CORTICAL CHANGES IN GROWING
OOCYTES AND IN FERTILIZED OR PRICKED
EGGS OF RANA PIPiens

NORMAN E. KEMP and NANCY L. ISTOCK

From the Department of Zoology, The University of Michigan, Ann Arbor, Michigan 48104

ABSTRACT
The folded cortex of the growing oocyte of the frog extends as microvilli into the substance of the developing vitelline membrane and, internal to the folds, possesses a layer of cortical granules. Free ribosomes, smooth-walled vesicles, coated vesicles, tubules, and electron-opaque granules are abundant in the peripheral zone of the cortex. Mitochondria, lipochondria, pigment granules, and electron-opaque granules are conspicuous between cortical granules and in the underlying endoplasm. Yolk platelets are restricted to the endoplasm. Cortical granules contain neutral and acid mucopolysaccharides, and possibly protein.

In the mature oocyte, microvilli are withdrawn and the surface folds eliminated. Cortical granules now lie close to the plasma membrane, sometimes contacting it. Fertilization or pricking causes a wave of breakdown of cortical granules lasting 1-1.5 min. Breakdown begins immediately after pricking but not until about 10-15 min after insemination, because the fertilizing sperm takes that long to penetrate the jelly and vitelline membrane. Cortical granules erupt through the surface and discharge their contents into the perivitelline space. Cortical craters left at sites of eruption soon disappear, and pseudopodial protrusions retract. By 30 min after insemination, the surface of the egg is relatively smooth.

INTRODUCTION
Among the inclusions which differentiate in the cortex of growing anuran oocytes are the cortical granules, first described for the frog by Motomura (36) and later for the toad by Osanai (41). Histochemical analysis has shown that these granules in anurans contain either neutral or acid mucopolysaccharides (22, 41, 46, 52) and possibly protein (22, 46). Electron microscopy has revealed that the cortical granules develop in the peripheral cytoplasm and then localize in a layer just outside of the zone of vitellogenesis (2, 7, 24, 52, 53).

According to Wartenberg (52, 53), oocytes of Triton alpestris and perhaps all urodeles lack cortical granules. Neither Hope, Humphries, and Bourne (18, 19) nor Wischnitzer (54) found cortical granules in oocytes of Triturus viridescens; but Detlaff (10) has referred to unpublished observations on extrusion of cortical granules after fertilization in both the anuran Rana temporaria and the urodele Ambystoma mexicanum. Among other vertebrates, cortical granules have been reported for the lamprey (30), for various bony fishes, in which yolk vesicles or cortical alveoli are homologous to cortical granules (32, 40, 56, 57, 58), and for several mammals. The latter include: hamster (5, 51); rat, mouse, coypus, pig (51); guinea pig (1, 51); rabbit (14, 59); and monkey (17).

After fertilization, the cortical granules of anuran eggs extrude their contents into the perivitelline space. Eruption of the granules has been estimated to occur sometime between 10 and 20 min after insemination (4, 21, 53). We have previously reported our estimate that eruption usually begins 10-15 min after insemination (28). If one
pricks an egg, breakdown of cortical granules begins at once and proceeds as a wave, spreading around the egg from the point of pricking in about 1 min (26). Since the reaction presumably proceeds at the same rate after the fertilizing sperm unites with the egg, one may infer that the sperm requires 10-15 min to burrow its way through the jelly and vitelline membrane investing the egg. This inference is supported by Katagiri’s (21) observation that, after pricking, ‘egg rotation and extrusion of the polar body apparently took place more rapidly as compared with those in fertilized eggs.”

Cytological and physiological aspects of the cortical reaction following fertilization or artificial activation of the egg in many species, principally echinoderms and vertebrates, have been reviewed by Rothscheld (47), Allen (2), Runnström, Hagström, and Perlmann (48), Pasteels (44), Monroy (35), and Austin (6). The present paper contrasts the cortex of growing oocytes and mature secondary oocytes (eggs) of the frog, and describes cortical changes following sperm entrance or pricking.

