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Studies on Rainbow Trout Egg (*Salmo gairdnerii irideus*) VI. Changes of Lipid Composition in Yolk during Development

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(Figs. 1–6, Tables 1–8)

The lipid is fully utilized to provide both energy and tissue more than the other substances. Changes of the lipid composition in the rainbow trout egg during development have been investigated by many workers[1–13]. However, there is very little information on each lipid class.

The lipid of the fish egg exists in both forms, free and bound to protein. The former occurs in the oil globule and the latter in the yolk globule[14]. In the rainbow trout the lipid consumed by the embryo during the development chiefly concerned the yolk globule with a small amount of oil globule[15].

We already reported that the lipid of lipoprotein in the yolk globule was markedly utilized after hatching[16]. But no examinations of the role of each lipid in oil- and yolk-globules have been made in connection with the development. The transport mechanism of yolk lipid to the embryo or alevin is complicated, since the embryonic growth is regulated under a certain control.

In this paper, the lipid composition, the fatty acid patterns of the yolk lipid of the rainbow trout egg and the way of supplying lipid to the embryo during the development are described.

MATERIALS AND METHODS

The rainbow trout eggs and alevins of three developmental stages were obtained in December of 1966 from the Zaoh Rainbow Trout Breeding Station, Miyagi-Prefecture.

The stages were as follows; mature egg before fertilization, 29th-day alevin immediately after hatching, and 42nd-day alevin in starvation period.

Preparation of HDF* : HDF which is mainly composed of lipoprotein was prepared from the yolk globule by the procedure described in the previous paper[16]. HDF of 42nd-day alevin was separated into two fractions by the solubility difference in water, such as HDF-P for the water-insoluble and HDF-S for the water-soluble fraction, respectively. However, the latter HDF-S was obtainable solely from the egg of the 42nd-day stage. All of them were lyophilized and stored at −20°C until used.

Extraction of lipid : The oil globule was separated from the yolk content by centrifuging and gently dissolved in petroleum ether. After dehydration, the solvent was removed by vacuum evaporation to obtain the lipid. The bound-lipid was obtained from HDF by extracting with a mixture of methanol and chloroform (1 : 2 v/v)[17].

Thin-layer chromatography : The lipid classes were fractionated on a silica gel plate (E. Merck, Kieselgel G) using a solvent mixture of petroleum ether, ether, and acetic acid (80 : 20 : 1 v/v/v). For the separation of phospholipids, a thin-layer plate was coated with Kieselgel H (E. Merck). The development was carried out with a mixture of chloroform, methanol, and water (65 : 25 : 4 v/v/v). The lipid fractions were detected by exposing them to iodine vapor or spraying 50 % sulfuric acid and charring. Ammonium molybdate in sulfuric acid, Dragendorf reagent, and ninhydrine reagent were used for the detection of phospholipids.

Quantitative determination of lipid composition : The lipid components were separated on a preparative thin-layer plate with a thickness of 0.5 mm, and detected by exposing them to iodine vapor. The lipid was extracted from the silica gel with a methanol-chloroform mixture. Then the composition of lipid classes was calculated from their weight. The composition of phospholipid extracted from silica gel by the procedure of SKIPSKI *et al.*[18] was represented as the distribution of phosphorus in each fraction. Phosphorus was determined by the method of ALLEN[19].

Fatty acid composition : Fatty acids were converted into their methyl esters by 3 % hydrochloric acid in methanol. The resulted methyl esters were then analyzed by a gas-chromatography. The copper column of 4 mm in diameter and 300 mm in length was packed with 10 % polydiethyleneglycol succinate on Diasolid (80–100 mesh). Shimadzu Gas-chromatography Model GC-1B with flame ionization detector was used at 190°C.

Enzymatic digestion of lipid : The lipid extracted from HDF of unfertilized egg was digested with a lipase from *Rizopus delemar* (Seikagaku Kogyo Ltd.), steapsin from pig pancreas (Kanto Kagaku Ltd.), and snake venom. Namely 3 mg of lipid substrate was suspended in 1.5 m*l* of 0.1 *M* $CaCl_2$ solution containing 7.5 mg of steapsin or lipase, or 75 μg of venom at pH 4.5. The reaction mixtures were incubated at 37°C

Abbreviation HDF*: High density fraction

within 1 hr. Then the lipid was extracted with a methanol-chloroform mixture.

