J. Fac. Fish. Anim. Husb.
Hiroshima Univ. (1971), **10**: 11~19

# Studies on Rainbow Trout Egg (*Salmo gairdnerii irideus*)

## III. Determination of Lipid Composition of Oil Globule and Lipoprotein

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(Figs. 1-6; Tables 1-2)

In a previous paper[1], the authors have made up a report on the three different fractions in rainbow trout eggs: the oil globule, the low density fraction (LDF) and the high density fraction (HDF). It is common knowledge now that the oil globule of the trout egg is formed at the end of the yolk vesicle stage[2], and appears as one of the vitellin elements in the course of the maturation. During the embryogenesis the oil globule seems to remain almost unabsorbable by the embryo. The presence of neutral fats in the oil globule has been observed by histochemical technique[3][4], but the chemical analysis of the oil globule itself has not yet been tried, as far as the authors know. On the other hand, after the vitellogenesis, the yolk globule contains a large amount of lipids. As previously reported, the main component of the yolk globule of the rainbow trout egg was found out to be a lipoprotein containing 22.8 per cent of lipids[1].

In the hen's egg yolk two kinds of lipoprotein, HDF and LDF, have been isolated by centrifugation[5]~[8]. They differ greatly from each other in their physical and chemical properties[9]~[12]. Since the lipid content of the LDF is higher than that of the HDF, the former seems to be insoluble in an aqueous solution. MARTIN *et al.*[6], however, found out that this LDF becomes soluble in an aqueous solution after an ether treatment.

On the other hand, there is little evidence of the lipid in lipoprotein of fish eggs. The lipid was supposed to have a nutritional significance for an embryo or alevin during the embryogenesis. This paper is meant to be a report on the lipid composition of oil globule and lipids of lipoprotein in rainbow trout eggs.

## MATERIALS AND METHODS

**Preparation of LDF, HDF and oil globule**: The fish egg yolk was treated as previously described[1]. After centrifuging of the yolk content, the oil globule of the hyperphase was dissolved in ether, filtered off and concentrated by vacuum evaporation. The hypophase was dispersed in a 2% NaCl solution and recentrifuged at 15000G for thirty minutes. The lower layer with a higher density, was collected, sealed in a cellophane tube and dialyzed against water for five days, then the precip-

itates resulting from the operation were collected as a sample of HDF. The floating layer with a lower density (LDF), was carefully washed with saline solution. The scheme of these procedures is shown in Fig. 1.

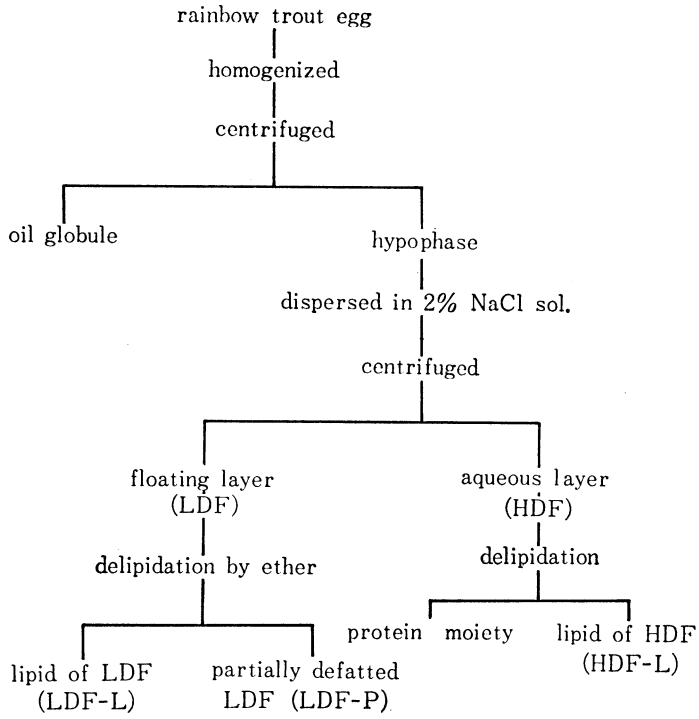


Fig. 1. Preparation of oil globule and lipid fraction of lipoprotein.

