It is known that within the egg plasm of several marine invertebrates a number of metachromatic granules are observed following supravital staining with basic dyes and that they evidently increase in number during the progress of the first egg segmentation and are found in each blastomere (IIDA 1942, PASTEELS 1955, 59, PASTEELS et al. 1957, 58, REBHUN 1956, 57, 58a, b, 60, KOJIMA 1959a, b). Independently, TAKASHIMA (1956, 61), KATSURA (1954, 55) and ITATANI (1957a, b) in our laboratory have pointed out applying Japanese sea urchin species that the metachromatic granules show similar behavior to that of the above noted granules. But the origin, fine structure and biological role of this kind of granules are nowadays unclear though PASTEELS et al. (1958, 63), PASTEELS (1951a) and REBHUN (1959b, 60) have recently studied the nature of such granules employing refined techniques of differential centrifugation and electron microscopy.

In the present work, the authors have proposed to investigate the structural and biological nature of this kind of granules in Temnopleurus toreumaticus applying not only a method of supravital staining but also careful electron microscopy.

Materials and Methods

Because of the transparency of the egg plasm, the sea urchin species, Temnopleurus toreumaticus, collected at the sea area of Naruto, Tokushima prefecture were used as materials. The supravital staining of the egg plasm from mature unfertilized eggs to four cell stage embryo was carried out employing toluidine blue O (Grübler) diluted with sea water to a concentration of $10^{-4}$.

The fresh and supravitally stained eggs were fixed with cold 1% OsO$_4$ solution in sea water buffered with veronal acetate at pH 7.8 and with 0.6% KMnO$_4$ (in sea water). Following fixation a routine dehydration and embedding were carried out with ethanol and epoxy resin 812, respectively. For the light and electron microscopy semi-thin (ca. 0.5—1.0 μ in thickness) and thin sections were made from the same block with LKB ultramicrotome and the former ones were stained with toluidine blue O and the latter ones with Millonig's method for electron staining. The electron microscopy was carried out with HU-11 (Hitachi).

* This work is dedicated to the memory of the late Prof. Masaji SEKI.

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Results

When the mature unfertilized eggs of the present sea urchin species are stained supravitaly with toluidine blue O (10^{-4} solution in sea water), one can perceive light microscopically a few metachromatic granules in variable sizes and stainabilities scattered among abundant yolk granules which show a weak affinity to the dye stuff as do other egg components (Fig. 1). The ground substance of the egg plasm, however, shows a slight metachromasia.

Following fertilization (Fig. 2, 3), especially during the first egg segmentation (Fig. 4, 5), however, the granules begin to increase in number and gather around the nucleus and within the astral region. In addition to the metachromatic granules, some of the yolk granules begin to disintegrate and to show variable intensities of metachromasia. These metachromatic and yolk granules are probably identical with the “β-granules” of PASTEELS because of their resemblance in size, stainability and localization as well. Therefore, it is noteworthy that the increased metachromasia of the ground substance of the egg plasm and of the metachromatic granules and the disintegrating yolk granules relate probably to some substantial changes in cytoplasm during the egg development, though the fine granules such as the “α-granules” called recently by PASTEELS can not be recognized.

Next, prior to the electron microscopy the eggs before and after fertilization were fixed with OsO_4 and embedded in epoxy resin. Following the procedure the fixed eggs were cut in 0.5—1.0 µ thick (semi-thin section) and stained with toluidine blue O (1% solution in distilled water). With regard to the light microscopy of the semi-thin sectioned specimen, the cytological features of the egg plasm are almost the same as those noted with the supravital staining of the living eggs. Namely, in the egg plasm of mature unfertilized eggs there can be seen a few small metachromatic granules of variable sizes and stainabilities, scattering among the bluish green yolk granules (Fig. 1). During the egg segmentation, however, there appear abundant metachromatic granules of variable sizes within and around the astral region among the yolk granules. The number of metachromatic granules was slightly decreased as compared with unfertilized eggs (Fig. 2).

These metachromatic granules are probably identical with the “β-granules” of PASTEELS and the “aster-associated particles” of REBHUN, and seem to correspond to the various phases of disintegration of the yolk granules as the present electron microscopy ascertains. In addition, the mitotic figure appears clearly within the astral region and the chromosomes are stained light bluish.

Now, referring to the present microscopy of the astral region and neighboring egg plasm in the first segmentation stage (Fig. 3), there can be perceived abundant granules in variable sizes and electron densities, as well as mitochondria and other cell components among the astral rays which show chains of stretched fine vesicles. However, at least within the astral region no fine granules such as the “α-granules” of PASTEELS and the Golgi complex can be found, while the ground egg plasm among these structures shows spongy structure accompanied with a few glycogen-like small particles of high electron density. Some of these granules appear to be ordinary yolk granules with complete limiting membrane with abundant yolk particles within them as those in unfertilized eggs, but others seem to be identified with the metachromatic granules observed in the supravital and semi-thin sectioned specimens because of their localization and variable sizes as well as fine structures (Figs. 1—4).