MATERIALS AND METHODS

Adult females of Rana pipiens (obtained from a E. G. Steinhilber, Oshkosh, Wisconsin) were induced to ovulate by injecting one fresh pituitary gland (from female frog) plus 5 mg of progestosterone in corn oil (55). Eggs obtained by stripping were inseminated with fresh sperm suspension or activated by pricking with a glass needle. Growing oocytes were obtained by feeding liver to stripped females for several weeks in order to stimulate ovarian growth.

Growing or mature oocytes, as well as inseminated or pricked eggs, were fixed for about 2 hr in Caulfield’s buffered 1% OsO₄ containing sucrose (9). Eggs in their envelopes of jelly were fixed initially for about ½ hr in vials resting in ice water. Partially fixed eggs and fixative were then poured into an operating dish so that most of the jelly could be removed with jewelers’ forceps. Eggs and fixative were returned to vials for the remainder of the period of fixation. To permit processing of animal and vegetative hemispheres separately, we transferred fixed eggs to an operating dish containing frog Ringer’s solution and transected them equatorially. Jelly and vitelline membrane usually separated from the egg fragments, but sometimes remained adherent. The isolated halves were dehydrated through an alcohol series and embedded in Epon 812 according to Luft’s technique (34).

Thin sections were cut with glass knives mounted on an LKB ultratome. After learning that pigment was difficult to section without leaving holes in the supporting Epon, we resorted to the expedient of sectioning mostly vegetative hemispheres containing little cortical pigment. Sections were mounted on uncoated, 200-mesh grids, stained for 1 hr in a saturated solution of uranyl acetate, and washed for 5 min in running tap water. Microscopy was performed with an RCA EMU 3E electron microscope operating at 50 kv. Micrographs were taken at initial magnifications of 3,125-9,360 and enlarged photographically.

Specimens for study by light microscopy were fixed in Bouin’s fluid, embedded in paraffin, and sectioned at 10 µ. Sections were stained with hematoxylin and eosin, bromphenol blue, Alcian blue, the PAS reaction with appropriate controls, and the triple stain of Himes and Moriber (16). The latter technique includes staining by the Feulgen reaction, the PAS reaction, and naphthol yellow S. A Spencer photomicrographic camera was used for taking photomicrographs on 4-X 5-in, Panatomic-X film.

RESULTS

Cortex of Growing Oocytes

Localization of cortical granules in a young oocyte at stage Y₂ (23), stained by the PAS reaction in the Himes and Moriber triple stain, is shown in Fig. 1. Both the cortical granules and the developing vitelline membrane (zona radiata, 52; zona pellucida, 54) were strongly positive for the Schiff reagent. Staining by the PAS reaction was abolished by acetylation with acetic anhydride-pyridine (45), but not by pretreating sections with diastase, bromine, or chloroform-methanol. These reactions indicate that the granules contain neutral mucopolysaccharide. They also stain positively with Alcian blue, thereby confirming Wartenberg’s (52) conclusion that anuran cortical granules contain acid mucopolysaccharide. Cortical granules were not conspicuously stained with cosin, naphthol yellow S, nor bromphenol blue, in contrast to the heavy staining of yolk platelets with these stains. Bromphenol blue does appear to stain cortical granules lightly, possibly indicating some protein content.