**Delipidation of LDF and HDF**: Ether and 2% NaCl solution were mixed to LDF by gentle stirring. Then the ether phase was collected and washed with water. After a treatment by anhydrous $Na_2SO_4$, the solvent was removed in order to obtain the lipids of LDF (LDF-L). The aqueous layer saturated with ether was sealed in a cellophane tube and dialyzed against water at 5°C for five days. The partially defatted LDF (LDF-P) which was precipitated in the tube, was collected by centrifugation and dissolved in a carbonate buffer solution at pH 9.8. A lipid portion of HDF (HDF-L) was also obtained by the HILLYARD's method[13] as previously reported.

**Electrophoresis of LDF-P**: The Tiselius and starch-grain zone electrophoresis of LDF-P were carried out according to the method described previously[1].

**Thin layer chromatography of lipid**: The oil globule, LDF-L and HDF-L were examined on the lipid class composition by the thin layer chromatography on plates covered with silicic acid (Merk kieselgel G and H). The solvent system used for the estimation of lipid class was petroleum ether, ether and acetic acid (90:10:1

v/v), and for the phospholipids methanol, chloroform and water (65 : 25 : 4 v/v). The spots were developed by spraying 50% sulfuric acid, 2.5% ammonium molybdate in 2.5 N sulfuric acid[14], 0.2% ninhydrin, Dragendorf reagent[15], and 0.5% 2,4-dinitrophenylhydrazine[16].

**Gas-liquid chromatography of fatty acids**: The fatty acids of the various lipid fractions were converted into the corresponding methyl esters by treatment of diazomethane in ether after saponification. The resulted methyl esters were then analyzed by gas-liquid chromatography using Shimadzu GC-2B, at temperature of 198°C. The column (300 mm) was packed with diasolid L containing 15 per cent ethyleneglycol-succinate on chromosorb W (80 to 100 mesh). A hydrogen ionization detector was employed. Fatty acid metyl esters were identified on the basis of the retention times compared with the standard fatty acids.

## RESULTS

Three components of LDF-P were measured by Tiselius electrophoresis at pH 9.8 (Fig. 2). They were determined by measuring their areas as 90.4 ($LDF\text{-}P_1$), 7.9 ($LDF\text{-}P_2$), and 1.7 ($LDF\text{-}P_3$) per cent, respectively. Comparing with those of HDF seen in Fig. 2, it appeared that the patterns of HDF and LDF-P were almost similar

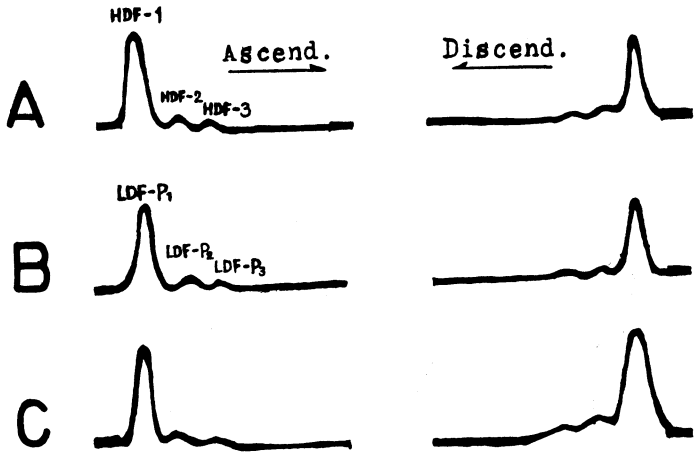


Fig. 2. Tiselius electrophoretic patterns of HDF and LDF-P at pH 9.8 in carbonate buffer solution.
A: HDF, after 60 minutes.
B: LDF-P, after 60 minutes.
C: mixture of HDF and LDF-P, after 60 minutes.

in their electrophoretic behavior. LDF-P was precipitated by dialysis against distilled water or by addition of ethanol, trichloroacetic acid and ammonium sulfate. The properties of LDF-P isolated by zone electrophoresis were examined with color tests. The results obtained are shown in Fig. 3.

