ON THE GRANULES IN CYTOPLASM IN RELATION
TO THE FORMATION OF ASCARIS EGG-SHELL*

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INTRODUCTION

On the egg-shell of ascaris only a few works were available yet. Zawadowsky (1914)1), Ida (1930)2), Chitwood (1938)3), and Jacobs (1939)4) made reports on the structure of the shell of eggs of Ascaris megaloecephala, A. suilla and A. lumbricoides. These authors stated that ascaride eggs were covered with three kinds of membranes, viz; outer proteinaceous membrane (protein coat), the shell proper (chitin membrane) and inner lipoidal vitelline membrane. But these results were obtained only from fully developed eggs at the end of uterus in female ascaris and had not concerned with the egg-shell formation. On this point Koizumi (1941)5) wrote in his text-book as follows: three layers of the egg-shell in A. lumbricoides had their origin in maternal body and inner lipoidal vitelline membrane had not been clarified in its origin. Fauré-Fremiet (1913)6) and Chitwood (1938) stated, however, that the shell proper and the vitelline membrane were secreted from the egg cell after fertilization.

In our previous work7) the structure of the reproductive organs in female ascaris was described. We studied on the the formation of the egg-shell of ascaris eggs and found certain granules appearing in cytoplasm in a stage of their ovogenesis. These granules were thought to play an important rôle in the egg-shell formation. Here the findings on the granules and their further changes in female germ cell in the course of their ovogenesis, are to be presented.

MATERIAL AND METHODS

Normal female Ascaris suilla were collected from the pig intestine at Shibaura Butchery. They were kept in 0.9 percent saline solution at 37°C. Only adult worms showing active movement were used in this investigation.

The reproductive organs were taken out from these worms, cut to pieces of about 2.0 cm. long, immersed in various fixatives and embedded in paraffine. A slice of section was 6 or 8µ thick. Aside with the above, a part of uterus and seminal receptacle were cut in 1 cm. long and from it female germ cells were squeezed out on a cover-glass and fixed in Schaudinn's and stained with Mann's solution.

Fixing and staining solutions used in this investigation were as follows:

* Biological Studies on Ascaris Egg VII

215
<table>
<thead>
<tr>
<th>Fixing Solutions</th>
<th>Staining Solutions</th>
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<tr>
<td>80 % alcohol</td>
<td>Mayer's hematoxylin-eosin</td>
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<tr>
<td>30 or 50 % formalin</td>
<td>Mayer's haematoxylin-azur II eosin</td>
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<tr>
<td>Schaudinn's solution</td>
<td>Giemsa stain</td>
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<tr>
<td>Regaud's solution</td>
<td>Cason's solution</td>
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<td></td>
<td>Mann's solution</td>
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<td>Tetra acid stain*</td>
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Material was dehydrated in isopropyl and butyl alcohol and xylol prior to paraffine embedding, and in acetone, acetone-xylol mixtures and xylol prior to mounting in Damar.

Germ cells in reproductive organs were divided into five stages: 1) small round oögonia stage, 2) oögonia growth stage, 3) oöcyte transformation stage, 4) fertilization stage, 5) egg-shell formation stage. In direct smears the position of the germ cells in oviduct and seminal receptacle was shown by noting the distance from the beginning of uterus in centimetre.

**RESULTS**

**Explanation of Stages:**

Stages above mentioned were described here briefly.

1. **Small round oögonia stage**: Oögonia in this stage were found in the initial part of ovary (archovary)\(^8\), corresponding to A-1\(^7\)\(^**\) and upper part of A-2 in ovary. Oögonia in this stage were round in shape and small in size and no axis yet appeared. At lower part of ovary in this stage several tens of oögonia were observed to be divided into several groups. The space among them were filled with fibrous intercellular substance. The axis were considered to be formed from that substance later.

