Studies on the Formation of Fish Eggs
III. Localization of Polysaccharides in Oocytes of *Liopsetta obscura* [1)]

By
Kiichiro Yamamoto

(Akkeshi Marine Biological Station, Akkeshi, Hokkaido)

(With 2 Plates)

Thanks to the cytochemical technique devised by Bauer (1933) and Hotchkiss (1948) for the examination of polysaccharides, many important papers have been published recently on the localization of carbohydrates and their function in the cell. Especially, Monné and collaborators (1950 a, 1950 b, 1951) have produced many interesting papers in this field of study; they pointed out that polysaccharides and some other carbohydrates are of great importance for the maintenance of cell function and transformation. Of late years several stimulating reports have appeared concerning the oocyte of the fish. Ihnuma and Tsukuda (1952) have made clear that in *Leiognathus* the yolk globules in an early stage of development contain a large quantity of low moleculed polysaccharides, while in an advanced stage they are composed of polymerized polysaccharides with much protein. The excellent studies of Kusa (1953, 1954) have made it clear that the cortical alveoli of some fishes hold sulfate-containing mucopolysaccharides. His studies have been extended by the work of Aketa (1954) resulting in the confirmation of the presence of polysaccharides in the cortical alveoli of *Oryzias* eggs in connection with those origin. The situation naturally requires the investigation of the localization of carbohydrate in the oocyte during oogenesis. This prompted the present author to take up a study of the change in localization of polysaccharides occurring during the growth period of the oocyte in the flounder, *Liopsetta obscrua*, with special concern for the chemical constituent of cell-elements.

Here, the author wishes to acknowledge his great indebtedness to Professor Tohru Uchida for his kind suggestions and encouragement. The author also takes much plaesure in expressing sincere appreciation to Professor Sajiro Makino for his kind criticisms and improvement of the manuscript for publication.

Material and method

The present study was based on the oocytes of the flounder, *Liopsetta obscura*.

1) Contributions from the Akkeshi Marine Biological Station, No. 74.
Jour. Fac. Sci. Hokkaido Univ. Ser. VI, Zool. 12, 1956.

The material was obtained in a similar way to that described in the preceding paper (Yamamoto 1956).

For the demonstration of polysaccharides in the oocyte, two different methods devised by Hotchkiss (1948) and by Bauer (1933) have been adopted (Glick 1949). The material subjected to these tests was fixed with Bouin's solution, Bouin-Allen's mixture and alcohol-formol solution. Serial sections, 10 μ in thickness, were prepared following the usual paraffin method. The coloration produced by these methods is said to be specific for vicinal OH or OH and NH_2 groups which occur only in carbohydrates and in some kinds of amino acids. However, there are two unusual features to be noted here ; first, unsaturated fatty acids of the tissue change into certain substances, though the mechanism is not sufficiently clear, and second, unidentified non-lipid substance localizes in elastic fiber (Gomori 1953). The non-carbohydrate substances which behave as an aldehyde give a positive Schiff's reaction even without oxidative pre-treatment. The control undertaken without oxidative pre-treatment proved that the oocytes of the flounder contain no such substances. For differentiation of glycogen from other Schiff-positive substances, it was subjected to the saliva test (Ichikawa 1953), and the results obtained were re-examined through Best's carmine staining. In general, many workers consider that glycogen is dissolved when the material is fixed with many aqueous fixatives (Monné and Slautterback 1950, Kusa 1953 ; for others refer to Gomori 1953). However, this seems not always to be the case. Glycogen in the oocyte was found preserved well in the material fixed with Bouin's solution or Bouin-Allen's mixture. Lison (1953) stated that glycogen was preserved well in the material fixed in Bouin's fluid and better in Bouin-Allen's mixture. Gomori (1953) also agreed with the results of this French worker. Judging from the results of the present study, Bouin-Allen's fluid seems to be better than alcohol-formol mixture as the fixative for polysaccharides in the oocyte, because the former not only preserves glycogen fairly well and other polysaccharides likewise ,but also gives better morpholcgical structure than the latter.

