;* Fig. 16).

The female pronucleus appears to be reduced in size and crenated, i.e. the pronuclear envelope possesses fewer pores than the controls, and becomes highly convoluted (Figs. 2 and 3, *FPN*). The convolutions of the female pronucleus interdigitate and produce an extremely irregular surface which increases in complexity with further exposure (10 min) to urethane (Fig. 4).

The Cortex after Insemination

1–6 MIN: Eggs treated with urethane do not undergo a typical cortical response at insemination. In most cases, the vitelline envolope separates from the surface of the egg, forming a thin amorphous layer referred to as the activation calyx (Anderson, 1968) (Figs. 5 and 6). Some eggs do not experience a separation of the vitelline layer and the formation of the activation calyx. In those eggs forming an activation calyx, the perivitelline space is variable in width but rarely as large as that observed in the controls (Figs. 5 and 6, *PVS*).

During the initial stages of fertilization, i.e. 1–6 min after insemination, almost all of the cortical granules appear to remain intact. On occasion, some cortical granules have been observed in various stages of dehiscence (Fig. 1, *Inset*, arrows). Development of a hyaline layer does not appear to take place during this period.

6 MIN TO THE TWO-CELL STAGE: By 6 min postinsemination various groups of cortical granules, located at apparently random loci along the surface of the zygote, break down in a manner analogous to that observed during artificial activation (Sachs and Anderson, 1970). The mechanism of cortical granule release proceeds as described by Anderson (1968) (Figs. 7 and 8, "*CG*"). The number of cortical granules that are released at any region along the cortex of the zygote varies. As development proceeds, greater numbers of cortical granules may dehisce; however, not all zygotes show the same degree of cortical granule breakdown. The result is a spectrum, i.e. most zygotes experience little if any cortical granule breakdown, some have a greater number, while few possess many large areas which have released their cortical granules. All zygotes examined had a substantially greater number of undehisced cortical granules than the controls.

Within the perivitelline space, rod-like structures and a thin hyaline layer, consisting of some amorphorous material, are observed during this period (Figs. 10 and 11). The rod-like structures originating from cytoplasmic vesicles are extruded into the perivitelline space by a process akin to exocytosis (Anderson, 1968; deDuve and Wattiaux, 1966).

After the initial series of pronuclear fusions (see below), the thin amorphorous activation calyx thickens and develops a trilaminar structure at various regions (see Anderson, 1968) (Figs. 9 and 10; arrows). In many cases the activation calyx is completely laminated by the two-cell stage, while in others it remains an amorphous layer with intermittent sections demonstrating the trilaminar structure (Figs. 9 and 10) (see also Anderson, 1968; Ito, 1969).

After insemination most of the pigment granules, which were previously dispersed throughout the cytoplasm, progressively migrate to the zygote's cortex (Figs. 9, 10, and 11, *P*). The migration of the pigment granules in the experimentally treated

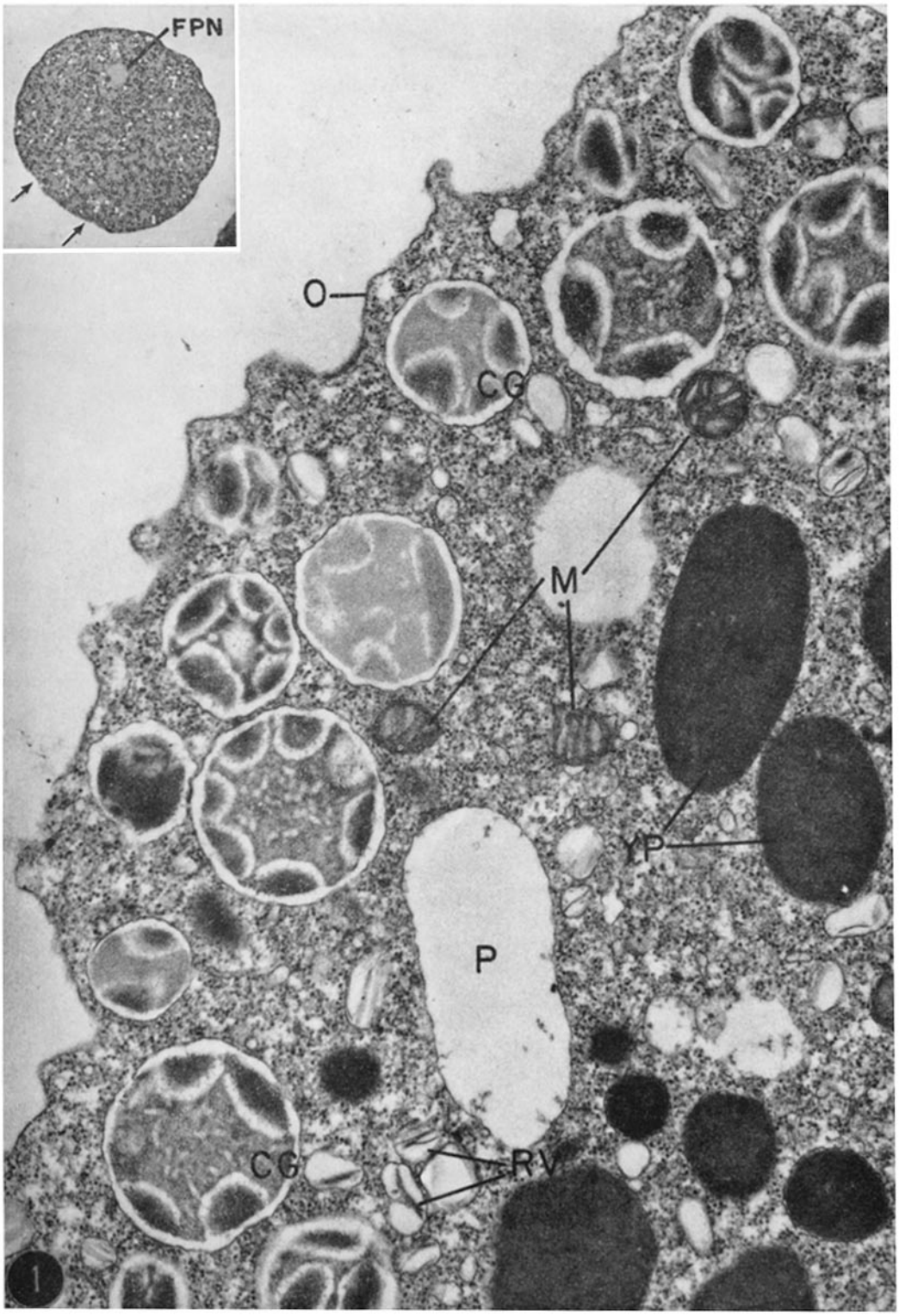
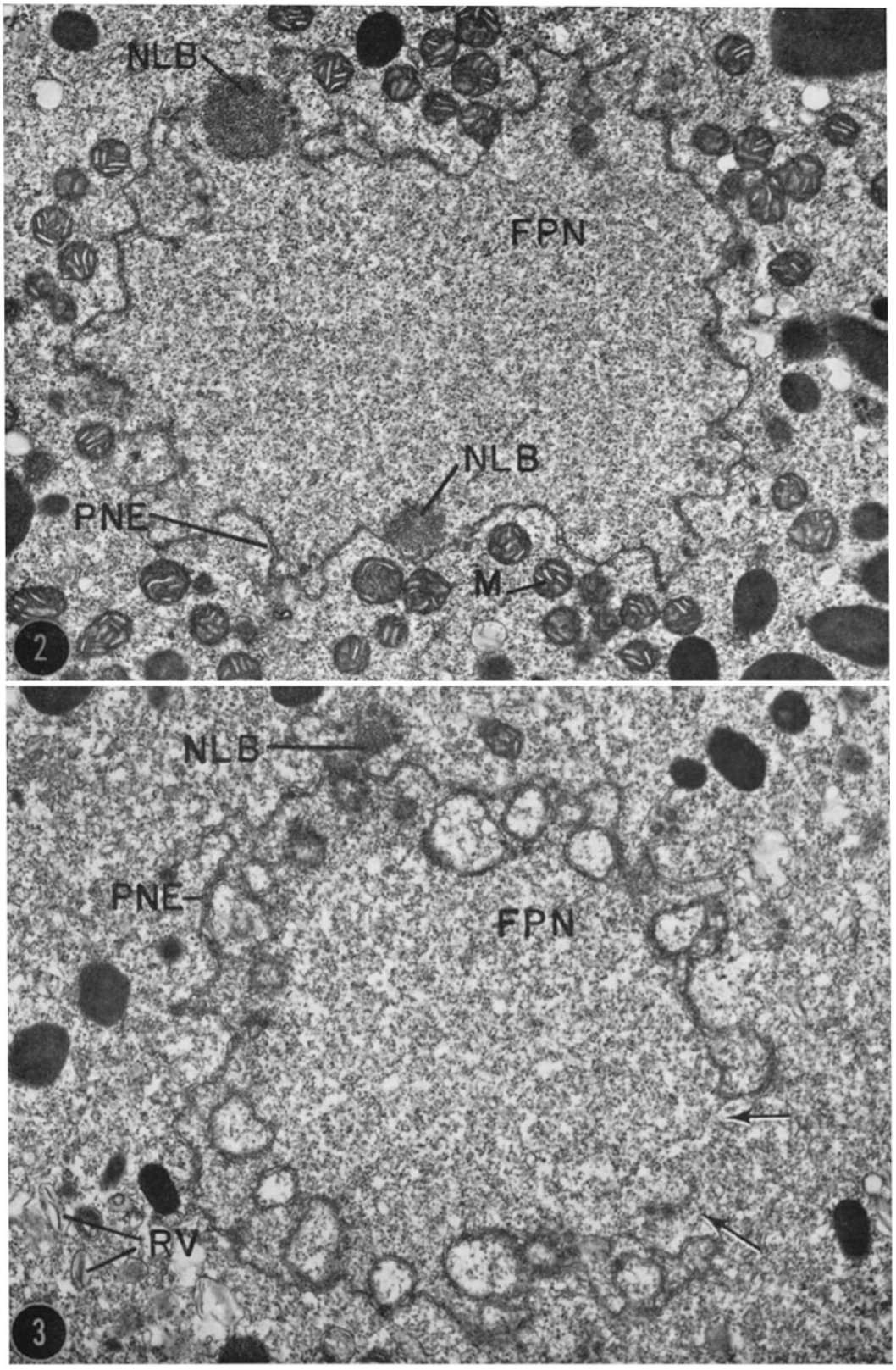


