BIOLOGICAL STUDIES ON ASCARIS EGGS
IV. ASCARIS ONTOGENESIS OBSERVED BY NUCLEIC ACID STAININGS, AND DISTRIBUTION OF FAT OR GLYCOGEN FOLLOWING THE DEVELOPMENT OF ASCARIS EGGS

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INTRODUCTION

The development of ascaris eggs, especially those of Ascaris megaloccephala, was precisely studied by T. Boveri. He cleared the mode of cleavage and formation of embryo. Several detailed reports have also been published on chromosomes of ascaris egg mainly in prefertilization stadium. However, the cytological and embryogical studies on ascaris eggs after fertilization seem to be relatively few.

The ascaris eggs has fairly strong resistance against chemicals as previously reported by the author, and it also reveals fair resistance against common fixative solutions. As the egg-cell contains many fatty granules, some special treatment such as freezing technic is indispensable for the preparation of samples. For the observation of the development of ascaris eggs, the present author applied both smear and sectioned specimens.

MATERIALS AND METHODS

(1) Materials

Ascaris eggs were collected from the uterus of a fresh adult of Ascaris suilla. The posterior end of uterus was cut off approximately 2 cm in length and the eggs contained were taken out by crushing the organ. After culturing them on titles or clay-plates, the eggs on various stages of development were used for observation.

Both fixing and staining of the specimen was conducted generally on a slide. Sometimes, the freeze sectioning after embedding materials in gelatin was applied for nucleic acid or fat staining (vide Izumi, Biological studies on ascaris eggs III).

The fixation technic on a slide was carried as follows. Eggs were scattered on a clean slide and covered with a cover-glass, a fixing or staining solution was placed on one end of the cover-glass drop by drop, and absorbed from the other end with a filter paper, thus making the solution to spread all over the materials.

(2) Method for fixing ascaris eggs

Bouin's solution, sublimate-acetic acid solution, 10% formal solution, etc. were not adequate for fixing ascaris eggs in so far as heat was not used. Only
Carnoy's solution was effective, by which eggs were fixed within a short time. This solution penetrated the protein coat and shell of eggs within 1–2 minutes and made egg-cells transparent being infused into it. The resistance of eggs against Carnoy's solution was proved to be different depending on the stage of their growth, namely, the eggs on an early stage of the development were much stronger than those on a late stage. On applying staining solutions upon the fixed materials, their penetrating activity were found very weak, and the differentiation of colors were not so clear, although it appeared that the stained part corresponded generally to the distribution of nucleic acid. Namely, in the eggs on an early developmental stage, pyronin stained at first the surroundings of a definite part and then methyl green gradually penetrated to the centre.

But such staining method was inadequate for the detailed observation. So the author removed the protein coat and external stratum of shell from eggs, and after such procedure Carnoy's solution was applied on them. This time the fixation of eggs was almost successful. The de-coating procedure of the protein coat and shell in the modification of the technic devised by S. Yoshida and partly by the author. Namely, after applying 5% caustic potassium to ascaris eggs, they were shaken, placed overnighted at 27°C, washed by tap water, and were put into 50% antiformin with shaking and left in it for about 1 hour.

After above treatments the external stratum or protein coat was broken down and a thin membrane, an inner part of the shell, remained around the egg cells. When these eggs were allowed to stand at moderate temperature, they started cleavage and developed to the larval stage, which proved the fact that the egg-cells were not injured at all.

If the de-coating is not sufficient, an increase in the concentration of caustic potassium and antiformin or a slight pressure on the cover glass will enable sufficient de-coating of the eggs.

After de-coating shell, eggs were washed by tap water 2 to 3 times and Carnoy's solution was applied on them. As a result of such procedures several eggs were broken down, but the perfectly fixed eggs with an inner shell membrane were still found occasionally in some part. The application of gelatin smearing on the de-coated specimens will prevent such destructions of eggs.

