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Basophilic Granules in the Oocytes of the Grass Frog, *Rana temporaria*[1]

By

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(With 1 Plate and 1 Table)

Although amphibian oogenesis has long been a subject of numerous cytological and cytochemical investigations, the basophilic and PAS-positive granules appearing in the oocytes have not attracted much attention until recently (Wartenberg '56, '59, Rosenbaum '58). On the other hand, the structure of the oocyte has come to be investigated with the electron microscope in relation to the problems of the cortical region, for example, of the surface coat (Holtfreter '43, Kemp '56, Wartenberg & Gusek '60). Further, it has been clearly ascertained that the cortical granules found in the cortical cytoplasm of amphibian eggs break down upon fertilization or activation (Motomura '52, Katagiri '59, Osanai '60).

In the present study, observations are made on the oogenesis of the frog, with special attention to the localization and the staining properties of basophilic granules occurring in oocytes.

Material and method : The grass frog, *Rana temporaria*, used in the present study was collected in the neighborhood of Sapporo from April to October. The ovaries removed and cut in small pieces in standard De Boer's solution[2] were fixed in Bensley's[3], Bouin's or Bouin-Allen's fluids. Then 3–7 μ thick sections were prepared by the ordinary paraffin method, or by the methylbenzoate-paraffin method in the case of heavily yolk-laden oocytes. The sections were stained with Delafield's hematoxylin and eosin, Heidenhain's azan stain, or with 0.1% aqueous solutions of various acid and basic dyes, *viz.*, acid fuchsin, alizarin red, basic fuchsin, Bismarck brown, Congo red, gentian violet, Janus green, malachite green, methylene blue, methyl green, neutral red, Nile blue, pyronin, safranin, thionin and toluidine blue. Cytochemical tests for polysaccharides were carried out by the use of the periodic acid-Schiff (PAS) method, Bauer's reaction, or the successive staining of 2, 4–dinitrophenylhydrazine (DPH) and Schiff's reagent after oxidation with periodic acid (*cf.* Monné and Slautterback '50). Millon's reaction and Salazar's iron-tannin method for protein, and Ciaccio's method for lipoid were also employed, when necessary.

Observations

Young oocytes measuring less than 250 μ in diameter have a spherical nucleus (germinal vesicle) with a few number of nucleoli (Fig. 1). The cytoplasm shows an intense basophilia, suggesting an accumulation of RNA (Brachet '47), but no basophilic granules can be observed anywhere. Tests for polysaccharides prove to be negative and a small number of lipoid droplets measuring about 10 μ in diameter are found in the cytoplasm.

In the oocytes about 300–400 μ in diameter, the nucleus is in an irregular form, showing so-called "sacculation", and the nucleoli have markedly increased in number. The basophilia of the cytoplasm is not so intense as that in the previous stage. Just beneath the surface of the oocyte fine granules stained deeply with basic dyes first appear in this stage (Fig. 1). They are very fine, less than 0.5 μ in diameter, and small in number at first, but soon increase in both number and size. These granules show both metachromasia with toluidine blue and strong polysaccharide reactions. Positive reactions for polysaccharides are for the first time demonstrated also in the cytoplasm of this stage. After saliva treatment, however, the reactions are very weak throughout the cytoplasm, whereas they still remain strong in the basophilic granules (Figs. 2 & 4). Accordingly most polysaccharides found in the cytoplasm, except those in the basophilic granules, are considered to be glycogen in this stage. Further it is noticed here that glycogen in this stage accumulates particularly rich around the nucleus (called "ring of perinuclear glycogen" by Brachet '47) with decrease in quantity toward the periphery of the oocyte, in contrast to the peripherally accumulated polysaccharides contained in the basophilic granules. Shortly after the first appearance of the latter, there appears an exceedingly thin membrane underlying the inner surface of the follicular epithelium (Fig. 5). It increases in thickness in the succeeding stages and forms so-called vitelline membrane. Lipoid is found as a number of droplets in both perinuclear and peripheral regions of the cytoplasm, especially in the latter region.

The oocytes measuring about 400–500 μ in diameter are characterized by the appearance of yolk platelets. The basophilic granules increase further in number and in size (about 1.5 μ in diameter) in the peripheral region of oocyte (Figs. 2 & 6) and the region of these granules roughly measures over 30 μ thick. In addition, a small number of basophilic granules are often observed to be scattered in the inner region of the cytoplasm, though their affinity to the dyes is somewhat weaker as compared with that of the granules in the peripheral region. In addition to the basophilic granules, yolk appears as fine granules in the peripheral region of the oocyte except in the narrow outermost region, *i.e.* cortical cytoplasm, where only basophilic granules are compactly distributed (Fig. 6). Even after saliva treatment, polysaccharide reactions are weakly positive in yolk and very strongly so in basophilic granules. Vitelline membrane has increased in thickness, and a narrow striated zone, the zona radiata, is clearly visible between

the vitelline membrane and the surface of oocyte.

