Studies on the Formation of Fish Eggs
II. Changes in the Nucleus of the Oocyte of *Liopsetta obscura*, With Special Reference to the Activity of the Nucleolus[1)]

By
Kiichiro Yamamoto

(Akkeshi Marine Biological Station, Akkeshi, Hokkaido)

(With 4 Textfigures and 3 Plates)

In a former paper of this series of studies published by the present author, the general history in growth of the oocyte of the flounder, *Liopsetta obscura*, was reported. It was noted that the nucleus of the oocyte shows marked changes during the growth period. The present study was undertaken to trace further the detailed changes of the nucleus in the growth period with particular concern with the activity of the nucleolus.

Before proceeding further, the author wishes to tender his cordial thanks to Professor Tohru Uchida for his encouragement and keen interest in the subject, and to Professor Sajiro Makino for his kind advice and the improvement of the manuscript for publication.

Results

1. Cytological study

The following investigation was carried out exclusively with the eggs of the flounder, *Liopsetta obscura*. For details of collection of the material and the technique for preparing sections one is referred to the author's former paper (Yamamoto 1956).

Observations: Oogonial division was not encountered at all in sections so far examined in this study. It seems probable that the oogonial division had been finished in the fish, about 11 cm in body length. The ovary of the fish larger than the above generally contains a large number of growing oocytes. A similar evidence was found in the ovary of the hake by Hickling (1935). The smallest oocytes which were observed in the present material are those in the "net-like" stage (Text-fig. 1, A). They are minute in size, and are found in great number lying just beneath the surface of the ovigerous lamellae. The nucleus occupies almost the entire space of the cell body and assumes an oval or slightly irregular form. It is surrounded by a very narrow zone of cytoplasm with an indistinct boundary. The chromatin elements in the nucleus show a little affinity for stain. They form an irregular

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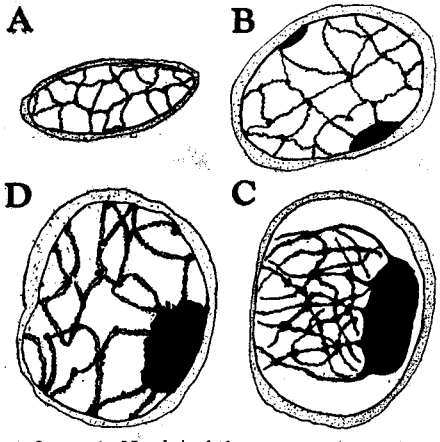


Text-figure 1. Nuclei of the youngest oocytes. ×2200. A. Earlier phase in the pre-synaptic stage. B. Later phase in the same stage as above. C. Synaptic stage. D. Post-synaptic stage.

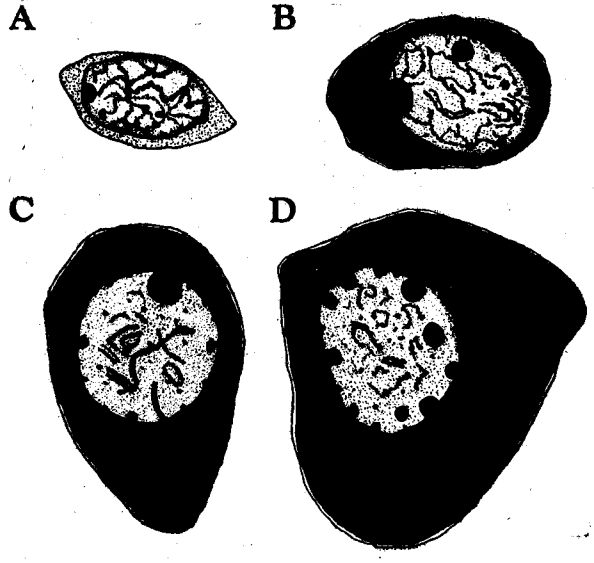
reticulum. On the meshwork of the reticulum are suspended several deeply staining chromatin-nucleoli of various sizes. This feature strictly resembles that described in the youngest oocytes of *Pleuronectes* by Franz (1910). With the growth of the oocyte the nucleus becomes spherical in form with a regular and distinct outline. The chromatin-reticulum shows a complicated anastomosis and is much more sharply defined than in earlier stages. The chromatin-nucleoli show no appreciable increase in number during the growth period, but there occur one or two bodies of conspicuously large size which are deeply stained (Text-fig. 1, B).

Oocytes of a similar nature were described by Scharff (1887) in the haddock, by Franz (1910) in the sole, and Hickling (1935) in the hake. This stage is followed immediately by a stage showing a gradual condensation of the nuclear contents characteristic to syanpsis. The general features of the oocyte at the synaptic stage are much like those recorded by Maréchal (1905) in *Gasterosteus*, by Franz (1910) in *Pleuronectes*, and by Hickling (1935) in *Merlucius*. Each oocyte of this stage shows a large chromatin-nucleolus lying in one side of the nucleus. The chromatin-reticulum at this stage is seen as a collected bundle of thick, intensely staining threads (Text-fig. 1, C).

After synapsis each spireme-thread seems to split longitudinally as usually occurs in other animals (Text-fig. 1, D). The next stage is represented by the enlargement of the nucleus containing the spiremes which are variable in length and interwined in various ways. There are also found a number of deeply staining chromatin-nucleoli in the nucleus as in the previous stage. The cytoplasm considerably increases in relative volume and becomes somewhat basophilic, being faintly stained with haematoxylin (Text-Fig. 2, A).

Along with the growth of oocytes the germinal vesicle likewise grows gradually. Each chromatin thread seems to double back to form a loop or to unit at the two ends to form a oval ring. In addition to the small nucleoli which are directly associated with the spiremes, there are one or two large and strongly basophilic nucleoli in the nucleus. Most probably they are the true nucleoli (plasmosomes), though there are some about which it is difficult to say with certainty whether they are true nucleoli or chromatin-nucleoli. The cytoplasm surrounded by a thin follicle layer increases much in its relative volume and becomes highly basophilic (Text-fig. 2, B). In the latter part of the early peri-nucleolus stage, the germinal vesicle is much enlarged and shows a regularly circular outline. The chromatin-threads

lie loosely in the central area of the nucleus and gradually lose their basophilic character. They become loose in both texture and outline, and gradually develop into the so-called "lamp-brush" chromosomes, characteristic of the middle growth period. At the same time the deeply staining basophilic nucleoli, spherical in shape and variable in size, gradually arrange themselves in the periphery of the nucleus. In addition to these peripheral nucleoli there are some minute ones scattered in the central area of the nucleus. They seem to have association with the chromosomes (Text-fig. 2, C).



