Cortical Granule Distribution and Cell Surface Characteristics in Mouse Eggs

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A cytological analysis of serially sectioned unpenetrated and penetrated mouse eggs revealed the existence of a marked polarity in the distribution of cortical granules and of microvilli in the egg cortex. Approximately 20% of the total cortex homolateral to the meiotic spindle was totally devoid of cortical granules and was covered by a smooth oolemma which appeared incapable of binding capacitated epididymal spermatozoa. In the remaining 80% of the egg, the cortex contained a heterogeneous population of cortical granules and was covered by short microvilli; sperm penetration of the mouse vitellus was restricted to this area. Quantitative estimates of cortical granule complements in eggs before (0 and 30 min), during (60 min), and after (60 and 120 min) sperm–egg fusion indicated that approximately 25% of the granules were released by exocytosis within the initial 30 min of insemination. No functional significance could, however, be attributed to such a premature release, since the fertilizability of eggs with reduced cortical granule complements was not affected.

INTRODUCTION

Cortical granules are a general feature of the mammalian egg, having been first identified in the hamster by light microscopy (Austin, 1956). The release of their contents at fertilization has been implicated in the block to multiple sperm penetration in eggs of diverse species (Austin, 1965). In most animals, including mammals, a block to polyspermy occurs at the oolemma and/or at the zona pellucida (vitelline membrane or envelope of lower vertebrates and invertebrates). Dramatic structural alterations have been associated with the vitelline-to-fertilization envelope transformation in amphibians (Grey et al., 1974) and sea urchins (Endo, 1961a, b; Anderson, 1968; Ito, 1969). More recently, differences in ruthenium red-staining properties, which may reflect the occurrence of a zona reaction, were described between zonae of unfertilized mouse eggs and those of embryos (Baranska et al., 1975). Available evidence suggests that the zona reaction and the transformation of the vitelline to fertilization envelope in sea urchins are related to the activity of cortical granule trypsin-like protease(s) (Vacquier et al., 1972a, b, 1973; Gwatkin et al., 1973; Schuel et al., 1973; Carroll and Epel, 1975a, b).

In examining sperm-excluding mechanisms in mouse eggs, we became interested in the temporal sequence of granule release. Although it is recognized that the egg contains few granules following sperm penetration, a precise definition of the temporal relationship between granule exocytosis and sperm entrance is unavailable. This process can be investigated in a well-characterized in vitro fertilization system where sperm penetration is highly synchronous and readily definable. Initial ultrastructural characterization of the unfertilized mouse egg suggested the presence of substantial gradients in cortical...
granule distribution and prompted a comprehensive examination of serially sectioned unpenetrated and penetrated eggs. The results presented in this study indicate that limited cortical granule release occurs prior to sperm penetration of the zona, although a functional significance could not be attached to this event. In addition, a correlation was established between the presence of granules in the cortex of unfertilized eggs and the site of sperm penetration, indicating a functional polarity in the egg surface. Preliminary accounts of this work have been presented (Wolf et al., 1974; Nicosia, 1975; Nicosia et al., 1975).

MATERIALS AND METHODS

Swiss mice (6-12 weeks old) were superovulated with pregnant mare serum gonadotropin (PMS) (Gestyl, Organon) and human chorionic gonadotropin (HCG) (Sigma), and unfertilized tubal eggs were recovered 12-14 hr following the injection of HCG. Eggs in cumulus were transferred to a modified Krebs-Ringer-bicarbonate medium, pH 7.4, for insemination or to a medium containing hyaluronidase (0.1% Sigma, Type 1) when cumulus dispersion preceded insemination. Zonae were removed mechanically by aspiration of cumulus-free eggs through micropipets. The recovery, handling, and in vitro fertilization of these egg types with capacitated epididymal sperm have been described previously (Inoue and Wolf, 1974, 1975; Wolf and Inoue, 1976; Wolf et al., 1976). Sperm incorporation into eggs was scored on aceto-lacmoid-stained whole mounts, as described by Toyoda and Chang (1974). Epididymal sperm supernatant solutions were prepared by mincing and suspending eight epididymides in 0.8 ml of culture medium for 15 min. Particulate material was removed manually and sperm were removed by centrifugation (1600g, 30 min).