Therefore, the metachromatic granules of ca. 700—810 µ in diameter are heterogeneous in their structure (Fig. 4, 5), though they show a complete limiting membrane as the ordinary yolk granules. But within them one can perceive a few small vesicles of ca. 540—690 Å
Fig. 1. A few metachromatic granules (Mg) of variable sizes and stainabilities appear scattering among the slightly stained yolk granules (Yg) (bluish). ×6,500

Fig. 2. In this picture (diaster stage of the first egg segmentation) the astral region is clearly shown, within which abundant metachromatic granules in variable sizes, shapes, and stainabilities are seen. Note that the chromosomes (Ch) are stained light bluish with toluidine blue O and the intact yolk granules are also slightly stained bluish. ×6,000.
Fig. 3. Electron micrograph of the astral region of the egg in the first segmentation stage. The astral rays (AS) show chains of fine stretched vesicles among which a few metachromatic granules and yolk granules in variable sizes, shapes and structures are found, while within the spongy ground egg plasm (Sp) appear a few glycogen-like electron dense particles. × 28,000
Fig. 4. Electron micrograph of the yolk and metachromatic granules within the astral region and neighboring egg plasma (monaster stage). Within the spongy egg plasma among the astral rays abundant metachromatic granules of variable sizes and structures are seen. × 58,000
in diameter and irregular or spherical masses of medium electron density among the ordinary yolk particles which decrease in number representing a sign of substantial changes within the granules. In addition to these structural changes some of the granules show often an incomplete limiting membrane and within them abundant small vesicles of ca. 560—590 Å in diameter (Fig. 6) similar to those observed by SOTELO and PORTER (1959) in the “multivesicular bodies” in rat ovum. Therefore, it is interest that at the broken down portion of the membrane the contents of the granules such as small vesicles and coarse substance seem to be in contact with the spongy structure and glycogen-like particles within the neighboring egg plasm.

On the other hand, the small ones of the metachromatic granules are of about 510 mÅ in diameter and show a particular fine structure (Fig. 7); namely, they are often partially or completely coated with an electron dense structureless material around their less electron dense cores. The cores show often a loose frame-like structure or coarse substance accompanied with a few electron dense glycogen-like particles connected with the materials within external spongy egg plasm. These structural changes of the aster associated metachromatic granules above mentioned, seem probably to represent variable phases of the disintegration of yolk granules, in other words, of the energy producing mechanism for the progress of egg segmentation.

Discussion

Stainability

Within the astral region of the first segmentation stage of living sea urchin eggs IIDA (1942) has already found abundant neutral red stainable granules attached to the astral rays
showing probably an intermittent growth of the astral fibers. With references to the Belgian group (Pastuels 1955, 59, Pastuels et al. 1957, 58, 63 and Mulnard 1958, Mulnard et al. 1959), however, there were perceived two kinds of granules supravitally stainable with toluidine blue O within the egg plasm of some invertebrates; of these granules the “α-granules” were fine in size and appeared uniformly among yolk granules while the “β-granules” were larger in size and were perceived within the astral region. Therefore, it was emphasized that the “β-granules” did not stain directly, but became stainable with the transfer of some substance to them from the α-granules and that the area within which the metachromatic granules appear showed abundant acid phosphatase activity and mucopolysaccharides reaction.

Otherwise, Rebhun (1956, 57) has pointed out that the yolk nuclei in living Spisula eggs could not be stained with toluidine blue O differing from the Golgi bodies in Mytilus eggs observed by Worley and Worley (1943) and Worley (1944a, b). But he found also two kinds of metachromatic granules stainable with methylene blue; some granules were less than 1/4 μ in diameter and appeared uniformly within the egg plasm, while after centrifugation they were found within the distal centrifugal hemisphere among yolk granules. The other ones, on the contrary, were over 0.5 μ in diameter and showed a localization within the astral region of cleaving egg and appeared in a centripetal sublipid cap. He called the latter ones “aster-associated particles”, and assumed that they might be identified with the β-granules of Pastuels and that they could be stained directly with the dye stuff unlike the result of Pastuels.