Text-figure 2. Nuclei of young oocytes. ×1000.
A. Later phase in the post-synaptic stage. B. Earlier phase in the early peri-nucleolus stage. C. Middle phase in the same stage as above. D. Later phase in the same stage as above.

As the oocyte grows further, the cytoplasm gradually loses its basophilic character and becomes stained faintly as already noted. In such a stage, it is noteworthy that some of the nucleoli are frequently found extruded from the nucleus into the cytoplasm. The nucleoli lying in the periphery of the nucleus are generally spherical in shape with some exceptional ones of spindle-shape. Some oocytes show black spots of nucleolar size in the cytoplasm lying close to the nucleus (Fig. 1). Undoubtedly these spot-like bodies may be the nucleoli which have migrated from the nucleus into the cytoplasm through the nuclear membrane. These extruded nucleoli are lost from vision after migration towards the periphery of the oocyte, due probably to their lack of staining capacity.

Following this stage, there occurs no appreciable change of the nucleoli in

the flounder's egg, in contrast to those of *Melanophares*, *Sterenoptyx*, and *Leiognathus* in which a marked change has been reported. In the early yolk vesicle stage the germinal vesicle still keeps a circular form. The nucleoli situated close to the nuclear membrane increase their size, being spherical or sometimes spindle in shape. Somewhat later, there are some nuclei which contain nucleoli in their central parts, in addition to those scattered in the periphery. In the latter part of this stage all nucleoli are found again situated close to the nuclear membrane. They are markedly meagre in number, being hemi-spherical in form and flat on the side toward the nuclear membrane. The extrusion of nucleoli into the cytoplasm is observable throughout this stage. The "lamp-brush" chromosomes distributed in the centre of the nucleus are loose in texture, staining faintly. In the primary yolk stage the germinal vesicle becomes distorted in form. Most of the nucleoli give up their peripheral arrangement and move towards the centre of the nucleus. Their arrangement is somewhat irregular, scattered at random in the nucleus. Usually they are spherical in form, still staining faintly (Fig. 2). As the yolk formation advances further, the yolk globules existing in the ooplasm seem to offer some pressure upon the nucleus, with the resulting change of the nuclear form into an irregular configuration. The nucleoli distributed in the nucleus becomes vesicular in struture and tend to stain weakly. In this stage the chromosomes are hardly observable. The nucleus has remained almost unchanged in either size or shape until the end of the secondary yolk stage, while the nucleoli change their size and form, becoming highly vesicular as shown in Fig. 3. At one month before spawning, the oocyte contains a well-grown germinal vesicle, somewhat circular in outline. The nucleoli show remarkable vacuoles, numerous in number (Fig. 4). The nucleus at the time of its migration becomes distorted to some extent, its contour being no longer smooth. Around the nucleus is found a narrow zone of apparently viscid substance, faintly stained with eosin. The nucleoli are very large in size and small in number, while the vacuoles within them disappear temporarily. The chromatin elements are still scarcely detected (Fig.5). After the migration of the germinal vesicle has been completed, the nucleus becomes again round in form with a smooth contour and enormous size. There are many spherical or oval nucleoli of varying sizes in each nucleus (Fig. 6). In addition to the large nucleoli there are many minute ones of a spherical form. The germinal vesicle is now ready for its breakdown. The vacuoles become marked again within the nucleoli (Fig. 7). Soon after, the nucleoli change their form. Some become amoeboid or oval in shape, but others still retain the spherical form. Along with the change of form they become somewhat basophlic. The nucleus exhibits no visible chromatin elements (Fig. 8). Suddenly, the nuclear membrane fades away. The nucleus now appears as a clear body, occupying an eccentric position in the ooplasm. The nucleoli become drawn into thick thread-like, chromosomal bodies which are convoluted and stained deeply with haematoxylin (Figs. 9 and 10). The chromatin elements which resemble strings of beads are now distributed around the

thread-like nucleoli. Then, the convoluted nucleoli become invisible due to the lack of affinity for stain. Along with this change the chromatin elements make their appearance as minute rod-shaped chromosomes (Fig. 11).

There are a few papers published on the behaviour of the nucleus at the time of its breakdown. Calderwood (1892) observed the eggs of the common dab, and reported that there are found more or less distinct congregations of nucleoli in the central area of the degenerating nucleus. The oocytes of the elasmobranch fishes exhibit a flock of nucleoil of spherical form varying in size at the late migration stage of the germinal vesicle (Kastschenko 1890, Ruckert 1892, Maréchal 1906). But the conversion of nucleoli into the thread-like bodies has not been demonstrated in these forms. The convolution of nucleoli was recorded by Jörgessen (1913) in *Melanophases*, by Nussbaum (1913) in *Sterenoptychus* and by Ito (1938) in *Leiognathus*, all of whom noted its occurrence in rather earlier stages. The significance of the nucleolar convolution remains unknown at present.

Along with the accumulation of the cytoplasm in the animal pole of the oocyte, the chromosomes arrange themselves in an area near the periphery of the animal pole, the metaphase spindle of the first maturation division being thus formed (Fig. 12). The "lamp-brush" chromosomes of this species assume vague outline in the latter stages of oogenesis, and therefore the behaviour of the chromosomes remains difficult to trace in the course of oogenesis.