2. **Oögonia growth stage**: this stage occupied the most part of uterus, corresponding to the lower part of A-2, B-1 and B-2. In this stage oögonia increased their cytoplasm and fibrous intercellular substance to which they attached, became clear. These intercellular substance united to a single axis gradually as they came down ovary. During this stage cytoplasm of the germ cells continued to increase in size, consequently the elongated fan-shaped oöcytes were arranged around the axis. At the end of this stage the oöcytes were measured about 200\(\mu\) in their maximum length.

3. **Oöcyte transformation stage**: oöcytes in this stage were found in the oviduct and the upper part of the seminal receptacle, corresponding to B-3, C-1 and the upper part of C-2. In this stage the transformation of grown oöcytes was

\* This staining solution was devised by Ishii and contains four acid dyes (methyl blue, orange G, fast green FCF and fuchsin S).

\** The following letters A-E showed parts of genital organs in our former paper.
observed. Here they transformed their shape gradually into ellipsoid up to the
time just before fertilization.

4. Fertilization stage: The entrance of the sperm to oöcytes took place
in the lower part of the seminal receptacle, corresponding to C-2; here the
formation of the egg-shell began.

5. Egg-shell formation stage: the whole process of the egg-shell forma-
tion was observed in the lower part of the seminal receptacle and initial part
of the uterus, corresponding to D-1, D-2 and D-3. In this stage the egg-shell
was formed on the surface of oöcytes just after fertilization. Following to the
egg-shell formation, the first polar body was observed at the end of this stage.

A. Observations on sections

1. On the preparations fixed in formalin

Small round oögonia stage: no granules were observed in oögonia of this
stage.

Oögonia growth stage: no granules were yet observed in this earlier stage
in cytoplasm of fibrous structure (Pl. I-a). When the structure of cytoplasm in
oögonia changed from fibrous to reticular and oögonia were measured about
100 μ in length, small follicular bodies appeared in the cytoplasm near the axis
(Pl. I-b). These follicular bodies became more stainable by acid dyes as
oögonia came down ovary. In the initial stage the distribution of these
bodies was limited to the part of cytoplasm, near the axis, later they continued
to increase in number and in size gradually until they were distributed in both
parts of cytoplasm near and distant from this axis. These granules were about
4.0–4.5 μ in diameter. The granules in the outer part of cytoplasm were more
dull in their outline than those in the inner part (Pl. I-c).

Oöcytes transformation stage: in this stage the follicular granules became
a little clearer in form and localized at the rivet part of cytoplasm. With the
process of transformation of oöcytes from fan-shape to ellipse, their localization
and further changes were varied. But in general, they were found scattering
in all the parts of cytoplasm (Pl. I-d).

Fertilization stage: when the entrance of the sperm into oöcytes took place
in lower part of the seminal receptacle, all scattered granules in cytoplasm localiz-
ed at the peripheral part of cytoplasm of oöcytes except the part near apexes
in major axis (Pl. I-e, I-f).

Egg-shell formation stage: just after fertilization the egg-shell formation
was observed. The thin first layer was formed on the surface of oöcytes, suc-
cessively the relatively thick second layer appeared beneath the first. After
the second layer was formed, granules were observed ejected between the sur-
face of oöcytes and the inner surface of the second layer, transforming their
shape into a new and thin layer gradually (Pl. I-g). This newly formed layer
was thick in earlier stage of its formation but became thinner afterwards (Pl,
Every space in cytoplasm where granules occupied before their ejection was filled with hyaline cytoplasm and in the central part of cytoplasm, numerous microsomes were observed. The first polar body was formed in ectoplasm, at the same time the ejection of granules to the surface of the oocytes took place (Pl. I-h).

After the formation of the polar body, the cytoplasm attaching to the inner surface of the shell, detached from it and the form of oocytes became a little smaller. Ejecting granules were stained double-ringed, especially with Cason’s solution (Pl. II-i).