Enzymatic digestion of HDF : HDF of the unfertilized egg was dissolved in 0.2 m*l* of a 2 % saline solution, mixed with 0.8 m*l* of the enzyme solution as mentioned above, and incubated at 37°C for 1 hr. Then 1.5 m*l* of 10 % trichloroacetic acid was added to the reaction mixture and centrifuged. Then the precipitates were extracted with a methanol-chloroform mixture and the lipid fraction was washed with water, dried with anhydrous sodium sulfate and concentrated under reduced pressure.

Thin-layer chromatography of digested lipid: The lipid composition was determined by the method of SODHI *et al.*[20] using thin-layer chromatography. It was firstly developed by the solvent system (chloroform, methanol, and water 65 : 25 : 4) for the phospholipid separation until the solvent front reached to the center of the plate. Then the plate was dried and developed further in a lipid class system (petroleum ether, ether, and acetic acid 80 : 20 : 1) until the solvent front reached to the top. The lipid fractions were detected by spraying 50 % sulfuric acid and charring at 120°C.

RESULTS

The visible light spectra of the oil globule showed the absorption maximum at 472 nm, and HDF at 450 nm. Fig. 1 shows the absorption spectra of both lipids dissolved in petroleum ether. HDF contained a little carotenoid, which gradually decreased as the development progressed (Table 1).

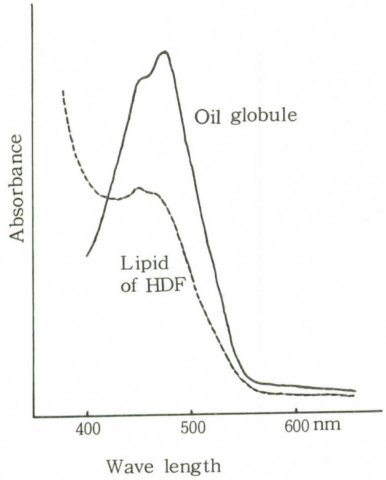


Fig. 1. Absorption spectra of the lipids in unfertilized egg.

Table 1. Change of carotenoid content in oil globule and HDF during development

	Developmental stage		
	Unfertil. egg	29th-day alevin	42nd-day alevin
Oil globule	0.49 μg*	0.25 μg*	0.14 μg*
HDF	+	±	–

*μg carotenoid per one egg

Carotenoid content was calculated from the value of 2,000 as the $E^{1\%}_{1cm}$ at 470 nm.

The oil globule was fractionated at six lipid classes, and retained its almost similar proportions throughout the development. It is noted that the triglyceride portion was always dominant, as seen in Fig. 2. The percentage composition of the lipid class

is shown in Table 2. No phosphorus was detected in the oil globule of the fish egg.

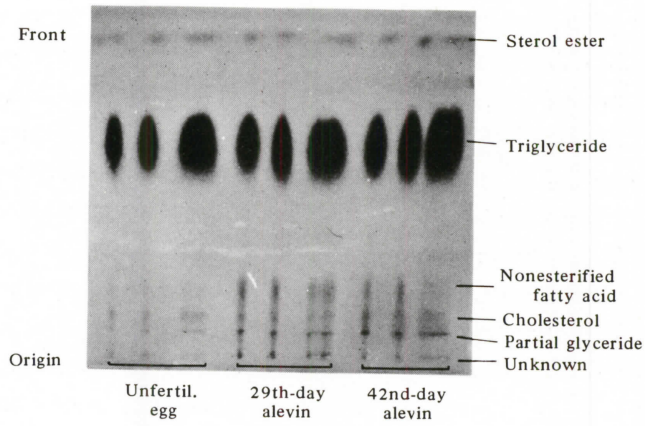


Fig. 2. Thin-layer chromatogram of the oil globule at three developmental stages. Kieselgel G, petroleum ether, ether, acetic acid (80 : 20 : 1).