2. Cytochemical study

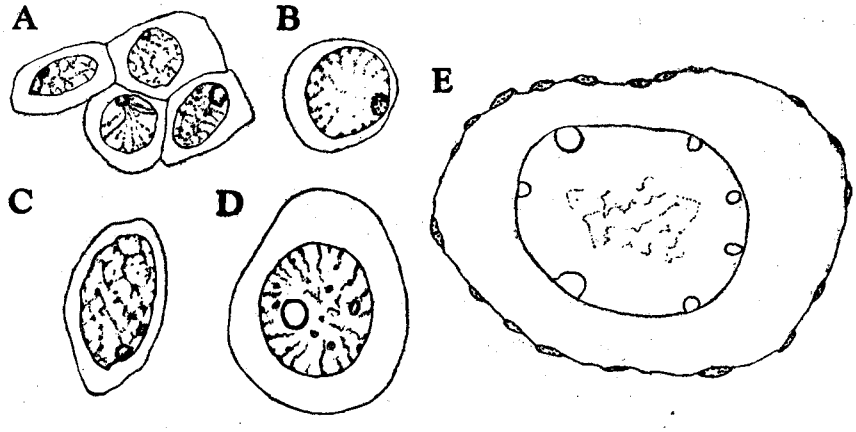
A) Feulgen nucleal reaction

The chemical nature of the chromosomes is the subject of prime concern in this study, since they undergo a very particular change during oogenesis. By means of the application of the Feulgen method the distribution of thymonucleic acid in the chromosomes was studied.

The ovaries of the flounder were preserved in one of the following fixing fluids, Bouin-Allen's mixture, Zenker's fluid, or sublimate acetic acid solution. The material fixed with Bouin-Allen's mixture gave the best results for satisfactory maintenance of both fine structure and good reaction to Schiff's reagent. After fixation the material was dehydrated through alcohol series, embedded in paraffin, and sectioned 8 to 10μ in thickness. Deparaffined sections successively were treated down through alcohol series up to tap water, because long maintenance in 95 % alcohol in order to remove the plasmal reaction produced some coloration of yolk even without hydrolysis. Twenty minutes' hydrolysis in N-HCl at 50°C proved suitable for this material. After the sections were stained for two hours with Schiff's reagent, they were rinsed carefully with aqueous solution of sodium metasulphaste, and then mounted in balsam as usual. As the control, one of the paired slides was stained with Schiff's reagent without hydrolysis.

Observations : The oocytes at the pre-synaptic and synaptic stages embrace densely stained nuclei in which the chromosomes are stained deep violet. A large chromatin-nucleolus is seen at one pole of the nucleus being surrounded by minute and deeply stained violet granules, together with several other flocks of Feulgen positive granules which correspond to minute chromatin-nucleoli (Text-fig. 3, A). The oocytes of the post-synaptic stage show the Feulgen-positive chromatin, being

stained violet. A large chromatin-nucleolus and many minute ones surrounded by minute violet granules are observable more distinctly than in earlier stages (Text-fig. 3, B). Observations of an oocyte in the early peri-nucleolus stage indicate that the chromatin elements of V-figure or oval ring are Feulgen positive, and further that there exists a true nucleolus of large size surrounded by a Feulgen positive ring together with many chromatin-nucleoli enclosed by granules stained violet (Text-fig. 3, C and D). A slightly more advanced



Text-figure 3. Nuclei of young oocytes, tested with the Feulgen nucleal reaction. ×1000, except Fig. E.
A. Pre-synaptic and synaptic stage. B. Post-synaptic stage. C. Earlier phase in the early peri-nucleolus stage. D. Middle phase in the same stage as above. E. Later phase in the same stage as above.

oocyte shows the chromatin-threads stained violet, faintly but distinctly. Several true nucleoli and small chromatin-nucleoli being enclosed by the Feulgen positive ring are also detected. A small oocyte including many peripheral nucleoli also contains the "lamp-brush" chromosomes which are Feulgen positive, though rather weakly. The ooplasm, nucleoplasm and nucleolus remain always unstained. Some of the peripheral nucleoli are surrounded by the ring weakly stained violet (Text-fig. 3, E). With the growth of oocytes the "lamp-brush" chromosomes become rather obscure in appearance, and finally completely negative in reaction. This condition seems to continue until the diakinetic stage is reached. According to Marza et al. (1937), in the oocytes of *Fundulus*, the zona radiata, the peripheral cytoplasm and the space between the yolk globules and the yolk all proved to be positive for the Feulgen nucleal reaction. In the oocyte of the flounder the zona radiata, the cytoplasm, and the yolk were found always to give a negative reaction even in the yolk globule stage. Exceptionally, after a long

maintenance of a deparaffined section in 95 % alcohol (24 hours or more), fairly violet coloration occurred in yolk without hydrolysis. A similar feature has already been demonstrated by Hibbard (1928) and Brachet (1937) in some amphibian eggs, by V. and E. Marza (1935) in the hen's eggs, and by Kugler (1953) in the bluegill's eggs. Brachet (1937) is of opinion that this coloration in the yolk is mainly caused by plasmologen, the acetaldehyde which contaminates the alcohol used in histological technique. After the breakdown of the germinal vesicle, the Feulgen reaction proved again to be positive in the chromosomes lying in the centre of the convoluted nucleoli. The ooplasm, the zona radiata, and the thread-like nucleoli always react negatively to the test, while the nuclei of the follicle cells are fairly positive in reaction.

The results obtained are fairly conclusive for the facts that the chromosomes and heterochromatins containing thymonucleic acid alone do give the Feulgen nucleal reaction, and that the cytoplasm and the yolk are probably free from thymonucleic acid. The response of the chromosomes to the Feulgen reaction in the flounder's oocytes in the present study was proved similar to that observed by Voss (1927) in frogs and lampreys, by Hibbard (1928) in *Discoglossus*, by Gresson (1930) in insects, by E. and V. Marza (1935, 1937) in hens and *Fundulus*, and by Gerush (1940) in frogs. In other words, the chromosomes of the young oocyte of this species are stained violet proving a positive reaction, while the "lamp-brush" chromosomes give a negative reaction. These negative results seem to be favourable to the interpretation that a chemical transformation of nucleic acid has taken place in the chromosomes. Brachet (1950) asserted that the negative results obtained by Gerush (1940) in the frog eggs were attributable to the reduced size of the chromosomes, since they are probably poor in thymonucleic acid contents. As mentioned above, the "lamp-brush" chromosomes of flounder's oocytes are small in size and indistinct in appearance at any time. It is probable that the Feulgen negative results may also be due to the reduced size of the chromosomes. In fine, the oocytes of the flounder are not favourable as material for the study of the chemical nature of chromosomes. Hence, the question whether or not the chemical nature of the chromosomes persists unaltered during oogenesis has remained unanswered.