FIGURE 1 A portion of the cortex of an unfertilized egg exposed to 3% urethane for 30 sec, showing cortical granules (*CG*), rod-containing vesicles (*RV*), mitochondria (*M*), pigment granule (*P*), yolk bodies (*YP*), and the oolemma (*O*). *Inset.* Photomicrograph of a zygote 1 min postinsemination. Arrows depict an area of the zygote's surface from which cortical granules have been released. *FPN*, female pronucleus. Fig. 1, × 27,000; *inset*, × 400.



FIGURES 2 and 3 Female pronuclei (*FPN*) of fertilized eggs exposed to urethane for 2 and 4 min, respectively. Arrows point to a break in the pronuclear envelope (*PNE*); *NLB*, nucleolus-like bodies; *M*, mitochondria; *RV*, rod-containing vesicles. Fig. 2, × 18,500; Fig. 3, × 14,000.

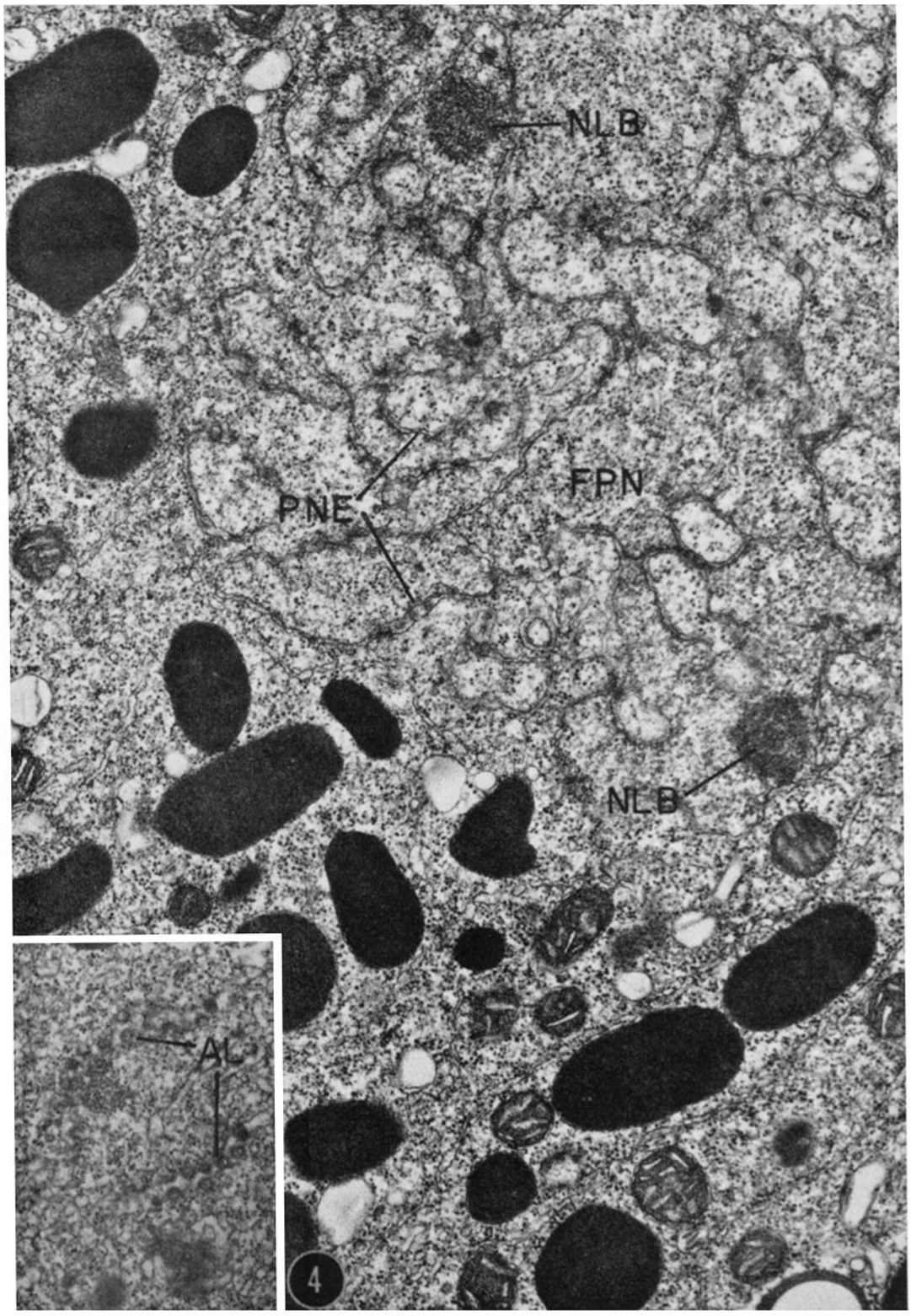
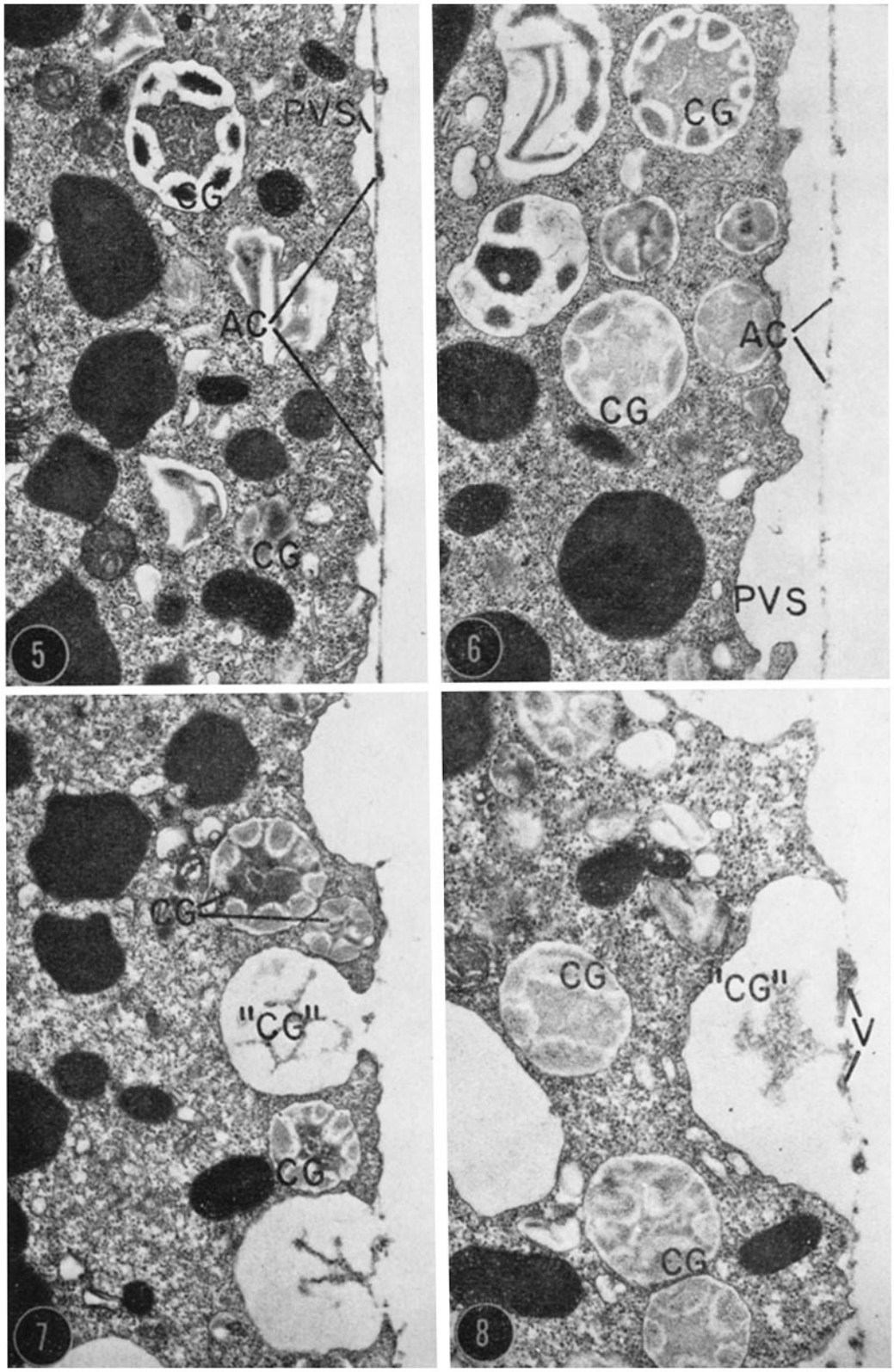
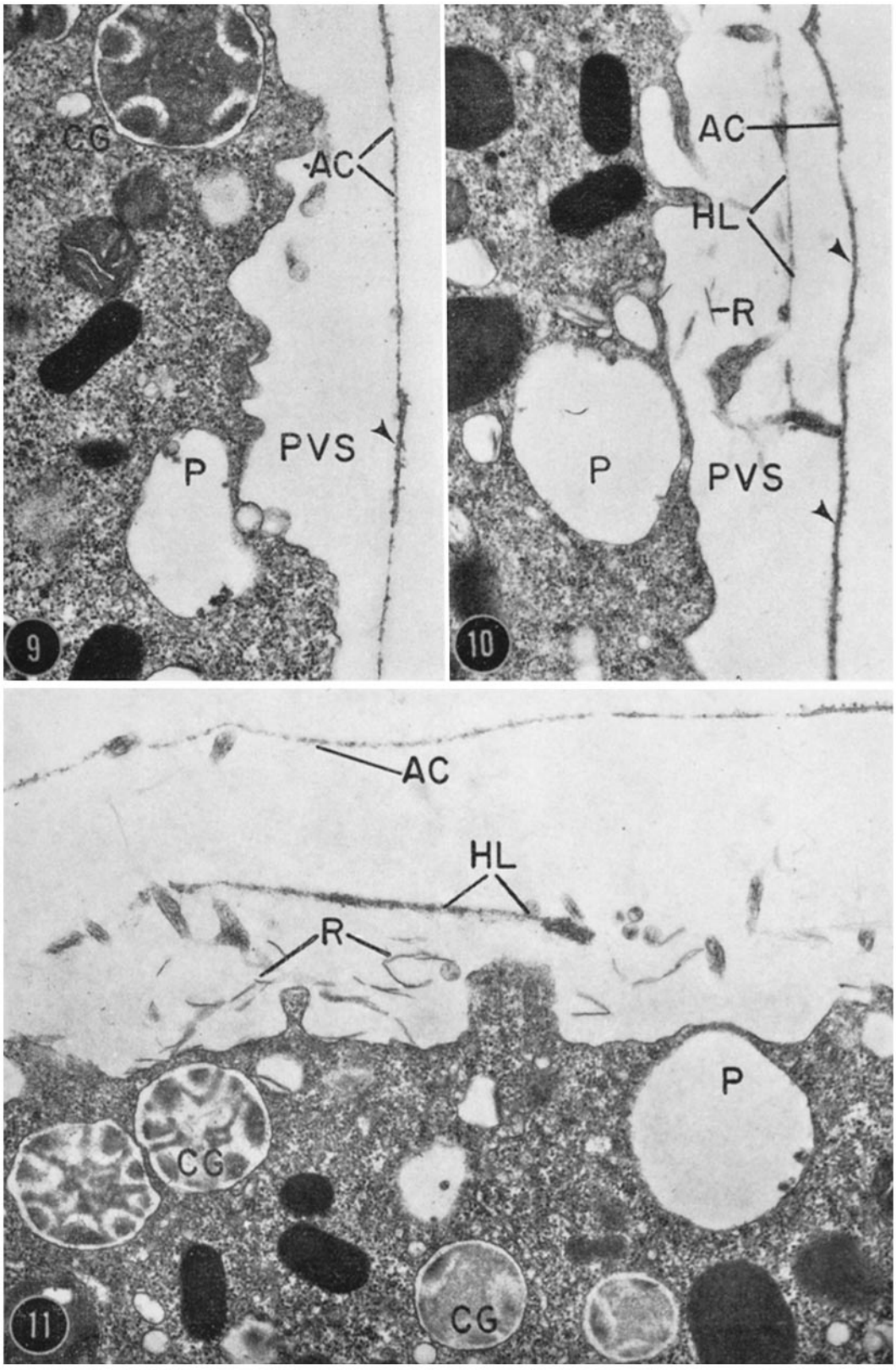


