Cortical Change at Fertilization in the Egg of the Grass Frog, *Rana temporaria*[1)]

By

Chiaki Katagiri

(Zoological Institute, Hokkaido University)

(With 8 Text-figures and 1 Table)

During the last decade, researches on fertilization from the view-point of developmental physiology have evidenced a series of cortical changes taking place in the just fertilized eggs; especially in the case of sea urchins. In the frog eggs, however, only very little information is available on the changes of the cortical cytoplasm at fertilization. Motomura ('52) first reported the existence of cortical granules in the eggs of *Rana nigromaculata* and their breakdown at fertilization. Similar observation was also reported in *Bufo vulgaris* (Osanai '58).

In the present paper, the author reports on the changes of the cortical cytoplasm, especially of the cortical granules, of the frog eggs at fertilization.

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Material and methods

The material used was the eggs of a grass frog, *Rana temporaria*, collected in the neighborhood of Sapporo. Mature eggs were obtained by vivisection of the female in amplectic embrace. Artificial insemination was performed by the method introduced by Rugh ('34). As the physiological balanced salt solution, standard De Boer's solution[2)] and its dilutions were used in the study of fresh materials, when necessary. For the morphological observations, the eggs fixed in Bouin's or Bensley's fluids[3)] for 4 hours were cut at 3–5 μ in thickness through methylbenzoate-paraffin method (*cf.* Romeis '48). The sections were stained with Delafield's hematoxylin and eosin or with 0.1 % aqueous solutions of the following dyes; acid fuchsin, alizarin red, basic fuchsin, Bismarck brown, Congo red, gentian violet, Janus green, light green, malachite green, methylene blue,

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2) Composition of standard De Boer's solution; M/8.8 NaCl 100 parts+M/8.8 KCl 1.2 parts+M/13 $CaCl_2$ 1.8 parts (pH 7.3).

3) Composition of Bensley's fluid used; 5 g sublimate and 2.5 g potassiumbichromate in 100 ml distilled water, with 10 ml formalin added before use.

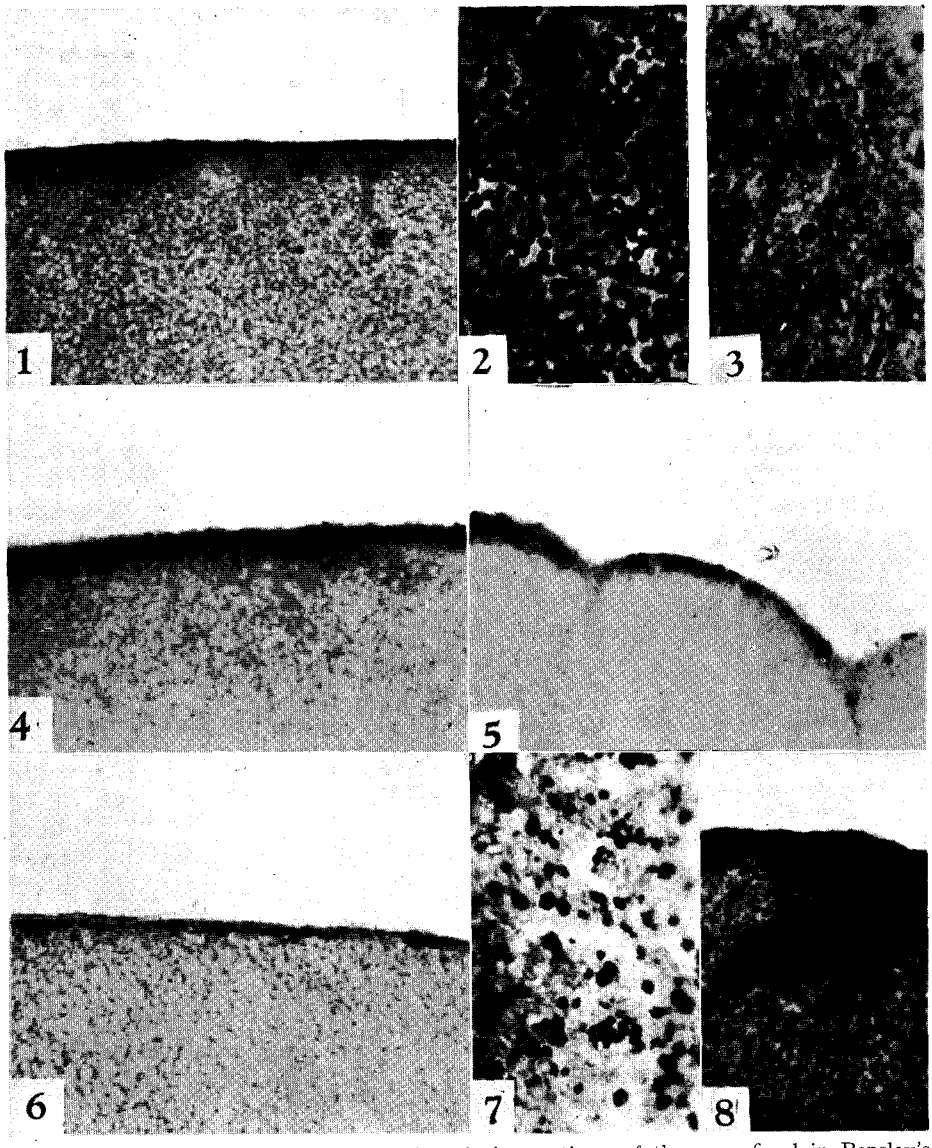
methyl green, neutral red, Nile blue, pyronin, safranin, thionin and toluidine blue. Heidenhain's azan stain and PAS- or Bauer's cytochemical reactions for polysaccharides were also applied. As pigment granules in the cortical cytoplasm of the egg prevented detailed observation, Kopsch's method for bleaching melanin (*cf.* Romeis '48) was adopted in some cases; the deparaffinized and hydrated sections were treated for 10–20 minutes with a mixture containing equal parts of 5% bleaching powder and 1% chromic acid. All the experiments on the fresh materials were made at the temperature 17–20°C.