Eggs were processed for electron microscopy by sequential fixation in 3% glutaraldehyde (280 mOsm) and 1% osmium tetroxide in 0.1 M cacodylate buffer. Following graded alcohol dehydration, eggs were embedded for 48 hr at 60°C in Epon 812 (Luft, 1961). Unfertilized tubal eggs (fresh or aged 120 min in culture medium or sperm supernatant) and eggs exposed to capacitated sperm for 30, 60, and 120 min were serially sectioned (18 eggs) or step-sectioned (one section every 2-3 μm; 18 eggs) with a diamond knife. Thin sections, 60-70 nm thick, were mounted on Formvar-coated, 100-μm-mesh copper grids (15-20 sections/grid), stained with lead citrate (Reynolds, 1963) and uranyl acetate, and examined with a Hitachi HU-12A electron microscope operated at 75 kV. As a guide for the selection of samples for electron microscopy, eggs derived from contralateral oviducts were also inseminated. The attainment of fertilization levels similar to those reported previously (Inoue and Wolf, 1975) was employed as a measure of the normalcy of the samples selected for study. The diameters of eggs examined varied from 58 to 65 μm (Konwinski et al., 1974). Seven of the 36 eggs processed showed signs of degeneration (distortion of the vitellus, focal interruption of oolemma and zona pellucida, zona penetration by granulosa cells, and extensive cytoplasmic vacuolization) and were discarded.

Cortical granule counts were made in approximately one thin section per micrometer of tissue and were normalized per 100 μm of plasma membrane in serially sectioned eggs or per egg section in eggs studied by step-sectioning. Egg circumferences were measured on low magnification micrographs of whole egg sections taken at regular intervals throughout the egg. A total of at least 30 artifact-free sections, representing approximately 5000 μm of egg circumference, were photographed in each serially sectioned egg. Circumferences in unphotographed sections were derived by extrapolation from adjacent photographed sections. The significance of the data was evaluated by the Student’s t test.
RESULTS

Cortical granules were identified as single, membrane-bound organelles measuring 0.2–0.6 μm in diameter and arranged rectilinearly or in irregular rows below the oolemma. Granules infrequently observed outside the cortical region (i.e., more than 2.0 μm away from the oolemma) were excluded from quantitative estimates of granule complements. In unfertilized eggs and in inseminated but unpenetrated eggs, granules were uniformly distributed throughout the cortex (Fig. 1A) with the exception of areas overlying or adjacent to the meiotic spindle (see below). The electron density of granules varied from uniformly dark or light (Fig. 1B) to, on occasion, irregularly dark (Fig. 1C), reflecting heterogeneity of contents. This phenomenon was not considered a technical artifact, since such variation was consistently observed within the same egg section (Fig. 1A, B). Some cortical granules were also characterized by orderly arranged crystalline structures, 1–2 nm in width with a center-to-center spacing of 20–22 nm (Fig. 1D, E), similar to those observed previously in lysosomes or related particles (Hruban and Rechcigl, 1969). Vesicles retained within the Golgi complex region and presumed to be cortical granule precursors also displayed differential electron density (Fig. 1F).

Cortical Granule Complements of Unpenetrated and Penetrated Eggs

Unfertilized eggs (N = 3), fixed within 10 min of removal from the oviduct, contained 32.72 ± 2.15 granules/100 μm of plasma membrane (mean ± SEM). The total number was approximately 4500 with a density of 50/100 μm² comprising approximately 0.5% of the total egg volume (for calculation, see Schuel et al., 1972). Fractional estimates of dark and light granules yielded the following results: dark granules, 20.53 ± 1.42/100 μm; light, 12.18 ± 0.96/100 μm. The granule complement of unfertilized eggs (N = 3) aged 2 hr in culture medium remained unchanged: total count, 30.57 ± 1.85/100 μm of plasma membrane; dark granules, 19.55 ± 1.17/100 μm and light granules, 10.77 ± 0.85/100 μm.