B) Staining with methylgreen-pyronine

In order to learn about the change of ribonucleic acid in differentiation of occyte, particularly the change in relation to the nucleolus, the method of Unna-Pappenheim for the examination of ribonucleic acid was applied in this study. This procedure enables one to determine the distribution of the ribonucleic acid on the basis of the difference in colour reaction. As the fixative, use was made of absolute alcohol and of alcohol-formol mixture consisting of 80 parts absolute alcohol saturated with picric acid and 20 parts neutral formol. Some other fixatives, such as Bouin's solution or Zenker's mixture, were also tested without good results. Deparaffined sections were stained with aqueous solution of methylgreen-pyronine for 20 minutes, in the manner of Sibatani (1949). Prior to preparation, both methylgreen (Gruber) and pyronine were purified. After staining, the slides were

differentiated in iso-propyl alcohol, dehydrated, and mounted in balsam. To study the digestive action of ribonuclease, one of the paired slides was placed in ribonculease solution for one hour at 65°C before staining. The ribonculease solution was prepared from beef pancreas following Brachet's method (Gomori 1953).

Observations : Since the fixatives failed to preserve well the fine structure of the small oocytes before the early peri-nucleolus stage, the results of the staining test of the chromatin and chromatin-nucleoli with methylgreen-pyronine technique were inconclusive. The peripheral nucleoli of the young oocytes were stained red with pyronine and the cytoplasm deeply purple. The coloration could not be detected in the chromatin even through careful observations, while the nuclei of the follicle layer cells were stained brilliantly green (Fig. 13). Along with the growth of the oocyte the cytoplasm becomes stained faintly and reddish. Throughout the peri-nucleolus stage the stainability of the nucleoli remains unchanged, being always stained reddish. Sometimes the oocyte of this stage contains some nucleoli which are extruded into the ooplasm from the nucleus. The extruded nucleoli lying in the vicinity of the nucleus are stained also fairly reddish (Fig. 14). Likewise in the oocyte of the yolk vesicle stage the nucleoli show fair affinity to pyronine, staining reddish (Fig. 15). The inner zone of the cytoplasm is stained deeply purple, whereas the yolk vesicle proper remains without being stained. As the yolk formation proceeds, the affinity of nucleoli to pyronine increases further. Fig. 16 taken from an oocyte of the primary yolk stage shows the reddish nucleoli scattered dispersedly throughout the nucleus. Within the nucleoli there are several vacuoles remaining uncoloured. Reddish cytoplasm is filled with colourless yolk globules. This condition continues for a short time. The germinal vesicle before migration is nearly the same as that of the later stage in staining reaction for methylgreen-pyronine, except that the nucleoli are strongly stained reddish. The occurrence of a narrow half ring stained deep purple is detected around the nucleus. The nucleus, ready for its breakdown, contains nucleoli also stained deeply with pyronine. The processes of the nucleoli take pyronine faintly (Fig. 17). The convoluted thread-like nucleoli show a coloration in reaction similar to that of the cytoplasm. In the material which has been digested with ribonuclease, both the nucleoli and the cytoplasm remain not stained (Fig. 18).

In order to ascertain the distribution of ribonucleic acid in the cell the combination method of methylgreen and pyronine staining is most useful giving an excellent contrast between DNA stained green and RNA stained red. But the test by means of Unna's mixture reflects the relative status of polymerization rather than any specific chemical difference in the molecules. Thus, the mixture does not stain depolymerized DNA or RNA in any form (Lum 1950, Sibatani 1952). Therefore it can be understood why the chromatin of the young oocyte scarcely takes methylgreen, although the presence of thymonucleic acid in the chromatin was indicated by the Feulgen test. A similar phenomenon has been observed by Pollister and Leuchtenberger (1949) who concluded that the loss of stainability

of the chromatin is due to a change in the physical state of the nucleic acid. It has been generally accepted that ribonuclease prepared by Brachet's method is highly active and that it shows a complete digestion of all basophilia due to RNA, but this crude preparation seems often to be contaminated by the protenase of some kind (Gomori 1953). In conformity with these considerations it would be concluded that the results obtained by methylgreen-pyronine method show rather fairly the distribution of nucleic acid, though not completely accurate.

The findings of the present study generally agree with those of Marza et al. (1937) in *Fundulus*, of Brachet (1950) in *Amphibia*, and of Kugler (1953) in *Lepomis*, though there are a few points in disagreement. In the oocytes of *Fundulus*, *Amphibia* and *Lepomis* the nucleoli take pyronine intensely during oogenesis, while the nucleoli of the flounder's eggs are stained faintly with pyronine in the earlier stages of oogenesis, and intensely in the later stages.

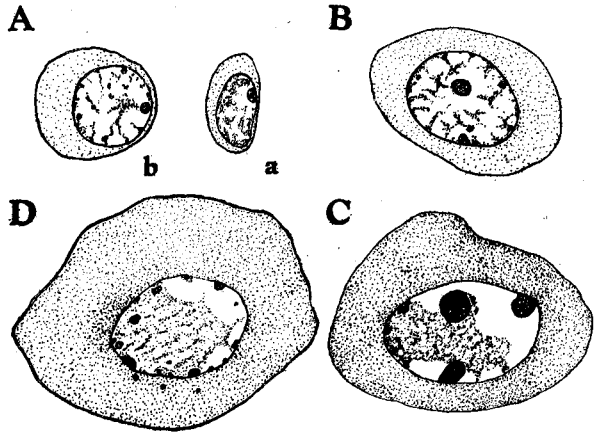
C) Ninhydrin-Schiff's protein reaction according to the method of Yasuma and Ichikawa

Yasuma and Ichikawa (1951) demonstrated protein by the application of ninhydrin-Schiff's reaction. This reaction is said to be based on the following principle that oxidative deamination of terminal amino acid with ninhydrin produces aldehyde groups which can be demonstrated with Schiff's reagent.

The material fixed with alcohol-formol and Bouin-Allen's solution was employed by the present worker for this test. Deparaffined slides prepared in a similar way as in the previous tests were treated with 0.1% solution of ninhydrin in absolute alcohol for three hours at 37°C. After rinsing in running water for one minute, they were stained with Schiff's reagent for 30 minutes. Subsequently, they were washed carefully with three changes of sodium metasulphate solution, being left ten minutes in each bath, and then were kept again in running water. Finally they were dehydrated and mounted in balsam as usual.

