Studies on the Formation of Fish Eggs
XI. The Formation of a Continuous Mass of Yolk and the Chemical Nature of Lipids Contained in it in the Oocyte of the Flounder, *Liopsetta obscura*[1)]

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(*With 10 Text-figures*)

Fish eggs can be classified, in conformity with the form of yolk, into two types. The eggs of the first type are characterized a continuous mass of yolk enclosed by a thin layer of cytoplasm, while those of the second type have a non-continuous mass of yolk composed of numerous, large yolk globules. *Pleuronectes* eggs (Cunningham 1894, Wheeler 1924), *Rhombus* eggs (Cunningham 1898), *Fundulus* eggs (Marza et al. 1937), *Oryzias* eggs (T. S. Yamamoto 1955), *etc.* belong to the first type; *Trigla* eggs (Cunningham 1898), *Osmerus* eggs (Lahm 1903), *Cyprinus* and *Gobio* eggs (Konopacka 1935), *Sacchobranchus* eggs (Narin 1937), *Perca* eggs (Mas 1952), *etc.* are of the second type.

As to the yolk formation of the first type eggs with which the present article deals, several divergent statements have hitherto been made as follows: (1) It has been assumed in the oocytes of several kinds of flatfishes that the yolk globules which are formed centripetally from the peripheral region of the ooplasm to the inner give rise to a continuous mass of yolk by their fusion (Cunningham 1894, 1898 : Wheeler 1924). (2) *Fundulus* oocytes show two kinds of yolk globules : One of them is the intravesicular yolk globules, which make their appearance as yolk vesicles arranged near the periphery of the oocyte and continue to be formed inwards along with the transformation into yolk globules due to the deposition of albuminous substance inside the vesicles. The other is the extravesicular yolk globules deposited directly in the extravesicular cytoplasm. These two kinds of yolk globules subsequently grow into a continuous mass of yolk by their confluence (Marza et al. 1937). (3) According to T.S. Yamamoto (1955) who has been working on the oocytes of *Oryzias*, yolk vesicles first appear in the peripheral region of the cytoplasm and then in the peri-nuclear region. Independently of the yolk vesicles, plate-like yolk is accumulated in the central part of the oocyte. The yolk then grows outwards, resulting in the formation of a continuous mass of yolk. The findings obtained in the present material are in agreement with

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none of the above results. Here, the writer wishes to report in detail the results of his observations on the formation of a continuous mass of yolk, with some accounts on the chemical nature of lipids contained in it.

It is the writer's great honor to dedicate this article to Professor Tohru Uchida, Director of the Akkeshi Marine Biological Station, under whose guidance the main part of this work has been performed, in commemoration of his 60th birthday. The writer is also indebted to Professor Sajiro Makino, Faculty of Science, and Professor Hisanao Igarashi, Faculty of Fisheries, for their kind advices.

Material and method

The oocytes of the flounder, *Liopsetta obscura*, were obtained in a similar way to that described in the former paper (K. Yamamoto 1956a).

Cytochemical staining :

a) Ciaccio's test (cited from Lison 1953) : Materials were fixed for three days with Regaud's solution containing acetic acid, and then immersed in 3 per cent potassium dichromate for 7 days. Deparaffined sections, 10 micra in thickness, were stained in Sudan black B or Scharlach R solution for one hour at 30°C.

b) Modified Sudan black B and alcohol method (cited from Lison 1953) : Use was made of 10 per cent formalin or Baker's formalin-calcium solution as fixative. Frozen sections, 15 to 30 micra, were prepared, and stained for 30 minutes with Sudan black B in 70 per cent alcohol.

c) Baker's test (cited from Lison 1953) : After being fixed with formalin-calcium solution and subsequently mordanted with potassium dichromate solution, the materials were embedded in gelatine. Frozen sections prepared from the material were stained with acid haematein, treated with borax-potassium ferricyanide, then mounted as usual. Slides treated with pyridine before haematein staining were used as control.

d) Schultze's test (cited from Lison 1953) : Frozen sections prepared from neutral formalin-fixed materials were exposed to direct sunlight for 4 days. Old acetic acid and sulfuric acid mixture was poured drop by drop on the slides, then they were covered and examined.

