ON THE STRUCTURE AND FORMATION PROCESS
OF THE EGG-SHELL OF ASCARIS OVA*

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Up to the present time, only a few works are available on the structure and formation process of ascaris egg-shell. Zawadowsky (1929)\(^1\), as the result of his detailed investigation, revealed that the egg-shell of Ascaris mealocepha\(\)la consists of five layers. Ida (1929)\(^2\) reported that he could recognize four layers on the egg-shell of A. lumbricoides and A. suilla by various chemical treatments of the eggs. On A. lumbricoides ova, chemical properties of the shell were studied by Chitwood (1938)\(^3\) who found chitosan in its “shell proper”. The “shell proper”, he called, was considered to be the whole shell layers except the protein coat. Details on the shell structure were not described in his report. Ohuchi (1951)** observed the effects of acids and alkalies on the protein coat of A. lumbricoides ova and recognized the swelling of the eggs by immersing them in inorganic acids and called this phenomena “intra-egg-shell swelling”. But no report has so far been published on the formation process of the egg-shell. In a previous paper, the present author and co-worker (1952)\(^4\) reported on the granules in the cytoplasm of oocytes, which formed the 3rd layer of the ascaris egg-shell. But in that report, the formation of the 1st and thick 2nd layer of the shell, especially that of the so-called inner vitelline membrane formed just beneath the 3rd one, was not dealt with.

In the present work, the author confirmed the structural elements of the shell by means of acid and alkali treatments and observed, furthermore, its formation process in A. suilla ova.

MATERIAL AND METHODS

Material used for studying the shell structure in this investigation was fertilized eggs in the end of the uterus (1–2 cm long) of Ascaris suilla. Methods for the treatment of ova with acids and alkalies were the same as those reported by Ida (1929)\(^2\). Chemicals were dropped at one end of the cover-glass under which eggs were mounted with water, and they were observed under the microscope. Water used for mounting was absorbed by placing filter-paper at the other end of the cover-glass. Such an operation was repeated until the reagents used completely replaced the water. When eggs were treated with two kinds

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** Private communication
of reagents, the eggs were immersed in a test tube containing one of these reagents, and after observation, they were washed with water by centrifuging (4 times, at 2,000 r.p.m.), then immersed into the other tube containing the other reagent. These procedures were carried out under room-temperature. Chemically pure acids and alkalies were employed as original solution unless especially remarked.

In order to study the formation process of the shell, both sections and smears were prepared with the eggs obtained from various parts of the uterus. The living specimens were directly observed on mounting with physiological saline solution. Procedures for these preparations were referred to our previous report. A different method from the former, potassium bichromate treatment was introduced; viz. the material fixed with Regaud's solution was immersed for a week in 3 percent potassium bichromate.

RESULTS

1) Treatment of eggs with strong acids and alkalies
   a) With acids

   Nitric acid: When eggs were immersed in nitric acid, the protein coat became suddenly swollen, contracted to the surface of the shell and was subsequently dissolved away. At the same time the thick middle layer of the shell was expanded and the outer layer of the shell was bulged by this expansion (Pl. 1–1). The outer layer bulged by the expansion and the thick layer expanded by the nitric acid treatment were called the 1st and the 2nd layer respectively. Another layer, the innermost one in the shell proper, which remained unchanged by the acid treatment and called the 3rd one and so-called inner vitelline membrane with uneven inside surface, were observed. The space between the 1st and 3rd layer was observed to be transparent and the 2nd one seemed as if disappeared. Observing in detail, very fine microgranules were recognized in that space. When the eggs treated with nitric acid were, furthermore, washed with water, the structural pattern in that space turned into an indistinct macrogranular one (Pl. 1–2). Such a macrogranular part of the egg-shell was easily separated from the 3rd layer by moving the cover-glass slightly.