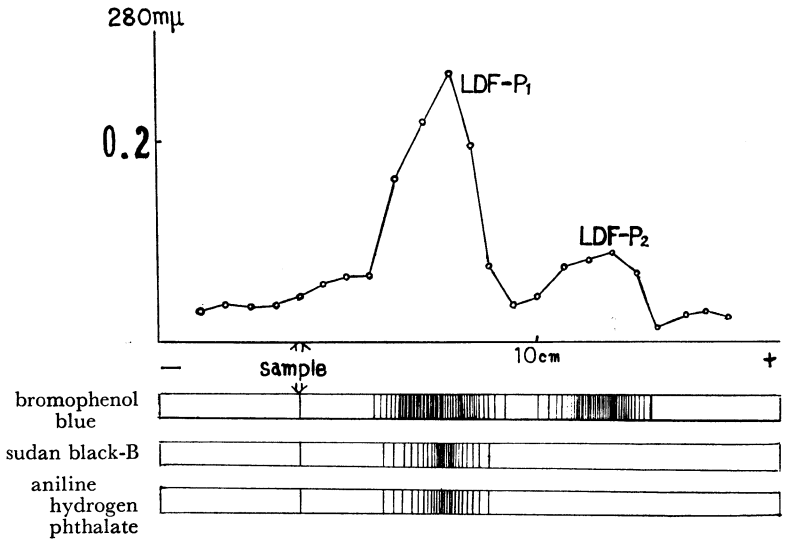


Fig. 3. Zone electrophoretic pattern of LDF-P (pH 9.8, 1.9 mA/cm², after 48 hours). The color tests were carried out on the filter paper by "finger printing".

LDF-$P_1$ showed a striking likeness as to the color reaction of HDF-1 which was a lipoprotein. LDF-$P_2$ was also similar in qualitative tests and electrophoretic mobility to HDF-2 (refer to Fig. 2). However, LDF-$P_2$ was a simple protein that did not contain any trace of lipid, phosphorus nor carbohydrate. In the case of LDF-$P_3$, the yield was too small to investigate minutely.

As shown in Fig. 4, the spectra of LDF-$P_1$ and LDF-$P_2$ showed the maximum absorption at approximately 280 m$\mu$, but not in visual region. The oil globule whose color was red, had a maximum absorption at 490 m$\mu$ which coincided with

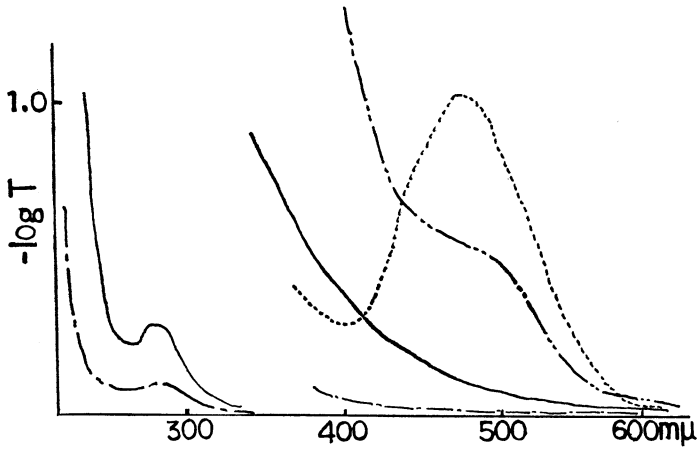


Fig. 4. Absorption spectra of LDF-$P_1$, LDF-$P_2$ and the lipid fractions. LDF-$P_1$ (——) and LDF-$P_2$ (– – –) were dissolved in buffer solution at pH9.8, the oil globule (·····) and LDF-L (—·—) in petroleum ether.

that of astaxanthin. LDF-L was orange and had a maximum absorption of nearly 500 mμ.