The results above mentioned were obtained from the fertilized oocytes. Those from unfertilized ones were to be described briefly. In oocytes without the sperm no localization of the granules in ectoplasm was observed. No remarkable difference was recognized between cytoplasm of unfertilized oocytes not passed seminal receptacle and those in uterus. In every part of uterus the latter were elongated ellipse in form and covered with one layer of the shell. The granules in unfertilized oocytes found in uterus were remained scattered in cytoplasm but less in number and large in size as compared with that of unfertilized oocytes not passed the seminal receptacle. These granules were stained double-ringed especially with Cason's solution (Pl. II-i).

2. On preparations fixed in alcohol

Small round oogonia stage: no granules were observed in this stage.

Oogonia growth stage: the time when granules appeared was the same as in formalin-fixed preparations but the process of their appearing was somewhat different from them. The follicular bodies with a fine grain in their center appeared in the cytoplasm near the axis. This fine grain became larger gradually. Thus the bodies were observed as the colorless ringed space around the large grain. At this time the diameter of this granules with colorless ring were measured about 4.0 μ in maximum.

The aspect of the distribution of the granules with colorless ring were as same as in preparations fixed in formalin (Pl. II-j).

Oocytes transformation stage: the form and distribution of granules in oocytes were about the same as in former preparations.

Fertilization stage: Egg-shell formation stage and unfertilized oocytes: no differences from the preparations fixed in formalin were observed.

3. On the preparations fixed in Schaudinn's solution

Small round oogonia stage: no granules were recognized.

Oogonia growth stage: the time when granules appeared was the same as in formalin-fixed preparations. In the earlier stage of their appearing, follicular bodies were observed in cytoplasm near the axis, and later distributed in cytoplasm near the nucleus. The granules became larger gradually and finally a clear outline was observed around them. At the lower part of the ovary the oocytes inceased in size considerably. In the cytoplasm near the axis, the large
folicular granules with dull outline were observed, whereas in the rest of cytoplasm, granules with fine outline were found. Distribution of these granules with fine outline was limited to the zone within about 100 μ from the outer margin of the oocytes (Pl. II-k).

Oöcyte transformation stage: in this stage oöcytes were observed to be detached from the axis. Localization of both kinds of granules in oöcytes just after freeing from the axis, was about the same as in those of the former stage (Pl. II-l).

Fertilization stage, Egg-shell formation stage and unfertilized oöcytes: granules showed no remarkable differences as compared with that in the above mentioned stage.

4. On the preparates fixed in Regaud's solution

Small round oögonia stage: no granules were observed.

Oögonia growth stage: the time when the granules appeared was as same as that in former three kinds of preparates. Granules in this stage resembled those in alcohol-fixed preparates (Pl. II-m).

Oöcytes transformation stage, Fertilization stage, Egg-shell formation stage and unfertilized oöcytes: distribution, localization, ejection and stainability of the granules in these stages were as same as in former preparates, and no difference was observed between unfertilized oöcytes in this preparates and in others. The appearance of the granules with the clear outline in this preparates was a remarkable difference as compared with that in others.

B. Observation on direct smears

The preparates were fixed in Schaudinn's solution only.

On the part 20–15 cm distant from the beginning of uterus: in this part oöcytes were found attached to the axis at their one end of cytoplasm. Here the observation was very difficult because of the summation of oöcytes in oviduct were seen free from axis. In its lower part two different figures of oöcytes were seen: the one was ordinary fan-shaped and the other elongated fan-shaped. This lower part was corresponded to that from the end of growth stage to early stage of transformation in sections.

In this part three kinds of granules were observed morphologically in cytoplasm of oöcytes. The first one was fine granules with dull outline stained red in centre and blue in outline. Their diameter was 2.5–1.0 μ. (Pl. II-n). The second was folicular granules with dull outline and stained light blue and in their centre part a clear outlined granules just like the first one, was found The second granules had the diameter of 4.0–5.0 μ. The third was folicular granules with dull outline and stained lightly blue. It had no tiny granules in centre (Pl. II-o). The first granules were chiefly observed in elongated oöcytes at the upper part of this stage and most of them were distributed in the
part of cytoplasm near the axis. As oocytes came down the oviduct, the second granules were observed more numerous. As the fan-shaped oocytes came down the oviduct further, the third granules were seen scattering in their cytoplasm. In the case that these three kinds of granules were observed in an oocyte, the first one was distributed in cytoplasm near the part which was attached to the axis, the third one in the opposite side of it and the second one in the area between them. Along with these three kinds of granules, numerous granules showing transitional aspects from the first to the third were observed. They were shown in Plate III-ii. The morphological changes in the course of transition of granules were as follows; around the first granules initially appeared, follicular part stained blue lightly, was formed. Thus the second one was formed. When the first granules in second disappeared gradually, then it transformed into the third itself.