   Hydrochloric and sulphric acids: Effects of hydrochloric and sulphric acids on the shell of ascaris ova were almost the same as that of nitric acid. The 3rd layer was observed to be uneven after washing the eggs treated with the acids. Effect of sulphric acid on the 3rd layer was the most remarkable among the various acids, and consequently the layer was torn and the egg cell was also destroyed after a short period of immersion.

   b) With alkalies

   Sodium hypochloride: When the eggs were treated with Na-hypochloride (antiformin), the protein coat was dissolved in a short period of time and a very thin membrane, which was light-blue in color and situated just inside of
the protein coat, was bulged slightly at both poles of the egg and soon began to be dissolved. This membrane was much thinner than the layer bulged by the acid treatment, and it seemed to be a part of the 1st layer. At the beginning of the immersion into antiformin, no changes were observed in the 2nd layer. But after immersion for 10 minutes, the 2nd layer was found swollen and became 1.5-2.0 times thicker than the normal one and looked to be semi-transparent with light greenish color (Pl. 1-3). The 2nd layer in this state was liberated from the 3rd layer by moving the cover-glass slightly (Pl. 1-4). At that time, the presence of the 1st layer was not distinguished.

Potassium hydroxide: When the egg was exposed to 50% KOH solution, the protein coat was coagulated immediately and adhered to the shell. Even 1 hour later, no changes were observed in these eggs (Pl. 1-5). Eggs were exposed to KOH solution of various concentrations for various immersion periods. Results obtained from these experiments were shown in Table 1. In each of 5, 10 and 15% KOH solutions, the protein coat was dissolved and a thin membrane dissolved by antiformin treatment was liberated from the 1st layer partly or wholly (Pl. 1-6). In each of the cases of 20, 30, 40 and 50% KOH solutions, on the contrary, the protein coat was coagulated partly or wholly on the shell.

Table 1. Effects of KOH on the protein-coat and the outermost layer of the shell

<table>
<thead>
<tr>
<th>Immersion time</th>
<th>Changes observed after immersion</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOH (%)</td>
<td>1 hour</td>
</tr>
<tr>
<td></td>
<td>outermost layer</td>
</tr>
<tr>
<td>5.0</td>
<td>a.</td>
</tr>
<tr>
<td>10.0</td>
<td>a.</td>
</tr>
<tr>
<td>15.0</td>
<td>b.</td>
</tr>
<tr>
<td>20.0</td>
<td>c.</td>
</tr>
<tr>
<td>30.0</td>
<td>?</td>
</tr>
<tr>
<td>40.0</td>
<td>?</td>
</tr>
<tr>
<td>50.0</td>
<td>?</td>
</tr>
<tr>
<td>Control</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Signs:  
a: liberated on the poles  
b: liberated on some parts of the shell  
c: liberated on all surface of the shell  
?: unobservable because of the coagulation of the protein coat  
A: dissolved  
B: almostly dissolved  
C: swollen and liberated from the shell  
D: swollen and liberated from some parts of the shell  
E: coagulated on the shell  
F: swollen slightly

These experiments were carried out at room-temperature.
c) Successive treatment of the egg with acid and alkali

Nitric acid and antiformin: Results obtained simply with nitric acid treatment were already stated in a preceding chapter of this paper. When the eggs were treated with antiformin after the treatment with nitric acid, the liberation of the thin membrane from the 1st layer bulged by nitric acid treatment was observed (Pl. 1-7). When this state was maintained for a long period of time, the 1st layer became gradually invisible, and that part of the shell showing the macrogranular structure, was softened, and then liberated from the 3rd layer by moving the cover-glass slightly.

Potassium hydroxide and nitric acid: After immersing the eggs in 15% KOH solution for 1 hour and washing, the eggs were further treated with nitric acid. In this case the 2nd layer was suddenly swollen and dissolved. In the case of 70% diluted nitric acid solution used in the successive treatment, swelling and dissolving phenomena of the 2nd layer were observed more clearly and slowly.