Table 2. Change of lipid class of the oil globule during development

Stage	Unfertil. egg	29th-day alevin	42nd-day alevin
Oil globule content	5.6 mg	5.3. mg	4.4 mg
Unknown	0.9 %	2.0 %	1.1 %
Diglyceride	0.6 %	2.0 %	1.5 %
Cholesterol	0.8 %	2.0 %	2.1 %
Nonesterified fatty acid	0.9 %	2.2 %	2.3 %
Triglyceride	94.5 %	88.0 %	89.2 %
Sterol ester	2.2 %	3.9 %	3.8 %

The chromatograms of the lipid and phospholipid from HDF are shown in Fig. 3 and 4, respectively.

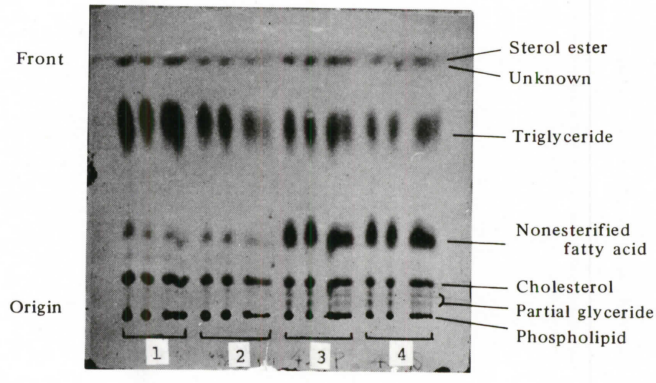


Fig. 3. Thin-layer chromatogram of the lipid of HDF at three developmental stages.
1 : HDF of unfertilized egg
2 : HDF of 29th-day alevin
3 : HDF-P of 42nd-day alevin
4 : HDF-S of 42nd-day alevin

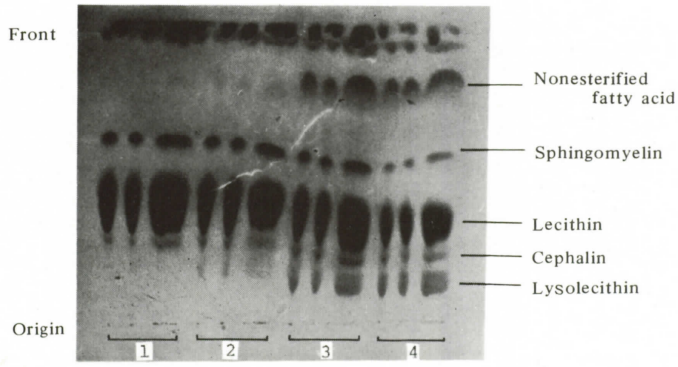


Fig. 4. Thin-layer chromatogram of phospholipid of HDF at three developmental stages.
Kieselgel H, chloroform-methanol-water (65 : 25 : 4)
1 : HDF of unfertilized egg
2 : HDF of 29th-day alevin
3 : HDF-P of 42nd-day alevin
4 : HDF-S of 42nd-day alevin

Table 3. Change of lipid class of HDF in the yolk globule during development

Stage	Unfertil. egg	29th-day alevin	42nd-day alevin	
			HDF-P	HDF-S
Lipid content*	9.8 mg	7.5 mg	4.4 mg	
Phospholipid	56.5 %	46.6 %	39.0 %	33.0 %
Monoglyceride	trace	trace	3.7 %	3.1 %
Diglyceride	3.7 %	5.5 %	6.6 %	3.1 %
Cholesterol	10.7 %	6.9 %	9.6 %	7.2 %
Nonesterified fatty acid	4.0 %	9.6 %	15.4 %	28.8 %
Triglyceride	20.8 %	28.8 %	19.2 %	18.8 %
Sterol ester) Unknown)	4.3 %	2.7 %	6.6 %	6.2%

* Lipid content of the yolk globule per one egg.