FIGURE 4 Female pronucleus (*FPN*) of a zygote 6 min postinsemination. *NLB*, nucleolus-like body; *PNE*, pronuclear envelope. *Inset*. Heavy body apparently in the process of breaking down. *AL*, annulate lamellae. Fig. 4, × 27,000; *inset*, × 18,500.



FIGURES 5 and 6 Regions of the cortex of zygotes 2 and 12 min postinsemination, demonstrating the activation calyx (*AC*), cortical granules (*CG*), and perivitelline space (*PVS*). Figs. 5 and 6, × 18,500.

FIGURES 7 and 8 Areas of the zygote's cortex (6 min postinsemination) depicting cortical granules ("*CG*") in various stages of dehiscence. *CG*, intact cortical granules; *V*, vesicles formed as a result of the fusion of the cortical granule membrane and the oolemma. Fig. 7, × 18,500; Fig. 8, × 27,000.



FIGURES 9 and 10 Sections of the zygote's surface, showing the activation calyx (*AC*) with laminated regions (arrows), rods (*R*) within the perivitelline space (*PVS*), and a hyaline layer (*HL*), at 14 min postinsemination (Fig. 9) and at the two-cell stage (Fig. 10). *CG*, cortical granule; *P*, pigment granules. Figs. 9 and 10, × 27,000.