For the purpose of better demonstration of Schiff-positive polysaccharides, a combination-stain consisting of fuchsin sulfite and 2-4-dinitrophenylhydrazine was applied, after oxidation with periodic acid (Monné and Slautterback 1950), to the oocyte. The preparation kept in phenylhydrazine for two days shows good differential staining ; certain cell components are stained yellow and others reddish-violet. According to Monné and Slautterback (1950) the yellow coloration demonstrates the presence of aminopolysaccharides and the reddish-violet coloration indicates the presence of mucopolysaccharides or closely related hydrophilic jelly-forming substance.

Further, metachromatic staining (Ichikawa 1953) was applied to differentiate mucopolysaccharides in the cytoplasm. The metachromatic substance of the cytoplasm fixed and stained with suitable basic dye is said to be due to sulfate-containing mucopolysaccharides existing in the cytoplasm (Lison 1953). For staining, toluidine blue and thionin were used in this study.

Observations

(1) *Nucleus* : Nucleoplasm in the young oocyte gave always the negative reaction for the test of polysaccharides either by Hotchkiss' or by Bauer's method. Just prior to the migration of the germinal vesicle, the nucleoplasm suddenly gave a Hotchkiss-positive reaction (Fig. 9). Then there appeared in the vicinity of the germinal vesicle a characteristic, large body which was strongly positive for

the polysaccharide test (Fig. 10). That body did not show appreciable structure, being stained deeply in homogeneously reddish-violet by the application of either Hotchkiss' or Bauer's test, and also by the combination staining method. Moreover, the digestion-test with saliva failed to digest this body, which showed no toluidine blue metachromasy (Fig. 11). From these results it may be said that the main components of this body are the mucopolysaccharides free from sulfuric acid residues, and further that the body resembles well the nuclear mucoid found in the cleaving egg of sea-urchin as demonstrated by Monn'e and Slautterback (1950). Hereafter, the body was visible for a considerable length of time in the vicinity of the nucleus until a period just prior to the breakdown of the germinal vesicle. As soon as the germinal vesicle broke down, the nuclear substance which occupied an eccentric position in the oocyte came to show deeply reddish-violet coloration with fuchsin sulfite (Fig. 12).

The nucleoli in the young oocyte were positive to the Hotchkiss test (Fig. 1). The treatment with saliva showed a decrease in staining capacity of the nucleoli in the Hotchkiss test and in the staining with Best's carmine, though not distinctly. With the growth of oocytes the reaction of nucleoli gradually decreased proving almost negative at the tertiary yolk stage. The threadlike nucleoli at the breakdown stage of the germinal vesicle appeared to be strongly positive to the Hotchkiss reaction, and they were hardly distinguishable from the cytoplasm, since both were deeply stained reddish-violet with fuchsin sulfite (Figs. 12 and 13). The results here presented agree with those reported by Ihnuma and Tsukuda (1952) in the oocyte of *Leiognathus*. On the other hand, Monné and Slautterback (1950) reported that in the sea-urchin egg the nucleolus of the oocyte showed the central part stained more intensely than in the periphery, when the material was fixed with Carnoy's fluid and treated with periodic acid and fuchsin sulfite. Based on these findings they assumed that a detergent-like mucolipid was present within the nucleolus. Different to the findings of Monné and Slautterback (1950), the nucleoli of the flounder's oocytes were always stained faintly and nearly homogeneously with application of the Hotchkiss test, without showing any differentiated central portion, when the material was fixed with the fixatives mentioned above.

The chromatin was found to give a negative reaction without exceptions in the test of polysaccharides.