A marked increase of lysolecithin and non-esterified fatty acid (NEFA), and a degradation of lecithin were observed in the advancing stage. The percentage composition of the lipid class and phospholipid are summarized in Table 3 and 4. Table 3 shows that the lipid content of the yolk glubule per egg decreased as the stage progressed. It is interesting to note that the compositional changes of phospholipid and NEFA were always contrary each other during the development. Concerning the yolk at the 42nd-day alevin, the lipid of HDF-S was characterized by an abundance of NEFA. The difference between HDF-P and HDF-S was distinctly discerned in the lipid composition, especially in the amount of lecithin, lysolecithin, partial glycerides, and NEFA.

The fatty acid composition of the oil globule and HDF are shown in Table 5. In all stages, palmitic, oleic, and docosahexaenoic acids appeared dominantly in the whole yolk. Moreover, they showed no noticeable change throughout the development. There

Table 4. Change of phospholipid composition of HDF in the yolk globule during development

Stage	Unfertil. egg	29th-day alevin	42nd-day alevin	
			HDF-P	HDF-S
Lysolecithin	trace	2.5 %	12.8 %	23.3 %
Sphingomyelin	7.1 %	2.4 %	7.8 %	10.7 %
Lecithin	84.5 %	88.3 %	73.0 %	60.0 %
Cephalin	8.4 %	6.8 %	6.4 %	6.0 %

was found a significant disparity of fatty acid composition between the oil globule and HDF. An abundance of unsaturated acids was characteristic of the oil globule. On the other hand, HDF was almost uniform in the fatty acid composition during the development with the exception of octadecadienoic acid, which considerably decreased from 12 % to 5 to 3.9 % between the unfertilized egg and alevin. However, it is natural that there is a minute increase or decrease in the fatty acid composition of the lipid class, especially in triglyceride, phospholipid, and NEFA of HDF (Table 6, 7, and 8).

Table 5. Percentage composition of fatty acid in the lipid of egg yolk during development

Fatty acid	Oil globule			HDF			
	Unfertil. egg	29th-day alevin	42nd-day alevin	Unfertil. egg	29th-day alevin	42nd-day alevin	
						HDF-P	HDF-S
14:0	2.2	2.0	2.0	2.0	1.6	1.4	2.4
16:0	13.8	14.6	12.0	19.0	17.7	15.7	15.7
16:1	9.9	11.3	9.6	9.8	10.5	8.5	9.9
17:1	1.4	1.9	1.5	2.1	2.4	1.7	2.4
18:0	4.0	4.3	3.7	7.8	8.2	8.5	9.7
18:1	25.5	26.3	32.7	22.9	22.4	22.5	21.5
18:2	4.6	6.6	6.1	12.0	5.0	5.3	3.9
20:1	2.2	1.8	2.5	2.2	2.3	3.1	2.6
20:3	1.4	0.8	0.9	trace	1.3	2.6	1.7
20:5	8.0	7.5	6.8	5.2	6.0	5.0	3.0
22:2	1.2	2.1	1.2	—	trace	0.9	trace
22:5	3.7	2.7	3.2	1.6	1.9	trace	1.8
22:6	16.4	10.8	12.2	11.0	12.9	12.9	8.9
Unknown	3.1	2.2	2.4	—	4.8	4.8	6.4

There was a marked difference in the fatty acid composition of phospholipid between HDF-P and HDF-S (Table 7). However, docosapentaenoic acid was quite unique in both the triglyceride and phospholipid, because of a great difference between HDF-P and HDF-S. The amount of NEFA was small at the unfertilized stage. But it's fatty acid composition revealed a marked variation as the development progressed. As far as the acids are concerned, the percentage of docosahexaenoic acid considerably diminished from 46.7 to about 8 % (Table 8). The results of the effect of enzymatic digestion on the fish egg lipid were illustrated in Fig. 5 and 6. The decomposition of triglyceride and the appearance of NEFA always corresponded with each other when the lipid was

Table 6. Fatty acid composition of triglyceride of HDF in the yolk globule

Fatty acid	Unfertil. egg	29th-day alevin	42nd-day alevin	
			HDF-P	HDF-S
14:0	1.7	2.8	1.9	1.3
16:0	9.2	12.0	11.0	6.9
16:1	8.3	11.9	8.7	6.7
17:0	1.4	2.6	1.0	1.9
18:0	1.4	1.5	2.1	1.5
18:1	19.2	27.3	22.9	16.4
18:2	1.4	4.5	2.7	4.1
20:1	1.4	1.8	1.6	1.2
20:3	1.5	3.0	4.4	3.4
20:5	13.2	5.5	9.4	9.7
Unknown	2.6	2.7	trace	4.5
22:5	3.4	trace	5.1	1.0
22:6	30.7	18.4	25.3	29.4