2) Formation process of the egg-shell

a) Changes on the surface of oocyte prior to fertilization

In the oviduct and the upper part of the seminal receptacle, no egg-shell was observed on the surface of the oocytes except the thin membrane observed in the upper part of the oviduct. This membrane turned to be a clear layer as the oocyte came down in the oviduct and recognized as a distinct structure of a layer on the surface of the oocytes when they reached at the upper part of the seminal receptacle (Pl. 1-8).

b) Formation of the 1st and 2nd layer

The formation of the egg-shell took place after the entrance of the spermatozoon into the oocytes. Just after fertilization, the above-mentioned thin layer (the 1st layer) was lifted from the surface of the oocyte. Such a lifting of the 1st layer was observed initially on a certain part of the surface of the oocytes and later on all part of it (Pl. 1-9). On careful observation at that time, semi-transparent and light yellowish substance was found between the 1st layer and the surface of oocytes in smears. Occasionally the 1st layer was lifted to a height of 5μ from the surface of eggs in smears. This layer was observed to be partly separated from the surface and appeared to be undulated but was no longer observed as such after its separation was completed. The 1st layer was separated completely from the surface by the secretion of semi-transparent and yellowish substance of which the 2nd layer was composed. The formation of these two layers, the 1st and 2nd layers, occurred in the oocytes after fertilization at the end of the seminal receptacle and the beginning of the uterus.

c) Formation of the 3rd layer and polar body

After the formation of the 2nd layer, the 3rd layer was formed beneath the 2nd layer by the ejection of cortical granules from the ectoplasm to the surface of the oocytes as already reported (Pl. 1-10).
In the ectoplasm of oocytes, the 1st polar body was formed as the result of maturation division. The time of its formation coincided with that of ejection of granules, of which the 3rd layer was composed. The 1st polar body, some part of which was buried into the 3rd layer, was observed attaching to the inner surface of it. In sections, the body was measured about 3.0–4.0 μ long and 1.0–2.0 μ wide (Pl. 1–11, 13 & 14).

d) Formation of inner vitelline membrane

The formation of the vitelline membrane took place after that of the 3rd layer, but this membrane could not be observed in ordinary sections. In the eggs treated with potassium bichromate solution, the aspect of its initial formation was observed: Not membraneous but fiber-like substance was found in the space between the 3rd layer and the surface of oocytes. On the contrary, in the living oocytes at this stage, a distinctive vitelline membrane was recognized. When eggs with vitelline membrane at the end of the uterus were treated with potassium bichromate, no membrane was observed in such a sectioned preparations. On account of these reasons, the formation of the vitelline membrane was studied by the observation on living specimens.

After the formation of the 3rd layer, the inner vitelline membrane was observed as a thin layer between the 3rd layer and the surface of the oocytes. At that time the point corresponding to that of the 3rd layer in the shell was recognized as a conspicuous dark line. As eggs came down in the uterus, the membrane became thicker (ca. 2 μ). When the membrane was completely formed, the egg cell was detached from the membrane at the poles and became round in its shape. Thus so-called semi-circular spaces were formed at both ends of long axis of the shell-shape. The figure of the 2nd maturation division was sometimes observed in the semi-circular space (Pl. 1–3).

The formation process of the shell and the inner vitelline membrane was schematically shown in Plate 2.

e) Egg-shell of unfertilized ova

The egg-shell of an unfertilized ova in the uterus was identical to the thin membrane on the surface of an oocyte in the upper part of the seminal receptacle. Findings on the surface of unfertilized oocytes in the oviduct have already been described. Unfertilized oocytes passed through the oviduct and came down into the uterus along with fertilized ones. Their surface became thickened slightly but the thickening of the surface of the unfertilized one was found ceased when they reached at the beginning of the uterus. Thereafter, no remarkable changes were observed on their surface until they came down to the end of the uterus. In general, no space was observed between the egg-shell and the oocytes. The unfertilized oocytes, therefore, were elongated ellipsoid in their shape. Some part of the ectoplasm of unfertilized oocyte was sometimes observed detaching from the shell. The shell on such a part was measured about 0.5 μ in thickness.
DISCUSSION