Observations : The nucleus of the young oocyte at the post-synaptic stage contains a minute body together with a certain number of smaller bodies showing positive protein reaction. In addition to these bodies there are several elements of fibrous structure faintly stained purple-red. Undoubtedly the small bodies are the chromatin-nucleoli as indicated by the cytological study (Text-fig. 4, A). The small oocyte at the early peri-nucleolus stage possesses a nearly similar structure, except that the stained bodies are larger in both size and number. The largest one, situated in the central region of the nucleus, is certainly the true nucleolus (Text-fig. 4, B). In a slightly larger oocyte of the same stage the true nucleoli stained purple red begin to arrange themselves around the nucleus. Within the nucleoli are found clear, colourless vacuoles, though the latter were not detected in the preparation stained with haematoxylin. In the central region of the nucleus occur the small bodies and fibrous elements which give positive protein reaction (Text-fig. 4, C). The extrusion of the nucleoli or nucleolar fragments into the cytoplasm was observed in the oocyte in the latter part of the early peri-nucleolus stage (Text-fig. 4, D and Fig. 19). Similar response of the nucleoli to the protein reaction was obtained in the preparation of oocytes of the late peri-nucleolus stage.

The nucleus of an oocyte at the yolk vesicle stage shows the large and worm-like nucleoli stained purple red. They contain several vacuoles. In the oocytes of the early yolk stage the nucleoplasm is stained as deeply as the nucleolus, so that the structure of the nucleoli in this condition is difficult to observe in detail. Then,



Text-figure 4. Nuclei of younger oocytes, tested with the ninhydrin - Schiff's reaction for protein.
A. Pre- and post-synaptic stage, ca. × 1000. B. Earleir phase in the early peri-nucleolus stage, ca. ×1000. C. Middle phase in the same stage as above, ca. ×660. D. Later phase in the same stage as above, ca. ×250.

the nucleoli of an oocyte at latter yolk stages show again the susceptibility to stain more deeply than the nucleoplasm. The nucleoli distributing in the nucleus increase in number and show a vesicular structure. The nucleus just before the stage of breakdown contains some amoeboid nucleoli together with many small bud-like ones. The protuberances of amoeboid nucleoli and of bud-like ones are stained more faintly than the main part of the nucleoli (Fig. 21). The convoluted thread-like nucleoli which appear after the breakdown of the germinal vesicle give a response to the protein reaction similar to that of the surrounding cytoplasm. Therefore, there lies some difficulty in distinguishing the nucleoli from the surrounding cytoplasm.

The above findings are fairly in harmony with those presented by the corresponding observations in *Leiognathus* by Ihnuma and Tsukuda (1952).

Discussion

The extrusion of the nucleoli or nucleolar fragments into the cytoplasm from the nucleus has been demonstrated by many investigators to occur in fish eggs of various species (Eimer 1875, Scharff 1887, Calderwood 1892, Eggert 1929, Narin 1937, Ito 1938, and Chaudley 1951). In the case of *Leiognathus*, Ito (1938)

has observed this feature morphologically in detail reporting that the nucleolar fragments are extruded into the cytoplasm successively through a period from the late peri-nucleolus stage to the secondary yolk stage. The present study has produced further evidence of that fact in the flounder's eggs through the investigation by observation of the protein reaction.

Recent interest centers round the mechanism by which the nucleoli or the nucleolar fragments are extruded from the nucleus into the cytoplasm. Interesting is the assumption of Ito (1938) that the nucleolar substance may be pushed out from the nucleus into the cytoplasm as a result of the dissolution of the nuclear membrane at the place where the nucleoli are in contact with it. Some certain substance derived from the vacuoles may serve to dissolve the nuclear membrane. The existence of such a substance was proved by Berg (1932, 1934) in the vacuoles of the nucleoli of liver cells. When the usual morphological staining method is employed, the nucleoli are stained homogeneously black without showing vesicular structure. But the treatment involving the protein test can clearly demonstrate the presence of the nucleoli which contain the vacuoles even in young oocytes, as shown in this study. The present observations appear to support the assumption offered by Ito (1938).

Knowledge has remained rather meagre on the origin of the peripheral nucleoli in the oocyte. Some authors, such as Cunningham (1894, 1898), Jörgessen (1913), Nussbaum (1913), Eggert (1929), and Subramanian and Aiyer (1935), have asserted that the nucleoli lying close to the nucleus originated from the fragmentation of a large nucleolus which had been present in the nucleus. The present author's findings indicated the presence of the chromatin-nucleoli in the youngest oocyte with no trace of the existence of the true nucleoli. This is also the same with other forms, so far reported (Maréchal 1905, 1906, Franz 1910, Hickling 1935, etc.). Here a question arises on the origin of the true nucleolus. As mentioned in the foregoing pages, the oocyte of this species shows chromatin-nucleoli which are positive to the protein reaction and are enclosed by minute Feulgen positive granules. On the other hand, the true nucleoli are also surrounded by a ring stained intensely with Schiff's reagent. Thus, the distinction between the chromatin-nucleoli and the true nucleoli are not marked in the early stage of their appearance, whether the observations are based on morphology or on cytochemistry. It seems highly probable, therefore, that the true nucleoli might have their origin from the chromatin-nucleoli. Furthermore, the fact that the chromatin-nucleoli, regardless of their size and position, are surrounded by the Feulgen positive ring seems to be strong evidence for the view that the nucleoli have originated from the chromosomes. Since there is no sign of dividng figures in the true nucleolus, the multiplication of the peripheral nucleoli is not the result of the division of the true nucleoli, but of the migration of the true nucleoli of chromatin-nucleolar origin towards the nuclear membrane. Then, it may be possible to conclude that the peripheral nucleoli are resulted from the chromatin-nucleoli of possibly chromosomal origin through their development and migration. Recently, Ris and Ruth (1952) working on the chemical nature of a substance eliminated from chromosomes in the first division of *Solenobia* reported that the eliminated chromosomal substance is essentially ribonucleoprotein because the Feulgen reaction and Hotchkiss test for polysaccharides proved to be negative, while the test for ribonucleic acid and protein were positive. This is very interesting in relation to the fact that the nucleolus which is assumed to be eliminated from the chromatin is negative to the Feulgen nucleal reaction, but positive to the ribonucleic acid and protein reaction.