(3) Staining method for nucleic acid of ascaris egg

In nucleic acid staining, 10% formol solution was commonly used for fixation. Formerly, it was presumed that the fixation by Carnoy's solution was adequate for the methylgreen-pyronin staining (Unna-Pappenheim). But this solution is not always suitable for fixing nucleic acid. In the present work, however, the author used Carnoy's fixing method aside from the discussion about these matters. A fresh solution was used for each staining. Methylgreen-pyronin used was a product of Grübler & Co.. For staining, Unna-Pappenheim's and Vogel's prescription were adopted. For thionin staining, 500 times water solution of this (Merck & Co.) was used. The stained materials was washed.
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with water after staining. The methylgreen-pyronin staining solution was
applied directly on newly fixed material. The staining solution was continuously
dropped till the fixing solution had been perfectly washed away. The methyl-
green should have a tinge of green but in this occasion it appeared rather blue
and a general aspect could roughly be estimated.

The fine structures of nucleus or cytoplasm could be differentiated more
precisely than in the specimens stained by other nucleus stainings. Feulgen's
and other similar reaction were not used because these were difficult to be
applied as original procedures.

When the above mentioned fixing procedure was carried out, the eggs in
one-cell stage were found to be fragile that they were easily broken and at
the same time their fat granules were dissolved away. Relatively good speci-
mens were obtained from the eggs on later stage of development, because the
structure of cells became steady.

(4) Staining methods for fatty substances and glycogen in ascaris egg1) 3)

For the fat staining, 75%-alcohol solution saturated with Sudan III or
scarla-red, water solution of osmic acid(0.1%), nile blue sulphate(0.5%) and
neutral red(0.2%) were used. The dyes were applied to the specimen which
was heated gradually by burner under the slide. When heating was moderate,
fat granules were stained relatively well without undergoing any destruction.
When overheated, however, the dissolved fat granules would spread between
the egg-cell and shell, and stagnate ther. In such cases, the spread fat was
stained well, but its tinge was somewhat different from the one within the cell.
The procedure of freeze sectioning was carried out according to the description
in chapter 2 of the proceeding report.

For the glycogen staining, Best's method and iodine reaction were used.
The specimen was fixed by warmed pure alcohol. Specimens suitable for obser-
vation were obtained when warmed at a moderate temperature. The cell thus
fixed were destructed and stained well.

ASPECTS OF STAINED NUCLEIC ACID IN EVERY DEVELOPMENTAL
STAGES OF ASCARIS EGG

(1) Polarity of ascaris egg

Observation of the ascaris eggs in one-cell stage revealed that the eggs
with relatively steady shell were stained without destroying the shell, but in
some cases nucleus staining was so bad that only the outline of nucleus was
found vaguely stained or only the position of nucleus was suggested by means
of penetrating stains. The tone of pyronin in nucleus was recognized evidently
by the intense light source.

A small spot stained densely by both methylgreen and pyronin was found
at a definite portion of the surface of egg-cell. It was irregular in form and,
generally, it was found situated about the middle portion between the pole and
the line of egg shell. Granular or club-like substances in this spot took the deep color of methylgreen and its central part was found stained well with pyronin. A particular structure of alveolar cytoplasm was recognized around this portion. It took a short club-like form when it reaches the lateral portion of the egg by moving the cover glass in order to rotate the egg.

This small spot is presumed to be one of the polar bodies or its end attached to the ovary which concerns the polarity of egg. In this spot, a shell structure different from the other portions of shell was also recognized. This spot might correspond to the so-called opening of shell.

Another small body was found in contact with egg-cell at one end of the egg, in which a part of this edge was crescent-shaped. Several granules in it took the color of methylgreen, while the other part took a tone of pyronin. This body was considered to be another polar body and to coincide with the body reported by T. Hasegawa.

(2) The structure and cleavage of ascaris egg (Fig. 1-9)

The nucleus was irregularly round in shape and generally situated somewhat apart from the centre of the cell. In most cases one nucleolus was found near the centre of the nucleus and in other parts of the nucleus chromatin granules were found scattered. The nucleus membrane, chromatin granules and the surrounding of nucleolus were all stained densely by methylgreen, taking a tinge of pyronin at the same time, so that, they appeared intensively violet in color. The alveolar structure in nucleus was differentiated clearly.