FIGURE 11 Electron micrograph of a zygote 65 min postinsemination, depicting the activation calyx (*AC*), hyaline layer (*HL*), rods (*R*), pigment granule (*P*), and cortical granules (*CG*). × 27,000.

eggs appears to be the same as that observed in the controls (see also Sachs and Anderson, 1970).

Sperm Incorporation, Formation of the Male Pronucleus, and the Sperm Aster

At the site of gamete fusion a confluence of the egg and sperm is established and a fertilization cone is produced. The fertilization cone of urethane-treated eggs may persist for as long as 14 min postinsemination and contains a plethora of cortical granules (Fig. 12). However, the fertilization cone is usually smaller than that of the controls and may not contain definite anterior and posterior regions (see Longo and Anderson, 1968).

Most sperm are incorporated into the egg within 1 min from the time of insemination, and they appear to be synchronous in their reorganization into male pronuclei. Occasionally, however, male pronuclei have been observed, at later stages of fertilization (30 min postinsemination), to undergo morphological events characteristic of recently incorporated spermatozoa, e.g., chromatin dispersion (Fig. 13, *Inset*) (see Discussion).

Usually, the spermatozoon rotates soon after it enters the egg; occasionally, however, it fails to do so. Sperm which fail to rotate appear to develop in synchrony with those that do. A similar situation has also been observed in eggs made polyspermic with nicotine (Longo and Anderson, 1970 *a*).

Albeit reorganization of the spermatozoon into a male pronucleus takes a course similar to that observed in the monospermic controls (see also Longo and Anderson, 1968), a few minor structural differences do exist. Dispersion of the sperm chromatin does not appear to be as extensive as that observed in the controls, for the periphery of the reorganizing nucleus consists of aggregates embedded within a meshwork of finely dispersed chromatin. Shortly after it begins to disperse, the sperm chromatin is surrounded by vesicles which coalesce and produce the male pronuclear envelope. This results in the

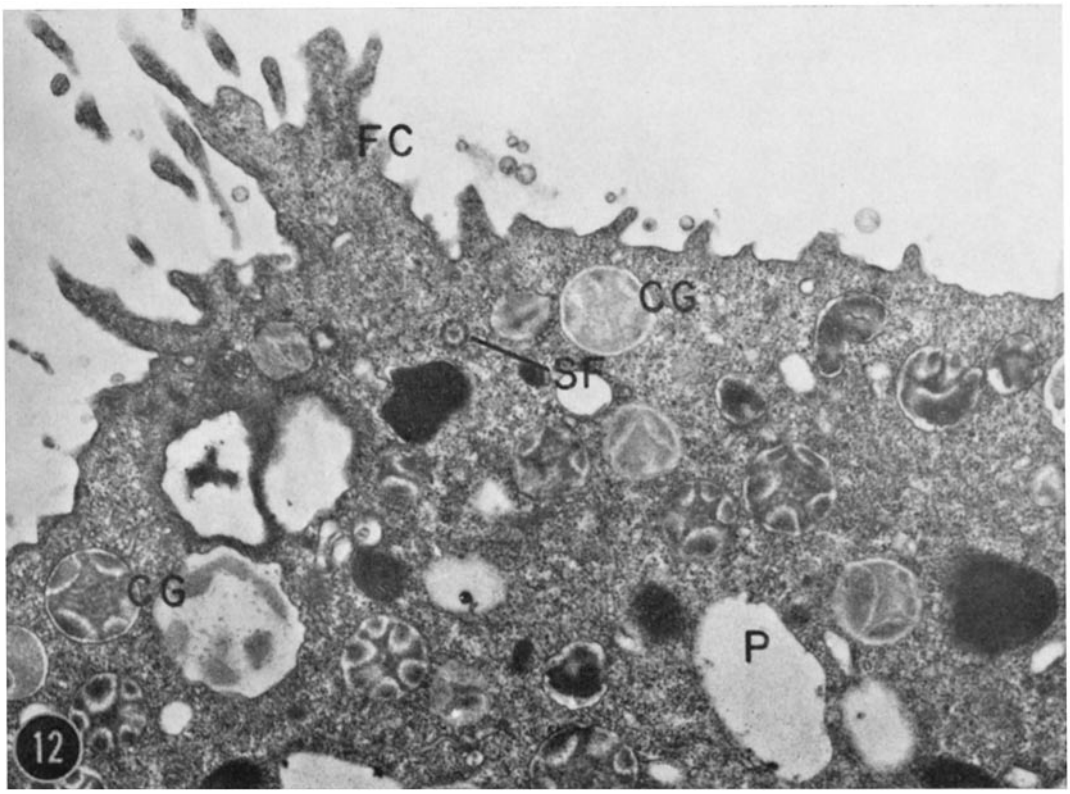


FIGURE 12 Section through a fertilization cone (*FC*), showing cortical granules (*CG*), pigment granules (*P*), and a portion of the sperm flagellum (*SF*). × 18,500.

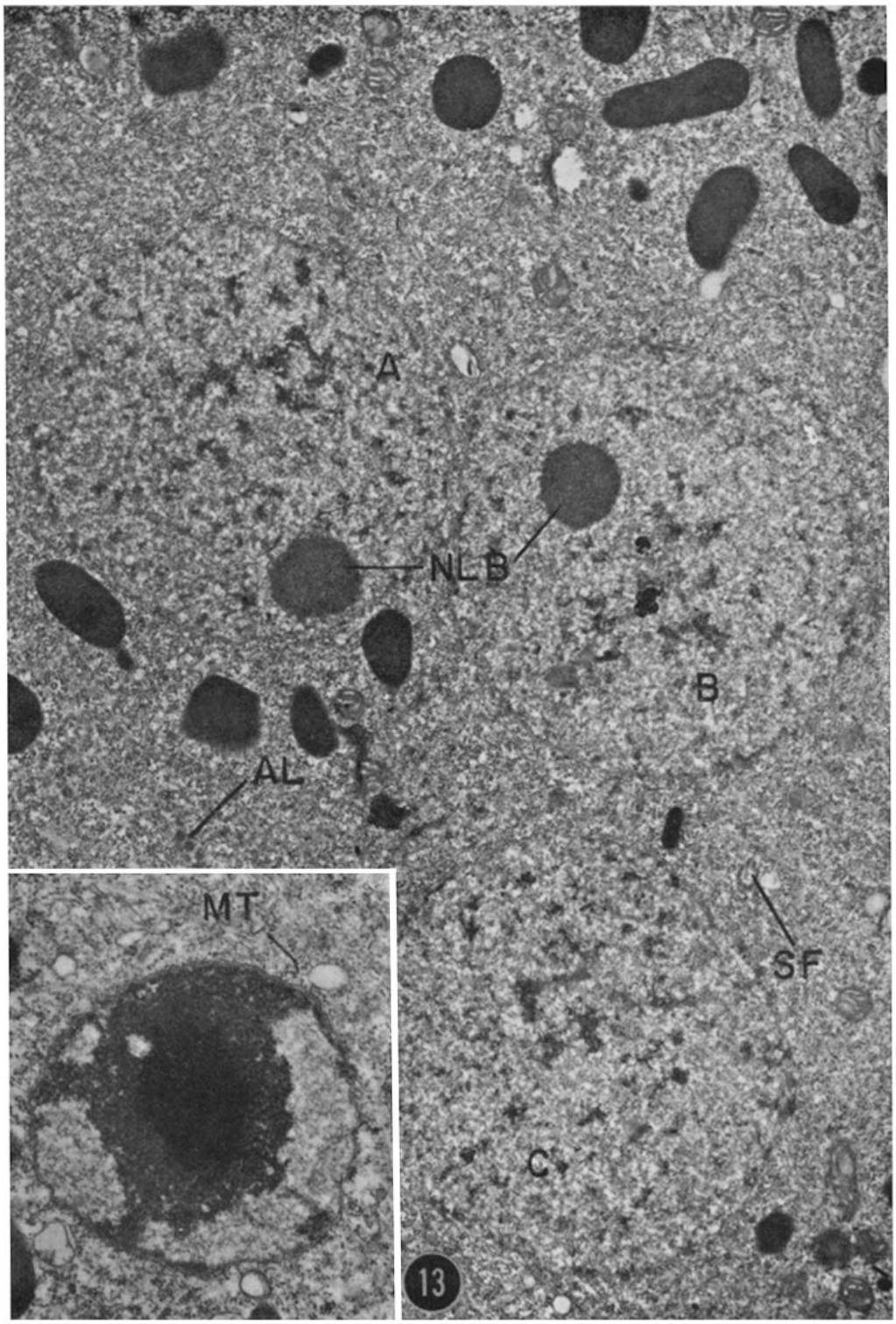


FIGURE 13 Three male pronuclei (*A*, *B*, *C*) within one large astral complex. *NLB*, nucleolus-like bodies; *SF*, portion of a sperm flagellum; *AL*, annulate lamellae. *Inset.* Male pronucleus exhibiting chromatin dispersion 30 min postinsemination. *MT*, microtubules. × 14,000; inset, × 18,000.