Light microscopic examination of mouse eggs following a 30-min exposure in vitro to capacitated sperm indicated that sperm had not yet penetrated the zona pellucida (Inoue and Wolf, 1975). This result was confirmed in the present ultrastructural study where sperm were confined to the outer surface of the zona following a 30-min insemination. However, the cortical granule complement of these ova (N = 3; 24.71 ± 1.07/100 μm of plasma membrane) was significantly reduced (P < 0.001) over unincubated controls. In two eggs, this reduction was due to a decrease in the number of "light" granules. Thus, dark granule counts of 17.61 ± 0.78/100 μm of plasma membrane were similar to controls (P < 0.1), while the normalized number of light granules was 7.21 ± 0.43/100 μm of plasma membrane, a highly significant reduction from unincubated fresh or aged controls (P < 0.001). Apparent release of cortical granules by an exocytotic process was observed in one egg following a 30-min exposure to capacitated spermatozoa (Fig. 2). Moreover, the oolemma of eggs inseminated for 30 min or more was characterized by the presence of numerous surface pits, a possible indication of previous granule discharge.

Following a 60-min exposure to capacitated sperm, all mouse eggs (N = 3) contained sperm in the perivitelline space and were penetrated or undergoing penetration. In one 60-min egg, suggestive evidence for incipient gamete fusion was restricted to a focal area where both sperm and egg membranes were closely juxtaposed and were separated by only a few nanometers at the level of the postacrosomal cap region (Fig. 3). This egg contained 15.96 ± 1.38 granules/100 μm of plasma membrane, of which 9.37 ± 0.76 were dark and 6.58 ± 0.67 were light. Unequivocally
FIG. 1. A. Cortex of an unpenetrated tubal egg. Cortical granules (cg) of different electron density are linearly arranged below the plasma membrane. Note microvillous processes (mv). × 26,000. B. "Dark" (dg) and "light" (lg) cortical granules in an unpenetrated egg following a 30-min incubation with sperm. × 47,500. C. Cortical granules showing heterogeneity of contents with focal "dark" deposits (arrows). A portion of a Golgi complex (go) can also be seen. × 25,600. D, E. Tubulo-lamellar arrays (arrows) of crystals within cortical granules in an unfertilized egg incubated for 2 hr in control medium (D) and in a fresh tubal egg (E). In the former, the granule is separated from the overlying oolemma by a distance of 5–8 nm. D, × 80,900; E, × 78,300. F. Peripheral Golgi complex. Note heterogeneity of presumed cortical granule precursors. × 9600.
FIG. 2. Unpenetrated tubal egg after 30 min of incubation with capacitated epididymal spermatozoa. Penetration of egg investments has not yet occurred, spermatozoa being only in contact (at a different level) with the outer margin of the zona pellucida. Note a surface pit still containing an electron-light filamentous material, suggestive of a cortical granule in the process of exocytosis (arrow). Note also the microvillar oolemma (mv), a peripheral Golgi complex (go) and a marginally sectioned "dark" cortical granule (dg). x 40,800.

penetrated eggs (i.e., with incorporated sperm head, midpiece, and postacrosomal membranes, Fig. 4A–C) contained cortical granule complements of 4.06 ± 0.16/100 µm of plasma membrane with 2.78 ± 0.11 dark granules and 1.27 ± 0.10 light granules per 100 µm of plasma membrane. The difference in granule complements between penetrated eggs and the egg presumed to be at an early stage of fusion suggested that a burst in granule release was associated with sperm penetration.