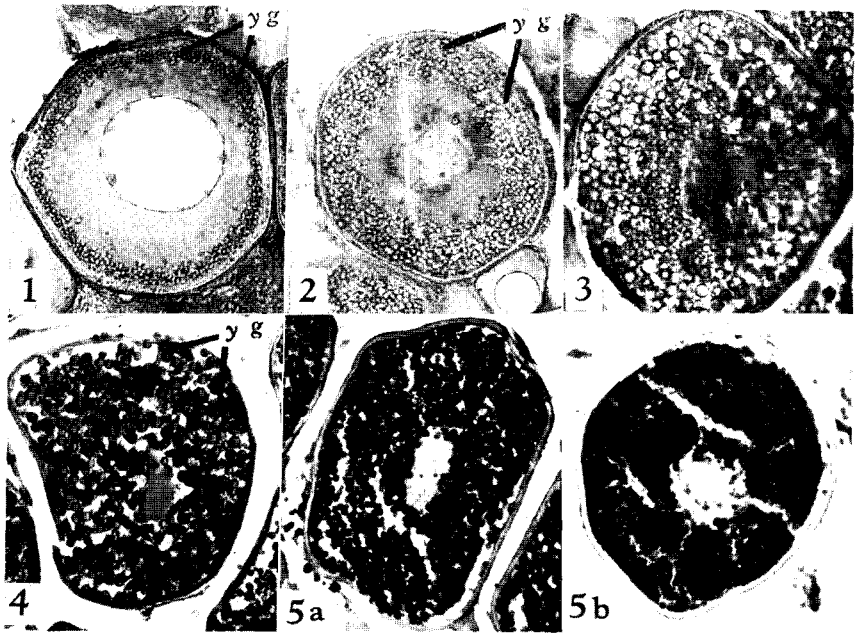
e) Smith's test (cited from Lison 1953) : Materials were fixed in 10 per cent neutral formalin and sections were prepared by freezing method. Slides were stained in one per cent Nile blue for 20 minutes, differentiated with one per cent acetic acid and then mounted with glycerine-gelatine.

Observations

1. Formation of continuous mass of yolk.

To trace the process of yolk-mass formation, Ciaccio preparations proved to be the most suitable. As was previously reported (K. Yamamoto 1956b), yolk vesicles exhibiting a negative Ciaccio's reaction are first formed in the peripheral region of the ooplasm. A little later than the formation of the vesicles, yolk globules giving a positive reaction to Ciaccio's test appear in the vicinity of the vesicles, the two type of bodies being intermingled (Fig. 1). As the oocyte grows, the two kinds of vitelline elements continue to be formed centripetally until the ooplasm comes to be choked with them (Figs. 2 and 3). During this

period, the globules grow rapidly in size and number, along with the increase of the affinity to Sudan black B. On the other hand, the vesicles grow slowly in size and remain sudanophobic. Hereafter, the yolk globules become step by step enlarged and numerous up to the tertiary yolk stage. Simultaneously, they become more and more evenly and deeply stained with Sudan black B (Figs. 4 and



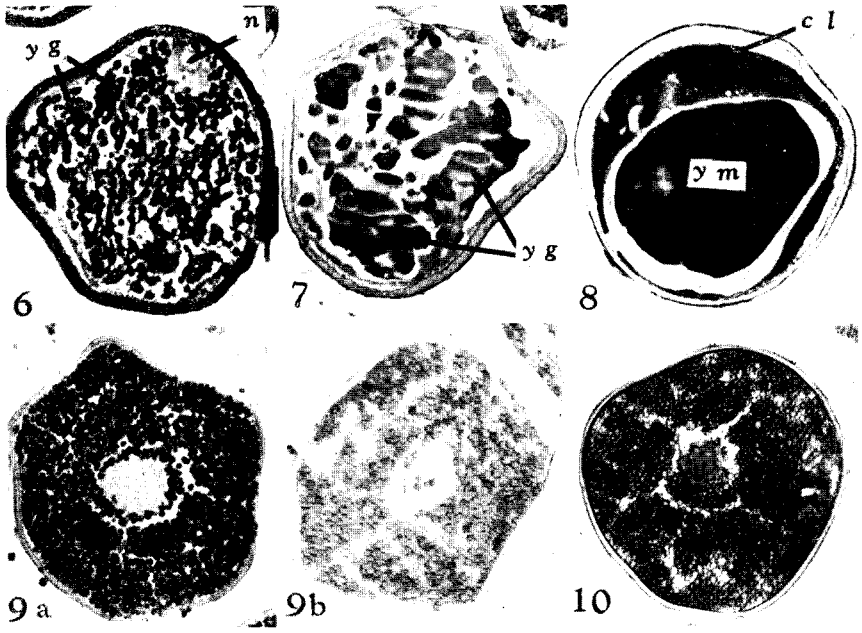
Photomicrographs from sections of flounder eggs.

Figs. 1 and 2. Yolk vesicle stage. Regaud and Sudan black B preparations. Fig. 3. Primary yolk stage. Preparation as above. Figs. 4 and 5a. Secondary yolk stage. Preparations as above. Fig. 5b. Same stage as above. Modified Sudan black B and alcohol preparation. *yg*, Yolk globule.