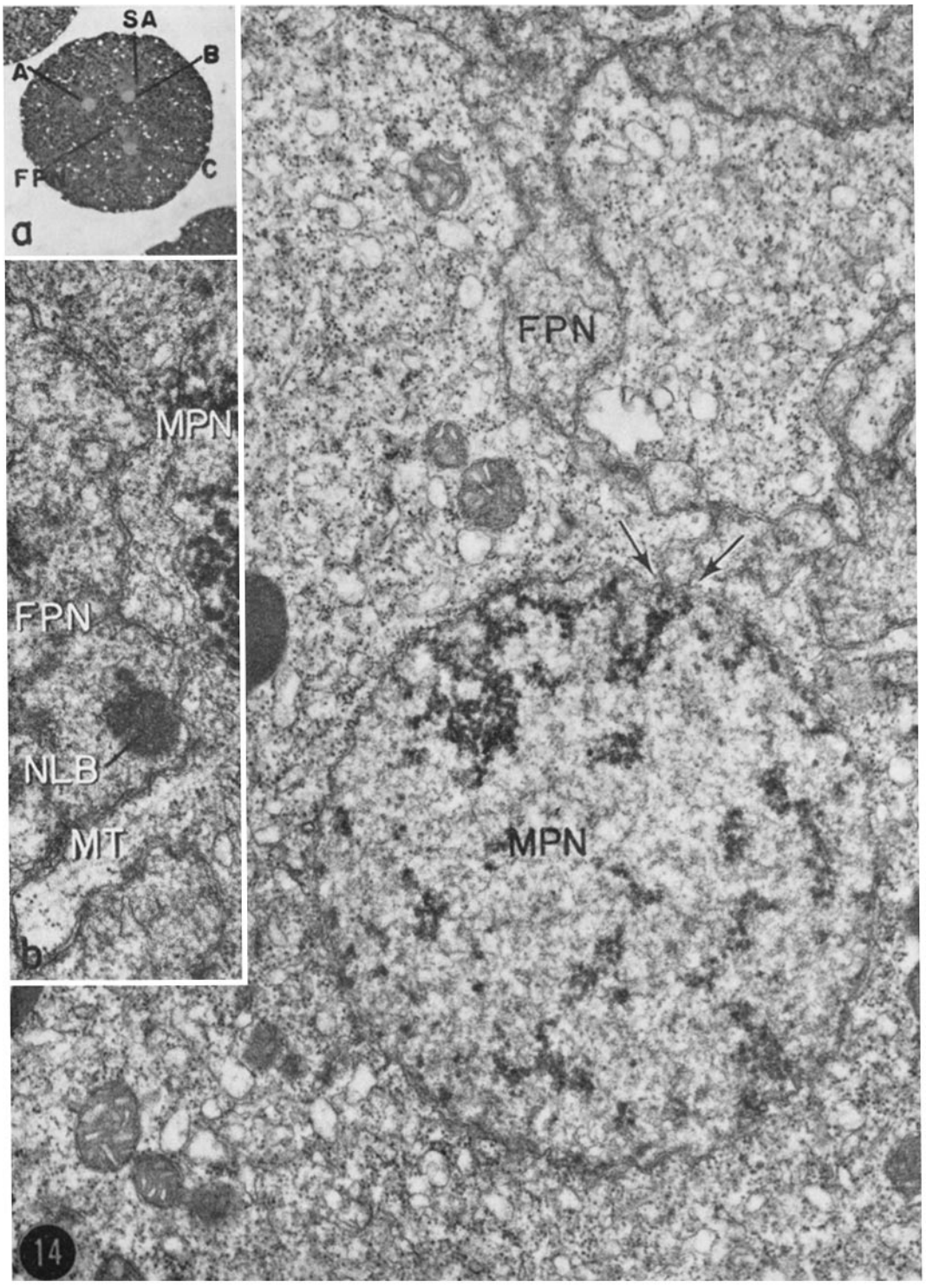


FIGURE 14 Male pronucleus (*MPN*) fusing (arrows) with a convoluted region of the female pronucleus (*FPN*). *Inset a.* Photomicrograph of a zygote containing three male pronuclei (*A*, *B*, *C*). *FPN*, female pronucleus; *SA*, sperm aster. *Inset b.* Electron micrograph of the male pronucleus (*MPN*) and female pronucleus (*FPN*) in apposition to one another. *NLB*, nucleolus-like body; *MT*, microtubules. Fig. 18, × 27,000; *inset a*, × 400; *inset b*, × 27,000.

formation of a heart-shaped male pronucleus (see also Longo and Anderson, 1968). Eventually, the male pronucleus becomes spheroid, enlarges, and forms nucleolus-like bodies (Figs. 13 and 14).

Just prior to its formation, each male pronucleus is associated with the development of a sperm aster. Morphogenesis of the sperm aster, which consists of fasicles of microtubules, endoplasmic reticulum, some annulate lamellae, and centrioles, appears to involve the same series of structural events as observed in the controls (see Longo and Anderson, 1968). Investigators studying the effects of anesthetics on dividing cells have frequently observed a reduction in the size of the mitotic asters (Rappaport, 1969; see also Cornman, 1954; Harvey, 1956); a decrease in the size of the sperm aster was not noted during this study (Fig. 14, *Inset a*).

Accompanying the male pronucleus and sperm aster during their migration are the sperm mitochondrion and flagellum. During later stages of development, several sperm asters may come together and fuse, thereby forming a large aster containing a number of closely associated male pronuclei (Fig. 13) (see Longo and Anderson, 1970 *a*).

Migration and Fusion of the Pronuclei

By 10–12 min postinsemination, several male pronuclei have migrated to the vicinity of the female pronucleus (Fig. 14, *Insets a* and *b*). Subsequently, the outer and the inner laminae of the male and female pronuclear envelopes contact and fuse, thereby forming a zygote nucleus (Fig. 14) (see also Longo and Anderson, 1968; 1970 *a*). Unlike the single locus of contact and fusion exhibited by the pronuclei of untreated zygotes, the male pronucleus may contact and fuse with several convoluted projections of the female pronucleus in urethane-treated eggs.

The regions of the early zygote nucleus that were formerly the male and female pronuclei are easily recognized. After the eventual increase in the diameter of the internuclear bridges, produced as a result of pronuclear fusion, the zygote nucleus possesses a spheroid or ellipsoid region (formerly the male pronucleus) and a highly convoluted region (formerly the female pronucleus) (Figs. 15 and 16). During later stages of development, the convoluted region of the zygote nucleus appears to be considerably reduced in size (Fig. 16, *Inset b*, and Fig. 17); however, this is apparently due to the relatively larger volume occupied by the paternal chromatin, after the fusion of many male pronuclei. In addition, the paternal chromatin is recognized by its increased density at the various sites of fusion (Figs. 15 and 16) (see also Longo and Anderson, 1968; 1970*a*). Occasionally, the zygote nuclear envelope that was once a portion of the female pronucleus is broken (Fig. 16, *).

After the initial series of pronuclear fusions the male pronuclei remaining in the cytoplasm continue to migrate to, and fuse with, the zygote nucleus (Fig. 16 and *Insets a* and *b*). Concomitantly, the male pronuclei, sperm aster, and nucleolus-like bodies increase in size (see also Longo and Anderson, 1970 *a*).