Effects of alkalies on the outermost thin membrane of the 1st layer have not yet been dealt with. According to the present investigation, the liberation of the thin membrane from the 1st layer was observed in the eggs treated with antiformin and potassium hydroxide. When antiformin is used for removing the protein coat, a thin membrane on the 1st layer was dissolved together with protein coat. The reason why the dissolution of this membrane has not so far been recognized, is that the strong action of the antiformin on the protein coat as well as on the thin membrane. In the case of KOH, the liberation of the membrane was easily observed in the eggs treated with KOH solution of less than 15%. On the contrary, in the case of more than 20% KOH solution, no changes were observed on the membrane because of the coagulation of the protein coat.

The fact that the 1st layer consisted of two membranes was clarified as the results of the treatments of eggs with nitric acid and antiformin. On the other hand, when the oocytes forming the 2nd layer at the beginning of the uterus were stained with Giemsa's solution, two membranes were differentiated on the outermost part of the shell. Formation process of this double-membrane structure was not investigated even in the serial sections. The author's idea that the 1st layer consists of two membranes is supported by the findings obtained from Giemsa's staining and successive treatments of eggs with acid and alkali.

Ida (1930) reported that the bulging of the 1st layer caused by the effect of inorganic acids (nitric, chloric acids & etc.) was interpreted as the separation of the 1st layer from the 2nd one by the penetration of the reagent into the space between them. According to his interpretation, the reagents existing between the 1st and 2nd layers may be washed away through the 1st one. But the layer bulged with acid was hardly shrunk by washing the egg with water, and therefore, the macrogranular structure observed after washing was not understood. Provided that this granular structure is composed of water-insoluble substance formed from that of acid-soluble one on the 1st layer by washing, through which the acid passes, macrogranular substance was too much in quantity and the resistance of the 1st layer against pressure was also too strong to originate this substance from the 1st one. When acid treatment of eggs was carried out slowly, the swelling of the 2nd layer could be observed under the microscope. Changes in the egg-shell treated with acid will be reasonably understood as follows: After the penetration of the reagents through the 1st layer they reacted on the 2nd layer to make it swollen. Consequently the 1st layer was bulged. By washing these eggs, the substance dissolved with acid in the 2nd layer was precipitated as a water-insoluble substance, which appeared as the macrogranular structure.
As already stated, the 2nd layer was not observed till the 1st one was separated from the surface of oocytes. The substance, of which the 2nd layer was composed, was presumed to be a kind of colloidal substance with low viscosity in the early stage of its secretion from oocytes. This presumption is based on the fact that the 1st layer was observed undulated in its form when the 1st layer was lifted from a part of the surface of eggs in smears. Afterwards the 1st layer was observed not to be undulated after completely lifted. The 2nd layer was apt to be compact as oocytes come down in the uterus. It is very easy to distinguish the 1st from the 2nd layer morphologically. In their stainability with various dyes, the latter was liable to be less stainable than the former.