Observations

Cortical granules in the unfertilized egg: When the bleached section of the unfertilized egg was stained with Delafield's hematoxylin, a narrow layer of the cortical cytoplasm with a few yolk platelets could be seen. This cytoplasmic layer measured 5–6 μ in thickness at the animal pole, and decreased in thickness gradually toward the vegetal pole where it measured only 1 μ. Detailed observation of this cortical cytoplasmic layer revealed that a layer of the granules which stained deeply with hematoxylin occupied the outermost part of the former (Fig. 1).

The staining properties of these granules were then investigated on the non-bleached sections. The granules were clearly demonstrated by Janus green through the method described by Motomura ('52). They had also strong affinity to the basic dyes. Their basophilic property was unaffected when the sections were pretreated with the method for removing nucleic acids (*cf.* Okamoto *et al.* '55). With toluidine blue and thionin, the granules showed a slight metachromasia. However, all the employed acid dyes, except anilin blue in azan stain, failed to stain the granules. Cytochemical tests proved that the granules were strongly positive in PAS- and Bauer's reactions for polysaccharides and that the reactions were unaffected by saliva treatment. On the basis of these results, it may be said that the granules contain some polysaccharides other than glycogen.

These basophilic and PAS-positive granules were found distributed in a layer between the plasma surface and pigment granule layer in the cortical cytoplasm. Each granule was spherical in shape and measured 1 – 2 μ in diameter. The average density of these granules was about 17–18 per 100 μ^2 (Fig. 2). Judging from the staining property and the distribution pattern, these granules may correspond to the 'cortical granules' described by Motomura ('52) in the eggs of *Rana nigromaculata*. Thus, in the present paper, too, these granules will be referred to as the 'cortical granules'. The cortical granules in the present material could hardly be observed in the animal hemisphere, since this region was almost entirely filled with pigment granules. Treatment of the sections with Kopsch's method followed by staining with Delafield's hematoxylin proved to be the best method clearly to demonstrate the cortical granules.



All figures are the photomicrographs of the sections of the eggs fixed in Bensley's fluid. Figs. 1–7 are sections stained with Delafield's hematoxylin after bleaching. Fig. 8. is the section stained with pyronin.