In some cases, by 60 min one large zygote nucleus is formed which is surrounded by microtubules, endoplasmic reticulum, and centrioles (Fig. 17 and *Insets a* and *b*). In addition, some annulate lamellae and sperm mitochondria may also be observed in the cytoplasmic region surrounding the zygote nucleus. Later the zygote nucleus enters into mitosis. In other cases some male pronuclei may remain unfused, and eventually they and the zygote nucleus undergo mitosis (Fig. 17, *Inset c*). We were unable to determine a clear relationship between the number of intact cortical granules possessed by a zygote and the above dichotomy. The urethane-treated eggs cleaved 10–20 min after cytokinesis in the controls (see Longo and Anderson, 1970 *a*; Harvey, 1956).

DISCUSSION

Absence of a Typical Cortical Reaction

Data presented in this study have demonstrated that eggs of the sea urchin, *Arbacia punctulata*, treated with urethane may be inseminated without a typical cortical reaction. The absence of the typical cortical reaction appears to be primarily due to the failure of cortical granule dehiscence which is accompanied by a decrease in the width of the hyaline layer and the perivitelline space. Previous studies utilizing agents other than urethane (e.g., heat, hydrostatic pressure, and uncouplers of oxidative phosphorylation) have also indicated a similar process at the light microscopic level of observation (Allen, 1958; Okazaki, 1956; Harvey, 1956; Whiteley and Chambers, 1960).

The absence of a typical cortical reaction in *Arbacia* does not appear to affect later events of fertilization, i.e., development of the male pronucleus, pronuclear and pigment granule migration, and pronuclear fusion are essentially the same as simi-

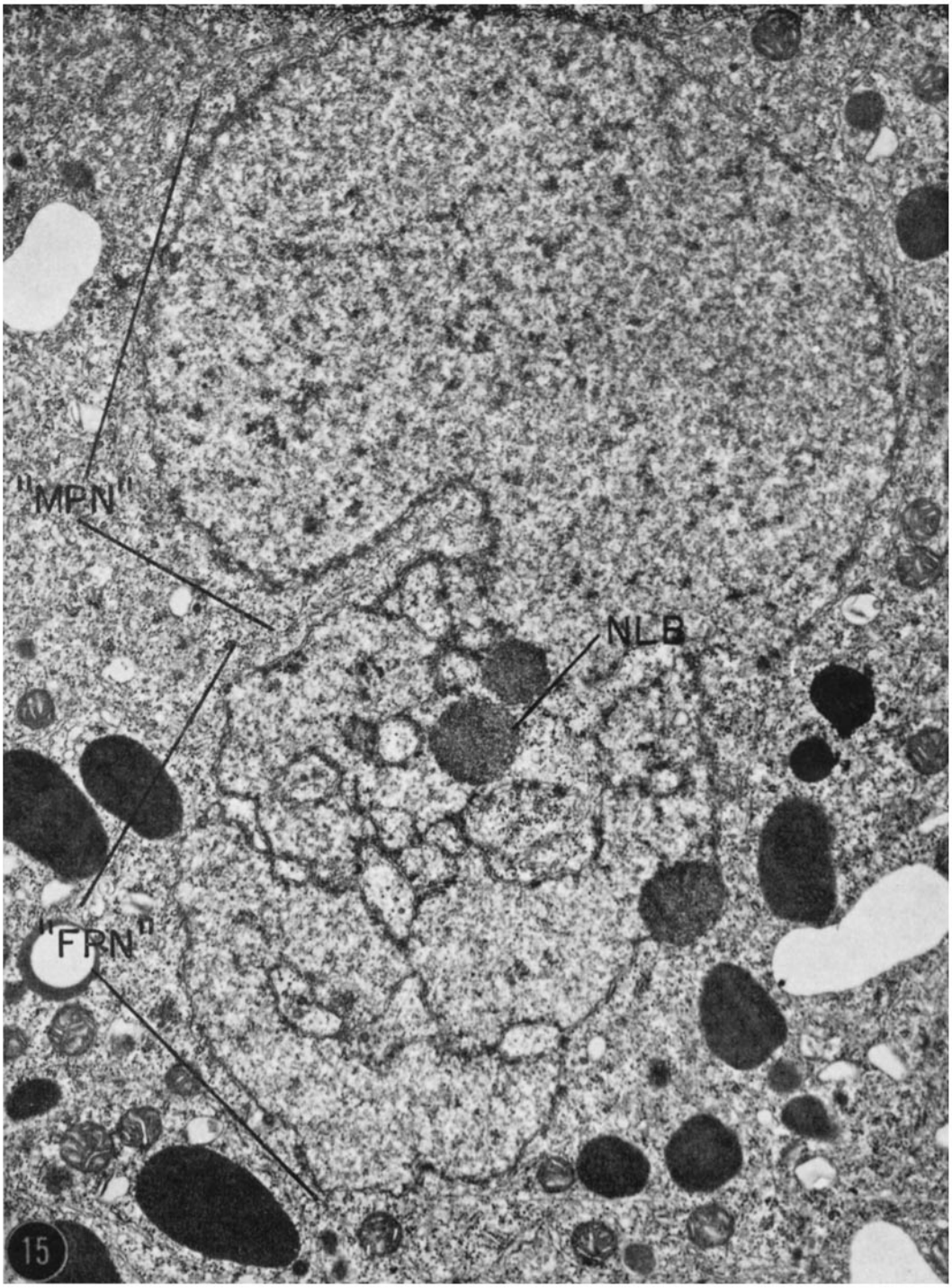


FIGURE 15 Section of a zygote nucleus showing portions contributed by the male pronucleus (*"MPN"*) and the female pronucleus (*"FPN"*). *NLB*, nucleolus-like bodies. × 18,500.