In the oocytes measuring about 500–1,000 μ in diameter, in which yolk platelets fill approximately the outer half of the cytoplasm, pigment granules make their first appearance in the periphery of the oocyte in such a way as to indicate an animal-vegetative gradient (Fig. 3). The peripheral region of the oocyte therefore can be divided into two parts in this stage; one is the part of the cortical cytoplasm containing only basophilic granules, and the other is the part showing intermixed basophilic granules with newly formed yolk platelets and pigment granules. With further increase of yolk and pigment there occurs gradual change in the distribution of basophilic granules. Namely, basophilic granules in the intermixing part with yolk and pigment gradually decrease in number so that in later stages they become observable only in the cortical cytoplasm (Fig. 7). The vitelline memberane is almost fully formed and lipoid is detected in the form of fine diffused granules throughout the cytoplasm except in the cortical cytoplasm which contains basophilic granules.

In the oocytes measuring about 1,000–1,400 μ in diameter, the cytoplasm is fully filled with heavily accumulated yolk platelets, except in the narrow perinuclear and cortical cytoplasm. Though pigment granules are heavily accumulated in the animal hemisphere, they are observed about 100 μ in thickness throughout the periphery of the oocyte. Basophilic granules in the form of one or two layers can now be found mostly in the cortical cytoplasm, but a few of them are still observed in another part of the periphery. Pigment granules have not yet compactly filled up the cortical cytoplasm, consequently basophilic granules are still easily recognizable even in the animal hemisphere.

In the fully grown ovarian oocytes measuring more than 1,500 μ in diameter, their morphological features are similar in principle to those of mature eggs except with respect to the zona radiata and nucleus. Basophilic granules are arranged only in the cortical cytoplasm, forming a layer. They are however hardly recognized in the animal hemisphere because of the marked increase of the pigment granules in the same hemisphere.

The basophilic granules in the fully grown ovarian oocytes are thus found to take their localization identical with that of the cortical granules in the mature eggs (Katagiri '59).

The staining properties of basophilic granules in the ovarian oocytes were then studied in comparison with those of cortical granules in the mature eggs, with additional attention to the properties of the cortical region including the vitelline membrane and the zona radiata.

The basophilic granules are easily stained with strong basic dyes, whereas they are not stained with acid dyes except with aniline blue in azan stain which is known to stain mucoidal substances. Such basophilic property of these granules is not due to the presence of nucleic acids since the granules are deeply stained with pyronin or toluidine blue after treatment with 5% trichloracetic acid

for 15–30 minutes at 90°C or with 1N HCl for 3 hours at 37°C. With toluidine blue or thionin the granules distinctly reveal β-type metachromasia; such metachromasia is also recognized even when the pH of the dye solution has been adjusted to below 4.0. When the bleaching of sections followed by staining with Delafield's hematoxylin, which is the method applied for demonstrating the cortical granules in the mature egg (Katagiri '59), is here also applied, it proves to bring a good result in staining the basophilic granules in the oocyte. That is, in detail, the basophilic granules and the vitelline membrane are clearly demonstrated by this method in contrast to the other cellular inclusions unstained. According to the cytochemical tests for polysaccharides, the granules are strongly positive to PAS- and Bauer's reactions even after saliva treatment. Positive reactions for polysaccharides are also strongly detected in the vitelline membrane and moderately in the zona radiata and in the cortical cytoplasm, but they are only weakly stained either with acid or with basic dyes and no metachromasia can be detected. However, once treated with 10% chromic acid for one hour, the vitelline membrane becomes strongly basophilic, showing metachromasia. Differential staining of polysaccharides can be obtained in the sections stained by the method described by Monné and Slautterback ('50). That is to say, after overnight staining with DPH the basophilic granules react still with Schiff's reagent. On the contrary, such reaction does not occur in the vitelline membrane and other cytoplasmic inclusions. Tests for protein and lipoid are negative in the basophilic granules, whereas the vitelline membrane, the zona radiata and the cortical cytoplasm give positive results only in protein reactions. The main results concerning the staining properties of the cortical region mentioned above are summarized in table 1.