Figs. 1 and 2. Cortical granules of the unfertilized egg. ca. ×1,000 and ×1,200, respectively.

Figs. 3 and 4. Cortical cytoplasm of the egg, 20 minutes after insemination. Most of

Behavior of the cortical granules at fertilization: The eggs were fixed at predetermined intervals of time after insemination and behavior of the cortical granules was studied during the process of fertilization. The cortical granules showed little or no change in density for 10 minutes, but almost entirely disappeared within 20 minutes and were scarcely visible 30 minutes after insemination (Figs. 3 and 4). Although most cortical granules had gone out of sight, a few were still observed occasionally in the cortical cytoplasm of the cleaving eggs. In some cases, unextruded cortical granules were found in the eggs even at the blastula stage (Fig 5). Existence of these unextruded cortical granules was recognized not only in the artificially fertilized eggs but also in the naturally laid eggs.

In the eggs fixed 20–30 minutes after insemination, a few cortical granules were found irregularly arranged in the somewhat sacculated cortical cytoplasm. In these eggs, some granules swollen and irregularly outlined were also observed (Figs. 3 and 4). The perivitelline space in these eggs, though more or less collapsed through fixation, was filled with a substance which showed a weakly positive reaction to the polysaccharide tests and stained with basic dyes. Positive reaction of the perivitelline fluid to the polysaccharide tests was also observed throughout the cleavage stages. The perivitelline fluid also showed distinct metachromasia when the eggs were vitally stained with 0.05% aqueous solution of toluidine blue.

In the fresh material, elevation of the vitelline membrane from the egg surface was clearly observed 15–20 minutes after insemination. The width of the perivitelline space measured about 5μ in the egg 20 minutes after insemination at the animal pole where the surface of the egg became rather flat. The egg rotation within the perivitelline space was completed within 50 minutes after insemination. From the observations mentioned above, it seems likely that the cortical granules are extruded from the egg surface within 20 minutes after insemination and participate in the formation of the perivitelline space.

Next, observation was made on the change of the egg nucleus during the process of fertilization in relation to the behavior of the cortical granules mentioned above. The uterine egg showed the metaphase spindle of the second maturation division. The same figure was observed in the fertilized egg 20 minutes after

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the cortical granules have already broken down. Note somewhat swollen and faintly stained cortical granules. ca. ×1,200 and ×1,000, respectively.

Fig. 5. Surface of the blastomeres of the egg at blastula stage, showing a few number of unextruded cortical granules. ca. ×1,000.

Fig. 6. Cortical cytoplasm of the egg pricked and immersed in tap water for 15 minutes, showing the disappearance of cortical granules. ca. ×1,000.

Fig. 7. Cortical cytoplasm of the egg pricked and immersed in De Boer's solution for 30 minutes. ca. ×1,200.

Fig. 8. Female pronucleus of the egg shown in Fig. 7. ca. ×1,000.

insemination. Anaphase took place about 30 minutes, telophase 40 minutes, and the extrusion of the second polar body about 50 minutes after insemination, respectively. By an hour after insemination, the spherical female pronucleus was formed; the fusion of male and female pronuclei occurred about 2.5 hours after insemination. Since the breakdown of the cortical granules is very likely to occur within 20 minutes after insemination, it may be safely said that the breakdown of the cortical granules is the first sign of fertilization to become evident before the completion of the second maturation division in the egg.

Breakdown of the cortical granules was also induced mechanically when the unfertilized eggs were stimulated by pricking with a fine glass needle and immersed in tap water. In the eggs fixed 15 minutes after such treatment, the cortical granules were found to have almost completely disappeared (Fig. 6). Behavior and fate of the granules after the breakdown seemed to be the same as in the case of the normally fertilized eggs. However, in the pricked eggs, such events as the egg rotation and the extrusion of the second polar body apparently took place more rapidly as compared with those in the fertilized eggs.