A similar distribution in cortical granule complements was obtained in 14 step-sectioned eggs in which counts were normalized per egg section (mean ± SEM/egg section); uninseminated controls (N = 3): total, 40.42 ± 1.05; dark, 24.83 ± 0.95; light, 15.58 ± 0.38; controls (N = 3) incubated for 2 hr in sperm-free medium: total, 39.79 ± 0.89; eggs (N = 3) inseminated for 30 min: total, 36.48 ± 1.15; dark, 26.12 ± 1.00; light, 10.34 ± 0.40; egg incubated with sperm for 60 min and probably undergoing penetration: total, 22.85 ± 1.45; eggs (N = 3) inseminated for 60 min and unequivocally penetrated: total, 6.21 ± 0.24; penetrated eggs (N = 2) after 120 min of insemination: total, 2.4 ± 0.08.

The possible significance of premature granule release to egg fertilizability was tested by the following experiment. Cumulus-free eggs were inseminated with 1 × 10^8 capacitated epididymal sperm/ml and
Fig. 3. Tubal egg incubated with capacitated epididymal sperm for 60 min. This egg has undergone a significant reduction in its cortical granule complement and its fusion with a closely juxtaposed spermatozoon appears incipient at the level of the postacrosomal cap region, from which it is separated only by a narrow intercellular space (arrows). Note underlying cortical granule (cg) and microvilli (mv). Only a few remnants (vs) of vesiculated plasma and outer acrosomal membranes can be seen on the upper side of the sperm head; es, equatorial segment. x 31,200.

Eggs were incubated for 30–40 min, at which time eggs would not yet be penetrated (Inoue and Wolf, 1975) but would contain approximately 25% fewer granules than their un-inseminated counterparts, based on our granule quantitation studies. Eggs were then removed and washed twice to dislodge loosely bound sperm, and aliquots were transferred to three insemination dishes containing fresh culture medium. One control group was reinseminated immediately, while a second was incubated in the absence of additional sperm. The experimental group was incubated for an additional 30 min, allowing time for cortical granule exudate-induced changes to occur, and then reinseminated with $1 \times 10^6$ sperm/ml. A reduced fertility level would be expected in the experimental eggs if the activity of cortical granule material led to the establishment of a vitelline block to polyspermy and/or to a zona reaction during this time. Eggs were incubated for a total of 4 hr before fertilization levels were scored. A low level of fertilization (11/87; 12.6%) was observed in eggs incubated in the absence of additional sperm, while the experimental group (97/158; 61.4%) was indistinguishable from reinseminated controls (94/137; 68.6%). It was thus concluded that premature granule release did not affect egg penetrability.

In order to investigate the specificity with which sperm exposure induced premature release of cortical granules from tubal eggs, epididymal sperm supernatant solutions were prepared. Cortical granule complements were determined on three eggs following a 2-hr exposure to these solutions at 37°C. Average values obtained were: total granules, $27.85 \pm 1.54/100 \, \mu m$ of plasma membrane; dark, $16.78 \pm 0.77$ and light granules, $11.07 \pm 0.87/100 \, \mu m$ of oolemma. These values, while lower, were...
FIG. 4. A–C. Each electron micrograph represents a different spermatozoon at a different stage of incorporation into mouse eggs. (A) The sperm head is fused with the egg except for a segment in which the subacrosomal region and the vesicular remnants of plasma and outer acrosomal membranes are seen (arrow head). The postacrosomal cap (pac) is symmetrically separated from the nucleus. Note granular aggregates of decondensing chromatin and vestiges of the nuclear membrane (rectangle). A portion of a Golgi complex (go) is seen near the site of penetration. × 19,200. (B) Sperm flagellum near a peripheral Golgi complex (go). Note dense granular material within the mitochondrial matrix (arrows). × 19,800. (C) Remnants of the postacrosomal cap region (arrows); go, Golgi complex. × 15,800.
not significantly different from those observed in fresh tubal eggs or in eggs aged 2 hr in culture medium.