As stated already, cortical cytoplasm is free from yolk platelets, but does contain basophilic granules to a great extent. When oocytes are centrifuged at

Table 1. Staining properties of the cortical region of the oocyte and those of the cortical granules of the mature egg. Intensities of reactions are indicated by marks − (negative) to ⧥.

	Basic dyes	Toluidine blue (meta-chromasia)	Basic dyes after extraction of nucleic acids	Acid dyes*)	PAS- & Bauer's reaction	Millon's reaction
Vitelline membrane	+	−	+	+	⧥	+
Zona radiata	±	−	±	+	+**)	+
Basophilic granules	⧺	+	⧥	−	⧥	−
Cortical cytoplasm	⧻	−	±	⧻	+**)	⧻
Cortical granules	⧺	+	⧥	−	⧥	−

*) Except aniline blue in azan stain.
**) The reaction is weakened by saliva treatment.

3,000 r.p.m. for 30 minutes in standard De Boer's solution, stratification takes place in the following order from the centrifugal to the centripetal side; yolk, basophilic granules, pigment, cytoplasm, nucleus and lipoid. Careful observation reveals, however, that the basophilic granules stratified by centrifugation are those previously localized in the inner part of the peripheral region; the localization of the granules in the cortical cytoplasm remains unchanged, which indicates the rigid nature of the cortical cytoplasm (Fig. 9). The same result is also obtained in the oocyte at the stage just before the formation of yolk. Except in the case of the fully grown ovarian oocyte which has the granules arranged in a layer, the basophilic granules in the cortical cytoplasm of the oocyte are moved by the greater centrifugal force as high as 10,000 r.p.m. for 30 minutes, resulting in the same order of stratification as noted above (Fig. 10). These facts may indicate that the basophilic granules in the oocyte are composed of material having relatively high specific gravity and that the cortical cytoplasm of the oocyte is early characterized by considerably high rigidity in the previtelline stage. The surface of the oocytes, however, is highly permeable to water even in the fully grown ovarian oocyte stage, because oocytes show marked shrinkage or swelling when immersed in 2 De Boer's (hypertonic) solution or in 1/10 De Boer's (hypotonic) solution, respectively. On the other hand, the mature eggs do not show any such remarkable changes with the same treatments. Further, the basophilic granules in the oocyte before ovulation are easily dissolved and dispersed throughout the peripheral region when the oocyte is immersed for one hour into 1/10 De Boer's solution, but that is not the case in the mature egg.

Discussion

Basophilic granules which appear in the young oocyte stage as mentioned above are finally found not within the endoplasm in the stage of fully grown ovarian oocytes, but are arranged in the form of a layer in the cortical cytoplasm. In view of their localization and staining properties, it can hardly be doubted that the basophilic granules appearing in the immature oocyte are the precursors of the cortical granules found in the mature egg which are known to break down upon fertilization and to participate in the formation of the perivitelline space. Description of such granules seems, however, to be restricted in the recent papers, so far as the present author is aware. From the results of his staining with Janus green, Motomura ('52) has briefly noted that granules like the cortical ones are found in the endoplasm as well as in the cortical cytoplasm of the immature ovarian egg of *R. nigromaculata*. With the electron microscope, Kemp ('56) has observed in *R. pipiens* oocyte the cortical granules of perhaps the same type. PAS-positive granules described by Rosenbaum ('58) and Wartenberg ('56, '59) in the oocytes of some anuran species, may also correspond to the basophilic granules of the present paper, though they did not investigate the mature eggs.

As mentioned already, the basophilic granules appear in the peripheral

region of young oocyte and are synthesized there. The origin of basophilic or cortical granules in amphibian egg has not been much studied. They seem, at least, not to be related to mitochondria, because, differing from the localization of the former, the latter are dispersed throughout the cytoplasm before the appearance of yolk, after which they accumulate in the periphery in the later stages. Kemp ('56) has stated that the cortical granules seem to emerge from the peripheral ground cytoplasm, but has given no further description. At any rate, the fact that their rearrangement and accumulation into a narrow cortical cytoplasm proceed with or after the appearance of pimgent might suggest that basophilic granules are formed before the synthesis of yolk and pigment. The gradual decrease in number of basophilic granules beneath the cortical cytoplasm might well be recognized as a consequence of their outward migration into the cortical cytoplasm of the growing oocytes. A tentative interpretation that the PAS-positive granules might become pigment granules was once offered by Wartenberg ('56) but has been recently abandoned that interpretation himself (Wartenberg '59).