The oil globule, LDF-L and HDF-L were respectively fractionated into several lipid classes by thin layer chromatography. They were identified by specific color

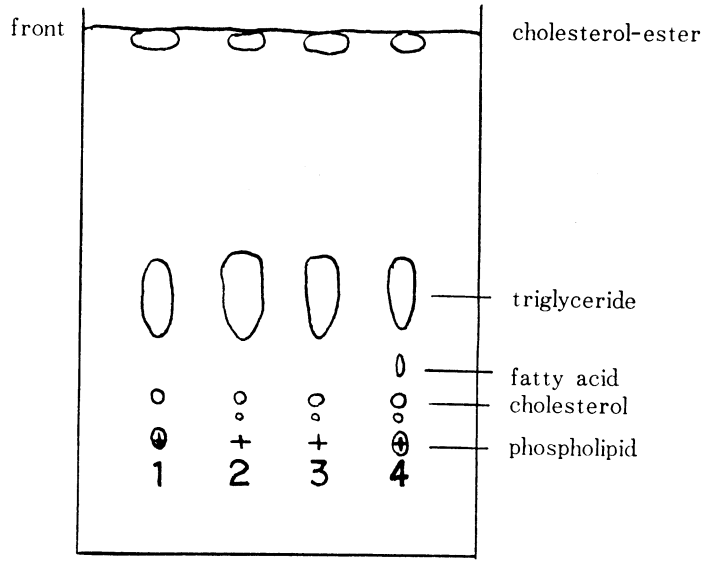


Fig. 5. Thin-layer chromatograms of lipid classes.
Kieselgel G, petroleum ether-ether-acetic acid (90: 10: 1).
1: lipid of lipovitellin, 2: oil globule, 3: LDF-L, 4: HDF-L.

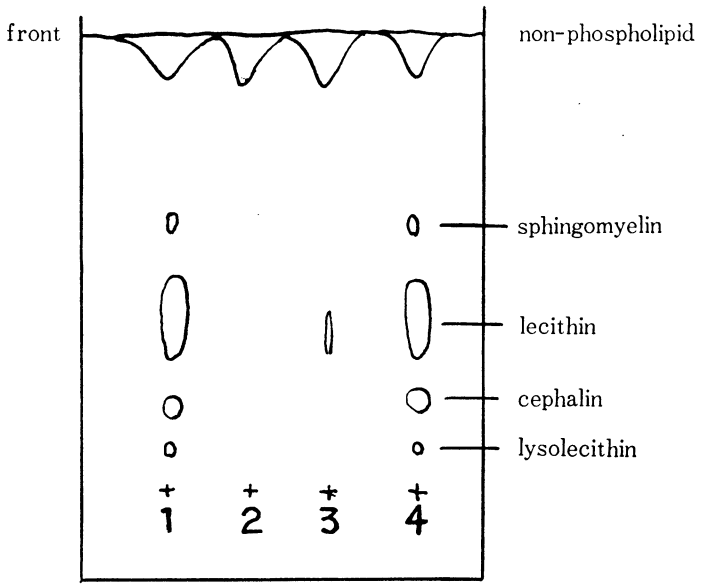


Fig. 6. Thin-layer chromatograms of phospholipid.
Kieselgel H, chloroform-methanol-water (65: 25: 4).
1: lipid of lipovitellin, 2: oil globule, 3: LDF-L, 4: HDF-L.

reactions and R*f* values. The lipid obtained from a hen's egg lipoprotein was fractionated into cholesterol esters, triglyceride, cholesterol and phospholipids. However, in HDF-L of the rainbow trout egg, cholesterol esters, triglycerides, fatty acids, cholesterol and phospholipids were found in the proportions shown in Fig. 5. But it was noteworthy that neither phospholipids nor fatty acids were detected in the oil globule. LDF-L was also absent from fatty acids. One or two unidentified spots were observed in the trout egg lipids.