(2) *Hyaloplasm* (*cortical layer*) : The cytoplasm in the oocyte of the early peri-nucleolus stage exhibited a very weak positive reaction in the Hotchkiss or Bauer test (Fig. 1). With the growth of the oocyte the reaction of the cytoplasm was found to increase through the late peri-nucleolus stage, reaching the maximum at the yolk vesicle stage (Figs. 2 and 3). This Schiff-positive substance occurring in the cytoplasm must be also non-sulfated mucopolysaccharides. The cytoplasm was always stained reddish-violet by the combination staining and showed no sign of metachromasy. Further, this coloration may be still retained after treatment

with saliva. Notwithstanding that the cytoplasm of oocytes at the yolk stages showed also a strong Hotchkiss-positive reaction, the Schiff-positive substance seems to be different in nature from polysaccharides detected in the younger oocytes. After pre-treatment with saliva the hyaloplasm of the yolk stages proved weakly positive in the Hotchkiss reaction (Figs. 6–8). The results seem to indicate that glycogen is being accumulated in the hyaloplasm in place of mucopolysaccharides during a period ranging from the primary yolk stage to the tertiary yolk stage. The cortical layer and the network of hyaloplasm in the oocyte of the maturation stage were also strongly positive in the Hotchkiss reaction, but pre-treatment with saliva resulted in a weak reaction (Figs. 12 and 14). The resistance of polysaccharides to the digestive action of saliva increased in the hyaloplasm step by step with the formation of homogeneous yolk (Fig. 14). As the formation of homogeneous yolk neared completion, the Hotchkiss reaction of the hyaloplasm were also found to be weak positive. The findings as above indicate that in the late maturation period the polysaccharides of the hyaloplasm show again a transformation from glycogen into mucopolysaccharides.

(3) *Yolk spheres* : As described in the preceding paper of this series (Yamamoto 1956), the yolk spheres make their first appearance as minute spherical granules distributed in the periphery of the oocyte in the form of a single thin layer. Such a yolk vesicle in a well-preserved condition gave a strong, positive reaction for the tests of polysaccharides according to either Hotchkiss or Bauer, staining deep reddish-violet with the combination staining method. The coloration produced by fuchsin sulfite was not different from the coloration before and after treatment with saliva. The vesicle indicated no sign of metachromasy (Figs. 2 and 3). In the oocytes fixed with Bouin's solution, the yolk globules were moderately positive in respect to Hotchkiss' reaction at the primary yolk stage (Fig. 7). They were also stained reddish-violet by the combination staining method, while in the latest part of this stage some yolk globules distributed in the periphery of the oocyte were stained yellow. The polysaccharides contained in the yolk globules were apparently difficult to digest with saliva. In addition to the yolk globules of large size, small granules stained deep reddish-violet were found in the ooplasm with a dispersed distribution.

In Fig. 6 is shown an oocyte of the secondary yolk stage treated by Hotchkiss' method. Three kinds of yolk globules were distinguished in the ooplasm in response to the polysaccharide test. The yolk globules in the outermost part of the ooplasm were stained very faintly reddish-violet by this method, and fairly yellow by the combination staining method. The middle part of the ooplasm gave a very strong, positive reaction to Hochkiss' test, and the innermost part showed a weak positive reaction. The two groups of yolk globules were stained reddish-violet by the combination staining method. The polysaccharides contained in these yolk globules seem to show a strong resistance against the digestive action of saliva.

At the tertiary yolk stage the oocytes contain a single kind of yolk globules which gave a very faint positive reaction to Hotchkiss test and were stained yellow by the combination staining method (Fig. 10). After the breakdown of the germinal vesicle the yolk globules gradually come together until the homogeneous yolk is formed. During this process the reaction of polysaccharides has remained unchanged in the yolk globules. In the oocytes with the homogeneous yolk mass one could see the margin of the yolk which was stained intensely with fuchsin sulfite. This coloration persisted almost unchanged even after treatment with saliva (Figs. 14 and 15).