5). Although it is difficult in Ciaccio preparations to trace fully the behavior of the yolk vesicles because of their small size and negative reaction to Ciaccio's test, there remains little doubt but that during this period the vesicles gradually move towards the periphery of the ooplasm as demonstrated clearly in the previous paper (K. Yamamoto 1956e).

In the migratory nucleus stage, the yolk globules located in the peripheral region of the ooplasm are larger in appearance than those located in the inner region. After the migration of the nucleus has been completed, the globules in-

crease rapidly in size and decrease markedly in number due probably to the fusion of the globules (Fig. 6). This activity subsequently seems to proceed quickly till a continuous mass of yolk is completely formed. In general, it appears likely that the globules situated on the opposite side of the migrated nucleus are larger in size than those of the nucleus side, in other words, the fusion of the globules



Photomicrographs from sections of flounder eggs.

Fig. 6. Migratory nucleus stage. Regaud and Sudan black B preparation. Fig. 7. Prematuration stage. Preparation as above. Fig. 8. Maturation stage. Preparation as above. Fig. 9a. Secondary yolk stage. Baker's acid haematein preparation. Fig. 9b. Same stage as above. Baker preparation with pyridine treatment. Fig. 10. Same stage as above. Smith's Nile blue staining preparation. *cl*, Cortical layer. *n*, Nucleus. *yg*, Yolk globule. *ym*, Yolk mass.

proceeds faster on the anti-nucleus side than on the nucleus side. In company with the confluence of yolk globules, the hyaloplasm covering the interstices between the globules gathers together in the periphery of the ooplasm, finally resulting in the formation of the cortical layer which is fairly thick at the animal pole and becomes thin towards the opposite pole. Throughout these periods the strong sudanophilic nature of the globules remains almost unaltered. The

hyaloplasm generally exhibited a very faint Ciaccio-positive reaction (Fig. 7), but in the maturation stage sometimes it was stained moderately with Sudan black B as shown in Figure 8, although the cause of this phenomenon has not yet been made clear.

From the findings mentioned above there remains no doubt that a continuous mass of yolk originates in the yolk globules. It may be also accepted that no yolk vesicles participate in the formation of a massed yolk, because the vesicles have a characteristic behavior and chemical composition independent of the yolk globules throughout vitellogenesis.

2. The chemical nature of lipids occurring in the yolk globules.

As already mentioned above, the yolk globules gave a positive reaction to Ciaccio's test throughout vitellogenesis. To recognize the chemical nature of lipids contained in the globules, the oocytes in the secondary yolk stage were subjected to several cytochemical tests following Lison's table for lipid-analysis (1953). The results obtained are as follows: By the application of the modified Sudan black B and alcohol method for the demonstration of lipids in general, the globules were stained deeply in blue-black (Fig. 5b). Unstained formalin-fixed frozen sections were almost colorless and proved to contain no carotenoids. Schultze's test for cholesterol was negative. Baker's test for phospholipids was fairly positive in the globules as illustrated in Figures 9a and 9b. Moreover, the globules were stained blue by Smith's Nile blue staining for the differentiation of blue-stained phospholipids from red-stained neutral fat (Cain, 1947) (Fig. 10). On the basis of the above data, it seems justifiable to consider that the globules contain large quantities of phospholipids, but no cholesterides, carotinoids and neutral fat in appreciable quantities. According to Baker (1947), only egg-lecithin globules are stained evenly in blue with acid haematein while other kinds of phospholipid globules, such as sphingomyelin and cephalin, have a blue-black rim and a yellow center. As the yolk globules of the present species were evenly blue-black throughout, it may be logical to presume that phosphatides present in the globules are composed mainly of lecithin.

However, there remains still a question of whether lecithin is always the main lipid-component of yolk globules throughout vitellogenesis. In Ciaccio preparations, each globule in the oocytes of early developmental stages has a light blue center and blue black layer lying a little below the surface (Figs. 1, 2 and 3). Although it becomes more and more stained deeply and evenly with the progress of vitellogenesis, such an uneven affinity for stains is retained in the yolk globules up to the late phase of the primary yolk stage (Fig. 4). Hereafter, the yolk globules always showed a strong and homogeneous reaction to Ciaccio's test. Thus it is evident that the intensity and mode of reaction are different in different developing globules, despite the fact that the globules gave always a Ciaccio-positive reaction. It may be, therefore, reasonable to suppose that the lipids contained in the globules are changed not only in amount, but also in nature,

during vitellogenesis.