In the stage of meiosis, the chromosomes, asters and spindles were found clearly in any stadium. The chromosomes took an intensive particular color.

Generally, in the eggs of early stages, figures of meiosis observed were mostly those horizontal to the line of spindle, but occasionally some of them were in the transverse sections.

After fat granules dissolved away the cytoplasm remained alveolar in structure and fine granules were found scattered in it. The components of cytoplasm were faintly stained taking tones of both methylgreen and pyronin. The proper reaction for this stain was indistinct. It might be either the result of surplus staining by the former or some of the nucleic acid in the cell might have disappeared when fat granules were removed from the cell.

In view of the above findings it was presumed that the chromatin substance or chromosome contained chiefly desoxyribo-nucleic acid, and the ribo-nucleic acid was distributed in and near the nucleus. The nature of granules within cytoplasm was not made clear by means of such staining procedure.

If methylgreen-pyronin solution was applied after fixing by Carnoy's solution without de-coating, pyronin penetrated at first into the nucleus of egg-cell and stained it intensively, especially in the stage of late morula. This aspect was distinctly recognized and only so-called animal pole was stained reddish. These
findings together with the above mentioned, will give clear pictures of the
distribution of nucleic acids.

The specimen stained with thionin, presented similar structures to those
stained with methylgreen-pyronin, though these were some differences in the
tinge and distinctness.

As to the cleavage of ascaris egg, each stage of mytosis was already recog-
nized in the egg of all developmental stages and a cell septum was also found
even in that of one-cell stage. In the egg of two-cell stage, there were found
many cells in which nucleus division was in process. In the egg of three-cell
stage, spindles appeared in the remaining cells. This spindle showed a hori-
zontal division in contrast to the other which took a vertical division, thus the
first cell was divided horizontally and the second vertically, and then the egg of
the common four-cell stage appeared. From four-cell stage to morula stage,
the mode of cleavage and its direction became so much complexed that the
observation of each process was not practicable. On eggs of all stages above
mentioned, no particular phenomena were found as for the shape of blastomere,
structure of nucleus and the staining of nucleic acid.

In the eggs on an early stage of morula, the cells considered to be as an
initial developmental stadium of ectoderm were observed, but the space between
those cells and internal ones became gradually indistinct following the advance
in the development of eggs and nuclei of them were distributed evenly in the
whole egg. However, it should be noted that the nuclei situated in the centre
of these cells took the tinge of pyronin densely, which could be observed
especially by means of the stronger light.

In the egg on the late stage of morula, both poles became clearly differ-
entiated. At one pole the divided nuclei assembled tightly together and a group
of cells stained intensively taking a tone of pyronin. At the other pole, the
conglomation of cells became more loose and contained many fat granules. In
this stage, the staining of both poles became more conspicuous, not only by
nucleic acid staining but also by Giemsa’s solution, neutral red etc.

From the tadpole stage to the larval one, the proximal as well as the
caudal part of the body were stained by methylgreen-pyronin more intensively.
Particularly the developing or young cells were found stained by pyronin more
intensively. Pyronin penetrated into the grandular organs surrounding esoph-
agus, to the portion suggested as an excretory cell, to the cell line corresponding
the ventral median line and also into the tail. But the portion of fat granules
along digestive organ and the nerve ring at the anterior part of the body
remained unstained.

**Distribution of Fat and Glycogen in Different
Developmental Stages of Ascaris Egg**

For the observation of fat or glycogen distribution in the ascaris eggs
on different developmental stage, the author used chiefly the smearing method,
but sometimes the freeze sectioning was utilized for a supplementary observation. Fixation plays an important role in the staining of samples prepared by smearing method (vide. Izumu, Biological studies on ascaris eggs II, 1952). It is inadequate to use fixative solutions penetrating into the shell, because those dissolve fat granules within cells. The author fixed the material by heating or by applying hot stained materials directly. In doing so, fat granules in their original place were kept in most desirable condition but in some cases fat granules were found melted together and flowed out of the body. A phenomenon in its turn was utilized to observe the character of fat drops.