Based on the above findings, the following assumption is possible—that polysaccharides such as mucopolysaccharides which are predominant components of the yolk vesicle gradually decrease in amount, and that in the place of the saccharides thus reduced the yolk globules produce another kind of polysaccharides, such as aminopolysaccharides.

(4) *Cortical alveoli* : In Figures 11 and 16 are shown photomicrographs from the preparations of an oocyte at the tertiary yolk stage prepared by Hotchkiss' method after treatment with saliva. In the peripheral region of the ooplasm there are found many bodies showing a strong Hotchkiss-positive reaction. They are spherical in form and variable in size. Each body consists of the outer layer stained deeply reddish-violet and the faintly staining central region. The features possibly indicate that these bodies are destined to develop into the cortical alveoli. In the latter part of the maturation division, the cotical alveoli are found arranged in the periphery of the cortical cytoplasm forming a layer (Fig. 17). They contain a small compact body in each. The contents were strongly positive to the Hotchkiss reaction with or without pre-treatment with saliva, showing no sign of thionin or toluidine blue metachromasy.

The presence of polysaccharide substance in the cortical alveoli of fish eggs was first shown by Kusa (1953, 1954) in the eggs of stickleback and of salmon after the cytochemical tests, and he stated that this substance is mucopolysaccharides with sulfuric acid residues, probably constituting a part of mucoprotein. A similar result has been obtained by Aketa (1954) in the cortical alveoli of eggs of the Medaka. Since the contents of the alveoli of the present material exhibited no sign of metachromasy, the mucopolysaccharides occurring in the alveoli of the flounder's eggs seem to differ from those in the alveoli of the salmon and stickleback.

(5) *Zona radiata* : The zona radiata is clearly visible in the oocyte of the early yolk vesicle stage (Fig. 2). At first it gave a very strong positive reaction to Hotchkiss' test, while it was weak to Bauer's test. The reaction of the zona radiata to the polysaccharide test has remained similar through the yolk vesicle stage (Fig. 3). The oocyte at the primary yolk stage also showed the zona radiata stained deeply by the periodic acid-Schiff method (Fig. 4). The reaction of the zona radiata gradually decreased as the membrane thickens.

At the secondary yolk stage there appeared beneath the zona radiata a radial striation, positive in Hotchkiss test. The condition continued unchanged until the late maturation stage (Fig. 16). In the ripe egg both the zona radiata and radial striation have proved almost negative to the test for polysaccharides. The coloration of the zona radiata produced by the periodic acid-Schiff method was the same as the coloration observed before and after the treatment with saliva, so far observed in the growth period. There was no sign of metachromasy in the zona radiata. As mentioned by Ihnuma and Tsukuda (1952), it seems probable that at the beginning of its formation the zona radiata is mainly composed of polysaccharides without differentiation into sulfated mucopolysaccharides, and that along with its growth protein is gradually produced, which takes the place of polysaccharides.

In this connection some mention should be made on a thin layer lying just outside the zona radiata and inside the follicle layer. It showed a strong positive reaction in the test for polysaccharides. The layer becomes visible in the oocyte at the secondary yolk stage. At the migratory nucleus stage it is found in a texture composed of long filaments and then shows a decrease in thickness until it is completely lost from sight. After treatment with saliva the layer was strongly positive to the Hotchkiss reaction (Fig. 13), and showed no evidence of toluidine blue metachromasy. To the best of the author's knowledge, nothing can be stated at present about the functional significance of this layer.