Discussion

The writer's statement as to the formation of a massed yolk resembles the reports of Cunningham (1894, 1898) and Wheeler (1924) working on other kinds of flatfishes, so far as yolk globules are formed centripetally and grow into a continuously massed yolk by their confluence. However, they did not refer to yolk vesicles, while the presence of the yolk vesicles is also expected in their materials with high probability, because Cunningham(1894) observed the appearance of minute refrangible globules distributed sparsely throughout the protoplasm of sole eggs a little before commencement of the spawning season. The observations of Marza et al. (1937) do not agree with the present author's findings, in the point that yolk vesicles are transformed into yolk globules by the accumulation of albuminous materials inside them and then they grow into a massed yolk by fusion with extravesicular yolk globules. As the result of closely examining his P.A.S. and Ciaccio preparations, the writer has concluded that the yolk vesicles of the flounder contain mucopolysaccharides throughout the course of vitellogenesis and that they give rise to the cortical alveoli independently of the yolk globules. Further findings obtained in the herring and the smelt (K. Yamamoto 1956 c, d) are also in favor of this conclusion, so that the work of Marza et al. seems to need confirmation. T.S. Yamamoto (1955) advanced an identical opinion with that of the present writer, that in *Oryzias* oocytes yolk vesicles give rise to the cortical alveoli and a continuous mass of yolk is derived from plate-like yolk alone. However, there can be found one noteworthy difference between the two species, that is, in *Oryzias* the formation of the plate-like yolk commences in the central region of the oocyte and proceeds outwards, while in *Liopsetta* globule formation commences in the peripheral region and proceeds centripetally. Although this difference might be attributed to the difference in species, there appears to remain some room for doubt before T. S. Yamamoto's statement on *Oryzias* is fully accepted, taking into consideration the data obtained hitherto, together with his photomicrographs.

As to the chemical nature of lipids contained in yolk globules, a few papers have already been published. T.S. Yamamoto(1955) has demonstrated the presence of lipids in the plate-like yolk of *Oryzias* eggs belonging to the massed yolk type by Ciaccio's test and the frozen Sudan III method. On the other hand, Konopacka (1935), who studied on *Gobio* and *Cyprinus* oocytes belonging to the non-massed yolk type, reported that the yolk plates give always a positive reaction to a formalin-fixed and Sudan III-haemalum method and Smith-Dietrish test, further that the relative amount of lipids to proteins in the plates is changed with the development of the oocyte. Moreover, the yolk plates of *Perca*, according to Mas (1952), were positive to the formalin-fixed Sudan III method and Ciaccio's test for the demonstration of lipids in general, but negative to the Liebermann-Burchardt test for cholesterol. Smith's Nile blue test was proved to stain these plates blue. From the above data together with the findings of the present study it may

be concluded that the yolk globules of many kinds of fishes, regardless of whether they give rise to a continuous mass, contain a large quantity of lipids, and further that the lipid components of yolk globules usually consist mainly of phosphatides, but of no cholesterol and neutral fat.

In the field of biochemistry, on the other hand, detailed analyses of fish egg lipids have already been made, although they, unfortunately, are concerned with the lipids of the egg as a whole, but not with their location in the egg. According to Fauré-Fremiet et Garrauelt (1922), fat fraction extracted by ether from the carp eggs consists of 12.3 per cent phosphatides, 6.1 per cent glycerides, 1.3 per cent cholesterides and 0.8 per cent unsaponifiable substances. Further, Igarashi et al. (1956a,b) have recently established the presence of much lecithin and some cephalin in the eggs of the pollack in connection with their publication of a detailed analysis of the lipid components. These biochemical results, therefore, seem to suggest that phosphatides, main component of the yolk globules, consist mainly of lecithin and within fish eggs other lipids such as glycerides and cholestrides are frequently located in the sites other than the yolk globules. Konopacka (1935) and T. S. Yamamoto (1955) gave an opinion holding that the lipids occurring in the yolk plates must be present in the state of lipoprotein. The present writer is of the same opinion.

Summary

1. The massed yolk of flounder eggs appears to be derived from yolk globules alone, independently of yolk vesicles. The yolk globules appear at first in the peripheral region of the ooplasm as minute granules and continue their centripetal arrangement until the whole ooplasm is filled with them. Subsequently, the globules increase in size and number, and finally develop into a continuous mass of yolk by their fusion.

2. With cytochemical techniques the presence of lipids was proved in the yolk globules throughout vitellogenesis. The globule lipids in the oocytes of the secondary yolk stage are regarded as phosphatides composed mainly of lecithin, but the change, in both amount and nature of the substances seems to occur during vitellogenesis.

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