In the next place, some of the unfertilized eggs were pricked with a fine glass needle and then immersed in the following solutions having different salt concentrations : one-twentieth, one-tenth and one-half strength De Boer's solutions, and standard De Boer's solution. Morphological changes which took place in these experimental lots were compared with those in the eggs pricked and immersed in tap water. The eggs fixed 30 minutes after pricking showed both breakdown of the cortical granules and the formation of female pronucleus in all the experimental lots at the same rate, while the eggs unpricked and immersed in these media, as well as in tap water, did not show such changes until they indicated cytolysis (Figs. 7 and 8). The results showed that the very same morphological changes occurred in both the pricked eggs immersed in tap water and in those immersed in isotonic salt solution. However, the rate of egg rotation showed a considerable difference depending on the concentrations of the media in which the pricked eggs were immersed. Firstly, counting was made on the pricked eggs which showed

Table 1. Rate of rotation of the pricked eggs immersed in media of various concentrations. Number in each column means the percentage of the eggs completed rotation by each time. Further explanation in text.

Time after immersion / Media	0 min.	30 min.	1 hr.	1. 5 hrs.	3 hrs.
Tap water	0	87.5	100.0	100.0	100.0
1/20 De Boer's solution	0	63.6	100.0	100.0	100.0
1/10 De Boer's solution	0	60.0	100.0	100.0	100.0
1/2 De Boer's solution	0	0	11.1	44.4	100.0
Standard De Boer's solution	0	0	0	0	41.7

the vegetal hemisphere upward at the time of immersion and then those rotated were counted at certain intervals for 3 hours. As will be seen in table 1, it took a longer time for the pricked eggs to rotate as the concentration of the surrounding medium became stronger. All the pricked eggs rotated within 16 hours after immersion even in standard De Boer's solution.

As described in the foregoing pages, no indication of the breakdown of the cortical granules was observed in the unfertilized and unpricked egg immersed in tap water nor in those in standard De Boer's solution. In the eggs immersed in tap water, the fertilizability was completely lost within 15 minutes. The eggs immersed in standard De Boer's solution, on the contrary, retained their fertilizability for a long time. In this connection, the effect was observed of pricking the unfertilized eggs which had lost their fertilizability by 6-hour immersion in tap water. Even in these eggs, pricking brought on breakdown of the cortical granules and the egg rotation.

Discussion

It was shown in the present study that the cortical granules, basophilic and PAS-positive, are embedded in the cortical cytoplasm of the mature unfertilized egg and that most of these granules disappear within 20 minutes after insemination. The perivitelline space which appears after fertilization proved to be filled with a substance of the same staining properties. This fact seems to suggest that the cortical granules are extruded from the egg surface at fertilization and accumulate in the perivitelline space. Direct observations also proved that the time of occurrence of elevation of the vitelline membrane from the egg surface, which causes egg rotation, corresponds approximately to that of the breakdown of the cortical granules. Thus, it may well be surmised that in the early stages of fertilization a series of three phenomena take place successively, *viz.*, breakdown of the cortical granules, formation of perivitelline space, and egg rotation.

The breakdown of the cortical granules was induced also by pricking the egg with a fine glass needle. As is well known, the frog eggs can be activated by pricking or electrical stimulation, inducing formation of perivitelline space and rotation of the egg as occurs in the case of the fertilized eggs (Bataillon '10 ; Ancel and Vintemberger '49). Considering these facts, it is clear that the formation of perivitelline space and the egg rotation are common events characteristic in the artificially activated eggs as well as in the fertilized eggs. It was ascertained in the present study that the cortical granules, as will be discussed in some detail later, contain mucopolysaccharide, a hydrophilic substance of high molecular weight. It is likely that the hydrophilic colloid of the cortical granules extruded into the interstice between the egg surface and the vitelline membrane may not pass through the vitelline membrane or jelly envelopes so that the uptake of water owing to the colloid osmotic pressure causes the formation of the perivitelline space. The same conclusion has been reached by Białaszewicz ('12) and Picken and

Rothschild ('48), who were unaware of the presence of the cortical granules in the unfertilized eggs. The conclusion may be supported by the fact that the egg rotation was retarded in the pricked eggs immersed in standard De Boer's solution, despite the fact that the cortical granules had disappeared in a short time. The conclusion drawn here may also coincide well with a supposed mechanism of the formation of perivitelline space in the eggs of sea urchins and the teleostean fishes (Loeb '08; Yamamoto '56).