Discussion

One of the noteworthy findings presented in the present study is the localization of glycogen. In the oocyte of the flounder, the accumulation of glycogen in the ooplasm takes place during the period ranging from the primary yolk stage to the tertiary yolk stage. Then it decreases in amount in the period ranging from the migration of the germinal vesicle to the maturation of the egg. It is generally accepted that in animal cells the greater part of carbohydrate used as fuel in a living organism is present in the form of glycogen, and that the dissimilation of glycogen liberates the energy necessary for endergoenic reaction. It was observed by the present author (1956) that the duration ranging from the secondary yolk stage to the tertiary yolk stage is considerably long, and that the oocytes show no marked change in either form or size within this period. On the contrary, there occur remarkable morphological changes in the oocytes during the period from the migratory nucleus stage to the prematuration stage. It is then likely that glycogen accumulated in the former stage is used as energy source for the changes taking place in the latter.

The other significant evidence as to localization of polysaccharides was that demonstrated in the egg membrane and the yolk spheres. The results obtained clearly show that the egg membrane consists mainly of mucoplysaccharides in the early developmental stage, and that in an advanced stage the membrane is

probably free from polysaccharides. Of late years it has been well established that the chitin-membrane of some invertebrates undergoes differentiation according to the following chemical gradations in its make up; carbohydrates →glycoproteins→proteins (Suzuki and Obata 1940). Most probably the egg membrane of fishes which is composed of scleroprotein according to Needham (1931), follows the same principle. Further, the fomation of yolk spheres may probably be interpreted on a similar basis.

The consideration should be extended to the mucoidal body found in oocytes at the time of migration of the germinal vesicle and of its breakdown. The nucleoplasm of the germinal vesicle before the migration stage gives a clear polysaccharide reaction. Later in the migration stage there appear irregularly coagulated bodies showing a mucopolysaccharide reaction in the vicinity of the germinal vesicle. They resemble very much in cytochemical nature the nuclear mucoid demonstrated in sea-urchin egg by Monné and Slautterback (1950). The nuclear mucoid of sea-urchin eggs is a substance existing in the interstices between the chromatin granules just at the time of cleavage. In the resting cells it appears as a substance present in one half of the nucleus. The above-named authors have stated that the nucleolus possibly contains a detergent-like mucolipid, and the nucleolus is dispersed during mitosis when the carbohydrate part of the detergent-like mucolipid begins to be hydrated. The present study has shown that the oocyte of the flounder contains many nucleoli which are always stained faintly and homogeneously by Hotchkiss' method, and that the nucleoli have remained without change in the nucleus until the germinal vesicle breaks down (Figs. 9 and 10). On the basis of these findings, the nucleolar origin of the nuclear mucoid must be improbable. Further, the mucoidal substance does not always occupy a definite place in the cytoplasm. In spite of this circumstance, the hypothesis of Monne' (1949) that the detergent-like mucolipid may be responsible for the dissolution of various structures during mitosis, is of importance for understanding the nature and function of the mucoidal bodies found in the present material. In this respect details will be published elsewhere after the completion of studies now in progress.

Summary

This paper deals with the localization of polysaccharides taking place in various stage of the growth period of the oocyte of the flounder, *Liopsetta obscura*, with special reference to the chemical constituents of the cell elements. The results are summarized as follows:

1. The nucleoplasm of the early growing oocyte always gives a negative reaction upon the application of the Hotchkiss test. By the migratory stage of the germinal vesicle, the nucleoplasm suddenly shows deep reddish-violet coloration with the Hotchkiss method. Then, hydrated jelly-like mucoid of a hemispherical form makes its appearance in the vicinity of the nucleus. The nucleoli contain a small amount of polysaccharides almost throughout the whole growth period.

2. With the growth of oocytes the polysaccharides existing in the hyaloplasm undergo a series of successive changs from mucopolysaccharides free from sulfuric acid residues into glycogen which changes again into mucopoly-

saccharides.

3. Non-sulfated mucopolysaccharides constitute predominant components of the yolk vesicle of the oocyte in an early stage of development. The yolk globules produce another kind of polysaccharides such as aminopolysaccharides.

4. The presence of a polysaccharide substance is demonstrated in the cortical alveoli of the oocyte. The saccharides in the alveoli show no sign of toluidine blue metachromasy.