Table 7. Fatty acid composition of phospholipid of HDF in the yolk globule

Fatty acid	Unfertil. egg	29th-day alevin	42nd-day alevin	
			HDF-P	HDF-S
14:0	1.2	0.8	1.5	0.8
16:0	20.0	23.8	22.2	12.5
16:1	4.3	5.3	7.0	3.9
17:0	0.9	0.8	2.0	trace
18:0	10.2	12.7	15.3	8.6
18:1	11.6	15.4	18.7	10.8
18:2	1.5	0.9	2.6	1.2
20:1	3.1	3.0	3.7	1.9
20:3	4.5	2.8	5.4	8.8
20:5	9.8	8.5	2.2	10.1
Unknown	2.6	2.0	2.4	4.3
Unknown	1.9	1.4	2.5	4.7
22:5	1.7	1.4	trace	5.4
22:6	19.7	15.6	5.1	22.6

allowed to react with lipase (Fig. 5). However, both lipid classes were rapidly decomposed without the appearance of NEFA in the case of HDF (Fig. 6). Snake venom seemed to act only upon phospholipid, while steapsin, which consists of several enzymes, hydrolized triglyceride showing an increase of NEFA. Of special interest was the fact that lecithin was reduced to lysolecithin but NEFA was indifferently incorporated into triglyceride (Fig. 6). It is probable that steapsin includes such a phospholipase-like enzyme. The pattern of the digestion of HDF by steapsin was somewhat similar to the change of the lipid composition during the development of the rainbow trout egg.

Table 8. Fatty acid composition of nonesterified fatty acids of HDF in the yolk globule

Fatty acid	Unfertil. egg	29th-day alevin	42nd-day alevin	
			HDF-P	HDF-S
14:0	0.8	1.0	2.0	2.7
16:0	5.1	6.9	22.0	19.5
16:1	2.9	1.7	8.5	10.3
17:0	1.2	trace	2.8	3.2
18:0	1.9	2.2	7.6	7.2
18:1	4.5	3.5	23.4	26.4
18:2	1.9	1.5	3.8	3.5
20:1	trace	trace	2.0	2.2
20:3	1.2	7.3	1.7	1.7
20:4	3.3	1.1	0.8	—
20:5	16.2	15.5	3.4	1.9
22:1	5.7	—	2.8	2.1
22:2	—	trace	2.2	3.2
22:5	1.9	5.8	1.3	trace
22:6	46.7	47.4	8.3	7.5

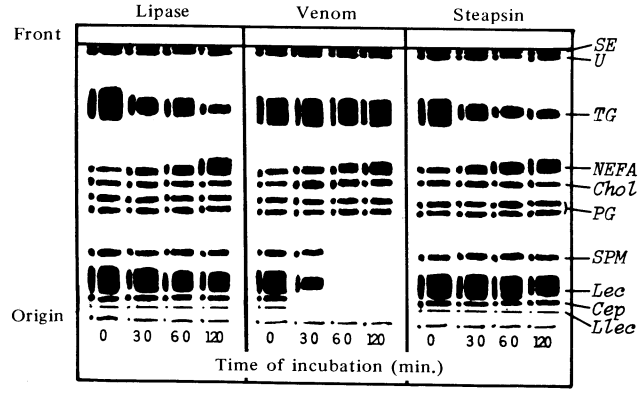


Fig. 5. Digestion patterns of the lipid from HDF with various enzymes.
SE: Sterol ester, *U*: Unknown, *TG*: Triglyceride, *NEFA*: Nonesterified fatty acid, *Chol*: Cholesterol, *PG*: Partial glyceride, *SPM*: Sphingomyelin, *Lec*: Lecithin, *Cep*: Cephalin, *Llec*: Lysolecithin.