Morphology of Egg Cortex in Relation to Cortical Granule Distribution

A significant polarity in granule distribution was observed in unfertilized eggs and in inseminated eggs that retained most of their granules. Such polarity was created by the absence of cortical granules in areas immediately overlying the meiotic spindle and also in cortical regions located throughout much of the egg hemisphere homolateral to the spindle (Fig. 5A–D). This characteristic distribution of cortical granules was observed consistently and was mimicked by a similar distribution of Golgi complexes (Fig. 6). The cortical granule-free area in any single section ranged from 0 to 100% of the egg circumference and, of course, was dependent upon the orientation of the section. Based on the number, thickness, and distribution of cortical granules in each section, 17–24% of the entire unfertilized egg cortex was calculated to be devoid of granules. Polarity in granule distribution was dramatically illustrated by two representative sections derived from opposite (hemisphere) ends of an egg (Fig. 7A–C).

Gradients in granule distribution could be correlated with configurational changes in the morphology of the overlying oolemma. Cortical regions containing granules always exhibited a “ruffled” oolemma with numerous, short microvillous processes, while regions devoid of granules were covered predominantly by a “smooth” oolemma (Fig. 7A–D). In recently penetrated eggs, there was a decrease in the density of microvilli covering cortical granule-rich regions. At later stages of fertilization, however, the entire egg surface resumed “ruffled” contours with numerous short processes throughout (Balinsky, 1966; Szollosi, 1967). Modulations in surface configurations were not apparent in eggs incubated for 2 hr in medium alone. The cortical granule-free area was also characterized by a 100- to 400-μm-thick, finely filamentous layer located immediately below the oolemma. Such a structure not only was present at the level of the meiotic spindle but also extended to adjacent cortical granule-free areas (Fig. 5A–D).

Polarity in Sperm Penetration

Based on our ultrastructural analysis (35 sperm, this study; Stark and Wolf, unpublished observations), sperm penetration of the vitellus was confined to egg regions which contained cortical granules and Golgi complexes, and which were covered by a “ruffled” oolemma (Fig. 8). The polarity in sperm penetration was also studied on whole mounts of zona-free eggs. Following 30–40 min of exposure of zona-free eggs to \(1 \times 10^5\) capacitated epididymal sperm/ml, the position of the penetrating sperm (tail and head remnants) could be evaluated relative to the meiotic spindle, i.e., the cortical granule-free area (Fig. 9). The experiment involved removal, by washing, of loosely bound sperm from inseminated eggs followed by mounting, fixing, and staining of eggs with aceto-lacmoid. For purposes of quantitation, the cortical granule-free area was considered as a circle centered on the meiotic spindle, with a diameter of three spindle lengths. Sperm entrance sites were considered to fall within the granule-free area only if the decondensed sperm head and sperm remnants were within this area and in the same plane of focus as the meiotic spindle. Such sites were localized within the granule-free area in only 2 of 190 ova (1.05%) examined. In the absence of polarity in sperm penetration, 10.6% of the eggs would have contained penetration sites within this granule-free area.

Morphology of Polar Body and Perivitelline Space

Twenty percent of the eggs examined exhibited intact first polar bodies which
FIG. 5. A-D. Cortical granule-free cortexes of unpenetrated tubal eggs. (A) Cortex above the spindle of the second meiotic metaphase (me). A continuous electron-dense layer can be seen immediately below the oolemma (arrows). Note that a variety of cell organelles but no cortical granules is present above the
Fig. 6. Fresh unpenetrated tubal egg. Note a marked polarity in distribution of cortical granules, Golgi complexes and microvilli (below broken line). The remaining egg section (above broken line) is homolateral to the spindle and is covered by a smooth and uneven oolemma. \( \times 5200 \).