Finally, the discussion should be extended to the rôle played by the extruded nucleolar material in the course of oogenesis. Many earlier workers have assumed that such nucleolar material may give rise to yolk spheres. A good example in support of that idea is offered by Hogben (1920) in insects. Here the yolk is said to arise from granules that are formed in vacuoles inside the nucleoli while still inclosed in the nucleus and that are later cast out into the cytoplasm, when they migrate towards the periphery and enlarge to form the yolk-spherules. But this seems to be a special case. There is no evidence to illustrate the direct relationship between the extruded nucleolar material and the yolk spherule. The view is thus only hypothetical. Recently, the extruded nucleolar substance has called the attention of many authors in the field of cytochemical study. The Caspersson school (1950) has emphasized that nucleic acid takes some part in the processes leading to synthesis of cellular protein, and further that the nucleolus plays an important rôle for the production of nucleic acid in the cytoplasm. Altmann (1949), on the basis of his findings in human liver tissue, has stated that as the nuclear vesicles which are extruded into the cytoplasm contain ribonucleic acid, new evidence is discovered for the idea of Caspersson concerning the connection between the nucleus and cytoplasmic nucleic acid. Further, Alfert (1950) working on the behaviour of the nucleolus in the course of oogenesis in the mouse, came to agree with the hypothesis of the Caspersson school regarding the role of the nucleolus in cellular metabolism. The findings of the present study indicate that a successive extrusion of the nucleolar substance into the cytoplasm has taken place during the period from the early peri-nucleolus stage to the secondary yolk stage, and further they demonstrate that the extruded nucleolar substance shows a reaction suggesting the presence of ribonucleic acid. The results here presented seem to be evidence in favour of Casperssons's hypothesis. The important rôle of nucleic acid in cell metabolism has been strongly emphasized by the work of the collaborators of Caspersson and Brachet.

Summary

Cytological and cytochemical observations were undertaken in order to make clear the activity of the nucleolus in relation to the egg formation in the flounder, *Liopsetta obscura*. The results obtained are summarized as follows:

1. The general configuration and behaviour of the chromosomes in the oocyte of the flounder are the same as those demonstrated in other osseous fishes. They appear at first as the "net-like" chromatin in the early stage of oogenesis and are found transformed into the "lamp-brush" chromosomes during the growth period. During the latter part of the growth period, the lamp-brush chromosomes are difficult to detect in the surrounding network configuration of the nucleus. Just after the breakdown of the germinal vesicle the chromatin elements are found like strings of beads distributed around the convoluted thread-like nucleoli. With the disappearance of the nucleoli, the chromatin elements develop

into rod-shaped chromosomes of minute size, and migrate as a group to the animal pole of the egg. Shortly the chromosomes form the metaphase spindle of the first maturation division.

2. Chromosomes of the young oocytes are positive to the Feulgen nucleal reaction, being stained deeply violet, while the "lamp-brush" chromosomes of the latter stage give a negative reaction. This condition is retained without change until the stage of diakinesis.

3. The oocytes of the pre-synaptic, synaptic and post-synaptic stages contain chromatin nucleoli only, with no trace of the existence of the true nucleolus. At the beginning of the early peri-nucleolus stage, one or two large nucleoli, strongly basophilic in nature, appear in the nucleus. Because of having no direct continuity with the spireme, they are regarded as the true nucleoli. As the oocytes grow, the nucleoli come to arrange themselves close to the periphery of the nucleus. During the latter growth period the peripheral nucleoli show a marked change in their form, size, number and stainability. Soon after the breakdown of the germinal vesicle the nucleoli become drawn into convoluted thread-like bodies which are deeply stained with haematoxylin. Shortly, these thread-like bodies become invisible because they lack affinity to stains.

4. The extrusion of the nucleolar fragments from the nucleus into the cytoplasm are demonstrated throughout the periods ranging from the early peri-nucleolus stage to the secondary yolk stage. It was found that the nucleoli assumed a vesicular structure throughout the stages noted above. Observations seem to present evidence in favour of the view that the extrusion of nucleoli is due to the dissolution of the nuclear membrane, probably through some certain action of vacuoles produced in the nucleolus.

5. Based on the results of both cytological and cytochemical studies here reported, the following conclusion is possible : that the peripheral nucleoli have their origin in the chromatin nucleoli which originated from the chromosmes.

6. The application of the method of staining with methylgreen-pyronine produced evidence that the extruded nucleolar fragments contain ribonucleic acid. Such evidence seems to support the view of Caspersson concerning the connection between the nucleus and cytoplasmic nucleic acid.

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Explanations of Plates

Plates XIII and Plates XIV

All figures in these plates are photomicrographs taken from the sections of the ovaries, prepared with Bouin or Bouin-Allen and iron-haematoxylin method.