Concerning the composition of phospholipids, four spots were detected in the HDF-L as seen in Fig. 6. They were identified as lecithin (phosphatidylcholine), cephalin (phosphatidyl ethanolamine), sphingomyelin and lysolecithin (lysophosphatidyl choline). As already stated, the oil globule lacked phospholipids, but LDF-L contained lecithin. Carotenoid pigments were present in oil globule and LDF-L but not in HDF-L as shown in Table 1.

Table 1. Presence of phospholipid and carotenoid in the lipids.

| | oil globule | LDF-L | HDF-L |
|---|---|---|---|
| phosphorus (%) | 0 00 | 0. 35 | 1. 98 |
| phospholipid | − | + | ++ |
| carotenoid | ++ | + | − |

The fatty acid composition of three lipid fractions are listed in Table 2.

Table 2. Fatty acid composition of the lipids (%).

| fatty acid | oil globule | LDF-L | HDF-L |
|---|---|---|---|
| 14:0 | 4. 6 | trace | 2. 7 |
| 16:0 | 17. 4 | 16. 6 | 21. 4 |
| 16:1 | 11. 9 | 11. 0 | 7. 7 |
| 18:0 | 5. 1 | 3. 9 | 9. 4 |
| 18:1 | 23. 4 | 22. 3 | 20. 1 |
| 18:2 | 4. 2 | 2. 1 | 1, 2 |
| 18:3<br>20:1) | 3. 8 | 4. 4 | 4. 1 |
| 20:5 | 11. 0 | 10. 5 | 8. 4 |
| 22:5 | 2. 9 | 6. 1 | 3. 2 |
| 22:6 | 6. 5 | 11. 1 | 13. 7 |

It indicated that saturated acids were somewhat higher in HDF-L than in the oil globlue or LDF-L. The decreasing order of the fatty acid contents were as follows: oleic, palmitic, palmitoleic, eicosapentaenoic and docosahexaenoic acids in the composition of the oil globule: while palmitic, oleic, docosahexaenoic, stearic and eicosapentaenoic acids in HDF-L. The comparatively lower content of docosahexaenoic acid was characteristic in the oil globule.

## DISCUSSION

HDF of hen's egg yolk has been examined in detail for many years. ALDERTON and FEVOLD[17] prepare lipovitellin from the hen's egg yolk. Later, FEVOLD and LAUSTEN[18] succeed in extracting a lipovitellenin containing 40 per cent of lipids by ether from the floating fraction of lipovitellin in an aqueous solution. In 1958, however, it was revealed that the lipovitellenin which is isolated from the LDF containing 90 per cent of lipids[8]. Recent investigations have shown that hen's egg yolk lipoproteins of HDF and LDF differ from each other in lipid composition[11)12)]. MARTIN *et al.*[19] report that the LDF of the hen's egg yolk is fractionable into some derived components by the degree of solubilization in aqueous solution. LDF obteined from the trout egg became soluble in aqueous solution by using the method of FEVOLD and LAUSTEN[18], but it was not precipitated like the flocks of lipovitellenin after removing off the organic solvent. The LDF of the rainbow trout egg was more separable from HDF than that of the hen's egg yolk. LDF-P produced from the LDF was a complex of three kinds of protein at a ratio of 90.4, 7.9 and 1.7 per cent. LDF became soluble in a saline solution after partial delipidation with ether, and the resulted lipoprotein (LDF-P) resembled in uni-electrophoretic behavior to the HDF.

The LDF-L contained a little amount of carotenoid pigment and phosphorus. Considering the fact that the pigment was rich in the oil globule and phosphorus in HDF-L, it was conceivable that the carotenoid pigment present in LDF-L was a contaminant resulted from the incomplete separation of oil globule. Although it is known that the egg protein is sometimes denatured in the course of ether delipidation[20], the procedure employed here showed no sign of denaturation.