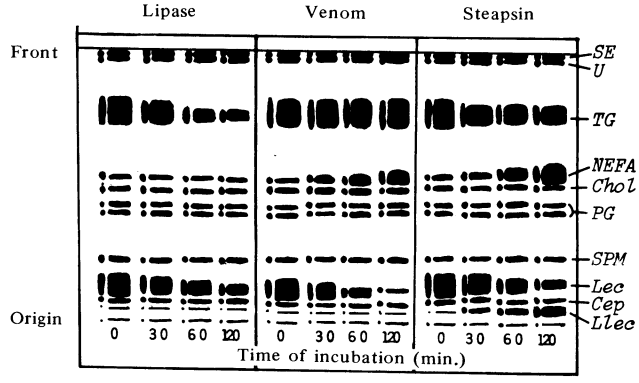


Fig. 6. Digestion patterns of HDF with various enzymes.

DISCUSSION

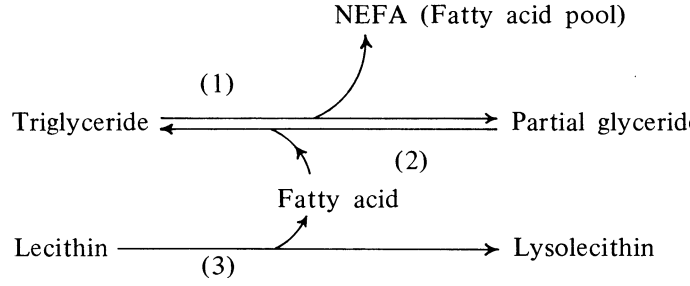
The changes of the content in the rainbow trout egg during development are not evident until after the hatching[15]. There is a preferential consumption of the lipid of the lipoprotein in the yolk globule at this stage. A gradual decrease of the oil globule after hatching was also seen, but neither the lipid composition nor the fatty acid composition themselves were variable. A fair amount of carotenoid was observed in the oil globule, but only a small one in the yolk globule. In an immature egg, carotenoid was not detected in the yolk globule[14]. The role of this pigment in the oil globule leaves many unsolved problems.

YAMAGAMI & MOHRI[7] reported that lysolecithin appeared in the egg yolk of a rainbow trout at a later developmental stage. In our experiments, it was characteristic of the HDF-S in the yolk of 42nd-day alevin that it contains a large quantity of lysolecithin and NEFA. FEENEY *et al.*[21] and MARRINETTI[22] reported that the lysolecithin produced by enzymatic digestion of the lipoprotein of a hen's egg yolk altered the solubility of the lipoprotein in water. Therefore, it is highly probable that the alteration of HDF of 42nd-day alevin in solubility is due to an amount of lysolecithin produced by the enzymatic hydrolysis of the lipid. At this stage, oleic acid in HDF increased, while palmitic and docosahexaenoic acids decreased. No selected consumption of unsaturated fatty acid during the development was reported by TAKAMA *et al.*[11] However, the polyunsaturated acid in both oil- and yolk globules was preferably consumed after hatching. This agrees with ANDO's results[5,6]. An attempt was made to know the mechanism of the lipid transformation in the yolk by analyzing the change of oil- and yolk globules, but it was unsuccessful. However, the following informations on this subject can be obtained. In triglyceride a larger quantity of unsaturated acid was found than in phospholipid. It is very interesting to note that there was no difference in the absolute amount of triglyceride per egg yolk until after hatching, but there occurred a great change in fatty acid composition. TURNER *et al.*[23] tried to explain the mechanism of lipid absorption from yolk to embryo with the use of a tracer. They reported that the yolk lipid was first degraded and than resynthesized to the embryo lipid. However, it is said that in the hen's egg triglyceride absorbed by the embryo is not subjected to any decomposition[24]. DEUCHAR[25] also says that the yolk content is degraded, pooled and absorbed by the embryo. Therefore, it was suggested that NEFA should be pooled before the resynthesis of the lipid of the embryo or the alevin.