5. The components of the zona radiata seem to differentiate through the following chemical processes: carbohydrates→glycoproteins→proteins.

6. The oocyte of the late growth stage shows a layer containing mucopolysaccharides which is located just outside the zona radiata and inside of the follicle layer.

Literature

Aketa, K. 1954. The chemical nature and the origin of the cortical alveoli in the egg of the Medaka, *Oryzias latipes*. Embryologia, vol. 2, p. 63.

Glick, D. 1949. Technique of histo-and cytochemistry.

Gomori, G. 1953. Microscopic histochemistry.

Ichikawa, M. 1953. Cytochemistry.

Ihnuma, M. and J. Tsukuda. 1952. Cytochemical studies on polysaccharide and protein of ovarian egg-cells of an osseous fish (*Leiognathus argenteum*). Fol. Anat. Jap., Bd. 24, s. 49.

Kusa, M. 1953. On some properties of the cortical alveoli in the egg of the stickleback. Annot. Zool. Jap., vol. 26, p. 138.

———— 1954. The cortical alveoli of slamon egg. Ibid., vol. 27, pl. 1.

Lison, L. 1953. Histochemie et cytochemie animales.

Monne, L. 1949. From Monne and Slautterback, 1950.

———— 1950. Structural changes of the cell resulting from clotting and liquefaction. Arch. f. Zool., Bd. 1, s. 101.

Monne, L. and D. B. Slautterback. 1950. Differential staining of various polysaccharides in sea urchin eggs. Expl. Cell. Res., vol. 1, p. 477.

Monne, L. and S. Harde. 1951. On the cortical granules of the sea urchin egg. Arch. f. Zool., Bd. 1. s. 487.

Needham, J. 1931. Chemical embryology.

Sokin, P. A. and A. G. Levene. 1952. Carbohydrate metabolism.

Suzuki, B. and Y. Obata. 1940. Biochemistry.

Yamamoto, K. 1956. Studies on the formation of fish eggs. 1. Annual cycle in the development of ovarian eggs in the flounder, *Liopsetta obscura*. Jour. Fac. Sci. Hokkaido Univ., ser. VI, vol. 12, p. 362.