spindle. \( \times 5600 \). (B) In this, as well as in other higher magnification micrographs, the cortical layer appears relatively homogeneous and fine filaments can be resolved only with difficulty. \( \times 17,400 \). (C) Cortical region overlying an area (ma) a few micrometers away from the meiotic spindle. Note the presence of the thin filamentous band (arrows). \( \times 3900 \). (D) Egg cortex in a region homolateral to but several micrometers away from the meiotic spindle. Note persistence of the filamentous band (arrows) and the absence of cortical granules. \( \times 6000 \).
FIG. 7. A–D. Opposite poles of an unpenetrated egg exposed to capacitated epididymal sperm for 30 min. In the pole containing the meiotic area (ma), cortical granules are absent, and a smooth oolemma overlies a filamentous ectoplasm (A, arrows; B). Numerous cortical granules (arrows), Golgi complexes (go) and microvilli (mv) are present in the opposite pole (C, D). A, × 3100; B, × 24,800; C, × 4900; D, × 12,000.
were almost entirely devoid of cortical granules (Fig. 10) and were characterized by a predominantly smooth plasma membrane and by the same filamentous layer (Fig. 10, inset) observed in the cortical granule-free regions of eggs (Figs. 5B and 7B). Two intact polar bodies also exhibited rhomboid-shaped crystalline inclusions with 16-nm-wide individual crystals uniformly spaced in parallel rows about 30 nm apart similar to those observed in human (Zamboni et al., 1966), rabbit (Van Blerkom, 1973), and mouse (Enders and Schlafke, 1965; Anderson et al., 1975) embryos.

The remaining 80% of eggs showed numerous aggregates of cortical granule-like organelles interspersed within the perivitelline space with a lesser number of lipid droplets and degenerating mitochondria. The cell organelles found within the perivitelline space could have derived from polar body breakdown (Austin, 1961) as a result of superovulation and accelerated
Fig. 9. Light micrograph of a zona-free egg exposed to capacitated sperm for 30 min. Similar preparations were used to score sperm entrance sites, as indicated by the position of a sperm tail and a decondensed sperm head (not visible here) relative to the meiotic spindle. x 950.

egg maturation (Donahue, 1972; Thompson et al., 1974) or, when coexistent with an intact polar body, from the liberation of ooplasmic components at the site of polar body constriction (Odor, 1960).

DISCUSSION

The major findings of the present study are summarized in Fig. 11 and include: (a) a definition of the polarity in cortical granule distribution and in vitelline surface structural characteristics in unfertilized mouse eggs; (b) the demonstration that a population of granules is released prior to sperm contact with the vitellus; and (c) the localization of sperm penetration of the vitellus to the "ruffled" oolemma overlying cortical granule-rich areas.

A cortical granule-free area associated with the meiotic spindle in mature mouse eggs has been noted previously, although the extent of this area apparently was not fully appreciated (Szollosi, 1967; Zamboni, 1970). The area, as defined here, extended over 20% of the entire cortical surface, creating substantial gradients in granule distribution. It follows that estimates of granule complements based on limited ultrastuctural examination may, therefore, be misleading if other species are similar to the mouse (Fraser et al., 1972; Fléchon et al., 1975).

Egg surfaces associated with cortical granule-free areas were devoid of microvilli and were characterized by a filamentous layer or "hyaline" band described previously (Stefanini et al., 1969; Zamboni, 1970, 1971; Thompson et al., 1974), albeit associated only with areas overlying the second meiotic spindle. The nature, origin, and significance of this layer are unclear. Its composition and its relationship with the meiotic apparatus suggest an involvement in the process of cytokinesis as a prerequisite to polar body extrusion. This possibility was advanced by Stefanini et al. (1969) and implies that the band plays an essential role in the development of the cleavage furrow by increasing the rigidity and gel strength of a specific cortical region. Equally plausible is an involvement in the cleavage of the zygote; however, the demonstration of actomyosin-like proteins and of a microfilament-related contractile system within this area has not yet been attempted. A filamentous layer, a smooth oolemma, and extensive granule-free cortical areas were also observed in the first polar body, suggesting that these characteristics were present in the egg sometime before polar body extrusion. Similar cytologic changes have been described in preovulatory rat eggs at the metaphase stage of first polar body formation (Odor and Renninger, 1960), and their origin could conceivably be related to the breakdown of the germinal vesicle and to the resumption of the first meiotic division.