The degradation pattern of HDF by steapsin was almost identical with that of the HDF lipid throughout the development, thus showing the disintegration of triglyceride and phospholipid with the increase of NEFA. However, steapsin hardly showed a decomposition of the phospholipid fraction when the enzyme was allowed to react with the lipid extracted from HDF. MARINETTI[26] found the conversion of fatty acid from

phospholipid to triglyceride in serum lipoprotein. This leads to the idea that the transformation of the fatty acid from phospholipid to triglyceride or from triglyceride to partial glyceride was carried out in the fish egg. The β-monoglyceride would play the role of a reservoir as a precursor of lecithin. Consequently the release of fatty acid might proceed *via* a lecithin-lysolecithin equibrium[27].

The steapsin seemed to aquire a phospholipase activity when it was allowed to react with HDF. The release of fatty acid from triglyceride seemed to stimulate the transesterification between phospholipid and triglyceride in the fish egg yolk. YAMAGAMI[28] reported that lecithinase activity was observed in neither yolk nor alevin, and the lecithin bound to lipoprotein was probably decomposed by a certain enzyme. The difference of fatty acid composition in NEFA between HDF-P and HDF-S was suggestive of a result of the transesterification in the lipoprotein. ANDO[13] already indicated that there was a transesterification between triglyceride and phospholipid in the course of the development of rainbow trout. Taking in account the above-mentioned facts, the fatty acid metabolism of a rainbow trout egg during the development is summarized in the following schema.



The degradation and resynthesis of glyceride was catalyzed by a certain enzyme (1) and (2). Lecithin changed to lysolecithin to let loose its component fatty acid (3), which was used for resynthesis of the triglyceride (2). Concerning the HDF of 29th-day alevin, the decrease of eicosapentaenoic and docosahexaenoic acids in triglyceride and a concomitant increase of these acids in NEFA prove to be within the possibility of its metabolic path as presented above. The fatty acid released from glyceride is pooled to become a fatty acid reservoir in yolk lipoprotein.

SUMMARY

In the three stages of development of the rainbow trout, the lipid composition of the oil globule and of the lipoprotein of the yolk globule was investigated.

1) A variation of the lipid class composition in the oil globule was not observed

in the course of the development, while the fatty acid composition was slightly altered.

2) In a later developmental stage, lysolecithin and NEFA appeared with the degradation of lecithin bound to the lipoprotein.

3) On analyses of the fatty acid, it was shown that polyunsaturated acid spreads in triglyceride more than in phospholipid. In NEFA, palmitic, palmitoleic and oleic acids were rich in the latter developmental stage, while docosahexaenoic and eicosapentaenoic acids were poor. A selective consumption of the polyunsaturated fatty acid was also observed.

4) A certain enzymatic action seemed to affect a transesterification between triglyceride and phospholipid within lipoprotein.

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虹鱒卵の生化学的研究
VI 胚発生過程における脂質の変化

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未受精卵，孵化直後，さい嚢吸収前の三段階の卵黄に含まれる脂質を油球および卵黄球リポ蛋白質にわけて，それぞれの組成をしらべた。また，*in vitro* の実験から卵黄球リポ蛋白質の脂質の胚への吸収経路を推定した。

1）油球の脂質組成には発生過程中に著しい変化は無いが，脂肪酸組成にはわずかながら18：1，18：2酸増加と 16：0，20：5，22：6酸の減少がみられた。

2）卵黄球リポ蛋白質の脂質に50%近く含まれるリン脂質は発生と共に減少し，リゾレシチンと非エステル脂肪酸の割合が増加した。

3）リポ蛋白質のトリグリセライドとリン脂質の脂肪酸組成は互いに異なり，特に前者に不飽和脂肪酸が多い。また，非エステル脂肪酸は発生にしたがい絶対量が増加し，未受精卵に多い22：6，20：5酸が減少したのに対し，16：0，16：1，18：1酸が増加した。これによって発生の進むと共に卵黄の高度不飽和脂肪酸が選択的に胚に吸収されることを認めた。

4）卵黄球の脂質は，ある酵素によりトリグリセライドが分解され，脂肪酸が遊離される。これは非エステル脂肪酸としてリポ蛋白質内に蓄積された後，胚へと移行する。一方，生じたモノまたはジグリセライドはレシチンから脂肪酸を得てトリグリセライドを再合成し，レシチンはリゾレシチンに変ずると考える。