Explanation of Plates

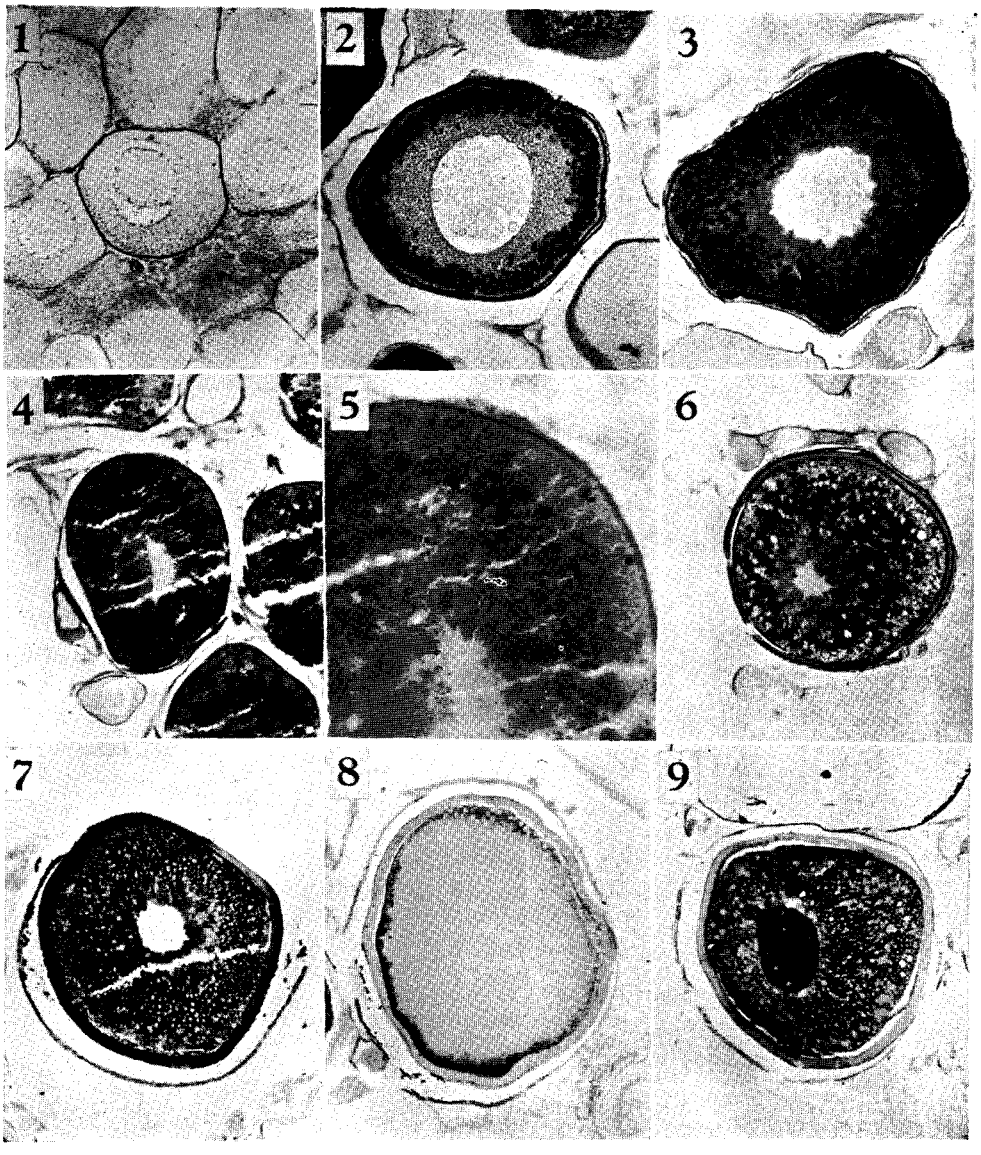
All are photomicrographs taken from sections of the cocytes, fixed with Bouin's fluid and Bouin-Allen's solution and examined with Hotchkiss method. The cellular components stained with fuchsin sulfite appear black in the photographs.