Regarding the cytochemical nature of basophilic granules, they are evidently positive to polysaccharide tests even after saliva treatment and show metachromasia with toluidine blue. Further, the granules have especially a strong affinity to the dyes such as pyronin, toluidine blue and Janus green. It is therefore concluded that the granules contain acid mucopolysaccharides, to which their strong basophilic property seems to be attributable. The cortical basophilia, which has been described by Kemp ('53) to be attributable to the presence of ribonucleic acid, is recognized to be different from the basophilia of these granules. The polysaccharides contained in the vitelline membrane, on the other hand, may be regarded as neutral mucopolysaccharides, because the membrane is stained with both acid and basic dyes and shows orthochromatic staining with toluidine blue. After extensive studies Wartenberg ('59) has reported the similar polysaccharide nature of baso-philic granules and vitelline membrane ; that is, the granules of *R. temporaria* are composed of acid mucopolysaccharides, whereas those of *Xenopus laevis* are of neutral mucopolysac-charides, and those of *R. esculenta* have the staining properties between the above two ex-treme forms. Judging from the differential uptake of toluidine blue, Rosenbaum ('58) has pointed out that the metachromasia in the cortical region is similar to that of the cell containing mucoitinsulfate. As has been noted by Wartenberg ('59), there seems some obscurity as to whether the metachromasia described by Rosenbaum was observed in the granules of the cortical region or throughout the cytoplasm of that region. In the present material, metachromasia was shown only by the basophilic granules both in the cortical cytoplasm and in the peripheral region of the oocyte. Although there is good reason to anticipate that the polysaccharides of the basophilic granules may be in the form of protein-polysaccharide complexes, the protein tests here used give negative results in the granules. The same is also true of Wartenberg's work. In this connection, it is of interest that Rosenbaum ('58) has not ascertained the protein nature of the granules by means of the cytochemical staining method, but has suggested it as a result of the digestion test by proteolytic enzymes. It seems therefore likely that even if the protein does exist in the basophilic granules, it may be so small in quantity or masked so strongly as not to be histochemically demonstrated.

The basophilic granules in the cortical cytoplasm of the present material

are movable under a considerably high centrifugal force, whereas those in the peripheral region can be moved by a moderate force. The results indicate that rigid cytoplasm is differentiated early in the stage of the first appearance of yolk.

Holtfreter ('43) has described for amphibian eggs and embryos the "surface coat" having significant functions in respect to physiological condition and morphogenesis and supposed it likely to correspond to the zona radiata described by earlier workers. Recent investigations on the fine structure of the cortical region in amphibian ooocytes by electron microscope have revealed that the granules corresponding to the basophilic granules in the present study are localized rather in an inner region of the cortex underlying the zone of microvilli which is the zone called "zona radiata" (Kemp '56, Wartenberg & Gusek '60). These facts may therefore lead to the conclusion that, at least in the present status of knowledge, there are no relationships between basophilic granules and the "surface coat" of Holtfreter.

Summary

Distribution and staining properties of the basophilic granules in the egg were studied during the course of oogenesis in the grass frog, *Rana temporaria*. Prior to the appearance of yolk, there appear basophilic and metachromatic granules in the periphery of young oocyte, at the stage when polysaccharide reactions can be demonstrated for the first time in the cytoplasm. The basophilic granules are mostly distributed in the periphery during the growth of the oocytes and are finally found to be arranged in a layer in the cortical cytoplasm of fully grown ovarian oocytes. Tests with various dyes and cytochemical methods prove that these granules are composed mainly of acid mucopolysaccharides. The results indicate therefore that the basophilic granules which appear in the young oocytes are the precursors of the cortical granules of the mature eggs. The origin and the cytochemical nature of these granules were discussed with the problems of the cortical region.

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Explanation of Plate XI

Fig. 1. Oocytes in the previtelline stage. Note the appearance of deeply stained granules in the periphery of larger oocytes. Bouin-toluidine blue. ca. ×67.

Fig. 2. Section of oocytes, showing the first appearance of basophilic granules (right oocyte) and their increase in number (left oocyte). The left oocyte is in the first stage of yolk formation. Bouin-Allen-PAS (treated with saliva). ca. ×67.

Fig. 3. Oocyte in the first appearance of pigment. Yolk has filled outer half of oocyte; basophilic granules in the inner part of periphery decreased in number. Bensley-pyronin (treated with trichloracetic acid). ca. ×67.