It is well known that the first visible change of the egg at fertilization or activation in sea urchins or fishes is the breakdown of the cortical granules or alveoli. Usually, the breakdown is completed in a short time (Endo '52; Yamamoto '56). Most cortical granules in the present material also break down in a short time. The similar granules have been reported also in the eggs of other anuran species, *R. nigromaculata* and *Bufo vulgaris* (Motomura '52; Osanai '58). A small number of unextruded granules were found in the blastula eggs of the present material. From the electron microscope study on the sea urchin eggs, Afzelius ('56) reported the existence of a small number of unextruded cortical granules in the developing larvae in the gastrula stage. The present observation of the unextruded cortical granules, therefore, may correspond to that described by Afzelius.

From the results of staining reactions, it is most probable that the cortical granules are predominantly composed of acid mucopolysaccharide. Similar polysaccharide nature has recently been reported in the cortical granules of toad (Osanai '58). The same is also true in the cortical granules of sea urchins (Monné and Hårde '51) and in the cortical alveoli of fish eggs (Kusa '53).

The basophilic and PAS-positive granules comparable to the cortical granules of the mature eggs were observed also in the immature ovarian eggs of the present material. The granules appeared first in the young oocytes prior to yolk deposition and were distributed mainly in a peripheral zone of the growing oocytes throughout the stages of oogenesis. The basophilic granules are likely to correspond to the granules described in the oocytes of *R. nigromaculata* (Motomura '52), *R. pipiens* (Kemp '56; Rosenbaum '58), *Xenopus laevis* and *R. fusca* (Wartenberg '56).

It was shown in the present study that the eggs, once pricked, can be activated either in tap water or in De Boer's solution. In the unpricked eggs immersed in these media, on the other hand, no breakdown of the cortical granules was observed and the egg nucleus remained unchanged for a long time. In *R. nigromaculata*, the cortical granules are reported to disappear as a result of mere immersion of the unfertilized eggs in tap water (Motomura '52). According to Picken and Rothschild ('48), the unfertilized eggs of European *R. temporaria* develop slowly a perivitelline space after immersion in tap water and a period of about 3 hours elapses before egg rotation takes place. Therefore, these eggs seem to be parthenogenetically activated by the mere immersion in tap water, which is inconsistent with the present observation.

Although the mere immersion of unfertilized eggs in tap water caused no breakdown of the cortical granules, the fertilizability of the eggs was lost within a short time. In standard De Boer's solution, on the other hand, the fertilizability

of the eggs was preserved for a considerably long time. The jelly envelopes covering the egg seemed to show different appearances in different salt solutions or concentrations. In addition, unpublished data revealed that the short treatment of unfertilized eggs with standard De Boer's solution prior to insemination raised rapidly the percentage of fertilization. These facts suggest that the rapid loss of fertilizability of the egg in tap water is possibly due to some changes of the jelly envelopes rather than any of the egg proper. The role of the jelly envelopes in amphibian fertilization in connection with the penetration of a spermatozoon into the egg has long been discussed by many investigators (Bataillon and Tchou '30; Tchou and Wang '56) and their view seems to support the present supposition. Further discussion on this problem will be found elsewhere.

Summary

The cortical change at fertilization or activation was studied in the eggs of the grass frog, *Rana temporaria*, and following results were obtained:

1) There is a layer of basophilic granules, the cortical granules, in the cortical cytoplasm of the mature unfertilized egg. The cortical granules are composed predominantly of acid mucopolysaccharide.

2) Most of the cortical granules are extruded from the egg surface within 20 minutes after insemination and participate in the formation of the perivitelline space.

3) The breakdown of the cortical granules can also be induced mechanically by pricking the unfertilized egg with a fine glass needle. The egg, once pricked, can be activated in standard De Boer's solution as well as in tap water.

4) The mere immersion of the unfertilized eggs in tap water or in De Boer's solution causes neither the breakdown of the cortical granules nor any change of the egg nucleus until the eggs show an indication of cytolysis. Fertilizability of the egg is preserved for a considerably long time in standard De Boer's solution, whereas it is rapidly lost in tap water. Concerning these results, discussion to some extent was offered on the role of the jelly envelopes in fertilization.

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