On the part distant less than 15 cm. from the beginning of the uterus: this part belonged to the lower part of ovary and seminal receptacle in which the oocytes were observed elliptic in shape as they came down genital organs. The granules scattering in cytoplasm in the oocytes of this part were stained blue lightly. Their distribution, however, varied somewhat according to the way of the transformation of the oocytes. It was very difficult to observe the contents of oocytes because the oocytes became thick and were, therefore, stained dark in the seminal receptacle. On the contrary, these smears in the earlier stage of the egg-shell formation as well as transformation were convenient to study the shape transformation of oocytes.

DISCUSSION

In even four kinds of fixed material especially in oogonia growth stage of them, the granules in sections showed different distribution and figures. The granules were seen most clearly and invariably in preparates fixed in Regaud's solution, and then those in alcohol. In formalin-fixed preparates the granules were observed rather dull in their form, and in Schaudinn's-fixed ones two kinds of different granules were observed; the one was in the inner and the other in the outer part of cytoplasm respectively. (Pl. II-k, & l). But these different granules seen in various fixed preparates were thought to be same in their nature. The basis of this thought was as follows; the time of appearing of granules in oocytes cytoplasm, of their localization in definite part of cytoplasm just after fertilization, and of ejection from the ectoplasm between the surface of oocytes and the inner surface of the second layer, were all the same in every kind of sectioned preparates. In cytoplasm of oocytes no other granules which might be confused with the present granules were recognized in every kinds of preparates. The reason why the granules were observed as if they were different with one another, would be attributed to the effects of various fixatives used, namely to the difference of infiltrating velocity of the fixing agents or
their components into material. The most reasonable example to support this idea was the figures of granules in Schaudinn's-fixed preparates, in which two sort of granules were seen. The one was the small and sharp granules in peripheral zone of cytoplasm and the other, folicular and large ones stained lightly in inner part of it. The distribution of these granules with a sharp outline was limited to the zone within about 100 µ distant from the outer margin of the oocyte arranging radially both in growth and early transformation stages. these phenomena would be explained by the difference of infiltrating velocity in each component of fixatives into material. In the oocytes less than 100 µ in length in the transformation stage, every granules took almost the same figure. This fact also serves as the testimony that all granules in different figures in various fixed preparates would be the same. As well known, alcohol contracts the cytoplasm and formalin swell up it generally. On the present preparates fixed in alcohol and formalin, granules with these characteristic figures were manifested. From these facts the apparently different figures and distribution of granules in each kind of preparates should be reasonably attributed to the effects of each fixative used.

Provided that all granules in the different shape and distribution were the same in nature, what is the function of the granules? Granules in oocytes prior to fertilization were observed scattered in cytoplasm. Just after fertilization they localized in peripheral parts near both apexes in major axis of the cell, and the first layer of the egg-shell was observed liberating from the surface of the oocytes immediately. Successively the layer was formed beneath the first one and the granules ejected from ectoplasm beneath the second. Consequently an innermost thin layer of the egg-shell was newly formed. From these facts it is clear that the granules play an important rôle in the formation of the egg-shell. It is doubtful whether the granules take part in the formation of the second layer directly. The fact that granules localized in ectoplasm during the period of the formation of the second layer, suggests that the material with which the second layer will be formed would be supplied from granules. But any morphological changes on the granules during the whole period of the second layer formation were hardly observed. Thus granules should be considered taking no part in the formation of the second layer directly.