As in the cases of above noted authors, such granules as appeared within the astral
Fig. 7. Electron micrograph of the metachromatic granules of smaller sizes. The granules are coated wholly with an electron dense material around the less electron dense cores accompanying a few of glycogen-like particles ($P$). $\times 68,000$
region and showed an evident metachromasia could be perceived in the present sea urchin species not only in the living but also in the semi-thin sectioned eggs. It is noteworthy that they could not easily be discriminated from such yolk granules found within the astral region and neighboring egg plasm among the metachromatic granules and showed an evident metachromasia during the progress of larval development as KATSURA et al. (1964) has recently pointed out. It may be, therefore, assumed that these structures may show an intimate relationship between the metachromatic granules and the yolk granules, suggesting probably a sign of energy producing mechanism.

The fine structure

As above cited, REBHUN (1959b, 60) has found two kinds of granules (α- and β-granules) in the living Spisula eggs, of which the β-granules were identified not only with the metachromatic granules which appeared within the astral region and neighboring egg plasm but also electron microscopically with the "multivesicular bodies" observed in the rat ovum by SOTELO and PORTER (1957). According to him the granules showed an incomplete limiting membrane being often curling back into the interior of the granules with its broken end, and within the neighboring egg plasm of the granules collectively appeared a few small vesicles of ca. 560—690 Å in diameter like the Golgi vesicles. From these results, he offered a working hypothesis that the small vesicles found within the granules were derived from the Golgi vesicles which later become enveloped by a membrane in some manner. It is noticeable that his interpretation shows no concurrence with that of SOTELO and PORTER. According to the latter authors, the vesicles could be recognized near and adherent to the limiting membrane of the multivesicular bodies and were derived from the latter following their breakdown.

Next, as revealed by the present electron microscopy, the granules within the astral region seem to concur with the metachromatic granules in living and semi-thin sectioned eggs and to be variable in size, structure and stainability. Of these granules, the larger ones are of ca. 700—810 nm in diameter and show externally an incomplete limiting membrane and internally a few small vesicles of ca. 560—590 Å in diameter like those within the multivesicular bodies of REBHUN and SOTELO et al. (Figs. 4—6). On the contrary, the smaller ones are of ca. 510 nm in diameter covered with a layer of structureless electron dense materials, but their central cores show a loose framework, suggesting existence of mucopolysaccharide within which a few glycogen-like particles are often perceived (Fig. 7). Besides, the substance of the cores are mixed often with the neighboring egg plasm accompanied by glycogen-like particles and small vesicles in variable sizes showing probably a breakdown and substantial changes of the granules.

Now, investigating the astral region, there can be perceived a few yolk granules in variable sizes with an incomplete limiting membrane and within them yolk particles which disintegrate and form the small vesicles similar to those within the multivesicular bodies as well as the larger metachromatic granules (Figs. 4—6). In other words, these structures show probably successive phases of resolution and absorption of the yolk substances during the progress of egg development.

According to the concept of TAKASHIMA et al. (1965, 66) the yolk substance might be originated from the mucopolysaccharide rich substance of the "giant vacuoles" in young oocytes, whereas the yolk granules in oocytes and mature unfertilized eggs show no metachromasia. But, as KATSURA et al. (1964) noted recently, the metachromasia become evident when the egg development proceeded where the yolk granules began to break down and to be resorbed being substantially changed in order to offer an energy for the egg development,
Briefly, it has been assumed that the metachromatic granules appearing within the astral region of cleaving sea urchin eggs might be identified with the “β-granules” of PASTEELS and the “aster-associated particles” of REBHUN, but they might be nothing but such granules in variable phases of disintegration and resorption which are related to the production of energy required for the egg development.

Summary

The morphological and biological nature of the supravitally recognizable metachromatic granules in living and fixed sea urchin egg following fertilization and during cleavage were investigated by means of light (supravital staining and semi-thin section) and electron microscopy.

Within the egg plasm of living unfertilized eggs one can perceive a small number of metachromatic granules stainable with toluidine blue O which appeared diffusely among yolk granules. But during the segmentation stage they appeared principally within the astral region and increased in number similar to the “β-granules” of PASTEELS as well as the “aster-associated particles” of REBHUN.

In the semi-thin sections (fixed with OsO4, embedded in epoxy resin, 0.5—1.0 μ in thickness, stained with toluidine blue O), there could also be recognized abundant metachromatic granules within the astral region. The granules seemed to be identified with the metachromatic granules in the living egg.

Furthermore, as revealed with the electron microscopy, two types of granules which were identified with the metachromatic granules were recognized. It has been suggested, thereafter, that these granules might be derived from certain yolk granules following structural and chemical changes contrary to the concept of REBHUN. Moreover, it should be emphasized that the biological role of the granules may be intimately related to an energy producing mechanism for the progress of egg segmentation and cell differentiation.
Aster Associated Metachromatic Granules in Sea Urchin Eggs

References


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