A premature sperm-accelerated release of 25% of the egg's cortical granules within 30 min of insemination was described here. Two fates have been recognized for these granules: (a) release in response to sperm contact with the vitellus, a process that leads to cortical granule exudate-induced alterations in the oolemma and/or the zona pellucida and that limits subse-
FIG. 10. A fragmented first polar body (pb) in an unpenetrated tubal egg. Note the chromosomal puffs (ch) and the cytoplasmic bridge (cb). No cortical granules are present in this and adjacent sections, and most of the smooth plasmalemma is underlined by an electron-dense filamentous band (arrows and inset) similar to that observed in the egg. × 7200; inset, × 39,000.

quent sperm penetration; or (b) reduced granule synthesis (Peluso and Butcher, 1974), inward migration from a cortical position to deeper cytoplasmic areas (Szollosi, 1971), and shedding into the perivitelline space together with other membranous structures (Longo, 1974) in the absence of sperm penetration and after the fertilizable life of the egg is exceeded. Although most authors suggest that a full complement of granules is present at ovulation, Zamboni (1970) reported that the synthesis and formation of granules in the mouse continues through and even after ovulation. While the possibility arises then that a dynamic equilibrium exists between production and release prior to penetration (Zamboni, 1974), the premature release of granules observed in the present study suggests a different mechanism involving gamete communication prior to sperm–vitellus contact. Equivocal results were obtained, however, when induction of granule release was attempted with sperm supernatant solutions. Unfortunately, the laborious nature of the assay discouraged a detailed study of these phenomena by the present approach. Experiments designed to test the functional significance of sperm-accelerated granule release were unsuccessful, despite their similarity to those utilized by Barros and Yanagimachi (1971) to demonstrate a zona reaction in hamster eggs. It is possible that in vitro fertilization conditions may mask any cortical granule-related effect on the egg, since a functional zona reaction does not occur in vitro in the mouse (Wolf and Inoue, 1976). An alternative role for premature granule release could be that of detaching and separating corona cell processes from the oolemma (Zamboni, 1974).

The functional significance of premature granule release could also be consid-
FIG. 11. Diagrammatic summary of cortical granule distribution and vitelline surface characteristics in mouse eggs. Ultrastructural findings (average estimates) are depicted at four different egg levels. Note the following: (i) the marked polarity in distribution of cortical granules in unfertilized eggs; (ii) the presence of cortical granule-free areas (approximately 20% of the total egg cortex) in association with the meiotic spindle; (iii) the existence of configurational differences in the vitelline surface with short microvilli covering cortical granule-rich areas and a smooth oolemma elsewhere; (iv) the localization of sperm penetration to the microvilli-rich oolemma; (v) the release of a cortical granule population between 0 and 30 min of insemination (arrows). The first polar body is shown with a broken outline, since it is found intact in only 20% of the eggs. Cumulus cell dispersal during insemination is also depicted. *, Presumed to be at the initial stage of fusion.

considered in the context of sperm modification. In fact, several recent observations point to a possible involvement of cortical granule exudate in the induction of the sperm acrosome reaction. First, most sperm observed at the outer margin of the zona 30 min postinsemination retain intact acrosomes (Nicosia and Wolf, unpublished ob-
servations), and penetration of the zona does not occur for another 15–30 min. The limiting event in this delay in zona penetration may, therefore, be the induction of an acrosome reaction. This conclusion is supported by the finding that, in the presence of zona-free mouse eggs, acrosome-reacted sperm are apparent within 5 min and sperm–egg fusion occurs within 15 min of insemination (Stark and Wolf, manuscript in preparation). Second, the induction of an acrosome reaction in hamster (Meizel and Lui, 1976) and in mouse sperm (Wolf, 1976) may involve a trypsin-like activity. In the former case, the ability of bovine follicular fluid to induce a morphologically distinct acrosome reaction was inhibited by trypsin inhibitors, while, in the latter, trypsin inhibitors were shown to decrease sperm penetration of zona-free eggs. Third, cortical granules in hamster (Gwatkin et al., 1973) and in sea urchin eggs (Vacquier et al., 1972a,b, 1973; Schuel et al., 1973) contain trypsin-like activity. Thus, it seems possible that cortical granules could be directly involved not only in the cessation but also in the initiation of sperm penetration.