Just after the presentation of the result of the present study at the 22nd meeting of Japanese Parasitological Society, we found the paper by R. Yama-guchi (1952) on the oogenesis of Ascaris, in which the granules in cytoplasm of its oocytes had been described. The granules he showed in his paper are considered to be the same as those we reported in this paper. But in detail these are some differences between his and our opinion concerned with granules. According to his report the time when the granules appeare in cytoplasm of oocytes was almostly same as one we recognized. Just after fertilization, however, he observed that some of the granules were localized in perivitelline space
formed by contraction of oocytes after fertilization between the surface of oocytes and the egg membrane which was present before fertilization. But we could not recognize the presence of the perivitelline space around the oocytes after fertilization. The granules in this space, therefore, were not observed. The present authors thought that his "perivitelline space" was a space that had been formed as the results of artifact caused by microtechnique. After the formation of the thin layer, he said that his minor granules were ejected to form the thick layer of the egg-shell, whereas we could not find any evidence that proved to be the direct relation between the granules and the thick layer.

In the unfertilized oocytes in uterus, granules were still present scattered in cytoplasm. These phenomena could be observed in our case too. But we could not recognize that the granules near the egg membrane which presented on the surface of oocytes before fertilization, were, afterwards, fused each other and thus a very thin and incomplete outer layer was formed in the unfertilized oocytes.

Motomura (1936, 1947)\textsuperscript{10} and Endo (1952)\textsuperscript{11} published interesting reports on the formation of the fertilization membrane in ova of various kinds of Japanese sea urchins. They observed the similar granules in them as those in ascaris eggs in the present paper. According to their reports these granules (0.5 \( \mu \text{m} \) in diameter) were stained with Janus Green B specifically (they called them janus green granule), observed scattering in cytoplasm of unfertilized eggs, localized in ectoplasm with the maturation process of eggs and ejected beneath the vitelline membrane when the sperm entered into the eggs. Then membrane was hardened by calcium ion in sea water. As for the fertilization membrane formation there have been two theories (vide. Shoyama 1940)\textsuperscript{12}; the one insisted that the antecedent substance of the fertilization membrane existed already on the surface of the eggs prior to fertilization and when the eggs were fertilized this substance became the fertilization membrane. The other said that prior to fertilization no antecedent substance existed on its surface and the fertilization membrane was newly formed by the entrance of the sperm into the egg. From the facts already quoted, Motomura insisted that the fertilization membrane was formed with the vitelline membrane which was antecedent substance and together with material from janus green granules in ectoplasm. Comparing those janus green granules in sea urchin eggs with those of ascaris eggs, a close resemblance was seen in the numerous points. The egg-shell of ascaris egg was not able to be observed in unfertilized oocytes in the oviduct as well as in upper part of the seminal receptacle. The first layer of the egg-shell was separated from the surface of the oocytes immediately after fertilization and was accompanied with the formation of the second layer beneath the first one and the third layer was formed just beneath the second one by ejecting of granules from ectoplasm to the surface of the oö-
EGG-SHELL OF ASCARIS

cytes. The facts that separation of the first layer from the surface was accompanied with the formation of the most part of the egg-shell in fertilized oocytes (Pl. II-p) and that such a process was not able to be seen in unfertilized ones, suggests that the egg-shell in ascaris eggs was a sort of fertilization membrane. On this point, Lillie (1919)\textsuperscript{13} already stated that a very thick and resistant \textquoteleft\textquoteleft fertilization membrane was formed as an immediate result of fertilization in \textit{Ascaris megaloecephala}.\textquoteright\ But it is unknown whether he called the whole egg-shell or only a part of it as \textquoteleft\textquoteleft fertilization membrane\textquoteright\ . According to our observation on eggs of \textit{Ascaris suilla}, the first and second layer of them were thought to be correspond to the fertilization membrane in their nature but it would be too hurry to insist that the third layer was doubtlessly a part of the fertilization membrane. If the fertilization membrane should be defined as a membrane which was formed on the surface of the egg just after fertilization, it would be possible to think the whole egg-shell as a fertilization membrane, because the third layer was made out of the granules above mentioned, successively after the formation of the second one, although the third one was formed considerably later. At that time the later formation of the third layer should be understood as a result of differentiation of the fertilization membrane by a long course of its parasitic life.