Plate XVI

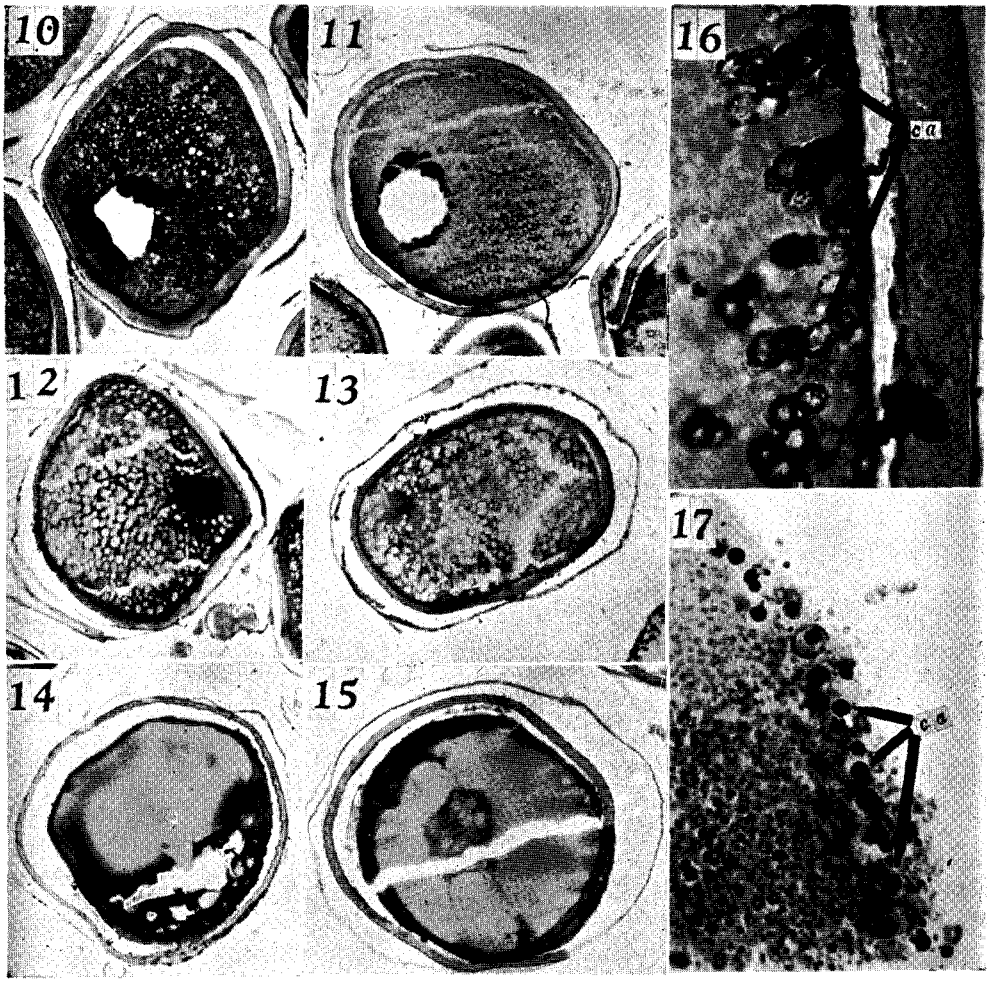
Fig. 1. Ocoytes of the late peri-nucleolus stage. ca. ×260.
Fig. 2. Oocyte at the early phase of the yolk vesicle stage. ca. ×260.
Fig. 3. Oocyte at the later phase of the yolk vesicle stage, pre-treated with saliva before the Hotchkiss test. ca. ×260.
Fig. 4. Oocyte at the primary yolk stage. ca. ×260.
Fig. 5. Portion of an oocyte at the same stage as above, pre-treated with saliva previous to the Hotchkiss test. ca. ×420.
Fig. 6. Oocyte of the secondary yolk stage. ca. ×70.
Fig. 7. Oocyte at the tertiary yolk stage. ca. ×70.
Fig. 8. Oocyte at the same stage as above, pre-treated with saliva prior to the Hotchkiss test. ca. ×70.
Fig. 9. Oocyte just before the germinal vesicle begins to migrate, in which the nucleus shows a positive Hochkiss reaction. ca. ×70.

Plate XVII

Fig. 10. Oocyte just the time when the germinal vesicle begins to migrate. ca. ×70.
Fig. 11. Ocyte at the same stage as above, pre-treated with saliva prior to the Hotchkiss test. ca. ×70.
Fig. 12. Oocyte just after the germinal vesicle has been broken down, ca. ×70.
Fig. 13. Oocyte at the same stage as above, pre-treated with saliva previous to the Hotchkiss test. ca. ×70.
Fig. 14. Oocyte at the maturation stage, pre-treated before the Hotchkiss test. ca. ×70.
Fig. 15. Oocyte at the same stage as above. ca. ×70.
Fig. 16. Cortical alveoli in an oocyte at the tertiary yolk stage, pre-treated with saliva previous to the Hotchkiss test. ca. Cortical alveoli. ca. ×1000.
Fig. 17. Cortical alveoli in an oocyte at the maturation stage, pre-treated with saliva prior to the Hotchkiss test. ca. Cortical alveoli. ca. ×1000.



K. Yamamoto: *Studies on the Formation of Fish Eggs*, III



K. Yamamoto: *Studies on the Formation of Fish Eggs*, III