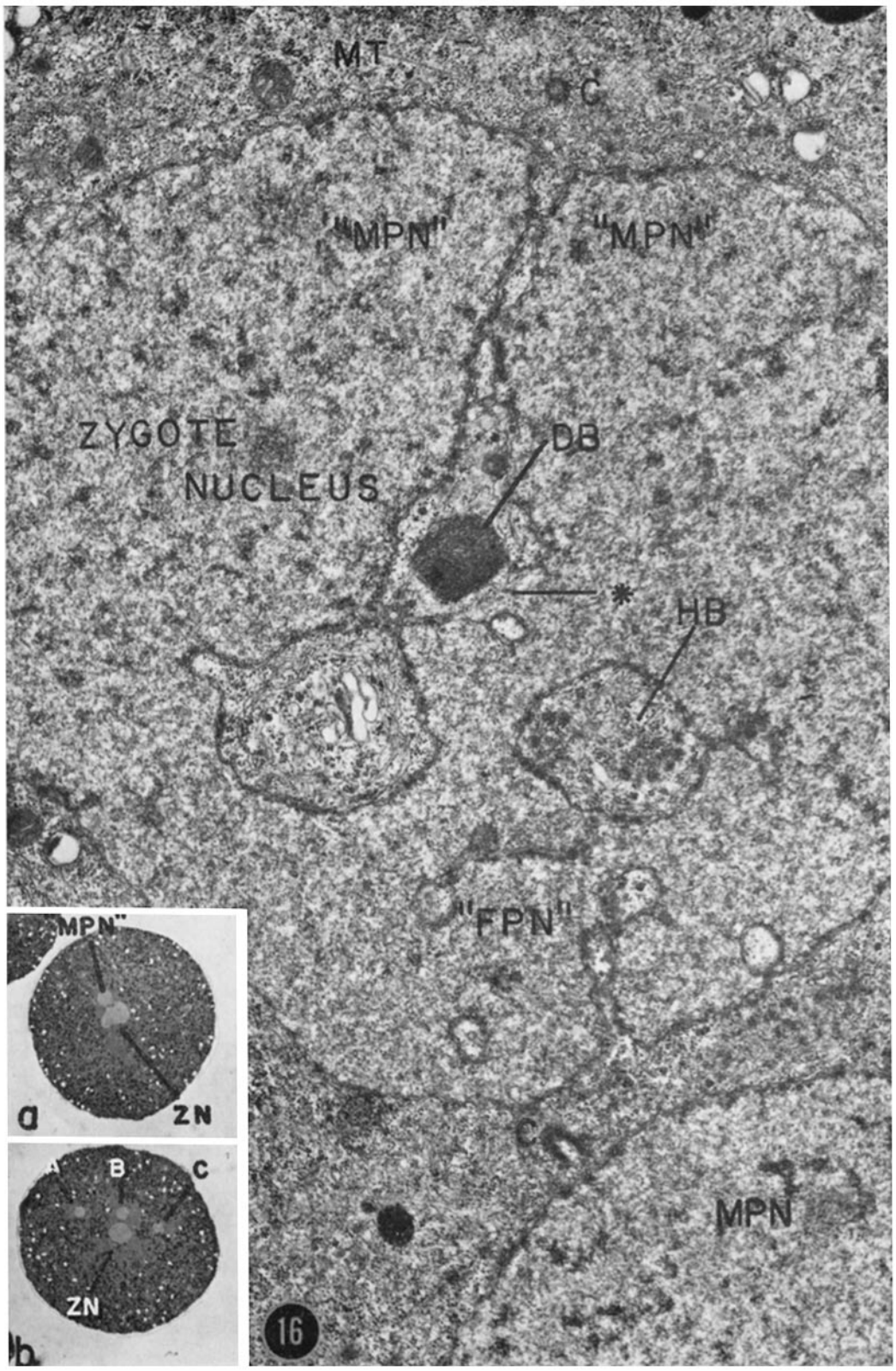


FIGURE 16 Electron micrograph of a male pronucleus (*MPN*) adjacent to the zygote nucleus. "*MPN*" and "*FPN*", portions of the zygote nucleus that were formerly male and female pronuclei; *C*, centrioles; *MT*, microtubules; *DB*, electron-opaque body; *, region of the zygote nucleus which is open to the cytoplasm; *HB*, heavy bodies apparently breaking down. *Inset a*. Photomicrograph of a zygote nucleus (*ZN*) following its fusion with a male pronucleus ("*MPN*"). *Inset b*. Photomicrograph of three male pronuclei (*A*, *B*, *C*) migrating to the zygote nucleus (*ZN*). Fig. 16, × 18,500; *insets a* and *b*, × 400.

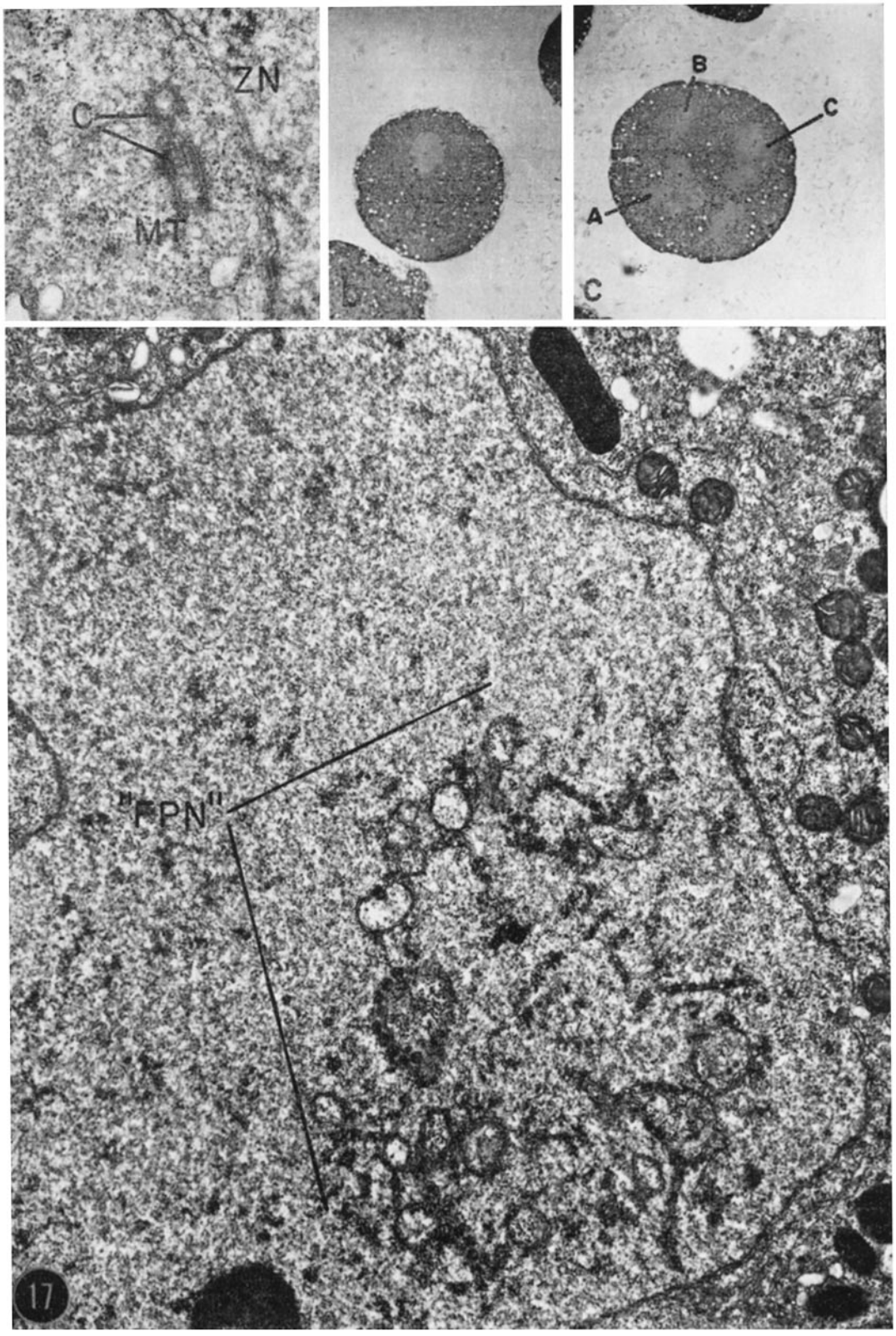


FIGURE 17 Zygote nucleus 70 min postinsemination containing the convoluted portion contributed by the female pronucleus ("*FPN*"). *Inset a*. Paired centrioles (*C*) adjacent to the zygote nucleus (*ZN*). *MT*, microtubules. *Insets b* and *c*. Photomicrographs demonstrating a zygote nucleus following the fusion of male pronuclei (*inset a*) and showing mitotic figures (*A, B, C*) containing chromatin derived from the zygote nucleus and male pronuclei (*inset b*). Many pigment granules (translucent spheroids) have moved to the zygote's cortex. Fig. 21, × 18,500; *inset a*, × 27,000; *insets b* and *c*, × 400.

lar events observed in the controls. In addition, the failure of cortical granule dehiscence does not appear to prevent mitosis or cleavage although these events did take place later than in the controls (see below).

The Vitelline Layer and Activation Calyx

The separation of the vitelline layer from the surface of the urethane-treated egg at insemination without the breakdown of the cortical granules suggests that these two events may not be causally related, i.e., the vitelline envelope may not be induced to detach from the egg's surface by the dehiscence of the cortical granules. This suggestion, however, does not exclude the possibility that the release of a limited number of cortical granules may induce the separation of the vitelline layer in a manner similar to that described by Costello (1959). In some cases the release of the cortical granules does not appear to initiate its complete withdrawal from the cell membrane, i.e., the vitelline layer remains attached to regions of the zygote's surface possessing undehisced cortical granules. How the separation of the vitelline layer comes about has not been determined (see Costello, 1959).

Investigations by Wolpert and Mercer (1961), Endo (1961 *a*), and Anderson (1968) have demonstrated that the activation calyx is derived from the vitelline layer and material released from the cortical granules. Together, these substances eventually form the trilaminar structure of the activation calyx (Anderson, 1968; Austin, 1968; Endo, 1961 *a*; Ito et al., 1967; see also Bryan, 1970). The observations made during this study indicate some relationship between the thickness and the amount of trilaminar structure of the activation calyx and the apparent extent of cortical granule breakdown, although this correlation is not apparent in all cases. Some embryos, which appeared to have experienced little if any cortical granule release, exhibited very little lamination of the activation calyx.