Details of the cortex of a growing oocyte at stage Y₂, after deposition of pigment had begun (23), are illustrated in Figs. 2 and 3. The surface of the oocyte is folded into ridges and narrow valleys or crypts. From the summits of the ridges, microvilli extend out into the substance of the developing vitelline membrane, occupying the zone between oocyte and follicular epithelial cells. Larger processes, “macrovilli,” extend toward the...
Cortical Granules in Mature Oocytes

Some time before ovulation, the follicular cell processes (macrovilli) and microvilli retract into their parent cells and the folded surface of the oocyte flattens (cf. 18). The mature oocyte is separated from the vitelline membrane by a narrow perivitelline space containing small vesicles and flocculent material. The contents of the perivitelline space apparently are adhesive, because the vitelline membrane sticks tenaciously to the cortex of fixed, unactivated eggs. After eggs have been fertilized or pricked, the vitelline membrane can easily be dissected free from the surface. Numerous short, blunt pseudopodia protrude into the perivitelline space surrounding the mature oocyte.

The peripheral cortical zone, which is promi-
nent in growing oocytes, is almost eliminated in the mature oocyte. As a result, cortical granules lie in a row close to the plasma membrane. Some appear to contact the cell surface, while others are separated from it by intervening cytoplasm (Fig. 4). We observed that the small cortical granules usually appeared more dense than the larger ones.

Cortical Reaction after Fertilization or Pricking

Early observations led us to estimate that cortical granules were not extruded until 10-15 min after insemination (25), but that the wave of extrusion was complete within about 1 min after pricking (26). The results of examination of the cortices of 117 inseminated eggs and 39 pricked eggs are recorded in Table I. Cortical granules are so closely packed at the surface of the mature oocyte that a single good section suffices to determine whether they are present (not extruded) or missing (extruded). We examined some sections from animal hemispheres, but our assay was made chiefly with sections from the vegetative hemisphere. The presence of cortical granules proved that the wave of breakdown had not reached the level sectioned, although the wave could have been under way closer to the point of sperm entrance or pricking. The absence of cortical granules proved that the wave of breakdown was well under way, although this wave might have been incomplete farther from the point of sperm entrance or pricking.

Table I shows that cortical granules were intact in all specimens fixed up to 7½ min after insemination. Some oocytes fixed at 10 min after insemination had already extruded their cortical granules. Nearly all oocytes fixed later than 15 min after insemination had extruded their granules. These results indicate that breakdown of granules may begin at various times, from somewhat under 10 min to about 15 min after insemination.

Pricking is a well known method for activating frog eggs. We have not observed directly the breakdown of cortical granules after pricking of eggs, but we assume that the reaction proceeds around the periphery from the point of pricking, as observed in the transparent egg of Fundulus heteroclitus (27). Breakdown had begun in some eggs fixed 45 sec after pricking (Table I) and appeared to be complete in all eggs fixed later than 1½ min after pricking.

Extrusion of the contents of an individual granule is evidently a rapid process. At first, our assay of sections merely revealed the presence or absence of cortical granules. After we had learned something of the timing of the cortical reaction, we chose the period between 12 and 13 min for fixing a series of eggs at 10-sec intervals. Figs. 5 and 6 illustrate the cortex of an egg fixed at 13 min after insemination, at a time when extrusion of granular contents was in progress. Cortical craters are formed after cortical granules perforate the plasma membrane. The structure that resembles a cortical granule in Fig. 5 is interpreted to be a newly formed cortical crater. Note that it is bounded by a membrane which looks like the plasma membrane. The contents of this crater are somewhat diffuse, but not discharged. Older craters (Figs. 5, 6) are smaller and appear to have discharged most of their contents, although they contain some light, diffuse material like that dispersed in the perivitelline space.

Long pseudopodia protrude into the perivitelline space at the time of breakdown of cortical granules (Figs. 5, 6). Flocculent material of low density and scattered, small vesicles are adherent to the under-
FIGURE 4 Cortical granules in mature oocyte fixed after stripping from cloaca. The largest cortical granule (cg) appears to contact the plasma membrane. Micrograph illustrates distribution of pigment granules (pg), yolk platelets (yp), mitochondria (m), lipochondria (l), and smooth-walled vesicles (v). X 28,500.

side of the denser vitelline membrane. This adherent material appears to consist of the contents of the perivitelline space before discharge of cortical granules. We have no evidence that extruded material forms a distinct layer against the vitelline membrane nor a separate hyaline layer as in sea urchin eggs (2).