Figs. 4–8. Enlargements of periphery of oocytes, showing gradual changes of distribution of basophilic granules with growth of oocyte. Bouin-Allen-PAS (treated with saliva). ca. ×670, respectively.
Fig. 4 : lower smaller oocyte in Fig. 2. Fig. 5 : oocyte before formation of yolk. Thin membrane external to the surface of oocyte is vitelline membrane. Fig. 6 : left oocyte in Fig. 2. Yolk platelets appear in the region indicated y. Fig. 7 : oocyte in the same stage as shown in Fig. 3. Fig. 8 : fully grown ovarian oocyte.

Fig. 9. Section of oocyte centrifuged at 3,000 r.p.m. for 30 min., showing basophilic granules (b) in the cortical cytoplasm which are not stratified. Bensley-pyronin (treated with trichloracetic acid). ca. ×100.

Fig. 10. Section of oocyte centrifuged at 10,000 r.p.m. for 30 min., showing basophilic granules (b) stratified between yolk (y) and pigment (p). Bouin-Allen- PAS (treated with saliva). ca. ×100.
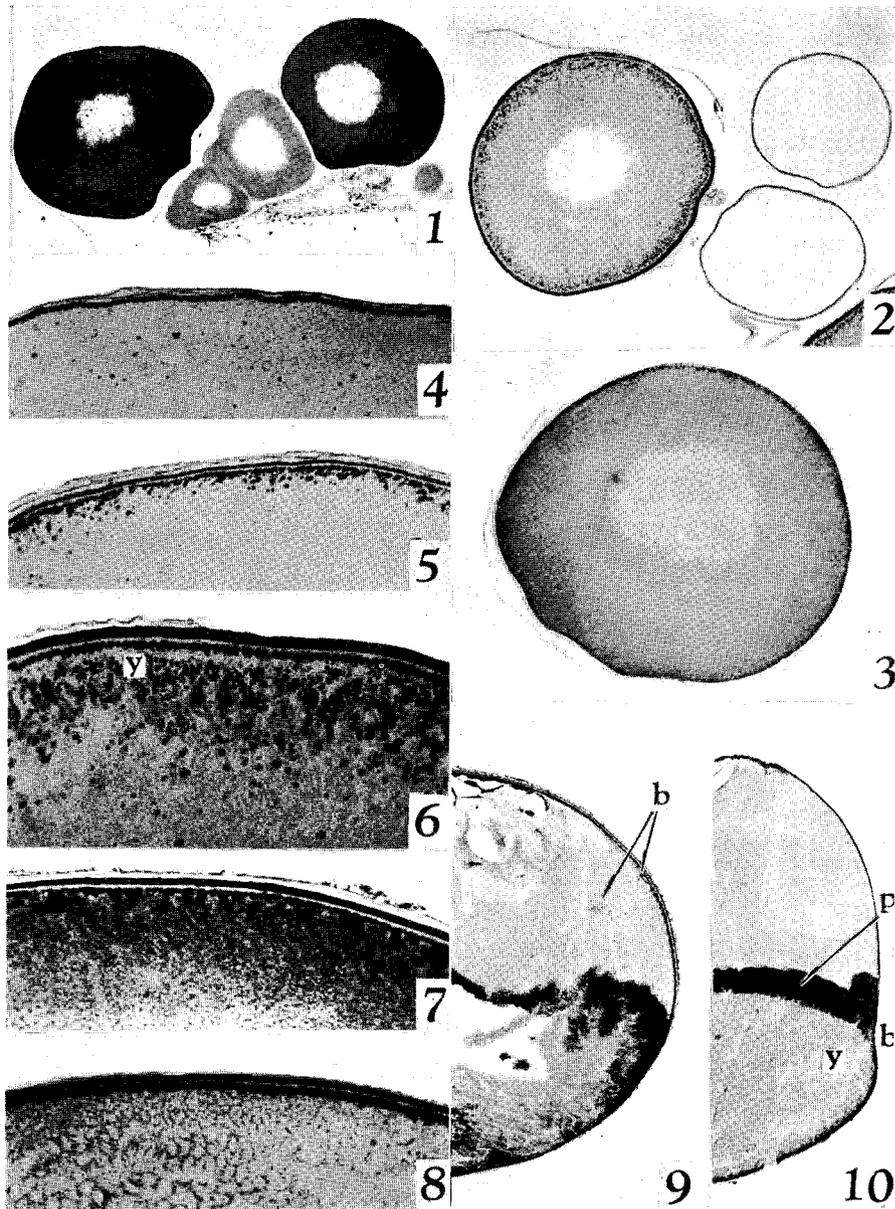
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