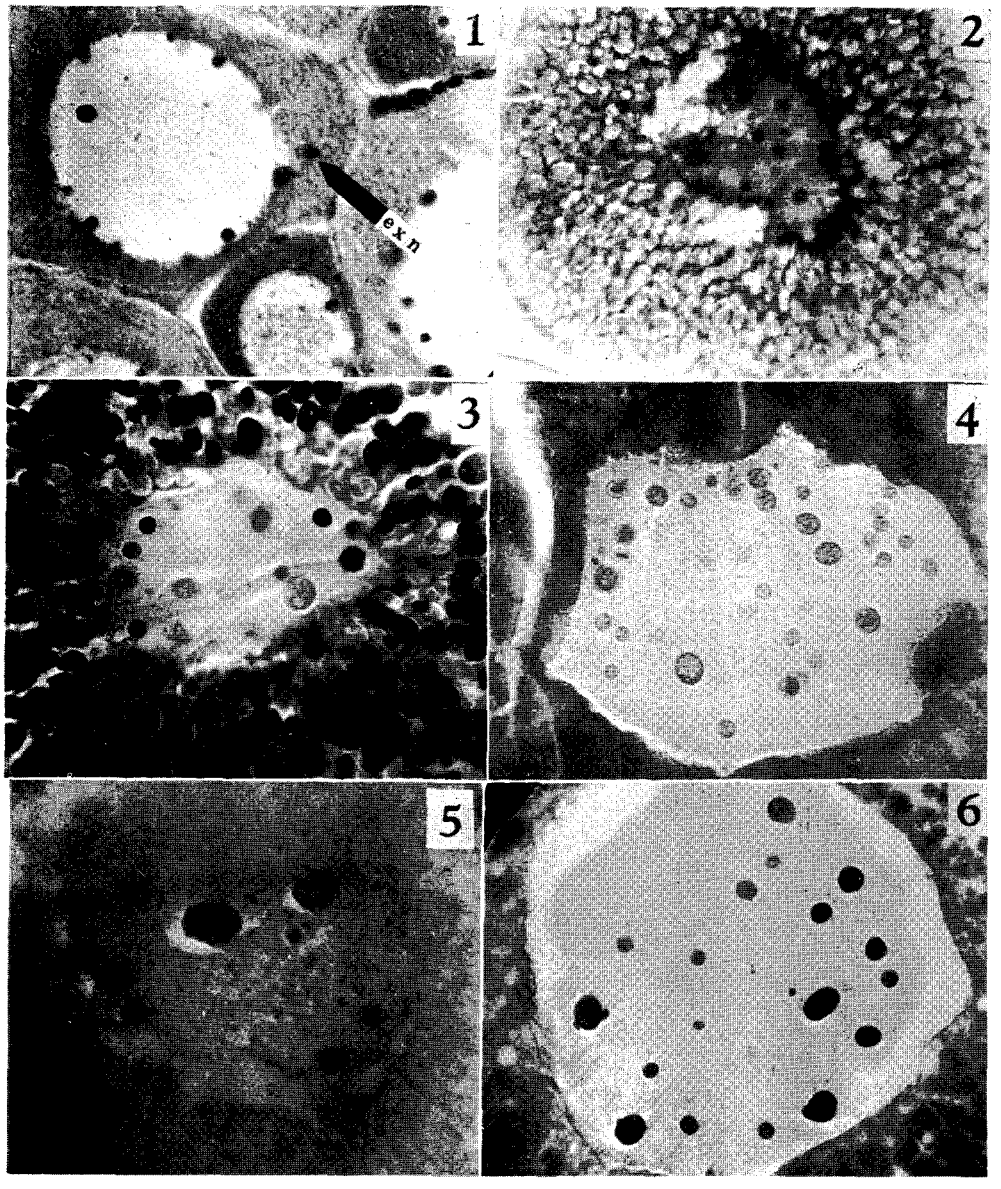
Fig. 1. Nuclei of oocytes at the late peri-nucleolus stage, showing the extruded nucleolus. ex. n. Extruded nucleolus ca. ×660.
Fig. 2. Nucleus of an oocyte at the primary yolk stage. ca. ×410.
Fig. 3. Nucleus of an oocyte at the secondary yolk stage. ca. ×410.
Fig. 4. Nucleus just prior to its migration. ca. ×410.
Fig. 5. Nucleus at the time of its migration. ca. ×410.
Fig. 6. Nucleus just finished its migration. ca. ×410.
Figs. 7–8. Nuclei at the preparatory stage of its breakdown. ca. ×350.
Fig.s 9–10. Convoluted thread-like nucleoli found just after breakdown of the germinal vesicle. ca. ×660.

Fig. 11. Chromosomes flocking in the central region of the thread-like nucleoli which have almost faded out. ca. ×1200.
Fig. 12. Metaphase spindle of the first maturation division. ca. ×1200.

Plate XV

The figures from 13 to 18 are photomicrographs taken from the sections, stained with methyl green-pyronine, and the figures from 19 to 21 photomicrographs from the sections with ninhydrin-Schiff's reaction for protein. Magnification in about 260 times with the exception of Fig. 19.

Fig. 13. Oocytes at the early peri-nucleolus stage.
Fig. 14. Oocyte at the later phase of the early peri-nucleolus stage, showing the extruded nucleoli.
y.n. Yolk nucleus. ex. n. Extruded Nuleolus.
Fig. 15. Oocyte at the yolk vesicle stage.
Fig. 16. Nucleus of an oocyte at the primary yolk stage.
Fig. 17. Nucleus at the preparatory stage of its breakdown.
Fig. 18. Nucleus at the same stage as above, treated with ribonuclease solution before staining.
Fig. 19. Oocyte at the early peri-nucleolus stage., showing the extruded nucleolus. ex. n. Extruded nucleolus. ca. ×660.
Fig. 20. Oocyte at the yolk vesicle stage.
Fig. 21. Nucleus at the preparatory stage of its breakdown.