As a result of discharge of cortical granules, other cytoplasmic components are brought close to the surface. Yolk platelets, mitochondria, lipochondria, pigment granules, and clusters of vesicles now may lie just beneath the plasma membrane (Figs. 5, 6).

Soon after eruption of cortical granules, pseudopodia retract and the cortical craters flatten and disappear. Fig. 7 shows the blunt pseudopodia remaining at the surface of an egg fixed 15 min after insemination. Eggs fixed at 20 min had similar surfaces. An egg fixed at 30 min after insemination is illustrated in Fig. 8. Its surface is relatively smooth, although it exhibits a few low protrusions.

DISCUSSION

Our finding that cortical granules of Rana pipiens contain both neutral and acid mucopolysaccharides confirms previous histochemical studies on anuran oocytes (22, 46, 52). Katagiri (22) has commented that "there is good reason to anticipate that the polysaccharide of the basophilic granules may be in the form of protein polysaccharide complexes." Light staining with bromphenol blue, which we have observed, may be due to protein in cortical granules.

It is significant that cortical granules extrude their contents into the perivitelline space with little or no loss of protoplasm. What probably happens is that those cortical granules not already
TABLE I
Breakdown of Cortical Granules in Inseminated or Pricked Eggs of Rana Pipiens as Detected by Electron Microscopy

<table>
<thead>
<tr>
<th>Time of fixation</th>
<th>No. examined</th>
<th>Cortical granules present (+), extruded (−), or partly extruded (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>After insemination</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 min</td>
<td>2</td>
<td>2 +</td>
</tr>
<tr>
<td>2 &quot;</td>
<td>1</td>
<td>1 +</td>
</tr>
<tr>
<td>5 &quot;</td>
<td>2</td>
<td>2 +</td>
</tr>
<tr>
<td>6 &quot;</td>
<td>1</td>
<td>1 +</td>
</tr>
<tr>
<td>7½ &quot;</td>
<td>1</td>
<td>1 +</td>
</tr>
<tr>
<td>10 &quot;</td>
<td>11</td>
<td>5 +, 4 −, 2 p</td>
</tr>
<tr>
<td>11 &quot;</td>
<td>9</td>
<td>6 +, 3 −</td>
</tr>
<tr>
<td>12 &quot;</td>
<td>13</td>
<td>9 +, 4 −</td>
</tr>
<tr>
<td>12 min 10 sec</td>
<td>6</td>
<td>4 +, 1 −, 1 p</td>
</tr>
<tr>
<td>12 &quot; 20 &quot;</td>
<td>8</td>
<td>3 +, 2 −, 3 p</td>
</tr>
<tr>
<td>12 &quot; 30 &quot;</td>
<td>6</td>
<td>3 +, 3 −</td>
</tr>
<tr>
<td>12 &quot; 40 &quot;</td>
<td>2</td>
<td>2 −</td>
</tr>
<tr>
<td>12 &quot; 50 &quot;</td>
<td>7</td>
<td>4 +, 3 −</td>
</tr>
<tr>
<td>13 &quot;</td>
<td>17</td>
<td>9 +, 7 −, 1 p</td>
</tr>
<tr>
<td>14 &quot;</td>
<td>8</td>
<td>6 +, 2 −</td>
</tr>
<tr>
<td>15 &quot;</td>
<td>12</td>
<td>6 +, 6 −</td>
</tr>
<tr>
<td>16 &quot;</td>
<td>1</td>
<td>1 −</td>
</tr>
<tr>
<td>17 &quot;</td>
<td>1</td>
<td>1 −</td>
</tr>
<tr>
<td>18 &quot;</td>
<td>1</td>
<td>1 −</td>
</tr>
<tr>
<td>19 &quot;</td>
<td>1</td>
<td>1 −</td>
</tr>
<tr>
<td>20 &quot;</td>
<td>7</td>
<td>1 +, 6 −</td>
</tr>
<tr>
<td><strong>After pricking</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8–15 sec</td>
<td>7</td>
<td>7 +</td>
</tr>
<tr>
<td>22 &quot;</td>
<td>1</td>
<td>1 +</td>
</tr>
<tr>
<td>30 &quot;</td>
<td>2</td>
<td>2 +</td>
</tr>
<tr>
<td>45 &quot;</td>
<td>9</td>
<td>5 +, 4 −</td>
</tr>
<tr>
<td>1 min</td>
<td>5</td>
<td>3 −, 2 p</td>
</tr>
<tr>
<td>1½ &quot;</td>
<td>4</td>
<td>1 +, 2 −, 1 p</td>
</tr>
<tr>
<td>2 &quot;</td>
<td>2</td>
<td>2 −</td>
</tr>
<tr>
<td>2½ &quot;</td>
<td>1</td>
<td>1 −</td>
</tr>
<tr>
<td>3 &quot;</td>
<td>1</td>
<td>1 −</td>
</tr>
<tr>
<td>5 &quot;</td>
<td>3</td>
<td>3 −</td>
</tr>
<tr>
<td>6 &quot;</td>
<td>1</td>
<td>1 −</td>
</tr>
<tr>
<td>11 &quot;</td>
<td>1</td>
<td>1 −</td>
</tr>
<tr>
<td>12 &quot;</td>
<td>1</td>
<td>1 −</td>
</tr>
<tr>
<td>15 &quot;</td>
<td>1</td>
<td>1 −</td>
</tr>
</tbody>
</table>