In this study, premature sperm-induced release was predominantly confined to "light" cortical granules. Morphologically distinct granules have been described in other mammals and in lower vertebrates (Austin, 1961; Balinsky, 1966; Hadek, 1963; Szollosi, 1971; Anderson, 1974; Grey et al., 1974; Campanella, 1975; Selman and Anderson, 1975), and the existence of functionally unique granule populations has been suggested on the basis of nonrandom distribution and release (Dandekar and Gordon, 1975). In the absence of identifiable functional differences, the possibility should also be entertained that these distinct granule populations may simply reflect different maturational stages, with the "light" granules representing immature or degenerated forms.

The existence of a gradient in the structural organization of the egg cortex and of a polarity in sperm penetration of the vitellus are new concepts as applied to mammalian eggs (Longo, 1973; Bedford, 1974; Fléchon et al., 1975), although mosaicism in the organization of concanavalin A receptor sites in the mouse oolemma has been described (Johnson et al., 1975). Polarity in the vitelline surface has been reported in amphibian eggs, where sperm entrance is limited to the pigmented animal hemisphere or to the animal dimple (Campanella, 1975; Elinson, 1975). In insects, cephalopods, and fish, polarity in sperm entrance results indirectly by the presence of micropyles in the surrounding egg chorions (Austin, 1965). In the marine invertebrates, Arbacia and Mytilus, Longo (1973) suggests that sperm entrance is unrestricted. In the mouse, sperm entrance sites were confined to the cortical granule-rich surface of the egg (80% of the total), and a similar polarity was observed in sperm binding to unfertilized zona-free eggs (Wolf, unpublished results). Similarly, Johnson et al. (1975) have indicated that sperm attachment to that portion of oolemma overlying the meiotic spindle is rare. These findings suggest that the "smooth" oolemma surface lacks specific sperm binding sites; alternatively, the absence of microvilli in these areas may preclude the juxtapositioning of gametes prerequisite to fusion. The presence of numerous microvilli is generally indicative of an active cell surface (Kemp, 1956), and microvilli have invariably been associated with sperm–egg fusion, leading to the suggestion that they provide the oolemma with an "anchoring" mechanism for the fertilizing sperm (Hadek, 1963; Pikó, 1969; Yanagimachi and Noda, 1970; Campanella, 1975). The significance to fertilization or to early development of polarity in sperm penetration of mouse eggs is unknown. In mammals, Johnson et al. (1975) have suggested that sperm incorporation over the meiotic spindle might impair second polar body extrusion. In amphibians, the success of syngamy may be related to
the proximity of penetration because of the potentially long pronuclear migration distances involved (Elinson, 1975).

The ultrastructural and analytical data presented in this report show that in the mature mammalian egg a characteristic distribution of cortical granules and microvilli exists that plays a major, if not absolute, role in determining the site of sperm penetration. In addition, cortical granules exist as a heterogeneous population of organelles, some of which are released prior to sperm penetration of the vitellus. Our interests are presently centered on the ontogeny of the cortical granule-free area, the cytochemical identification of the filamentous layer and of the different granule types, and on confirming, by scanning electron microscopy (Wolf et al., 1976), configurational differences in the egg surface and their relation to the site of sperm penetration.

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REFERENCES