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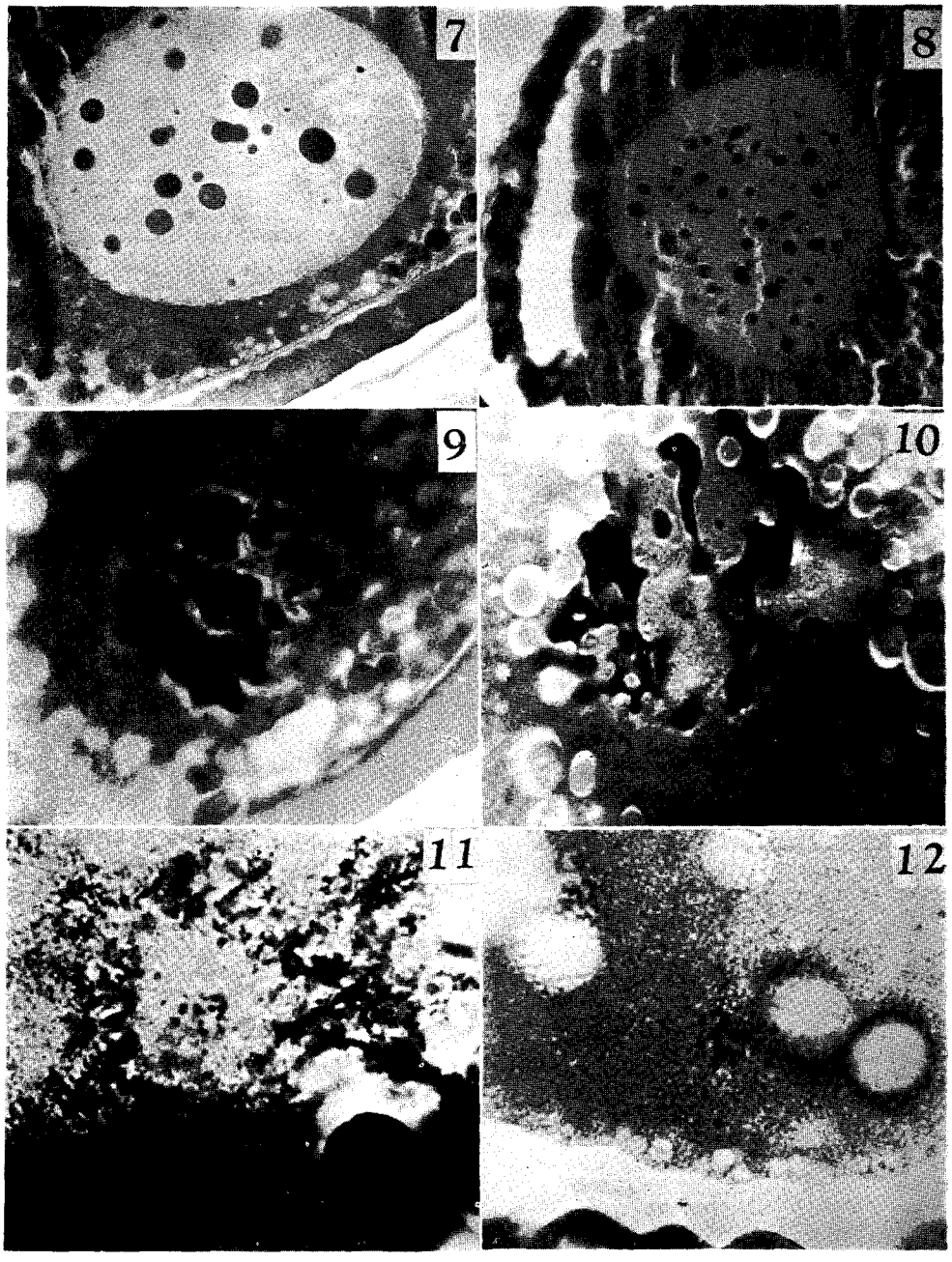
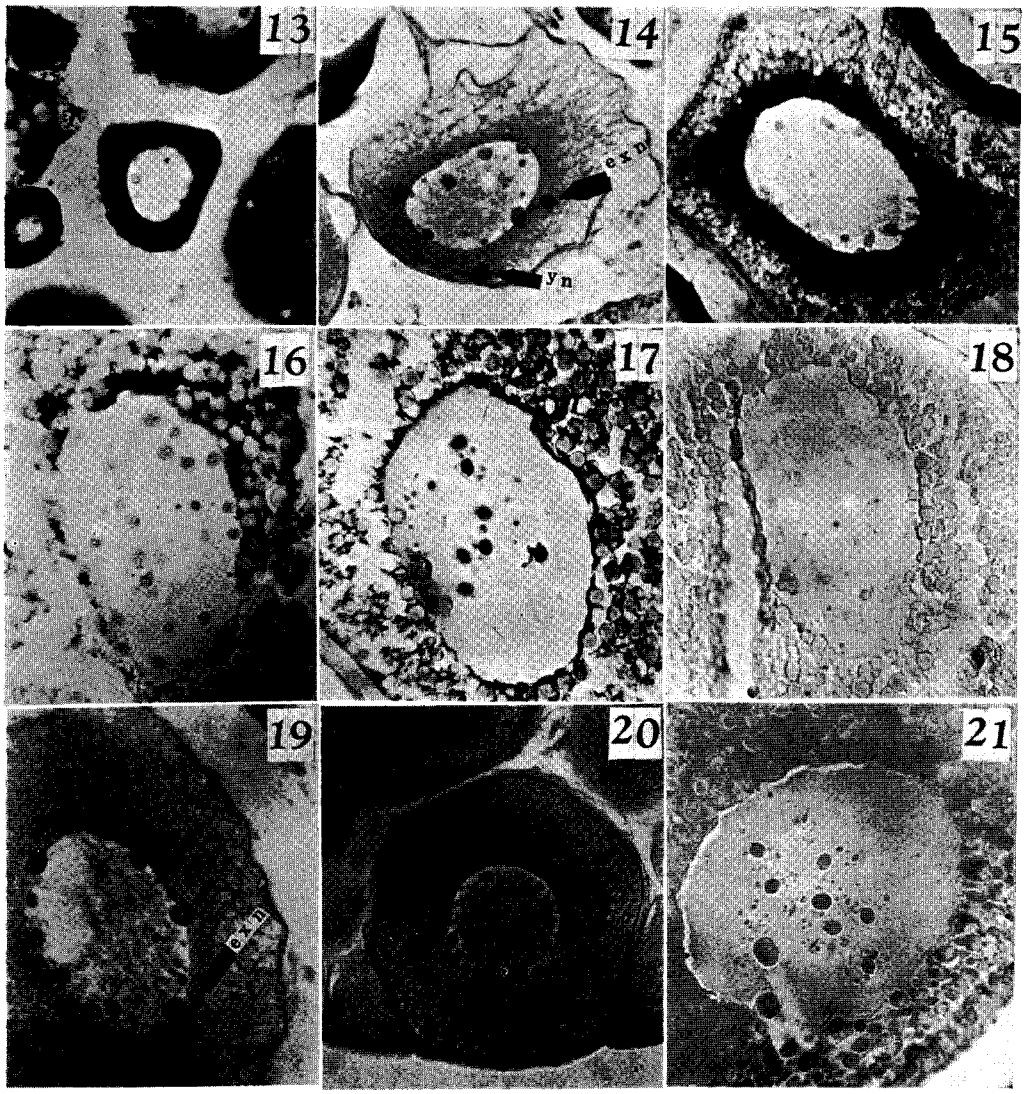


K. Yamamoto: *Studies on the Formation of Fish Eggs, II*



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