in contact with the plasma membrane before fertilization move up to a position of contact as the fertilization wave sweeps around the egg. Perforation probably occurs in the zone of contact between plasma membrane and the membrane surrounding a cortical granule. Rapid fusion of the torn margins of the apposed membranes around the rim of a perforation apparently occurs, thereby preventing leakage of protoplasm. The contents of cortical craters are then pushed into the perivitelline space. This hypothesis to explain discharge of cortical granules in frog oocytes is similar to that of Endo (12) for the sea urchin egg.

Several questions remain unanswered at present: whether the membrane surrounding a cortical granule is structurally like the plasma membrane before perforation, whether this membrane is altered to become plasma membrane after perforation, or whether the membrane of the cortical granule dissolves and new plasma membrane is deposited around the cortical crater. Fusion of plasma membranes at the surface of the oocyte and around cortical craters must result in temporary increase of surface area. The increase is evidently counteracted to some extent by shrinkage of cortical craters as they discharge their contents.

Extrusion of the contents of cortical granules appears to be a secretory process like that observed in various merocrine glands (42, 43). Discharge of adhesive material from the oral sucker of the frog tadpole (11) and of the jelly from frog oviducal glands (33) resembles closely the discharge of cortical granules.

The properties of the material extruded from the cortical granules into the perivitelline space of the frog’s egg are largely unexplored. Ginsburg (13) has shown that fluid from the perivitelline space of fertilized trout eggs could agglutinate sperm cells, and, furthermore, that supernumerary sperm could fertilize the egg if perivitelline fluid were removed. Sakai (49) has reported polyspermy of eggs of Oryzias latipes inseminated after removal of their chorions. It seems likely that perivitelline fluid helps to block polyspermy in the frog. The contents of the cortical granules of sea urchin eggs (2, 6, 35) or the homologous cortical alveoli of eggs of fishes (39, 58) have been implicated in the process of hardening of the fertilization membrane, although Ohtsuka (39) believes that hardening of the chorion of fish eggs is an oxidative process independent of the release of alveolar contents. Osanai (41) has demonstrated that a PAS-positive layer develops in the perivitelline space around the surface of the toad’s egg about 30 min after insemination. We have not tested for such a layer surrounding the fertilized frog’s egg.