MARTIN *et al.*[12] detect that lipovitellin is composed of 22 to 26 per cent of lipids, of which 61 per cent is phospholipids, and that the LDF consists of about 89 per cent of which 27 per cent is phospholipids. ITO *et al.*[21] report that the lipoprotein of the rainbow trout egg has 22 per cent of lipid containing 58 per cent of phospholipids. Our results were almost identical to those of ITO *et al.*[21], and the same is true for the hen's egg yolk. Phospholipids and triglycerides were found in the HDF-L and the latter was dominant in both the oil globule and LDF-L of rainbow trout egg. There occurred at least four spots in the phospholipids of the HDF-L. Among them, lecithin was relatively dominant. YAMAGAMI and MOHRI[22] discover one cephalin, four lecithins which are different in column-chromatographical behaviour, one sphingomyelin and one unknown component in the trout egg protein, and moreover, lysolecithin in the later stage of development. Though LDF-L contained a small amount of phosphorus, a faint spot of phospholipid was detected. It is suggested that the presence of lysolecithin was related to the maturity of egg. We shall report on this matter elsewhere.

The fatty acid composition of three fractions were somewhat different from each other. Palmitic and docosahexaenoic acids were dominant components in HDF-L. It was noteworthy that the saturated fatty acids in HDF-L were relatively higher

than LDF-L or oil globule. The percentage of some unsaturated fatty acids of LDF-L was found to be in the middle between the oil globule and HDF-L. As LOVERN[23] reports that the highly unsaturated fatty acids resulted from phospholipid, the high content of docosahexaenoic acid in HDF-L might be attributed to the presence of phospholipids. Thus it is suggested that the different fatty acid composition of the lipid fractions showed the difference of nutritive importance for embryogenesis.

## SUMMARY

1. The low density fraction of the rainbow trout eggs which was obtained by centrifugation was separated into the partially defatted lipoprotein and its lipid moiety by ether treatment. This lipoprotein was divided into three components by electrophoresis. The slowest component in mobility was a lipoprotein similar to the slowest component of high density fraction previously reported.

2. The oil globule, characteristically rich in carotenoid and lacking in phosphorus, comprised mainly triglycerides, a small amount of cholesterol, cholesterol esters and a fairly amount of higher fatty acids.

3. The lipid of low density fraction contained triglycerides, cholesterol, cholesterol esters, higher fatty acids, phospholipids and trace amount of carotenoids.

4. The lipid moiety of high density lipoprotein consisted of about 50 per cent of phospholipids, in which lecithin, cephalin, sphingomyelin and lysolecithin were contained. The rest were triglycerides, cholesterol, its esters and higher fatty acids.

5. The fatty acid composition of the oil globule, the lipids of low density fraction and the lipids of high density fraction of the fish egg were determined.

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# 虹鱒卵の生化学的研究

## III. 油球及びリポ蛋白質の脂質組成について

中川平介・土屋靖彦

1. 卵黄を遠心分離して油球，低比重画分及び高比重画分（卵黄球）にわけ，それぞれの脂質の分析を行なった．

2. 油球は主にトリグリセライドから成り，他にコレステロール，そのエステル及び遊離の脂肪酸を認めた．リン脂質を全く含まないがカロチノイドに富む．

3. 低比重画分はエーテル処理により，エーテル可溶部と塩溶液可溶部を生じ，前者はトリグリセライド，遊離脂肪酸，コレステロール，そのエステル，リン脂質及びわずかながらカロチノイドを含む．後者は電気泳動によりリポ蛋白質のほか二成分の蛋白質より構成されていることを認めた．

4. 高比重画分の脂質，即ち第1報で報告したリポ蛋白質の脂質は約50%がリン脂質で，他にトリグリセライド，遊離脂肪酸，コレステロール及びそのエステルを含む．リン脂質はレシチン，セファリン，スフィンゴミエリン，リゾレシチンで構成される．

5. それぞれの画分の脂肪酸分析の結果，各画分で脂肪酸組成に相違のあることを認めた．