At any late the granules of sea urchin eggs found by Motomura and those in ascaris in the present paper are considered to have the same function; both of them take part of the formation of the fertilization membrane directly. It is very interesting to find the similar granules on the oocytes of \textit{Eimeria}. The granules in \textit{Eimeria} were reported by Hosoda (1928)\textsuperscript{14} and Roudabush (1937)\textsuperscript{14}. On \textit{Eimeria avium} Hosoda reported that the plastic granules and hematoxylinophilic granules appeared in cytoplasm of macrogametes before fertilization. The former appeared in the peripheral part of cytoplasm and the latter inside the former. They were 2.0 and 1.5 $\mu$ in diameter respectively. After fertilization these granules were ejected on the surface of macrogametes and made themselves into a kind of wall of oocytes. The same phenomena were observed in \textit{Eimeria nieschulzi} by Roudabush.

It would be very important to study out the chemical nature of the granules because it would reveal the nature of the egg-shell. The result obtained on this point, however, were as follows; granules in ascaris eggs were stained in acid dyes (eosin Y, fast green FCF, methyl blue, fuchsin S and orange G) and not stained in basic dyes (methyl green, toluidine blue and pyronin Y). They were stained also in Hematoxylin. When stained by one-step-stain consisted of several acid dyes, a difference in their affinity to the granules was found. Between the stainabilities of granules of ascaris egg and those of sea urchin egg, a considerable difference was seen.
SUMMARY

1. In ova of Ascaris suilla granules stained with various acid dyes appeared in cytoplasm on the process of ovogenesis. They increased in size in the whole stage of growth and were measured about 3–4 μ in diameter just before fertilization.

2. The localization of the granules were confined to the area of the ectoplasm when the sperm entered into oocytes. During the formation of the first and second layers of the egg-shell, they were observed without any morphological changes.

3. Just after the formation of the second layer, granules disappeared from ectoplasm and ejected to be interposed between the surface of oocytes and inner surface of the second layer. Thus the third layer was formed.

4. In unfertilized eggs in uterus, granules were scattered in cytoplasm and became less in number and larger in size as they came down the uterus. Granules were found still remained in cytoplasm of oocytes at the end of uterus.

5. The relation of the granules to the janus green granules of sea urchin egg was discussed.

ACKNOWLEDGEMENT

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EXPLANATION OF PLATES

Plate I–a: Fibrous structure of the cytoplasm in oocytes in growth stage.
   b: Reticular structure of the cytoplasm in growth stage.
   c: Distribution of the granules in oocytes in later growth stage.
   d: Granules of oocytes in transformation stage.
   e: Granules localizing in the ectoplasm of fertilized oocytes.
   f: Granules localizing in the ectoplasm and sperm in oocytes.
   g: Ejecting and disappearing granules in egg shell formation stage.
   h: Formation of the third layer and first polar body at the ectoplasm.

Plate II–i: Granules stained double-ringed with Cason's solution in unfertilized egg in uterus.
   j: Granules in growth stage fixed with alcohol.
   k: Granules in growth stage fixed with Schaudinn's solution.
   l: Same to above.
   m: Granules in growth stage fixed with Regaud's.
   n: Fine granules in smears.
   o: Large granules in smears.
   p: Formation of the first layer just after fertilization in smears.

Plate III–i: Disappearing process of granules, I, II, & III).
   ii: Transitional process of granules (from a. to e.) and other transitional aspects (f. & g.) in direct smears.
Plate III.

i)

ii)

\[ a \quad b \quad c \quad d \quad e \quad f \quad g \]