Newport (38) was the first to observe living spermatozoa penetrating the jelly surrounding the...
frog's egg. He saw sperm cells reaching the zona pellucida (inner jelly layer) within the first minute after insemination, and "sticking in the vitelline membrane" at 5 min after insemination. His observations indicate that penetration of jelly is fairly rapid and that fertilization is delayed principally by the time necessary for a sperm cell to penetrate the vitelline membrane. The latent period between insemination and fertilization is approximately 10–15 min in *Rana pipiens*, compared with 3–4 min for the toad *Bufo* (31), 12–22 sec or 17–23 sec for the sea urchins *Psammechinus* or *Arbacia* (3), and 3 sec for the fish *Acipenser* (13) in which the egg has a micropyle.

It has long been known (15, 37) that frogs' eggs inseminated in the white vegetative hemisphere may be activated but will not cleave normally. Bataillon (8) discovered that eggs pricked with a glass needle anywhere on the surface could likewise be activated, but would not cleave normally unless a "second factor" were introduced at the time of pricking. Shaver (50) later referred to this factor as a "cleavage-initiating substance." Currently, Huff et al. (20) are attempting to characterize this substance.

Our observations (Table I) indicate that breakdown of cortical granules is complete by 1–1 1/2 min after pricking the frog's egg. We have performed
FIGURE 6 Another view of the cortex illustrated in Fig. 5, from fertilized egg fixed 13 min after insemination. A small, late-stage cortical crater (arrow) communicates with the perivitelline space (PV). Yolk platelets (yp) are developing within mitochondria at lower right. Portion of large yolk platelet at lower left. Protoplasmic salients and vitelline membrane (VM) are as described for Fig. 5. X 24,500.

some experiments in which eggs were first pricked and then inseminated. Regardless of where the eggs were pricked, they could not subsequently be fertilized. We surmise that the cortical reaction proceeds to completion in pricked eggs and creates a block to sperm entrance, probably in the perivitelline space, before a sperm can penetrate the jelly and vitelline membrane. Our estimate that a fertilizing sperm requires 10–15 min to penetrate these envelopes is consistent with Katagiri’s observation (21) that cleavage occurred about 10 min earlier in pricked eggs than in inseminated eggs of Rana temporaria.

This investigation was supported by grants from the National Science Foundation (NSF G-19447 and GB-1486), the United States Public Health Service (USPHS GM-05867-05, 06 and 07), and the United States Atomic Energy Commission (AEC AT(11-1)-1080). We are indebted to Dr. William Bachop for assistance with histochemical procedures and to Mrs. Judith A. Hoyt for assistance in photography. Received for publication 20 July 1966.

Note Added in Proof: Two papers by P. Van Gansen were called to our attention during editorial review of our manuscript. The first (1966. J. Embryol. Exptl. Morphol. 15:355.) describes the ultrastructure of the peripheral cytoplasm of mature ovarian oocytes and unfertilized eggs of Xenopus laevis. The second (1966. J. Embryol. Exptl. Morphol. 15:365.) describes changes after fertilization. Particular emphasis is given to ribosomal size and distribution.

REFERENCES


3. Allen, R. D., and J. L. Griffin. 1958. The time


12. Enyo, Y. 1961. Changes in the cortical layer of sea urchin eggs at fertilization as studied with

Figure 7 Cortex of egg fixed at 15 min after insemination. Cortical craters are no longer present. Short, blunt pseudopodia protrude into perivitelline space (PV). Mitochondria (m), yolk platelets (yp), and vesicles (v) are close to surface. × 32,500.

Figure 8 Cortex of egg fixed at 30 min after insemination. Pseudopodia have regressed so that only occasional blunt hillocks protrude into the perivitelline space (PV). Yolk platelets (yp), mitochondria, and vesicles are still relatively close to surface. × 24,000.


45. Pease, A. G. E. 1960. Histochemistry Theoreti-