Above procedure was chiefly conducted after de-coating the shell as mentioned before.

(1) Findings by fat staining (Fig. 10–12)

In eggs from one-cell stage to the early morula one, fat granules were distributed equally and densely in each cells. They took a tone of yellowish red by staining of sudan III. Fat drops stagnated outside of the cell had a tinge of intensive red. In eggs of the late morula stage, fat granules were found situated in one pole and the other pole remained either unstained or stained in faint yellow. But following the development from tadpole to larva, fat granules were found around the digestive organs only. On double staining by thionin and sudan III, those parts which stained either by sudan III or thionin became gradually distinct, particularly it became quite distinct after the late stage of morula. The part stained by sudan III was found clearly separated from that stained by thionin. Similar results were obtained when scarla-red was used in the place of sudan III.

On staining by osmic acid, fat granules were found as granules of dark gray or black in color. The animal pole of a late morula and the anterior part or tail of tadpole or larval embryo were scarcely stained being only slightly gray in color.

On staining by neutral-red, fat granules were scarcely colored. When heated, only those fat drops stagnated between the shell and the embryo, stained yellow, but the animal pole of late morula and the anterior portion or tail of tadpole or larval stage were found stained red.

On staining by nile blue sulphate, fat granules were found scarcely stained. By heating, above mentioned stagnant fat drops took blue color tinged red and the cells themselves were colored simply blue.

The animal pole of the late morula and the anterior part or tail of larva were stained blue, whereas, fat granules were hardly stained, but some time they took reddish tone.

When eggs were stained after heating by others, i.e. eosin, trypan blue, bismarckbrown, etc., the entire part of cell stained evenly in each proper color of stains and was easily decolourized by washing with water.
(2) Findings of glycogen staining

Ascaris egg were fixed by warm pure alcohol and stained by Best's method. The space between fat granules was stained red in early developmental stages. The animal pole of late morula, the anterior portion or tail of tadpole or larval stage were also stained red. When Bauer-Feulgen's method was applied after completely removing fat granules treating with ether-alcohol solution, the egg-cells were found stained reddish purple. But the position of the stained portion in cells were not so distinct, that nothing was proved but the reaction was positive. Also the result of iodine reaction was indistinct.

COLOR REACTION FOR PROTEIN IN ASCARIS EGG

Xantoprotein and biuret reaction for ascaris egg were tried.

Eggs were smeared on an object glass and treated with alcohol-chloroform mixing solution, then washed with water, and they were given the same treatment as used commonly in histo-chemical or in-vitro experiments. Here the cytoplasm was stained orange yellow in xantoprotein reaction and purple in biuret reaction.

In these procedures the rupturing of both ends of egg shells was recognized in several eggs. On such occasion, the internal mambrane was found swollen out of the shell. Thirty minutes after treating by nitric acid and potassium causticum, the percentage of eggs expanding at one end 25%, at both ends 26% and the eggs remained normal.

SUMMARY

For observing the structure of ascaris egg and nucleic acid distribution during cleavage, eggs of ascaris were fixed with Carnoy's solution after removing the protein coat and a part of shell. They stained with methylgreen-pyronin in two kinds of method (Unna-Pappenheim's or Vogel's staining technic), sometimes with thionin. The outline of results obtained can be summarized as follows.

(1) A small apparatus at a certain place of the surface of egg-cell was found. It stained intensively by both methylgreen and pyronin and was considered as one of the polar bodies or the end attached to the ovary which concerned polarity of egg. Also, a small body was found in contact with egg cell at one end of this. It stained intensely by the stain and was considered as another polar body.