Ida (1929) reported that in a certain part of the 2nd layer a pitlet was observed, which played an important rôle in the emergence of larvae from the shell. Regarding the special structure on the shell of parasitic nematode ova, Baylis (1929), Dorman (1931) and Ackert (1931) reported on the special structure of a certain part of the shell in Ascaridia, Heterakis papillosa and Ascaridia lineata respectively. Dorman (1931) stated that the apparatus (concerned with pitlet) on the shell played an important rôle in the emergence of larvae, and Ackert (1931) was also of the same opinion about it. Although most of investigators have been of the same opinion on the functional rôle of this apparatus, it is very difficult to find the reports describing its finer structure and formation. Izumi (1952) reported that he found the pitlet in the part of the shell corresponding to that of the surface of the cell stained dark with methyl-green pyronin in A. suilla ova. And the part of the ectoplasm which had adhered to the axis in the ovary, could not secret the substance, which the 2nd layer was composed of. The pitlet, therefore, was formed due to the partial deficiency in the secretory function of the shell substance on the surface of the egg cell. The author considers as follows: The 1st polar body produced as the results of the first maturation division was buried in the shell substance. Consequently this part of the shell was optically observed as the pitlet. In sections, the formation of the first polar body in a certain part of the ectoplasm in oocytes was often observed after the ejection of the granules, which the 3rd layer was made of. This first polar body showing Feulgen positive reaction was recognized adhering to the inner surface of the 3rd layer and partly being buried in it (Pl. 1–13). In sections, the pitlet as described by Ida (1930), was not found on the 2nd layer of both A. suilla and A. megalcephala. If the pitlet was really present on the 2nd layer, it will be natural to be able to discover the pitlet itself or something else showing its presence on the 2nd layer. According to Ackert (1931), one end of the egg of Ascaridia lineata was a structure bearing somewhat resemblance to a micropyle, but which was proved to be a solid conical appendage and was able to be freed from the shell with the aid of Chamber's micromanipulator. These results obtained from the egg of Ascaridia lineata, belong-
ing to the same family as *A. suilla* and *A. megalococephala* will greatly support the author’s idea. Baylis (1929), who considered the apparatus to be an internal thickness of the shell on the egg of genus *Ascaridia*, and Dorman (1931) who described it as an opercular plug in the egg of *Heterakis papillosa*, thought it no pitlet. In addition to these findings on the egg of *A. megalococephala*, the author was able to observe a relatively large solid body (ca. 5μ long) between the 2nd layer and vitelline membrane. The part of the 2nd layer where the body adhered to was slightly thin. Provided that the polar body is sooner or later destined to disappear, the place where the body disappeared in the shell will be observed optically as the conical structure like a pitlet. At any rate, it is very reasonable to think that this apparatus in the shell plays an important rôle in the emergence of the larvae as stated by Ida and Dorman, because this part of the shell was liable to be broken by the movement of the larvae.

The inner vitelline membrane was not observed in the ordinary sections as described already. Chitwood (1938)\(^3\) considered that the vitelline membrane was composed of a kind of sterol, and Izumi (1952)\(^10\) who made investigation on the penetrating activity of the chemicals into the ascaris eggs, assumed that lipid substance would be one of the components of the shell, basing on the fact that fat-soluble substance had a high penetrating activity into the egg. The author, therefore, employed the chroming method of potassium bichromate on the material fixed with Regaud’s solution. But the vitelline membrane was observed only in the early stage of its formation. This fact will be attributable to the insufficient penetration of various chemicals into the egg after the complete formation in quality of the 2nd and 3rd layer. In other words, chemicals will penetrate through the 2nd and 3rd layers in the early stage of the formation of the vitelline membrane and react with it and make it observable by fixing. After the complete formation of the 2nd and 3rd layer in quality, chemicals will no longer pass through them and react with the membrane. Thus the vitelline membrane will be dissolved away with the hydration agent used in preparation procedure. On the other hand, the surface of the egg cell was found by observing the living specimens, adhered to the inside of the shell or vitelline membrane till the membrane formation was completed. While in section, the cell shrunk and liberated from the inside of the 3rd layer was observed at a corner inside the shell after the formation of the 3rd layer or the first polar body (Pl. 2–12 upper, 13, & 14). However, this was not observed in the living specimens, and therefore this will support the author’s idea already expressed.

Concerning the origin of the shell, Koizumi (1939)\(^11\) stated that the 1st, the 2nd and the 3rd layers of the shell in *Ascaris lumbricoides* ova had their origin in its maternal body, but the inner vitelline membrane had not been clarified in its origin. From the author’s observation, the origin of the vitelline membrane as well as the shell proper is considered to exist in the egg cell itself. Namely, the 1st layer was the outermost one of unfertilized oöcytes and lifted
by the substance secreted from the oocytes, of which the 2nd layer was composed. The 3rd layer was formed by ejecting the granules in the ectoplasm as reported previously by the present author and co-worker. The innermost vitelline membrane is hardly thought to originate from other sources than that of the egg cell.