The Cortical Granules

It is difficult to unequivocally state that no cortical granules are released upon insemination of *Arbacia* eggs treated in urethane. During later stages of fertilization, many zygotes do possess a limited number of dehiscing granules; however, as far as we were able to determine, some zygotes appear to exhibit very little to almost no cortical granule breakdown throughout development. Furthermore, all embryos treated with urethane contain considerably more undehisced cortical granules than normally observed in the controls.

The presence of dehiscing cortical granules adjacent to ones that are intact in urethane-treated egg suggests that the breakdown of a cortical granule does not induce its neighbors to dehisce. A similar situation has also been described in artificially activated eggs of *Arbacia* by Sachs and Anderson (1970). In addition, the "intermediate zone" (Allen, 1954 *b*; Allen, 1958; Allen and Hagström, 1955 *a*), which consist of cortical granules, decreased in number and in size and separate areas of released and intact cortical granules were not observed during the course of this study.

Experiments by Sugiyama (1956), who examined the effects of various activating agents on sea urchin eggs, have also indicated that the wave-like breakdown of cortical granules is not a result of a product diffused from one granule to another (see also Kacser, 1955). Sugiyama (1956) suggests that the chain-like release of cortical granules is due to a subtle yet undetectable change, referred to as the fertilization wave or impulse, which is propagated under normal conditions over the surface of the egg (see also Monroy, 1965). In the case of eggs narcotized with agents such as urethane, the irritability of the cortex is decreased, thereby inhibiting the fertilization impulse and consequently cortical granule release (Sugiyama, 1956). The results of the present report are not entirely consistent with this suggestion, but this may be due to differences in the organisms used and the experimental design. For example, Sugiyama (1956) treated eggs of *Hemicentrotus* with 6% urethane at 9°C, and his principal method of analysis was at the light microscope level of observation on whole specimens. Observations of living and fixed preparations of unsectioned, inseminated, urethane-treated *Arbacia* eggs during the initial stages of fertilization revealed only subtle changes along the egg surface, which could conceivably be overlooked unless additional investigation was carried out at the ultrastructural level of observation.

A possible mechanism to explain the failure of cortical granule breakdown is that urethane alters the viscosity of the eggs, thereby disrupting the intimate association of the cortical granules and the oolemma. Thus, at insemination, the membrane of the cortical granule is unable to fuse with the egg

plasma membrane in order to affect membrane vesiculation and the dehiscence of the cortical granules (see Anderson, 1968). Several facts support such a suggestion: (*a*) The increase in the diameter of the urethane-treated egg indicates an alteration in its osmotic properties which may result in an influx of water (see Davson, 1964). The possible influx of water may then effect a decrease in cytoplasmic viscosity. (*b*) Investigations by Heilbrunn (1920 *a*, 1920 *b*, 1927; see also 1928) demonstrate a reduction in viscosity of the sea urchin egg after urethane treatment. (*c*) Urethane induces the movement of cortical granules from the egg cortex, whereby they become distributed throughout the zygote. Studies by E. Anderson (unpublished observation) which deal with the centrifugation of urethane-treated eggs have illustrated the ease with which the cortical granules may be dislodged from the cell's cortex. In addition, the present study has shown that cortical granules found outside of the cortical region were never observed to break down.

These observations suggest a possible mechanism involved in the random release of cortical granules during various stages of fertilization in urethane-treated eggs: the cortical granule membrane must be within a specific distance from the oolemma in order to initiate membrane fusion. In the untreated egg the cortical granule-oolemma distance is maintained and the cortical granules are free to dehisce upon activation. On the other hand, by the time the urethane-treated egg is inseminated, the cortical granules have been dislodged. However, they may randomly move to within the critical distance in order to initiate fusion with the plasma membrane, thereby effecting cortical granule release.

The decrease in the width of the hyaline layer in urethane-treated eggs presumaly is due to the absence of a general breakdown of the cortical granules, which form a considerable portion of this stratum (Anderson, 1968; Kane, 1969; Stephens and Kane, 1970; see also Monroy, 1965). In addition, there appears to be a direct relation between the extent of cortical granule breakdown and the thickness of the hyaline layer. Zygotes having a thick hyaline layer invariably contained fewer intact cortical granules than ones having a relatively thin hyaline layer.

The Female Pronucleus

Urethane treatment greatly affects the morphology of the female pronucleus, whereas little change in the male pronucleus is observed. This dissimilarity may be due to some inherent difference in the pronuclei or to a technical variation involved in the present experimental protocol. Although it would be difficult to determine the basis of this dissimilarity, structural (e.g., size) and morphogenetic (e.g., the production of convolutions at the region of the female pronucleus proximal to approaching male pronuclei) differences have been observed in untreated eggs (Longo and Anderson, 1968; see also 1970 *a*). Therefore, the morphological differences exhibited by male and female pronuclei in eggs treated with urethane may depend upon a dissimilarity in the pronuclei themselves. Disruption of the nuclear surface has also been observed in embryonic neural tube cells after exposure to urethane (Kauffman and Herman, 1968).

It is interesting to note that the annulate lamellae, a membranous system apparently originating from the nuclear envelope of oocytes (Bal et al., 1968; see also Kessel, 1968), are disrupted and reduced in quantity during urethane treatment. Breakdown of the zygote nuclear envelope and of the annulate lamellae normally occur concomitantly during the first mitotic division (Harris, 1967; Tilney and Marsland, 1969).

Development of the Male Pronucleus

Although other alternatives are possible, eggs treated in urethane are polyspermic, possibly due to the absence of a cortical response. The lack of a well developed hyaline layer and activation calyx and the failure of many eggs to initiate the separation of the vitelline layer provide morphological evidence for such a contention. In addition, the occasional presence of male pronuclei in early stages of development late in fertilization (30 min) lends further support to this suggestion. However, several possibilities may account for such an observation: (*a*) The male pronucleus may in fact represent a recently incorporated spermatozoon, or (*b*) the male pronucleus may be abnormally delayed in its development. The latter suggestion is somewhat unlikely since most sperm appear to be incorporated within 1 min of insemination and are rather synchronous in their development (see also Longo and Anderson, 1970 *a*).

All sperm incorporated into urethane-treated eggs appear to differentiate into male pronuclei and were never observed in cleaving zygotes or blastomeres. A similar situation has also been re-

ported for eggs made polyspermic with nicotine (Longo and Anderson, 1970 *a*). In addition urethane-treated eggs, like those incubated in nicotine, experience an extended period of pronuclear migration and fusion and cleave later than their respective controls. For a further discussion on this matter and polyspermy, the reader is referred to Harvey (1956), Cornman (1954), and Longo and Anderson (1970 *a*).

Enlargement of the male pronucleus and its nucleolus-like bodies eventually yields a pronucleus which possesses many characteristics of a female pronucleus. The attainment of "feminine" qualities by male pronuclei and the entrance of many male pronuclei into prophase prior to their association with the female pronucleus (or zygote nucleus) are normally characteristic of the *Ascaris*-type of fertilization (see Wilson, 1925; Longo and Anderson, 1969 *a*, *b*, 1970 *a*, *b*). The converse of the *Ascaris*-type of fertilization is the sea urchin-type which is characterized by the dramatic inequality of the male and female pronuclei at the time of fusion (see Longo and Anderson, 1968, 1970 *a*; Wilson, 1925). Experiments by Wilson (1902) and Longo and Anderson (1970 *a*) indicate that the difference in the two types is temporal and is due, at least in part, to the length of time the male pronucleus spends within the cytoplasm prior to its encounter with the female pronucleus (or zygote nucleus). Thus, the artificial prolongation of the interval between sperm incorporation and the association of the male pronucleus with the zygote nucleus by the urethane treatment may have induced the sea urchin-type of fertilization to take on, more or less, the character of the *Ascaris*-type of fertilization (see also, Longo and Anderson, 1970 *a*).

Although the eggs treated in urethane are polyspermic and demonstrate some heterogeneity with respect to the extent of cortical granule breakdown, separation of the vitelline layer, and the fusion of all of the male pronuclei, the events of fertilization are essentially similar to those previously reported for monospermy (Longo and Anderson, 1968). Thus, it appears that many of the events of fertilization, e.g. aspects related to the pronuclei, are independent of a typical cortical response and the release of the cortical granules.

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