(2) As to the structure of the cell, the alveolar components in nucleus and the nucleolus were clearly recognized, although these were not stained by methylgreen, whereas the chromatin granules scattered surrounding or within the nucleus were stained intensive purple in color. These were considered to contain a great quantity of desoxyribo-nucleic acid. On the eggs without removing the shell, the intensive penetration of pyronin near the nucleus was
recognized. In cytoplasm, the alveolar structure remained after fat granules were dissolved away by Carnoy's solution. Along this structure fine granules stained slightly were found, but these granules were not evident whether they were proper basophil substance.

(3) The form of cleavage and division spindles were also recognized clearly by the above staining. In two-cell stage, a division was found at first at one of the cells and a spindle appears pararell to the septum of cells. In late three-cell stage, division occured in the remained cell, in which the spindle took a different direction from the former, thus resulting a common four-cell stage. In late stage of morula, the divided cells assembled closely together observed at one end of it. Among these were observed one group of other cells being stained distinctly by methylgreen-pyronin. In the tadpole and larval stage, the anterior portion or tail were stained good. Especially the parts where the development or division of cells actively took place, were intensively stained by pyronin.

(4) The aspect of fat distribution in every developmental stage of ascaris egg was as follows. From one cell stage to early stage of morula, fat granules were distributed evenly and densely in the body, but in the later stage of morula they gathered to a pole opposite to that stained by thionin intensely. In the tadpole or larval stage, fat granules were formed remained only arround the digestive organs. The parts where fat granules gathered in these stages were quite different from the parts stained by thionin and Best's method. In otherword, in the part stained by thionin etc. no fat granules were found. Judging from tones of each fat-stain in later morula stage, the vegetative pole seemed to contain mainly neutral fat, lipoid and the animal pole only lipid.

(5) In early developmental stages glycogen was found distributed in the space between fat granules and in larval stage in animal pole, anterior portion or tail of it.

(6) Xantoprotein- or biuret-reaction was positive on ascaris egg-cell.

(7) As the result of the 3rd and 4th issues of this study the author attained the following conclusion. Namely, throughout the whole process of development of ascaris eggs substances stainable by nucleic acid- and glycogen-staining procedures were found increased in parts where the cell division and tissue formation were actively taken place, whereas the role of fat granules was considered to remain rather inactive, excepting the early stage of it. Also it is considered, judging from findings of fat- and glycogen-stainings during the ontogenesis of ascaris egg, that those two substances, i.e. stand antagonistic with each other.

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REFERENCES


EXPLANATION OF MICROGRAPHS

Fig. 1: One-cell stage of ascaris egg, Ascaris suilla (U-P staining). Fig. 2: Two-cell stage, ibid. Fig. 3: Two-cell stage, ibid, the mytosis is found in one nucleus. Fig. 4: Three-cell stage, ibid, the mytosis occurring in one nucleus. Fig. 5: Four-cell stage, ibid. Fig. 6: Eight-cell stage, ibid. Fig. 7: Chromosomes and asters, ibid. Fig. 8: Morula, ibid. Fig. 9: Larval stage, ibid, the shell was broken during the fixation. Fig. 10: Early stage of morula, ibid (sudan III staining). Fig. 11: Larval stage, ibid, the portion about digestive organs is especially stained. Fig. 12: Larval stage, ibid, stained after de-coating.
Fig. 13: Fat granules in ascaris ovary (sudan III-hematoxylin staining), Ascaris suilla. Fig. 14: Transverse section of ascaris ovary (U-P staining), ibid. Fig. 15: Transverse section of seminale receptacle (U-P staining), ibid. Fig. 16: Ascaris ovary (Feulgen's reaction), ibid. Fig. 17: Seminale receptacle of ascaris (Feulgen's reaction), ibid. Fig. 18: Germ cells at the initial part of the ovary, ibid. Fig. 19: Germ cells at the posterior end of the ovary, ibid. Fig. 20: Y-form stage of egg cell, ibid. Fig. 21: V shape, ibid. Fig. 22: Spindle shape, ibid. Fig. 23: Farther developed stage than the formers, ibid. Fig. 24: Egg cell just before fertilization, ibid. Fig. 25: Egg after fertilization, ibid.