**SUMMARY**

The fine structure of the egg-shell of *Ascaris suilla* was investigated from the specific changes of its respective layers after the treatment of eggs with acids and alkalies. The formation process of the egg-shell was also studied by observing sectioned and smeared preparations and living specimens. The result obtained were as follows.

1) The egg-shell of *Ascaris suilla* ova was composed of three layers; the 1st layer (outermost) bulged by inorganic acids, the 2nd layer (thick) became swollen by acids, and softened and liberated from the 3rd one by the treatment with alkalies, the 3rd layer remained unaffected even with both alkalies and acids.

2) A sheet of thin membrane, furthermore, was liberated from the 1st layer by the treatment with alkalies. Therefore, the 1st layer was composed of two membranes. The outer one of the 1st layer, was dissolved by exposing to antiformin but remained to be liberated from the inner one in the case of the eggs treated with 5.0–20.0 % KOH solution.

3) The 1st layer was identical to that of an unfertilized oocyte, which lifted by the substance secreted from oocytes. This substance turned to the 2nd layer afterwards. The 3rd layer has already been described in our previous paper.

4) The inner vitelline membrane was observed beneath the 3rd layer on the living specimens. But it was not recognized in sections.

5) The shell of an unfertilized egg at the end of the uterus was homologous to the surface of the oocytes prior to fertilization. The shell of an unfertilized egg in the uterus was thicker than that in the oviduct.

6) The so-called “pitlet” on the shell was the first polar body buried between the 3rd layer and the vitelline membrane. This body was observed optically as the pitlet.

7) The shell proper (the 1st, the 2nd and 3rd layers) as well as the vitelline membrane were originated from the egg cell (oocyte).

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REFERENCES


EXPLANATION OF PLATES

Pl. 1-1. Egg treated with nitric acid prior to washing
-2. Egg treated with nitric acid after washing (macrogranular structure)
-3. Egg treated with antiformin, showing swelling and softening of the 2nd layer
-4. Egg treated with antiformin, showing liberation of the 2nd layer from the 3rd one
-5. Egg treated with conc. KOH sol. (30%), showing the coagulation of the protein coat
-6. Egg treated with diluted KOH sol. (10%), showing the liberation of the 1st layer
-7. Egg treated with nitric acid and antiformin, showing the thinner membrane liberated from the first layer
-8. Oocyte prior to fertilization (in smear)
-9. The 1st layer lifted just after fertilization (in smear)
-10. Localization and ejection of cortical granules, of which the 3rd layer was composed
-11. The first maturation division in the ectoplasm of the oocytes just after ejecting granules (in section)
-12. Egg (lower) just after ejecting granules and egg (upper) with the 1st polar body adhered to the shell (in section)
-13. The 1st polar body adhered to the shell (in section)
-14. Ibid. at the end of the uterus (in section)

Pl. 2- Formation process of the egg-shell and vitelline membrane in A. suilla ova (schematic drawing)
-a. Unfertilized oocyte in the upper part of the oviduct
-b. Unfertilized oocyte in the upper part of the seminal receptacle
-c. Lifting of the 1st layer by secretion of the substance of which the 2nd layer is composed
-d. Compact 2nd layer and ejecting granules
-e. The first maturation division after the formation of the 3rd layer
-f. The first polar body and vitelline membrane formation
-g. Detaching of the cell surface from the vitelline membrane after its formation
-h. Complete formation of the semi-circular space

Signs: 1. cortical granules; 2. microsome in cytoplasm; 3. 1st layer; 4. substance secreted from cell; 5. 2nd layer; 6. 3rd layer; 7. 1st polar body; 8. vitelline membrane; 9. so-called semi-circular space; 10. egg cell